INVESTIGATIONS INTO THE ROLES OF *BACTEROIDES FRAGILIS* THIOREDOXINS DURING THE OXIDATIVE STRESS RESPONSE

by

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(Under the direction of C. Jeffrey Smith, Ph.D)

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The Gram-negative *Bacteroides fragilis* is the most frequently isolated bacterium from anaerobic infections. Native to the human intestinal tract, it serves a symbiotic role breaking down complex polysaccharides and restricting the growth of potentially harmful organisms. However, if translocated into the peritoneal cavity, *B. fragilis* can induce abscess formation; a potentially life-threatening condition. Although an obligate anaerobe, this bacterium is capable of surviving aerobic environments for extended periods of time, and its role as an opportunistic pathogen depends on this capability. Aerobic survival relies on an oxidative stress response of genes activated either dependently or independently by the stress response regulator OxyR. An important subset of OxyR-independent genes are the thioredoxins (Trxs). *B. fragilis* contains an extensive catalog of six *trx* genes, and analyses indicated each is differentially regulated during oxidative conditions. Single and multiple *trx* deletions

were produced to determine functional differences of the proteins. It was found that *trxA* was essential for growth while no other single *trx* deletion conferred anaerobic growth defects. The *trxD* gene was found to be induced by the thiol oxidant diamide and TrxD was shown to be protective during exposure to diamide as well. In a *trxD* mutant strain, diamide-induced expression of *trxC, trxE, and trxF* increased significantly, suggesting compensatory effects in the Trx system. TrxD and TrxE were determined to be the only two *B. fragilis* Trxs capable of reducing the aerobic ribonucleotide reductase (NrdAB) of *E. coli*, suggesting specificity of Trx targets in *B. fragilis.* Further investigations into TrxD determined the *trxD* promoter region and transcriptional start site as well as demonstrating TrxD to be the Trx primarily responsible for the reduction of specific oxidative stress-induced proteins including AsnB (asparaginase II) and Tps (thiol peroxidase scavengase). These data provide initial insight into both the specific and overlapping functions of Trxs in *B. fragilis*.

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CHAPTER ONE: INTRODUCTION

The impetus for this work was derived from recent discoveries involving the regulation of genes responsible for aiding in the survival of *Bacteroides fragilis* during oxidative stress. Experiments utilizing a mutant that lacks a functional gene for the redox sensing transcriptional regulator, OxyR, showed that it is important for controlling the production of a specific set of proteins during oxidative stress (such as AhpC and KatB) but there were other proteins being upregulated in the oxidative environment in an OxyR-independent manner (137). One group of proteins shown to be OxyR-independent was the thioredoxins. The goal of this current research was to identify which thioredoxins are most important for survival during oxidative stress and to elucidate the roles and regulation of specific thioredoxins in *B. fragilis.* To recognize the significance of this research it is important to first discuss basic information regarding *B. fragilis,* its host environment, the functions and roles of thioredoxins in bacterial systems, and how they may operate in *B. fragilis*.

1.1 *Bacteroides* in the Human Gut

The *Bacteroides* genus consists of Gram-negative, nonsporeforming, nonmotile, anaerobes which make up nearly 30% of all bacterial isolates found in the human gastrointestinal (GI) tract (35). As a member of the indigenous gut flora, *B. fragilis* provides several advantages to the host by aiding in physiology and normal function of the GI tract; such as assisting in the breakdown of complex polysaccharides, providing valuable nitrogen cycling, and utilizing nutritional resources which helps prevent the colonization of the gut by potentially harmful bacteria (83, 127). Further studies have demonstrated that this symbiosis is due in part to the use of a two-way communication

system allowing strains of *Bacteroides* to directly modulate functions of the gut (41). For example, *B*. *thetaiotaomicron* is capable of inducing production of terminally fucosylated glycoproteins and glycolipds by intestinal epithelial cells which the bacterium then cleaves the L-fucose moieties off of to internalize and use as an energy source. While the *B. thetaiotaomicron* signal for intestinal fucosylation is still unknown, it is suspected that FucR, the repressor of the L-fucose-utilization gene cluster *fucRIAK* acts with L-fucose as a corepressor of this signal. When fucose is low in concentration, the production of this signal is unrepressed and can then act on the host to increase the level of fucosylated glycocogugates (15, 59). Other research has shown that *B. thetaiotaomicron* can also act to modulate the expression of host genes involved in nutrient absorption, mucosal barrier fortification, and the production of angiogenic factors (58). This relationship is likewise beneficial for the bacteria as the colon provides them with an essentially continuous nutritional supply in a reduced and anaerobic environment.

While this relationship between the host and *Bacteroides* is advantageous for both organisms, there are situations where these bacteria can cause serious injury to the host. Although the majority of the anaerobic species that are indigenous to the GI tract do not cause disease, *Bacteroides* species are opportunistic pathogens, and are the most frequent anaerobic bacteria isolated from infections. They are important causes of such life-threatening conditions as intra-abdominal abscesses (IAA) and bacteremia, which require rigorous antimicrobial treatment and surgical intervention for successful resolution (36). The most frequent anaerobic isolate from these infections is *B. fragilis,* which has a role in nearly one third of all intra-abdominal abscesses that

occur in the peritoneal cavity even though it only accounts for 0.5% of the human colonic flora (14, 105). While abscesses are typically polymicrobial in nature and usually consist of organisms found in the normal flora, *B. fragilis* is one of the few bacteria known to be able to induce abscess formation as the sole infecting organism in experimental animal models (153).

1.2 Intra-Abdominal Abscess Formation

The formation of abscesses in response to bacterial infections is actually a crude, and often inadequate, host immune response that attempts to isolate and contain the invasive organisms. While certain types are rare, such as those found in the brain, IAAs are quite common. Abscesses represent a serious medical condition and a critical clinical and financial issue, with treatment costs estimated at $500 million every year in the United States alone (23). IAA formation requires several steps involving both the entrance of the organism into the intra-abdominal area from the GI tract as well as the subsequent regulation of virulence and survival factors by the bacteria.

Typically, the manner in which microorganisms gain entrance into the normally sterile intra-abdominal space is via a rupture or opening into the cavity due to disease, surgery, or abdominal trauma. Upon entry into the intra-abdominal space, bacteria encounter stress conditions due to the increased oxygen partial pressures in the peritoneum, at around 6%, as well as components of the host immune response. Following entrance into the peritoneal cavity, most of the invading organisms are removed by the lymphatic system or phagocytized by the resident macrophages or incoming polymorphonuclear cells (PMNs) (23). However, initial host immune responses may be averted, in part, by strong adherence of the bacteria to surrounding

tissue upon invasion, preventing efficient bacterial cell elimination. Examples of this phenomenon, in which adherence ability is correlated to survival, can be seen with the protein adhesins on the outside of *S. aureus* cells (38) as well as with the polysaccharide capsule of *B. fragilis* (26).

The formation of an IAA by *B. fragilis* has been demonstrated to rely heavily on the capsule of the bacterium; in fact the capsule alone has been shown to be sufficient to induce abscess formation in experimental models (26). It has also been shown that protection against abscesses in animals can be achieved by systemic injection of the capsule; presumably from antibody development (145). Evidence suggests that the high incidence of involvement of *B. fragilis* in IAAs could be due in part to an enhanced adherence ability of its capsule. This capability could allow better attachment to the peritoneal mesothelium and prevent *B. fragilis* from being cleared from the site after entry, compared to unencapsulated organisms or organisms with less adherent capsules (44). Furthermore, while the *B. fragilis* capsule has been shown to aid in mucosal tolerance in the gut by inducing anti-inflammatory cytokines from T-regulatory cells (122), it also has been shown to induce abscesses in the peritoneal cavity through regulating the production of cytokines as well. Studies have shown that the *B. fragilis* capsule was capable of inducing tumor necrosis factor α (TNF-α) and interleukin 1α (IL-1α), which cause the production of the cell adhesion molecule ICAM-1 by mesothelial cells (44, 45). The ICAM-1 produced by these cells can then act as a ligand for the infiltrating PMNs to bind to, which is an important step in the initiation of an abscess. These PMNs are further recruited to the site by the production of IL-8, which is also induced by the *B. fragilis* capsule.

The host immune response plays an even further role in abscess formation by increasing the release of vasoreactive substances during infection of the peritoneum, resulting in fibrin deposition which traps the adherent bacteria and progresses abscess development (1). This issue is confounded by the fact that intra-abdominal infection also reduces fibrinolytic activity, increasing the potential for fibrin accumulation (147). Furthermore, the core of an established abscess, consisting of necrotic debris and bacteria, is surrounded by host immune cells that continually attempt to destroy the infecting organisms and drives these organisms to then upregulate virulence and survival factors. These circumstances create a feedback loop in which the more the host attempts to resolve the abscess, the more the bacteria attempt to survive against the host response. The unintentional, and dangerous, result of this effort to isolate and contain the infecting organisms is the potential for these mature abscesses to rupture. Rupture of an abscess initiates the entrance of bacteria, already primed to contend with the stresses of a host immune system, into a normally sterile body cavity which typically results in bacteremia and disseminated infection (153).

B. fragilis is able to persist both in the oxidative environment outside of the GI tract and inside the abscess where host immune cells are producing reactive oxygen species, and other antimicrobial compounds, to combat the infection. This ability requires a highly controlled and coordinated system, involving the regulation of many genes in order to function effectively. In *B. fragilis,* this robust mechanism of survival is known as the oxidative stress response (OSR).

1.3 Oxidative Stress and *B. fragilis*

Protective mechanisms for dealing with oxidative stress are an integral part of any organism that lives in, or is exposed to, an aerobic environment. While aerobic organisms have developed highly effective systems to contend with the constant threat of destructive oxygen radicals, anaerobic organisms introduced to an aerobic environment are at an elevated risk for damage. Oxygen toxicity in anaerobes is a complex phenomenon involving many aspects of cellular physiology that are impaired as oxidative damage occurs. For example, upon persistent exposure to oxidative stress, the aerotolerant *B. thetaiotaomicron* is growth impaired, in part due to the oxidation of iron-sulfur clusters located within metabolic enzymes (98). To combat this problem, some anaerobic bacteria have evolved multifaceted strategies to manage the production and effects of reactive oxygen species (115).

B. fragilis is unable to multiply in the presence of air (21% O_2); however it is highly resistant to oxidative stress and can survive for extended periods in a fully aerobic environment. In this regard, *B. fragilis* is one of the most aerotolerant anaerobes known, and has been observed to survive at least 120 hours in the presence of atmospheric oxygen. By contrast, oxygen sensitive anaerobes survive less than 2 hours in atmospheric O_2 conditions (141). This remarkable resistance to oxidative stress is mediated by the OSR which involves a wide array of genes that are tightly regulated to ensure an appropriate level of physiological response for the stress level encountered.

An ever growing set of genes and their cognate proteins induced in response to oxidative exposure have been discovered, and while the function of some have been

deduced, many of their contributions to aerotolerance remain to be clarified (53, 113-115, 137). In *B. fragilis,* the induction of gene expression in the OSR is due, in part, to the LysR family regulator, OxyR. OxyR is an oxidative stress sensor and transcriptional regulator activated by disulfide bond formation between a pair of conserved cysteine residues (C199 and C208) (158). OxyR has been shown to be vital for dealing with oxidative stress in *B. fragilis* (114) and contains several important genes in its regulon; including *katB* (catalase)*, ahpCF* (alkyl hydroperoxidase)*, dps* (DNA binding protein)*, and tpx* (thiol peroxidase), which are all positively regulated in response to oxidative stress (113). Furthermore, OxyR has been shown to indirectly regulate several other genes, such as *ftnA* (ferritin), as part of the OSR process (118). In addition, studies have shown that an *oxyR* mutant was less virulent than wild type *B. fragilis* in a mouse abscess model suggesting the importance of this regulator in the ability of *B. fragilis* to cause disease (137).

While significantly deficient in many oxidative stress response factors, studies also showed that the OxyR mutant was still capable of inducing limited *dps* expression during exposure to air, helping to demonstrate that *dps* was under dual regulation. This suggested another arm of the OSR system existed that was OxyR-independent (114). The differences between these two systems appeared to be in the levels of regulation of their respective regulons when exposed to an array of oxidative stress conditions. When the expression levels were compared between the OxyR- dependent and -independent genes it was shown that only the OxyR-dependent regulon was highly induced by H_2O_2. Additional evidence for the OxyR-independent response was from further experiments which showed the OSR genes *osuA* and *fho*, encoding for a starch

binding protein and an elongation factor G homolog respectively, were upregulated by air in an *oxyR* mutant. Microarray analysis utilizing *B. fragilis oxyR* mutants also produced data revealing many other genes that appeared to be regulated by an OxyR-independent pathway. Thioredoxin (Trxs) genes, that code for a family of proteins important in the stabilization of the reduced cytosolic environment (57), were found (by microarray results and confirmed by real-time RT PCR), to be induced by oxidative stress conditions independently of OxyR (109, 119, 137).

1.4 Sulfhydryl Group Redox Management and Thioredoxins

Sulfhydryl groups play amazingly diverse roles within a cell, and the redox state of cysteine residues can affect both function and structure of many enzymes, as well as receptors and transcription factors. Upon exposure to reactive oxygen species like H_2O_2, hydroxyl radicals, and the superoxide anions, the sulfhydryl groups (-SH) of proteins can become oxidized, resulting in conformational changes that can lead to subsequent inactivation or improper functionality of these proteins within the cell. In order to combat this issue, both prokaryotic and eukaryotic organisms utilize the small, heat-stable oxidoreductase Trx proteins in order to minimize the oxidation of sulfhydryls within their cytoplasm. In many species there also exists a second system similar to Trx, known as the glutaredoxin (Grx)/glutathione (GSH) system, and while these two systems overlap considerably, they are differentially regulated.

Trxs are ubiquitous redox active proteins (~12 kDa) found in all phylogenetic branches. The first Trx was discovered in *Escherichia coli* and was proposed to be a hydrogen donor in the enzyme-catalyzed reduction of ribonucleotides to deoxyribonucleotides (75). Further investigations have shown that while small yet

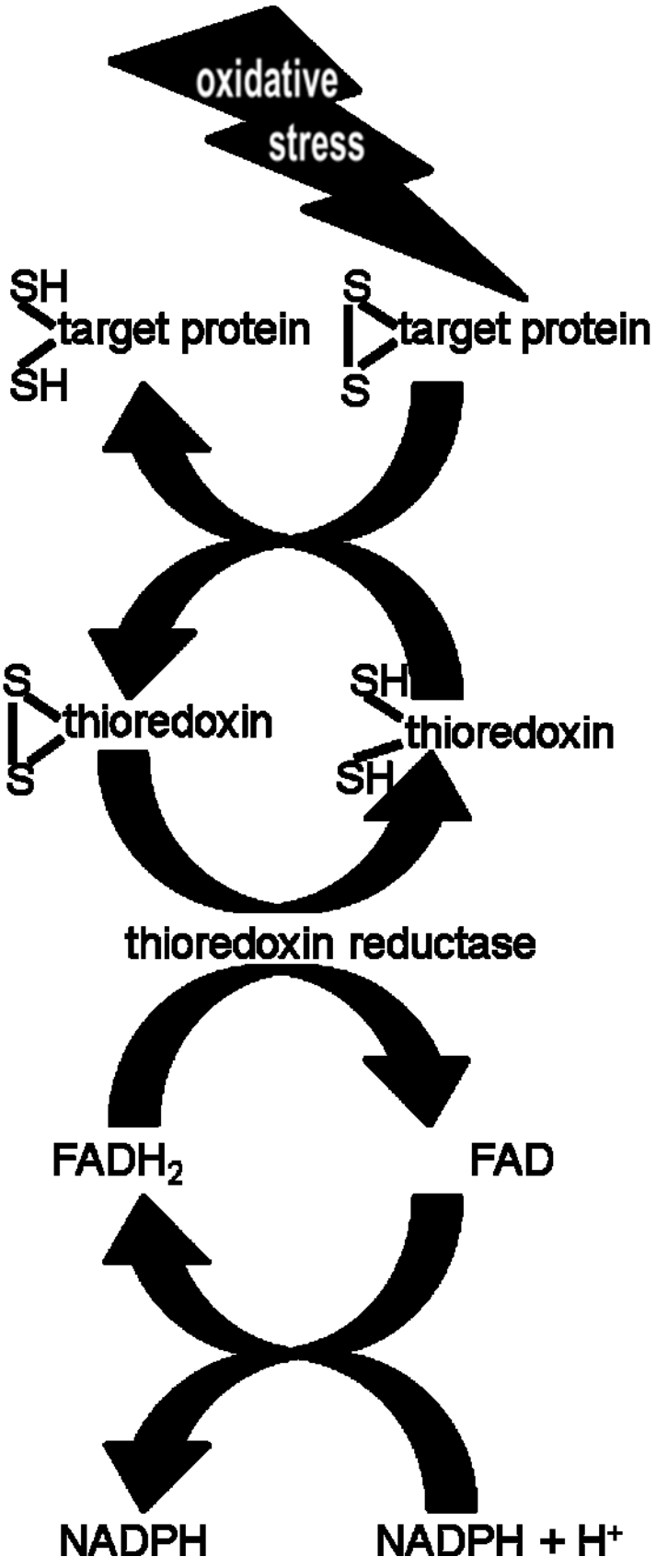
important differences in their amino acid sequences and enzymatic roles are present in typical bacterial, fungal, and animal Trxs, plants have an unusually complex complement of distinct Trxs. The Trxs in plants are composed of six well-defined types (type- *f, m, x, y, h,* and *o*); with the type-*m* being most closely related to archetypal bacterial Trxs and the type-*x* and type-*y* being most closely associated to Trxs found in cyanobacteria (86, 101, 144). These different types of Trx in plants are found in different cellular compartments and have highly specialized roles ranging from aiding in metabolism and photosynthesis, to intracellular communication (86, 138, 140).

Trxs contain a highly conserved Cys-X-X-Cys motif at their active site, allowing for catalysis of thiol/disulfide reactions (3, 119). The first step of the normal Trx-catalyzed reduction pathway between a Trx and its target involves the cysteinyl residue nearest the N-terminus in the CXXC motif acting as the primary nucleophile that attacks the disulfide of the oxidized substrate. The second cysteine in the motif serves to cleave the mixed disulfide intermediate, allowing the release of oxidized Trx and reduced target protein (13). Sulfhydryl groups are normally only reactive when ionized to the thiolate species, and the acid dissociation constant (pKa) of an undisturbed cysteine thiol is 8.7 (134). This means that at neutral pH, uncatalyzed thiol/disulfide exchange reactions are relatively slow. However, since the reactive thiol group found in the Trx family of proteins has a pKa shifted to around 6.7, this allows the thiol to be almost fully ionized and reactive at physiological pH values (66). This fact explains how the two-electron oxidation of the conserved cysteines in Trxs occurs, which allows them to reduce their target proteins (46, 104).

The reduction of Trx proteins is mediated by the flavin adenine dinucleotide-dependent Trx reductase which converts oxidized Trx to its free thiol form (Figure 1.1) (3). In *B. fragilis,* the single Trx reductase is known as TrxB and appears to act as the primary reducing enzyme of the Trxs in the organism. A recent study has shown that TrxB is induced in *B. fragilis* during aerobic and oxidant stress conditions and experiments utilizing a TrxB mutant demonstrated that this protein is required for maximal growth during oxidative stress. This aerobic growth defect could be rescued by providing a reducing agent, such as dithiothreitol (DTT), in the growth media. These observations suggested that the TrxB/Trx system functions as the major system for maintaining the thiol/disulfide cellular equilibrium in *B. fragilis* and likely plays a substantial role in the OSR (119).

Since the discovery of the role of Trxs in DNA synthesis and in maintenance of the reduced state of intracellular protein disulfides, Trx proteins have been shown to play a vital role in defense against oxidative stress (57). Trxs aid in the regeneration of oxidatively damaged proteins, modulate the activity of redox stressors, aid in nucleotide metabolism, and act as hydrogen donors for detoxification enzymes important during the OSR (18, 34, 90, 93, 94). Studies have also shown that in some bacteria both the Trx and the Trx reductase can even act as redox-powered chaperone machines able to assist in the re-folding of proteins after stress conditions have caused them to be oxidized (70). Many bacteria have evolved mechanisms which make use of the Trx system, and other related proteins containing the Trx domain, to combat and manage oxidative stress. The following examples illustrate the importance of the Trx system for the prolonged survival and proliferation in a variety of organisms.

Figure 1.1. Oxidation/reduction pathway of the thioredoxin system. Oxidative stress induces disulfide bond formation in a target protein that is then reduced by a thioredoxin. The thioredoxin is then reduced by thioredoxin reductase, restoring reducing power to the enzyme. Thioredoxin reductase is then reduced by $FADH_2$, which is subsequently reduced by NADPH allowing for the reduction cycle of the system to recur.


oxidative
stress
SH
target protein
SH
S
target protein
S
S
thioredoxin
S
SH
thioredoxin
SH
thioredoxin reductase
FADH2
FAD
NADPH
NADPH + H+

1.4.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobic bacterium frequently associated with the human skin and nose flora. While able to cause a wide variety of minor to severe illnesses including skin infections, food poisoning, pneumonia, meningitis, osteomyelitis, and toxic shock syndrome (80), approximately 20% of people are long-term carriers, most without incident. As a facultative anaerobe, *S. aureus* is capable of growing in the absence of oxygen by fermentation or using alternative terminal electron acceptors. The regulation of genes in response to the availability of oxygen could be a key factor in *S. aureus* pathogenicity and survival as they may be involved in cell adherence and invasiveness necessary for colonization of tissues and other environments (28, 76). The ability of *S. aureus* to counteract and eliminate a variety of reactive oxygen intermediates allows it to adapt to changes in oxygen concentration, and indicates the existence of a robust OSR system in *S. aureus*.

While *S. aureus* has two *trx* genes and one Trx reductase gene in its genome; *trxA, trxB* (encodes for Trx reductase), and a largely unstudied putative *trx* labeled SACOL1794, it does not possess a glutathione system and so must employ Trx and similar systems to carry out redox functions (56). The importance of the Trx system was suggested by the mutational analysis studies investigating oxidative stress in *S. aureus* in which the inability to create a *trxB* disruption mutant suggested that TrxB is essential to the survival of the organism (146). Interestingly, transcriptional analysis showed that the *S. aureus trx* genes were equally expressed in both aerobic and anaerobic conditions, demonstrating that the *trx* genes are not significantly regulated by oxygen concentrations. However, when *S. aureus* was treated with oxidative stress

compounds such as diamide, a thiol-specific oxidant that reacts with free thiols to promote disulfide bond formation (73), rapid induction of the transcription of the *trx* genes occurred. This suggests an important role of Trx in *S. aureus* during conditions of high cytoplasmic oxidative damage in which toxic oxygen species cause disulfide bond formation to occur, preventing proper function of cytoplasmic proteins. Additional studies have shown that the *trx* genes in *S. aureus* also are upregulated in response to acid stress. This upregulation is potentially due to the fact that low pH conditions increase iron ion toxicity, and that an excess of these ion levels can cause oxidative damage in the bacterium (12). These studies in *S. aureus* have helped to strengthen the link between oxidative stress responses and acid stress responses that has been suggested in several other studies, and further display the importance of the Trx system in such conditions (21, 22, 84).

1.4.2 *Treponema pallidum*

Treponema pallidum, a Gram-negative spirochete, is an obligate parasite and the causative agent of the chronic human disease, syphilis. *T. pallidum* is classified as a microaerophilic bacterium due to its limited tolerance of oxygen in vitro. However, in order for it to effectively disseminate throughout an infected host, it must cope with oxidative stressors from a variety of sources; such as reactive oxygen species produced by commensal flora to fight against noncommensal intruders or macrophages and neutrophils that combat invading bacteria (25, 31, 51, 81, 87, 123).

Interestingly, while able to readily disseminate and survive in well oxygenated tissues and inflammation sites, *T. pallidum* lacks many of the proteins involved in the pathways that other bacteria utilize to combat oxidative stress. This bacterium lacks

superoxide dismutase (SOD), catalase, and glutathione peroxidase, and only encodes one known peroxide scavenging enzyme, an alkyl hydroperoxide reductase, known as AhpC, but lacks its typical dedicated reductase, AhpF (40, 106). However, other data have shown that while AhpF usually acts as the highly efficient electron donor to AhpC in other organisms, Trx can act as a substitute in this role (106).

A recent study has shown that in *T. pallidum* this is indeed the case and Trx does provide the reducing power for AhpC. This system was shown to constitute a very active and robust mechanism for handling oxidative stress in the spirochete and possessed broad substrate specificity (99). Researchers also found that this system seemed to be important for survival of *T. pallidum* during active infection, as the two enzymes were highly abundant in the bacteria isolated from inflamed rabbit testes but not in in vitro co-culture growth conditions where exogenous antioxidants were plentiful (99). These new findings of a highly capable and broad-acting Trx/AhpC system have helped provide a potential explanation to the longstanding paradox in which *T. pallidum* was shown to both display oxygen sensitivity in vitro and yet was still capable of thriving in oxygenated tissues in its obligate human host (25, 95).

1.4.3 *Synechocystis* sp.

Synechocystis sp. PCC 6803 is a Gram-negative photosynthetic cyanobacteria often used as a model for photosynthesis research and was one of the first prokaryotes to have its genome be fully sequenced. This sequence analysis revealed that *Synechocystis* contains a total of four *trx* genes; *trxA, trxB, trxC,* and *trxQ*, as well as two *trx* reductase genes; *ftr* and *ntr* (67, 68). Studies into the oxidative stress system of cyanobacteria have shown several peroxidase proteins to be important in the OSR of

these species; including catalase-peroxidases, 2-Cys peroxiredoxins (2-Cys Prx), and NADPH-dependent peroxidases (42, 61, 156). Early experiments showed that a loss of functional 2-Cys Prx in *Synechocystis* eliminated H_2O_2 tolerance, suggesting the necessity of this protein in dealing with oxidative stress (156). Later studies showed that all five Prx proteins found in *Synechocystis* (1-Cys Prx, 2-Cys Prx, PrxII, and PrxQ) utilized Trx as their electron donors and were thus termed "Trx-dependent peroxidases."

The researchers also noticed a high degree of functional overlap between the Trxs in their ability to reduce the Prxs; suggesting that each Prx could potentially be reduced by several different native Trxs. Additionally, it was shown that *Synechocystis* was unable to utilize the Grx/GSH redox system to reduce the Prx proteins after oxidative stress was applied, which further suggested the importance of the Trx proteins in this capacity (102). In similar research, the importance of Trxs in the role of Prx turnover in other photosynthetic bacteria has been further demonstrated in the purple bacteria *Rhodobacter sphaeroides*, which has been shown to rely significantly on the anti-oxidative response of Prxs reduced by Trxs (150).

1.4.4 *Desulfovibrio spp.*

Desulfovibrio species are Gram-negative, sulfate-reducing obligate anaerobes often found in anoxic marine sediments in which sulfate is used as a terminal electron receptor in their respiratory chain (27). While these bacteria are obligate anaerobes, they can survive prolonged exposure to oxygen, suggesting the presence of a strong and effective OSR system. This is a beneficial adaptation, as marine sediments are often disturbed and aerated. (27). One species, *D. desulfuricans* has been implicated in corrosion of metal equipment and petroleum reservoir souring, but their ability to reduce

toxic metals has shown significant importance in bioremediation. *D. desulfuricans* is also known to be associated with disease in humans, causing liver abscesses and bacteremia in rare cases (85, 143). While many *Desulfovibrio* species possess intact SOD, catalase, and rubrerythin genes, certain strains, like *D. desulfuricans* strain Essex 6, are catalase negative, providing evidence for the presence of an alternative system for managing oxidative stress (24, 29, 30, 39, 50, 82).

The genome of *D. desulfuricans* contains two thioredoxin genes *dstrx1* and *dstrx2* as well as the reductase gene *dstrxR.* Interestingly, no homologue to *dsrtrx2* was found in *D. vulgaris*, a congener of *D. desulfuricans,* implying a potentially specific role for its gene product (129). Research into understanding the functions of the two *trx* genes in *D. desulfuricans* has not been actively pursued until recently, and the early data suggest that each may have overlapping, yet potentially partially unique, roles in the cell during oxidative stress and survival (129). The close proximity of *dstrx1* to *dstrxR* suggests that both the genes may be expressed together as part of an operon with their action being tightly controlled similarly to other operons (64). However, the absence of *dstrx2* from the *dstrx1–dstrxR* locus may indicate that it is expressed independently under specific stress conditions and Dstrx2 may act as a more general disulfide reductase enzyme. Furthermore, pattern search analysis indicated the presence of a paraquat binding domain from the 6^{th} to 48^{th} amino acids in DsTrx2. This is interesting because a gene with a similar domain found in *E. coli*, *pqi-5*, is a member of the *soxRS* oxidative stress regulon, and the *dstrx2* gene in *D. desulfuricans* is present within a locus that comprises genes actively participating in stress response (129). These data suggest that binding of compounds similar to the superoxide radical

generator paraquat under natural conditions may activate DsTrx2 as part of the OSR for this organism, and that the synthesis of DsTrx2 may occur not only for the maintenance of proteins in the reduced state but also for reducing harmful free radicals (129). New studies conducted in the related species, *D. vulgaris,* also found data strongly suggesting the involvement of Trx-dependent systems in the OSR as well, further illustrating the importance of Trx in redox regulation in the *Desulfovibrio* genus (159).

1.4.5 *Clostridium spp.*

Clostridium species are ubiquitous Gram-positive, spore-forming obligate anaerobes found throughout nature, including the GI tract of animals and humans. The *Clostridium* genus is interesting in terms of aerotolerance because many members can actually utilize oxygen proficiently and grow in microaerobic environments. For instance, *C. glycolicum* has been shown to grow in the presence of oxygen in up to 6% headspace O_2 in static culture with oxygen-consuming activities, in which it switches to a fermentative metabolism that is not as sensitive to oxygen as acetogenesis (74). This ability to persist in aerobic conditions is partly responsible for another member of this genus, *C. perfringens*, being associated with several serious diseases in both humans and animals, and thus not surprisingly, the most studied organism in the genus (16). The relationship between this pronounced aerotolerance and Trx however, is intriguingly highlighted in the species *C. acetobutylicum,* which has an interesting feature in its genome; two sets of Trx/Trx reductase genes, known as *trxA1/trxB1* and *trxA2/trxB2* respectively, in two different operons. The first set, *trxA1/trxB1* is located in an operon with the glutathione peroxidase *gpx3* and is expressed within 10 minutes after O_2 stress, while the second set, *trxA2/trxB2,* forms its own two gene operon and is

expressed constitutively (69). This differing transcription is indicative of well controlled OSR regulation of Trx production and suggests an important role for *trxA1* during the onset of oxidative stress. The evidence for this is further demonstrated by data showing that the genes for two thiol peroxidase family proteins found in *C. acetobutylicum*, BCP and TSA, are both strongly upregulated in response to O_2 stress. They also were shown to act as Trx-linked peroxidases and are probably recycled primarily by TrxA1 as its gene was upregulated during oxidative stress (69).

1.4.6 *Bacteroides fragilis*

Analysis of the genome of the obligate anaerobe *B. fragilis* has revealed the existence of one Trx reductase gene (*trxB*) and six *trx* homologs; *trxA*, *trxC* (also known as *trxP* (130)), *trxD*, *trxE*, *trxF,* and *trxG*. The TrxA, TrxC, trxD, and TrxF belong to the m-type Trx class, but are associated with two different lineages. TrxE and TrxG are most closely associated to the cyanobacteria y-type class. This extensive catalog of *trx* genes appears unusual when compared to the typical number of two or three *trx* genes found in most other species (49, 63, 69, 71, 110, 129). Previous research has shown that the Trx system is the major thiol/disulfide redox system in *B. fragilis* and is important for survival in vitro and in vivo (119). The Trx reductase, TrxB, was shown to play in major a role in the aerotolerance of *B. fragilis*, with experiments demonstrating its importance for maximal growth during oxidative stress conditions. Furthermore, the lack of a functional TrxB in a *B. fragilis* mutant prevented IAA formation in an animal model, presumably due to the inability of the bacteria to grow in the presence of oxidative stresses. These data provided evidence that the TrxB/Trx system is an important factor in the ability to survive and multiply in the peritoneal environment long

enough to induce abscesses (119). The current study stems from this work investigating TrxB and has shown that the Trx system in *B. fragilis* is highly redundant, with individual *trx* genes being differentially regulated under oxidative stress, but able to compensate for other *trxs* under stress conditions (109). This research has also helped to demonstrate the significance of TrxD in protection against diamide-induced disulfide stress, which is an important and specific subset of oxidative stress. Additionally, this study provides data suggesting that some Trxs in *B. fragilis*, like TrxD, have specific roles in the OSR. These findings support the hypothesis that TrxB is required for normal function of the Trx system, by providing the reducing power for enzymatic turnover of the Trx proteins, and each Trx is important for specific, but sometimes overlapping, roles in survival against oxidative stress in *B. fragilis*.

Evidence from these experiments have shown that TrxD has a major role in managing thiol-oxidation and the upregulation of *trxD* during disulfide stress suggests that it is the preferred electron donor for the repair of inadvertent disulfides, and may be a partner in a specific disulfide repair pathway (109). In the current study we present evidence that *B. fragilis* possesses a highly redundant Trx system in which individual *trx* genes are differentially regulated during oxidative stress, but able to compensate for other *trx* genes under stress conditions. We also present evidence suggesting that TrxD has a major role in managing thiol-oxidation and is the Trx primarily responsible for reducing a specific subset of OxyR-independent OSR genes. We also demonstrate that both TrxD and TrxE appear to function as co-factors for aerobic ribonucleotide reductase and that TrxA is an essential gene.

CHAPTER TWO: THIOREDOXINS IN REDOX MAINTENANCE AND SURVIVAL DURING OXIDATIVE STRESS OF *BACTEROIDES FRAGILIS* (109)

2.1 Summary

The anaerobe *Bacteroides fragilis* is a Gram-negative, opportunistic pathogen that is highly aerotolerant and can persist in aerobic environments for extended periods. In this study, the six *B. fragilis* thioredoxins (Trxs) were investigated to determine their role during oxidative stress. Phylogenetic analyses of Trx protein sequences indicated that four of the six Trxs (TrxA, TrxC, TrxD, and TrxF) belong to the M-type Trx class but were associated with two different M-type lineages. TrxE and TrxG were most closely associated to Y-type Trxs found primarily in cyanobacteria. Single and multiple *trx* gene deletions were generated to determine functional differences between the Trxs. The *trxA* gene was essential, but no anaerobic growth defects were observed for any other single *trx* deletion or for the Δ*trxC* Δ*trxD::cfxA* Δ*trxE* Δ*trxF* Δ*trxG* quintuple mutant. Regulation of the *trx* genes was linked to the oxidative stress response, and all were induced by aerobic conditions. The Δ*trxC* Δ*trxE* Δ*trxF* Δ*trxG* and the Δ*trxC* Δ*trxD::cfxA* Δ*trxE* Δ*trxF* Δ*trxG* multiple deletion strains were impaired during growth in oxidized media, but single *trx* gene mutants did not have a phenotype in this assay. TrxD was protective during exposure to the thiol oxidant diamide, and expression of *trxD* was induced by diamide. Diamide-induced expression of *trxC*, *trxE*, and *trxF* increased significantly in a *trxD* mutant strain, suggesting that there is some capacity for compensation in this complex Trx system. These data provide insight into the role of individual Trxs in the *B. fragilis* oxidative stress response.

2.2 Introduction

Protective mechanisms for dealing with oxidative stress are an integral part of any organism that lives in, or is exposed to, an aerobic environment. While aerobic organisms have developed robust systems to contend with the constant threat of destructive oxygen radicals, anaerobic organisms introduced to an aerobic environment are at an elevated risk for damage. Oxygen toxicity in anaerobes is a complex phenomenon involving many aspects of cellular physiology that are impaired as oxidative damage occurs. For example, aerobic exposure of the aerotolerant *Bacteroides thetaiotaomicron* inhibits growth, in part due to the oxidation of iron-sulfur clusters located within metabolic enzymes (98). To combat this problem, some anaerobic bacteria have evolved multifaceted strategies to manage the production and effects of reactive oxygen species (115). *Bacteroides fragilis*, is a commensal anaerobe found in the human intestine, but also is the most frequently isolated anaerobe from human infections (36). *B. fragilis* is unable to multiply in the presence of air (21% O_2); however it is highly resistant to oxidative stress and can survive for extended periods in a fully aerobic environment. In this regard, *B. fragilis* is one of the most aerotolerant anaerobes known, and is able to survive for at least 72 hours in the presence of atmospheric oxygen. By contrast, intolerant anaerobes survive less than 2 hours in air (141). This remarkable resistance to oxidative stress is mediated by an oxidative stress response (OSR) which involves a wide array of genes activated during exposure to air or H_2O_2. An ever growing set of genes and proteins have been discovered that are induced in response to aerobic exposure, and while the function of some have been deduced, many of their contributions to aerotolerance remain to be clarified (53, 113-

115, 137). In this regard, a recent expression microarray showed that the *B. fragilis* thioredoxin genes were induced by aerobic conditions but their role in the OSR has not been adequately explored (137).

Thioredoxins (Trxs) are small redox active proteins (~12 kDa) found in all phylogenetic branches. Trxs contain a highly conserved Cys-X-X-Cys motif at their active site, allowing for catalysis of thiol-disulfide reactions (3, 119). The reduction of Trxs are mediated by flavin adenine dinucleotide-dependent Trx reductases (TrxB) which convert oxidized Trxs to their free thiol form (3). Since the discovery of their role in DNA synthesis and in maintenance of the reduced state of intracellular protein disulfides, Trxs have been shown to be involved in defense against oxidative stress (57). Trxs regenerate oxidatively damaged proteins, modulate the activity of redox stressors, and act as hydrogen donors for detoxification enzymes important during the OSR (18, 34, 90, 93, 94).

Analysis of the *B. fragilis* genome revealed the presence of a single Trx reductase (TrxB) and six Trx homologs. This large repertoire of *trx* genes appears unusual when compared to the typical smaller number of *trx* genes (two to three) found in other anaerobes (49, 63, 69, 71, 110, 129). Previously, Rocha et al. (119) showed that the TrxB/Trx system is the primary thiol/disulfide redox system in *B. fragilis* with an important role in aerotolerance and essential for survival in an in vivo mouse abscess model. These findings prompted us to propose that while TrxB is required for the function of the system overall, each Trx has important, specific roles in survival and defense against oxidative stress. In this study we present evidence that *B. fragilis* possesses a complex Trx system in which individual *trx* genes are differentially

regulated, but have some capacity to compensate for other *trx* genes under stress conditions. We also present evidence suggesting that TrxD has a major role in managing thiol-oxidation and that *trxA* is an essential gene.

2.3 Materials and Methods

2.3.1 Bacterial Strains and Growth

B. fragilis strains used in this study are listed in Table 2.1. The *trx* gene homologs in strain 638R correspond to the following genes in the genome sequence (http://www.sanger.ac.uk/Projects/B_fragilis/) : *trxA*, BF638R-0680, *trxC,* BF638R-2717, *trxD*, BF638R-2296; *trxEF*, BF638R-3044, 3045; *trxG,* BF638R-1282. Strains were grown anaerobically in brain heart infusion (BHI) broth supplemented with hemin and cysteine (BHIS) for routine cultures. Rifampicin (20 µg/ml), gentamicin (100 µg/ml), tetracycline (5 µg/ml), erythromycin (10 µg/ml), and cefoxitin (25 µg/ml) were added to the media when required. Disk diffusion assays to test for sensitivity to oxidative stress were performed by spreading overnight cultures on plates of either BHIS (without cysteine) or on Defined Minimal Media (DMM) (148), allowing the plates to dry and then adding a sterile 6 mm filter disk to the center of the plate (136). Ten µl of 2 M diamide was added to the disk and then the plates were either placed in the anaerobic incubator or exposed to air (at 37°C) for 6 h prior to anaerobic incubation. Following overnight incubation the diameter of the zones of growth inhibition were measured and the results are the average of three independent experiments done in triplicate.

For the oxidized media experiments, sterile BHIS broth with no cysteine was split and either maintained in the anaerobe chamber (anaerobic media) or shaken in an aerobic incubator at 250 rpm for 24 h (oxidized media). Overnight cultures grown in

Table 2.1. List of strains used in this study. The relevant phenotypes are listed in the center column.

Strain or Plasmid	Phenotype and/or genotype[a]	Reference or source
B. fragilis		
638R	Clinical isolate, Rif^r	(107)
ADB77	Strain 638R *ΔthyA*, Rif^r, Tp^r	(8)
IB298	Strain 638R Δ*oxyR*::*tetQ*, Tet^r, Rif^r	(114)
IB458	Strain 638R *ΔtrxC*, Rif^r	This Study
IB473	Strain 638R *ΔthyA*, *ΔtrxA* single crossover into *trxA*, Rif^r, Tet^r	This Study
IB469	Strain 638R *ΔtrxD*, Rif^r	This Study
IB490	Strain 638R *ΔtrxE*, Rif^r	This Study
IB491	Strain 638R *ΔtrxF*, Rif^r	This Study
IB471	Strain 638R *ΔtrxG*, Rif^r	This Study
IB492	Strain 638R *ΔtrxE*, *ΔtrxF*, Rif^r	This Study
IB498	Strain 638R *ΔtrxC*, *ΔtrxE*, *ΔtrxF*, *ΔtrxG*, Rif^r	This Study
IB483	Strain 638R *ΔtrxC*, *ΔtrxD*::*cfxA*, *ΔtrxE*, *ΔtrxF*, *ΔtrxG*, Rif^r, Cfx^r	This Study
IB499	IB498 *ΔoxyR*::*tetQ*, Rif^r, Tet^r	This Study
IB500	IB483 *ΔoxyR*::*tetQ*, Rif^r, Cfx^r, Tet^r	This Study
E. coli		
DH10B	F$^-$ *mcrA* (*mrr-hsdRMS-mcrBC*) Φ80*lacZ*M15 *lacX74 deoR recA1 endA1 araD139* (*ara leu*)*7697 galU galK rpsL nupG tonA*	Invitrogen
HB101::RK231	Kan^r Tet^r Sp^r; HB101 containing RK231	(48)
Plasmids		
pFD340	*Bacteroides-E. coli* expression shuttle vector, (Amp^r), Erm^r	(132)
pFD516	*Bacteroides* suicide vector, derived by deletion of *Bacteroides* replicon pBI143 in pFD288, (Sp^r), Erm^r	(133)

[a]Erm^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance; Tp^r, trimethoprim Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance. For *Bacteroides-E. coli* shuttle vectors, parentheses indicate antibiotic resistance expression in *E. coli*.

BHIS were diluted 1:20 in fresh anaerobic BHIS, grown to mid-log phase (A_{550} 0.3–0.5), inoculated at 1:50 into 5 ml of “anaerobic” or “oxidized” media, and placed in the anaerobic incubator at 37°C. The A_{550} of the cultures was followed and the results represent two independent experiments performed in triplicate.

2.3.2 Construction of *trx* Deletion Mutants

Briefly, chromosomal fragments containing an N-terminal portion of the *trx* gene, (upstream from the conserved Trx cysteine residues) was amplified by PCR with oligonucleotides containing nucleotide modifications to create sites for BamHI at the 5’ end, and PstI at the 3’ end, and then cloned into pUC19. The same approach was applied to create fragments for the C-terminal end of the constructs except PstI was at the 5’ end and HindIII at the 3’ end. The amplified fragments were then ligated together to create the mutated gene fragment which was inserted into the *Bacteroides* suicide vector pYT102 (8). These plasmids were mobilized into *B. fragilis* ADB77, and exconjugates where selected on BHIS plates containing rifampicin, gentamicin, and tetracycline (8). Sensitivity to tetracycline, resistance to trimethoprim, and PCR were used to confirm the double-crossover allelic exchange into the *B. fragilis* chromosome to create the in-frame, unmarked *trx* deletion mutants. Table 2.2 shows the amino acids deleted for each mutant generated. Multiple *trx* mutations were constructed by subsequent rounds of mutagenesis resulting in strain IB492 and strain IB498 (Table 2.1). All *ΔthyA* strains were reverted to *thyA*$^+$ prior to phenotypic characterization as described previously (8).

Table 2.2. Deletions in Trx ORFs.

Trx	# amino acids in ORF	amino acids deleted
TrxA	104	24-82
TrxC	161	33-136
TrxD	118	19-50
TrxE	98	14-81
TrxF	139	23-72
TrxG	107	1-107

In all cases the CxxC active site was removed by the deletions indicated.

2.3.3 Construction of a Marked *trxD* Deletion Mutant

Briefly, a 308-bp chromosome fragment containing the C-terminal portion of *trxD* was amplified by PCR using oligonucleotides containing restriction sites for SstI and EcoRI and cloned into the *Bacteroides* suicide vector pFD516 (133). Next, a 993-bp chromosome fragment containing the N-terminal portion of *trxD* was amplified by PCR using oligonucleotides containing restriction sites for BamHI and SstI and then cloned into the plasmid. The resulting plasmid contained a 215-bp *ΔtrxD* allele with a 139-bp deletion which encompassed the conserved cysteine residues. Next a 1.1-kb SstI cefoxitin (*cfxA*) resistance gene cassette was cloned into the unique SstI site to create the plasmid pFD*trxDcfx*. This plasmid was mobilized into *B. fragilis* strain IB498, and exconjugants were selected on BHIS containing rifampicin, gentamicin, and cefoxitin. Sensitivity to erythromycin was determined, and PCR was performed to confirm the double-crossover allelic exchange of the *trxD*::*cfxA* mutation into strain IB498 to create the quintuple mutant designated strain IB483.

The *ΔtrxC ΔtrxE ΔtrxF ΔtrxG ΔoxyR::tetQ* and a *ΔtrxC ΔtrxD::cfxA ΔtrxE ΔtrxF ΔtrxG ΔoxyR*::*tetQ* mutants were constructed by mobilizing suicide vector pFD754 containing the *ΔoxyR*::*tetQ* mutant allele (114) into *B. fragilis* as described above. Exconjugants were selected on BHIS containing rifampicin, gentamicin, tetracycline, and cefoxitin (when necessary). Sensitivity to either tetracycline/cefoxitin (when necessary) or erythromycin was used to identify recombinants that were tetracycline and cefoxitin resistant and erythromycin sensitive. These two strains were designated strain IB499 and strain IB500, respectively.

2.3.4 Trx Overexpression Constructs

Plasmids constitutively expressing specific *trx* genes were constructed by PCR amplification of promoterless *trx* genes. The promoterless *trx* gene fragments containing the ribosome binding site were cloned into the BamHI and SstI sites of the *Bacteroides-E. coli* shuttle expression vector pFD340 (132) in the same orientation as the IS*4351* constitutive promoter. The new constructs; p*trxA*, p*trxC*, p*trxD*, p*trxE*, p*trxF*, p*trxG*, and p*trxEF* were individually mobilized into *B. fragilis* strains as described above. Transconjugants were selected on BHIS containing rifampicin, gentamicin, and erythromycin. The primers used for these plasmid overexpression constructs are listed in Table 2.3.

2.3.5 RNA Isolation and cDNA Synthesis

RNA was isolated using the hot acid phenol method (116). Fifty micrograms of total RNA was precipitated with ethanol and contaminating DNA was removed by treatment with Turbo DNA-*free*™ DNase (Ambion). The RNA concentration was determined by measuring the A_{260}/A_{280}. Synthesis of cDNA was as follows; 0.75 µg of RNA was added to reaction mixtures containing 10 ng / µl random hexamers, 0.5 mM dNTPs, first strand buffer (Invitrogen, Carlsbad, California) and 1 µl Superscript II RNase H-Reverse Transcriptase I. Reactions were incubated at 42°C for 50 min. Superscript II was heat inactivated by incubating the reaction mixture at 70°C for 15 min.

2.3.6 Quantitative PCR

Quantitative real-time PCR was performed essentially as described previously using a Bio-Rad iCycler with the real-time PCR Detection System (Bio-Rad, Hercules,

Table 2.3. Primers used for cloning of the *trx* genes into pFD340 and pFD516.

gene	forward (5')	reverse (3')
trxA	cagt**ggatcc**cgcataacagtgatagatact	cagt**gagctc**atatttgtttatgggactgg
trxC	cagt**ggatcc**ctggaaaaaagaaatcatcc	cagt**gagctc**gaaacaatgattcctgacgc
trxD	cagt**ggatcc**tcctatctttgcaacatcaa	cagt**gagctc**accggatcaatcatttaatg
trxE	cagt**ggatcc**tatttgccccctgtaaacga	cagt**gagctc**ttatcattagtagctttggccat
trxF	cagt**ggatcc**cattagtagctttggccatg	cagt**gagctc**ggcatggttttctcaacgga
trxG	cagt**ggatcc**gcattgtttcggagtgcagt	cagt**gagctc**cggattatcaagacaagcactgc

All primers contained restriction endonuclease sites (in boldface) for BamHI (forward) and SstI (reverse).

California) (136). The primers used were designed to amplify products of 100–150-bp. All products were verified by agarose gel electrophoresis and by melting point analysis according to the Bio-Rad iCycler software. The reaction mixture contained 12.5 µl 2× iQ SYBR Green Supermix, 1.5 µl of 5 µM of forward primer, 1.5 µl 5 µM of reverse primer, 8.5 µl H_2O and 1 µl of cDNA template (diluted 1/100) per well. All samples were run in triplicate and RNA with no reverse transcriptase was run as a control to monitor for genomic DNA contamination. Relative expression values were calculated using the Pfaffl method (103). Fold induction relative to the wild type in anaerobic conditions was determined for each gene using sigma-54 modulation protein as the reference gene, which does not vary significantly under conditions tested (137). All results were the average of at least two independent experiments in triplicate with freshly isolated RNA.

2.3.7 Northern Hybridization

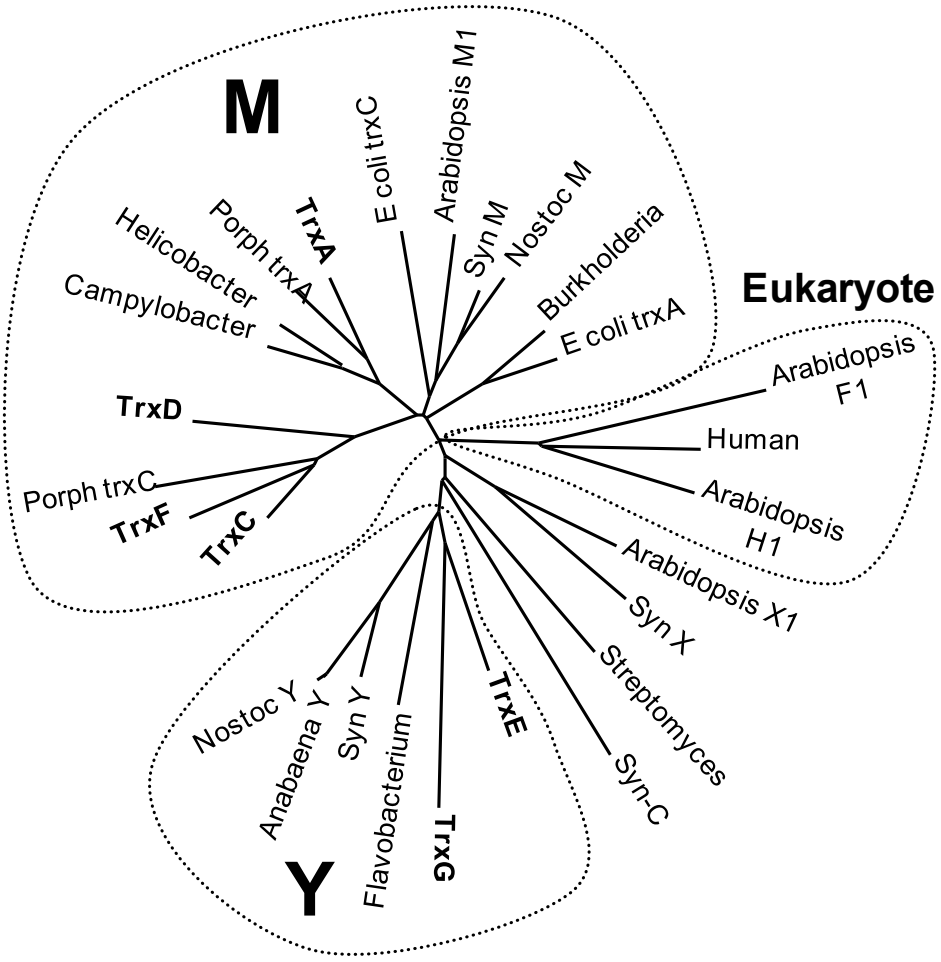
Cultures were grown in BHIS to early logarithmic phase and either maintained anaerobically for 15 min, treated with 50 µM H_2O_2 for 15, or shaken aerobically at 250 rpm for an hour as previously described (137). RNA was then isolated and Northern blot analysis was carried out as previously described (116). The entire open reading frame of each *trx* gene was radiolabeled with ^{32}P dCTP and used as the hybridization probe. Densitometry analysis 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.

2.4 Results

2.4.1 Trx System in *B. fragilis*

Previous studies demonstrated that *B. fragilis* possesses an extensive Trx system consisting of thioredoxin reductase (TrxB) and six Trx orthologs each of which has the classic CXXC redox-active center (119). The work showed that this is the primary mechanism for controlling the intracellular thiol/disulfide equilibrium and that in the absence of TrxB, cells required an exogenous reducing agent for growth (119). Since there is no glutathione system it is likely that *B. fragilis* Trx proteins play diverse roles in cellular metabolism, some of which may overlap with classic functions associated with glutaredoxins or glutathione in other organisms. The presence of six distinct Trx proteins in a heterotrophic prokaryote is quite unusual. Therefore, we used phylogenetic comparisons to gain insight into the evolution and potential roles of these Trxs. The results in Figure 2.1 suggest that the *B. fragilis* Trxs are comprised of several discrete types, analogous to cyanobacteria where six or more Trxs are common and at least four types, M, X, Y, and C have been described (37, 55). Four of the *B. fragilis* Trxs grouped with the archetypical prokaryotic/mitochondria Trxs referred to as the M type, but within this group they formed distinct subgroups. TrxA was associated with *Campylobacter*, *Helicobacter* and *Porphyromonas gingivalis* in one divergent group whereas TrxC, TrxD, and TrxF, which appear to share a common ancestor, formed a second M subgroup that included a second *P. gingivalis* Trx. The greatest divergence was observed for TrxE and TrxG which usually clustered with the Y type of prokaryotic Trx isoforms. The Y isoforms have been found in cyanobacteria, single cell algae, and plants and are known to have unique in vitro target specificity (77).

Figure 2.1. Phylogenetic comparison of 27 Trx proteins from diverse sources. ClustalW was used to align protein sequences for 27 Trx proteins. The unrooted bootstrap consensus tree was inferred using the minimum-evolution method with 500 bootstrap replicates (120). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (133). The following sequences with accession numbers were used: *Burkholderia*, YP_333769; human, NP_003320; *E. coli* TrxA, AAA67582; Porph TrxC, *P. gingivalis* AAQ65495; Syn M, *Synechococcus* ZP_01124485; *Anabaena* Y, ABA23368; *Flavobacterium*, CAL43878; *Nostoc* Y, NP_485933; Syn Y, *Synechocystis* NP_442168; *Campylobacter*, YP_178167; *Helicobacter*, NP_223481; Porph TrxA, *P. gingivalis* NP_904389; *Streptomyces*, CAB72414; TrxA, YP_210347; TrxC, YP_212311; TrxD, YP_211860; TrxE, YP_212629; TrxF, YP_212630; TrxG, YP_210941; *Arabidopsis* H1, CAA78462; *Arabidopsis* F1, AAD35003; *Arabidopsis* X1, NP_564566; Syn X, *Synechocystis* NP_440611; Syn C, *Synechocystis* NP_439965; *E. coli* TrxC, NP_417077; *Arabidopsis* M1, AAF15948; *Nostoc* M, NP_485906.


M
E coli trxC
Arabidopsis M1
TrxA
Porph trxA
Helicobacter
Campylobacter
Syn M
Nostoc M
Burkholderia
E coli trxA
Eukaryote
Arabidopsis F1
Human
Arabidopsis H1
Arabidopsis X1
Syn X
Streptomyces
Syn-C
TrxD
Porph trxC
TrxF
TrxC
Nostoc Y
Anabaena Y
Syn Y
Flavobacterium
TrxG
TrxE
Y

Several of the branches in the tree shown in Figure 2.1 had low bootstrap values (see Figure 2.2) presumably because of the small size of the Trx proteins. However, we have a high degree of confidence in the tree topology for the following reasons: 1) consensus trees constructed by four different methods yielded very similar results. Methods used were Neighbor Joining, Minimum Evolution, UPGMA, and Maximum Parsimony; 2) consensus trees constructed using 81 Trxs and protein disulfide isomerases yielded similar results; 3) results from interior branch tests were 98% confidence probability for the TrxA group, 99% for the TrxC, D, F group, and 84% for the TrxE, G group (data not shown). In summary, the *B. fragilis* Trxs fall into three divergent groups resulting from at least two independent lines of descent. The phylogenetic relationships and sequence divergence of the Trxs is consistent with evolution toward specialized functions which will need to be determined for a better understanding of their roles in the physiology of anaerobic bacteria.

2.4.2 Generation of *trx* Mutants in *B. fragilis*

The first step for analysis of the Trxs, was construction of unmarked, deletion mutants using the two-step, positive selection vector, pYT102 (Figure 2.3). Deletions were successfully obtained for all genes except *trxA* in which case it was possible to obtain the initial single crossover event but selection for the double crossover event always resulted in the isolation of colonies with the wild type locus (Table 2.4). By comparison, between 20-40% of *trxC, trxD, trxE, trxF,* and *trxG* double crossovers were deletion mutants. This result suggested that *trxA* might be essential, so we set out to determine if *trxA* cloned into a constitutive expression vector could rescue deletion mutants. As shown in Table 2.4, in the presence of p*trxA*, deletion mutants were

Figure 2.2. Evolutionary relationships of 27 Trx proteins from diverse sources. The evolutionary history was inferred using the Minimum Evolution method (125). The bootstrap consensus tree inferred from 500 replicates (157) is taken to represent the evolutionary history of the taxa analyzed (157). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (157). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (161) and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (121) at a search level of 1. The Neighbor-joining algorithm (126) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 85 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (142).

The following sequences with accession numbers were used: *Burkholderia* [YP_333769], Human [NP_003320], *E. coli* TrxA [AAA67582], Porph TrxC [*P. gingivalis* AAQ65495], Syn M [*Synechococcus* ZP_01124485], *Anabaena* Y [ABA23368], *Flavobacterium* [CAL43878], *Nostoc* Y [NP_485933], Syn Y [*Synechocystis* NP_442168], *Campylobacter* [YP_178167], *Helicobacter* [NP_223481], Porph TrxA [*P. gingivalis* NP_904389], *Streptomyces* [CAB72414], TrxA [YP_210347], TrxC [YP_212311], TrxD [YP_211860], TrxE [YP_212629], TrxF [YP_212630], TrxG [YP_210941], *Arabidopsis* H1 [CAA78462], *Arabidopsis* F1 [AAD35003], *Arabidopsis* X1 [NP_564566], Syn X [*Synechocystis* NP_440611], Syn C [*Synechocystis* NP_439965], *E. coli* TrxC [NP_417077], *Arabidopsis* M1 [AAF15948], *Nostoc* M [NP_485906].

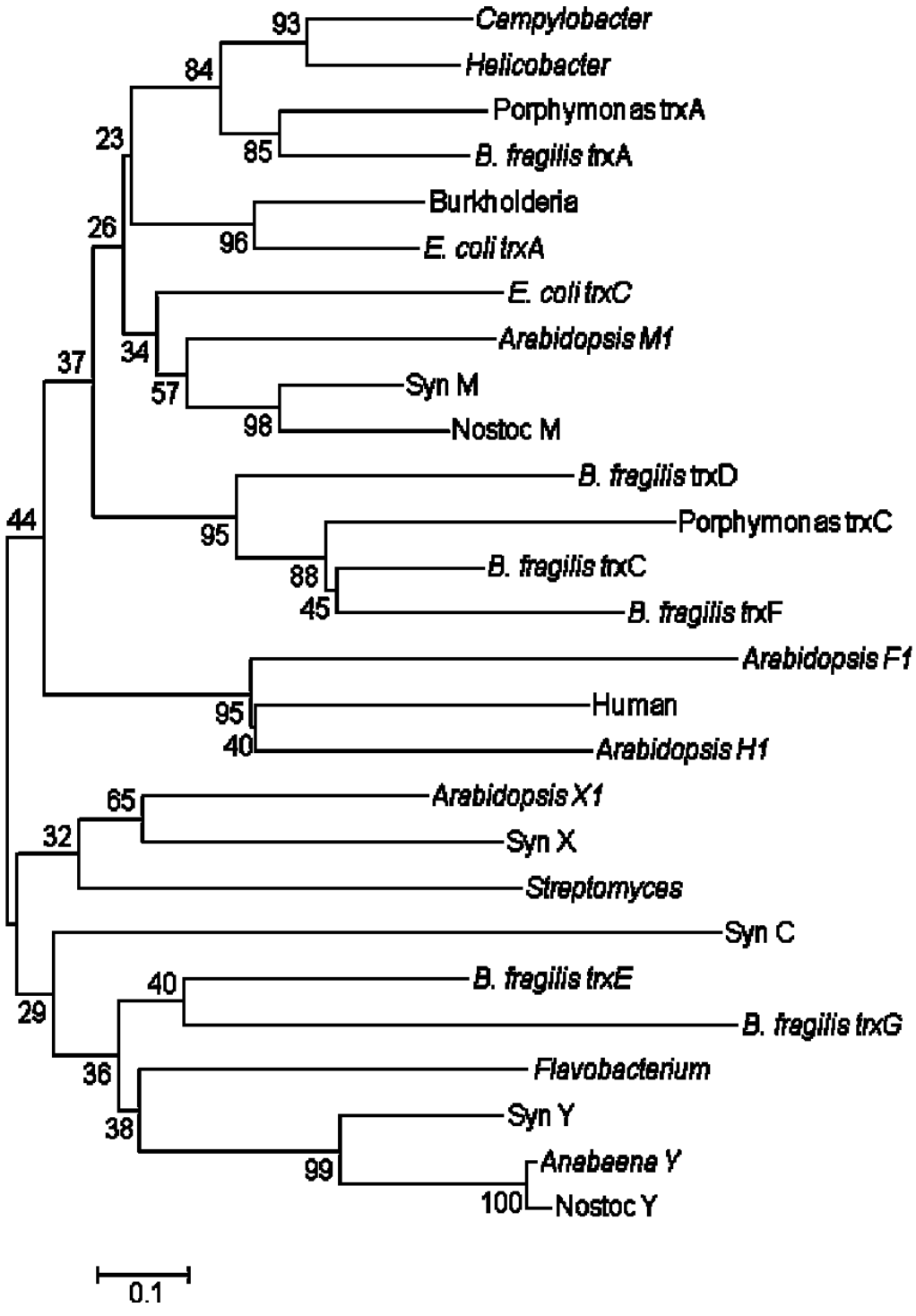

93
Campylobacter
Helicobacter
84
Porphymonas trxA
23
85
B. fragilis trxA
Burkholderia
26
96
E. coli trxA
E. coli trxC
Arabidopsis M1
34
37
Syn M
57
98
Nostoc M
B. fragilis trxD
44
Porphymonas trxC
95
88
B. fragilis trxC
45
B. fragilis trxF
Arabidopsis F1
Human
95
40
Arabidopsis H1
65
Arabidopsis X1
32
Syn X
Streptomyces
Syn C
40
B. fragilis trxE
29
B. fragilis trxG
36
Flavobacterium
38
Syn Y
99
Anabaena Y
100
Nostoc Y
0.1

Figure 2.3. Genetic loci of the six *B. fragilis trx* genes. The maps are drawn to scale; the dashed lines above the *trx* genes show the regions deleted in each *trx* mutant, and the black lines under the *trx* genes represent the approximate sizes of the mRNAs observed in Fig. 3. Genes: unk, unknown with no matches in database; *dnaE*, DNA polymerase III; *fldA*, flavodoxin; *doxDA*, thiosulfate quinone oxidoreductase; *mauG*, tryptophan tryptophylquinone synthesis; *cztBC*, heavy metal efflux pump; *hel*, DNA helicase; hyp-Ptase, hypothetical phosphatase; *nfnB*, oxygen-insensitive nitroreductase; *rbr*, rubrerythrinlike; *per*, peroxide response regulator homolog; hyp-reg, hypothetical DNA binding protein; *spoU*, SpoU-like RNA methylase; *arsF*, sulfatase.

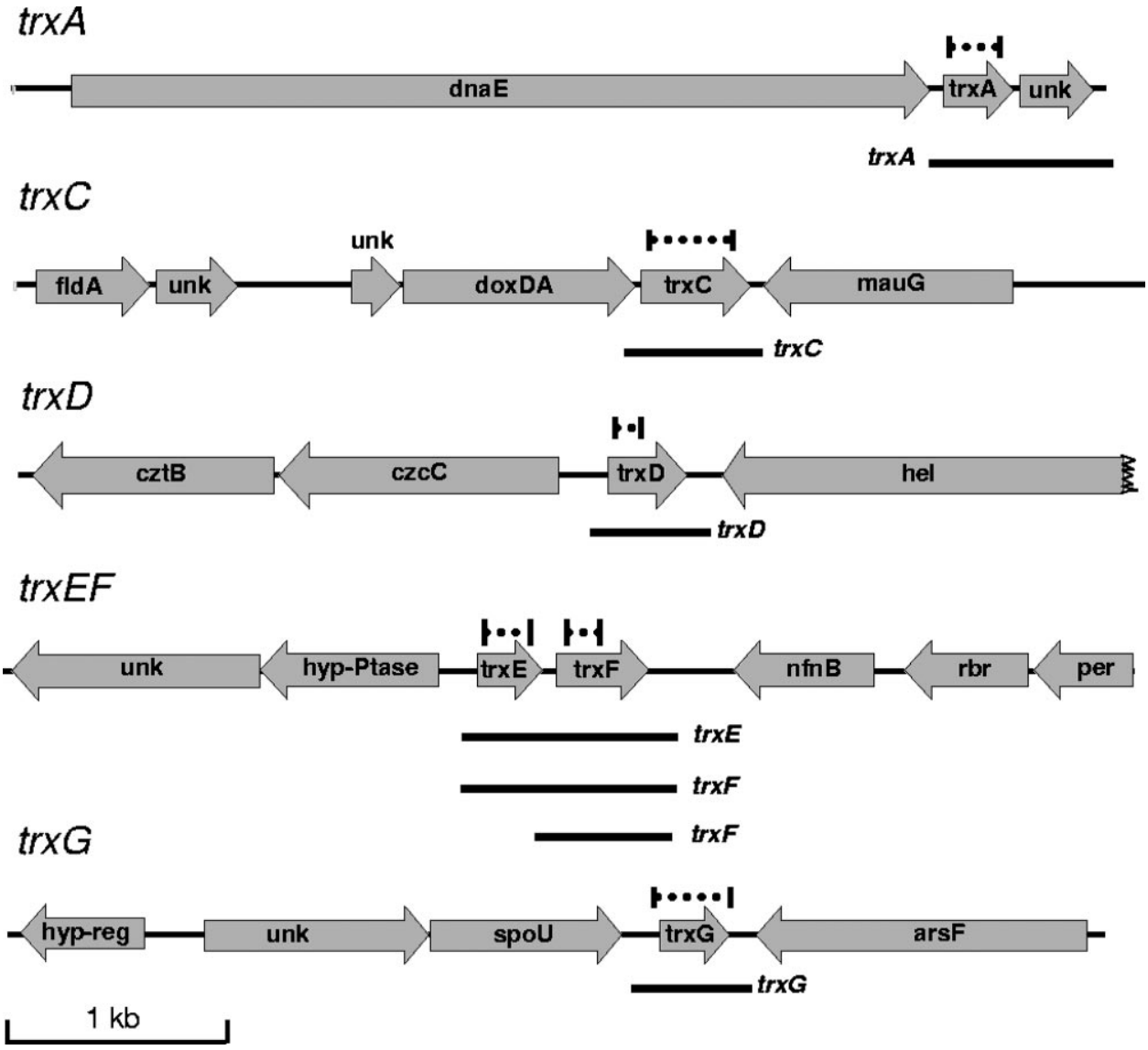

trxA
dnaE
trxA
unk
trxA
trxC
unk
fldA
unk
doxDA
trxC
mauG
trxC
trxD
cztB
czcC
trxD
hel
trxD
trxEF
unk
hyp-Ptase
trxE
trxF
nfnB
rbr
per
trxE
trxF
trxF
trxG
hyp-reg
unk
spoU
trxG
arsF
trxG
1 kb

Table 2.4. Isolation of *trxA* deletion mutations in the presence of complementing plasmids.

Plasmid No.	(%) of trxA deletions observed/total no. tested
None	0/468
ptrxA	27/84 (32)
ptrxC	0/23
ptrxD	0/20
ptrxE	0/51
ptrxF	0/47
ptrxG	0/72

Strain IB473 containing the trxA deletion construct single crossover integrated into the trxA gene was the recipient for all trx-containing plasmids. Deletions were identified by PCR analysis of the genomic DNA isolated from individual colonies that had resolved the trxA deletion construct (see Materials and Methods for more details).

recovered at a frequency similar to that observed for the other *trx* genes. In contrast, none of the other cloned *trx* genes were able to rescue *trxA* deletion formation. This was the first indication of Trx target specificity in *B. fragilis*.

Multiple *trx* gene knockouts were constructed from single deletion mutants by applying multiple rounds of the pYT102/ABD77 strategy. In this way an unmarked Δ*trxC* Δ*trxE* Δ*trxF* Δ*trxG* quadruple mutant (strain IB498) was constructed but multiple attempts to construct the quintuple mutant using the unmarked Δ*trxD* were not successful with IB498. Finally the quintuple mutant was constructed by double crossover insertion of a Δ*trxD::cfxA* construct containing a cefoxitin resistance cassette. This mutant, strain IB483, had only an intact *trxA* gene but it did not display any anaerobic growth defects in either complex or defined media (data not shown).

2.4.3 Induction of *trx* Genes by Oxidative Stress

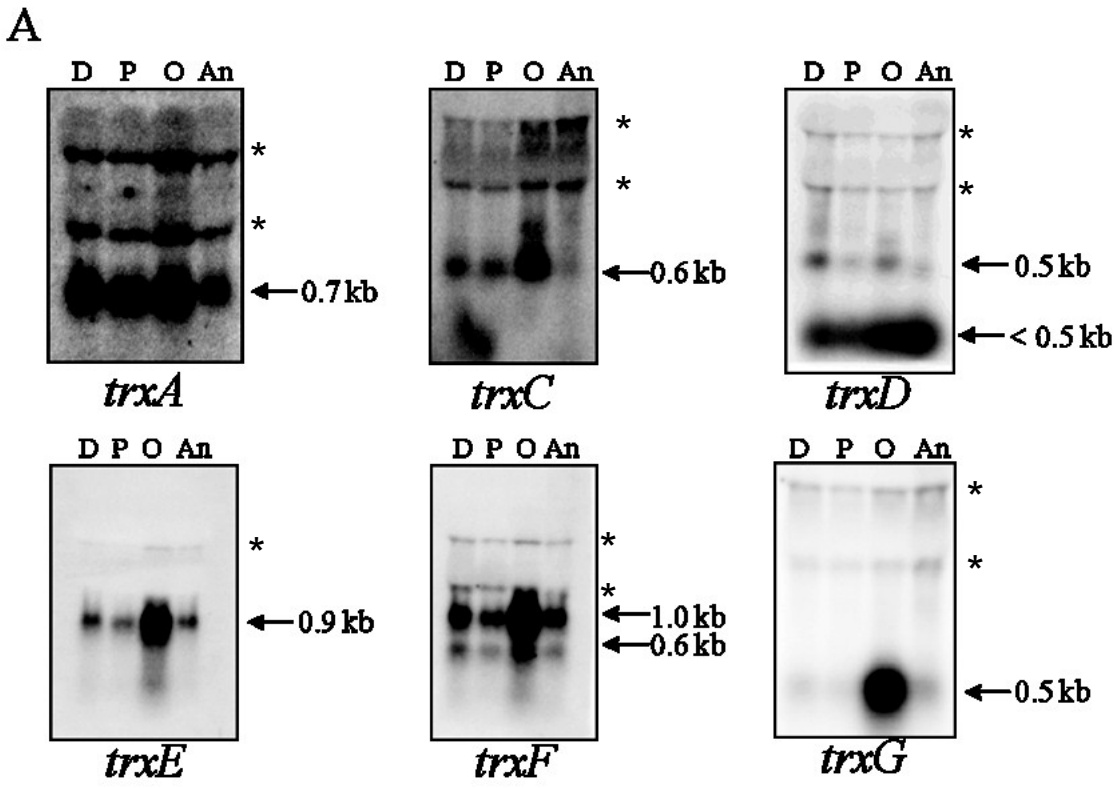
Trxs have been shown to be important during oxidative stress as a source of reducing power for detoxification reactions and the regeneration of inactivated proteins (18, 34, 90, 93, 94). Consistent with this, a previous study using expression microarray data showed *trx* gene induction in *B. fragilis* exposed to aerobic conditions (137). To verify this induction and determine *trx* gene organization we performed Northern blot hybridizations using RNA isolated from cultures exposed to atmospheric oxygen, hydrogen peroxide, and the thiol-specific oxidant, diamide. The analysis revealed differential expression of the *trx* genes during each of the conditions tested (Figure 2.4). Aerobic conditions induced the expression of all *trx* genes. The *trxC, trxD, trxF,* and *trxG* transcripts were monocistronic whereas the *trxA* transcript was part of an operon with a hypothetical gene and *trxEF* was a bicistronic mRNA. The *trxG* transcript

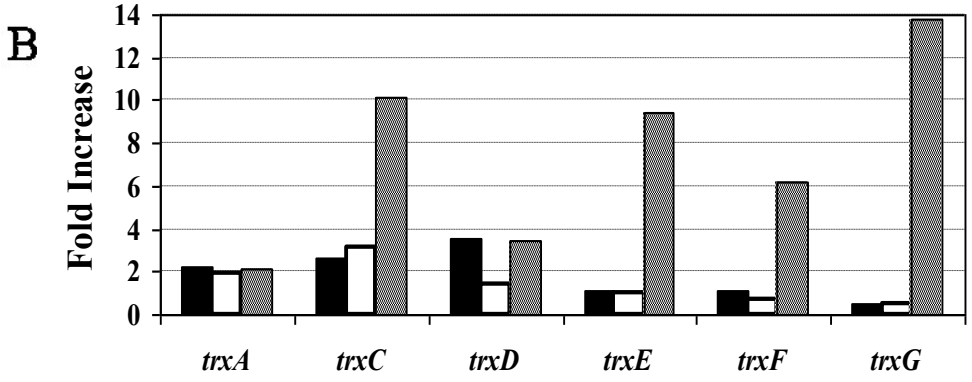
showed the highest fold induction at nearly 14-fold over anaerobic control. The *trxC* and *trxD* genes were induced during diamide exposure, and *trxC* showed substantial induction (3-fold) during hydrogen peroxide exposure. Interestingly, a second RNA species, which was less than 200-bp, was observed to hybridize strongly to the *trxD* probe. This RNA was in greatest abundance during anaerobic growth and may be a sRNA species. Alternatively, this fast migrating RNA band may be the product of premature *trxD* termination or post-transcriptional regulation. The *trxA* transcript was constitutively expressed during anaerobic conditions and only increased about two-fold during the stress conditions tested. Finally, the Northern blot hybridizations confirmed previous *in silico* analysis that *trxE* and *trxF* are in a two gene operon and are expressed primarily in a polycistronic message (Figure 2.4).

2.4.4 Growth in Oxidized Media

In order to determine if the Trxs were important for growth during oxidative stress we examined the ability of *trx* mutants to initiate growth in oxidized media. This assay is used to determine if there is a defect in the ability to rapidly reduce the media and initiate growth in the presence of low levels of oxygen. Typically, the expected phenotype is an extended lag period prior to the start of growth. When compared to the wild type strain, the single *trx* mutant strains had no significant defect either in anaerobic growth or the ability to initiate growth in the oxidized media (data not shown). However, as in seen Figure 2.5, the Δ*trxC* Δ*trxE* Δ*trxF* Δ*trxG* (strain IB498) and Δ*trxC* Δ*trxD::cfxA* Δ*trxE* Δ*trxF* Δ*trxG* (strain IB493) mutants were somewhat impaired in the ability to grow in the oxidized media. We hypothesized that the OxyR regulon might have masked a greater growth defect of the *trx* mutants due to its role in the rapid removal of oxygen

Figure 2.4. Northern hybridization analysis of total RNA of *B. fragilis* strain 638R (wild type). RNA was isolated from cells grown to midlogarithmic phase in BHIS and then treated as described in the text: 500 μM diamide (D), 50 μM hydrogen peroxide (P), exposed to air (O), or untreated (An). (A) Autoradiographs of blots hybridized to radiolabeled probes containing the entire open reading frame of each *trx* gene as indicated. The approximate sizes of the transcripts are shown. The apparent bands (*****) at about 1.5 and 2.5 kb are a commonly observed compression artifact caused by the 16S and 23S rRNAs. (B) Fold increase of transcript levels under each condition compared to the anaerobic control based on densitometric values. Black bars, 500 μM diamide; white bars, 50 μM H_2O_2; hatched bars, aerobic exposure.


A
D P O An
*
*
0.7kb
trxA
D P O An
*
*
0.6kb
trxC
D P O An
*
*
0.5kb
< 0.5kb
trxD
D P O An
*
0.9kb
trxE
D P O An
*
*
1.0kb
0.6kb
trxF
D P O An
*
*
0.5kb
trxG


B
Fold Increase
14
12
10
8
6
4
2
0
trxA
trxC
trxD
trxE
trxF
trxG

radicals (137). Thus, *oxyR* deletion derivatives of both strain IB498 and strain IB493 were constructed by allelic exchange and tested in the oxidized media. These mutants had a reproducibly longer lag period in oxidized media when compared to the wild type, multiple *trx* mutant strains or the *oxyR* single mutant but there were no anaerobic growth defects (Figure 2.5). When in combination with *oxyR*, the multiple *trx* mutants also grew at a slower rate, taking longer to reach maximum growth suggesting a cumulative decrease in the ability to combat oxidative stress when both the Trx and OxyR systems are impaired.

2.4.5 Sensitivity to Diamide

Diamide is a thiol-oxidizing agent that mimics damage due to oxygen exposure (91). Therefore, sensitivity to diamide was used to establish if any of the Trxs were important for thiol/disulfide homeostasis. As shown in Figure 2.6A, the Δ*trxD* mutant was more sensitive to diamide in disk diffusion assays than the parent strain, other single *trx* mutants, and the Δ*trxE* Δ*trxF* double mutant. Furthermore, the parent strain harboring the multicopy plasmid with *trxD* expressed from the constitutive IS*4351* promoter (p*trxD*) was less sensitive to diamide than the parent strain or strains with any of the other *trx* gene-containing expression plasmids (Figure 2.6B). Although, the effect of the single Δ*trxD* mutation alone was small, but statistically significant, a more dramatic difference was observed with the strain IB483, which lacked all functional *trx* genes except for *trxA*. In this mutant background the addition of p*trxD* also restored diamide sensitivity back to a wild type level. This complementation with *trxD* is consistent with the observation that the quadruple mutant, IB498, which lacked all

Figure 2.5. Growth analysis of *B. fragilis trx* and *oxyR* mutant strains in anaerobic and oxidized media. Strains were grown overnight in BHIS and then inoculated into either fully oxidized medium (Ox) or anaerobic medium (An). Growth was measured on a spectrophotometer at 550 nm. The results shown are the averages from triplicate observations in two growth experiments. Strains IB101 (wild type, ♦), IB298 (*oxyR*, ◊), IB498 (*trxC trxE trxF trxG,* ■), IB499 (*trxC trxE trxF trxG oxyR*::*tetQ,* □), IB483 (*trxC trxD*::*cfxA trxE trxF trxG,*▲), and IB500 (*trxC trxD*::*cfxA trxE trxF trxG oxyR*::*tetQ*, ∆) were used.

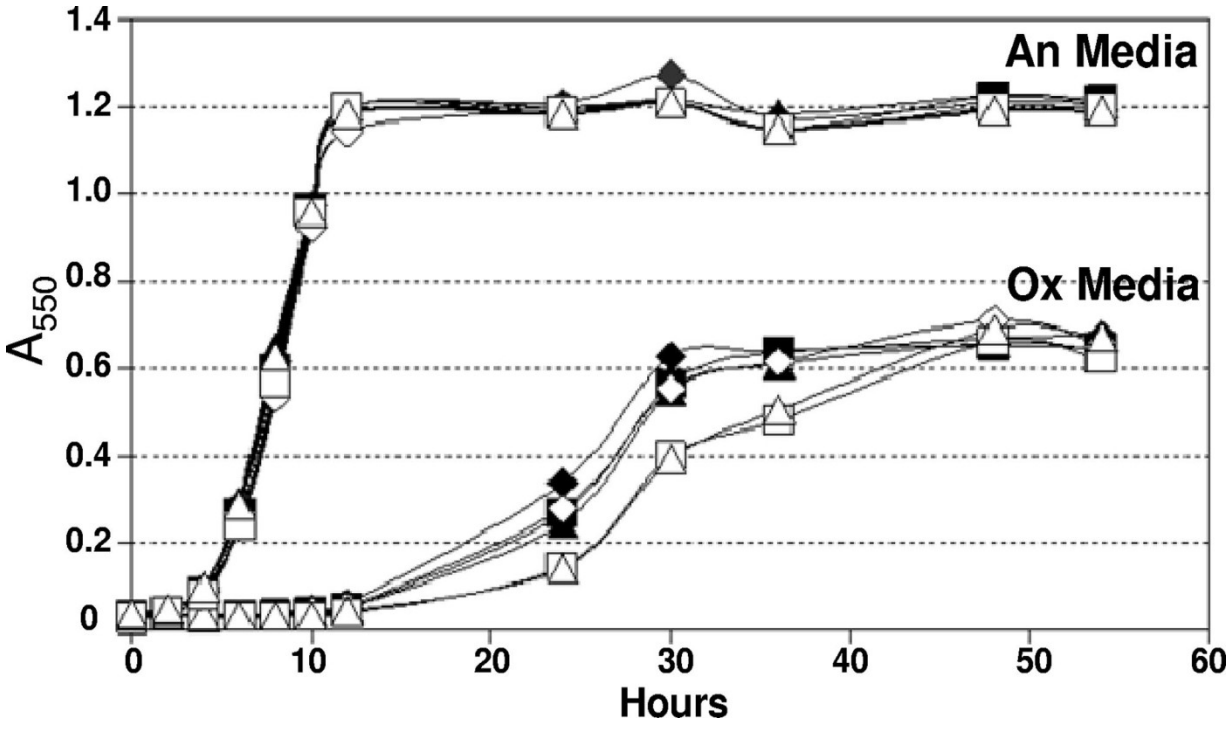
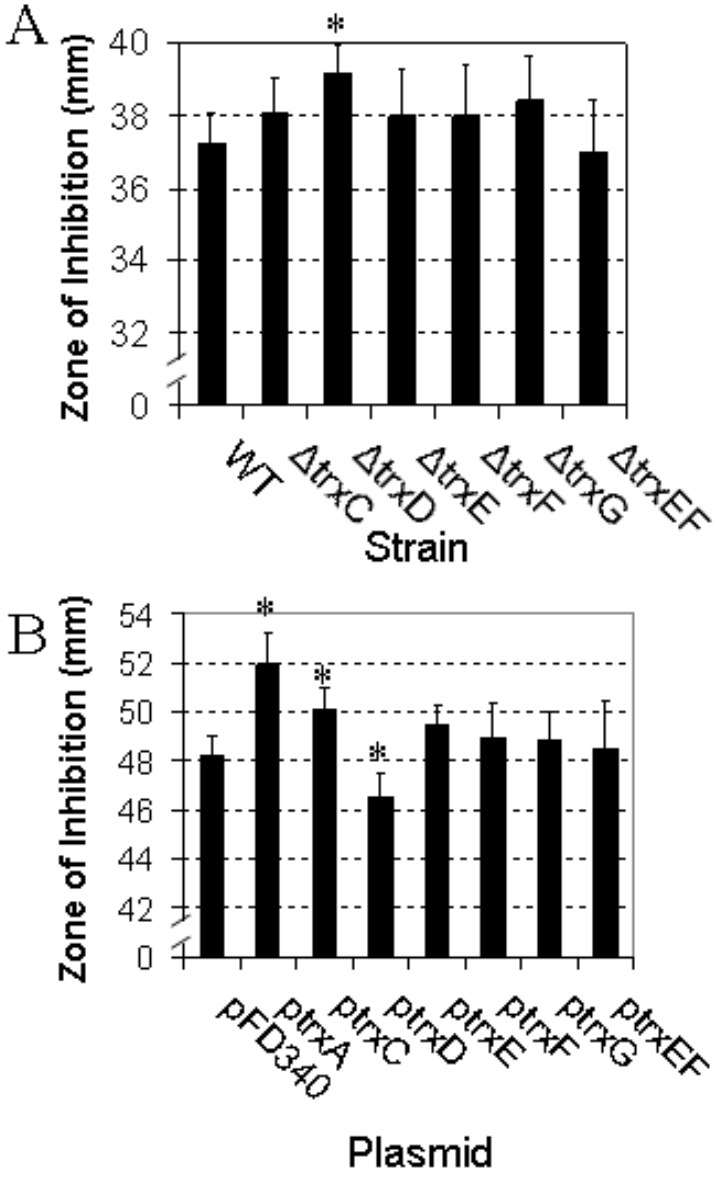

An Media
Ox Media
A_{550}
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Hours

Figure 2.6. Effect of each Trx on survival during oxidative stress. (A) Wild type strain 638R was compared to *trx* mutant strains in diamide disk diffusion assays on BHIS plates with no added cysteine. (B) Strain 638R harboring the empty expression vector pFD340 was compared to 638R strains harboring pFD340 containing *B. fragilis trx* genes in diamide disk diffusion assays on defined minimal medium. The values are mean diameters of growth inhibition zones measured in three independent experiments performed in triplicate and are given in millimeters. The error bars indicate standard deviations. *, $P < 0.01$ compared to wild type strain. Strains in panel A: wild type (WT), 638R; Δ*trxC*, IB458; Δ*trxD*, IB469; Δ*trxE*, IB490; Δ*trxF*, IB491; Δ*trxG*, IB471; and Δ*trxEF*, IB492


A
Zone of Inhibition (mm)
40
38
36
34
32
0
*
WT
ΔtrxC
ΔtrxD
ΔtrxE
ΔtrxF
ΔtrxG
ΔtrxEF
Strain
B
Zone of Inhibition (mm)
54
52
50
48
46
44
42
0
*
*
*
pFD340
ptrxA
ptrxC
ptrxD
ptrxE
ptrxF
ptrxG
ptrxEF
Plasmid

functional *trx* genes except for *trxA* and *trxD,* was not significantly more sensitive to diamide than wild type (Figure 2.7).

2.4.6 Real-time RT-PCR Analysis of *trx* Gene Expression During Diamide Exposure

The presence of multiple *trx* genes in the genome suggested the possibility that there may be some overlap in their roles managing thiol stresses. Consistent with this is the observation that the single *trx* mutations generally did not result in strong oxidative stress phenotypes (Figure 2.6). To investigate the potential for compensatory regulation of *trx* genes, real-time RT-PCR analysis was performed to determine specific differences in activation of these genes after exposure to diamide oxidative stress. As shown in Figure 2.8, the wild type strain 638R demonstrates a pattern of *trx* gene expression after 5 minute exposure to diamide similar to what was observed in the Northern blot hybridization analysis (Figure 2.4), with *trxD* having the highest induction of any *trx* gene under this stress. The fold induction of *trxD* was somewhat greater than seen in the Northern Blot experiments but this is likely due to the greater sensitivity and dynamic range of real-time RT-PCR. Interestingly, in the Δ*trxD* mutant, there was a dramatic increase in the induction of *trxC, trxE, and trxF* after exposure to both 500 μM and 100 μM diamide, suggesting that these genes were upregulated in response to the cell now lacking a functional TrxD.

2.5 Discussion

It has been known for some time that Trxs play important roles in the virulence and survival of a wide array of pathogenic bacteria, yeast, and protozoa but there has been very little information available on Trxs in anaerobes (11, 89, 155). In this regard,

Figure 2.7. Rescue of strain IB483 diamide sensitivity phenotype by plasmid p*trxD*. Diamide disk diffusion assays were used to compare sensitivities of the wild type strain 638R, the quadruple *trx* mutant strain IB498, the quintuple *trx* mutant strain IB483, and IB483 expressing *trxD* on plasmid p*trxD*. Black bars represent plates placed directly into an anaerobic incubator after plating, and open bars represent plates placed in an aerobic incubator for 6 h prior to being placed into the anaerobic incubator. The values are mean diameters of growth inhibition from three independent experiments performed in triplicate, and are given in millimeters. The error bars indicate standard deviations. *****, $P < 0.001$ compared to wild type strain.

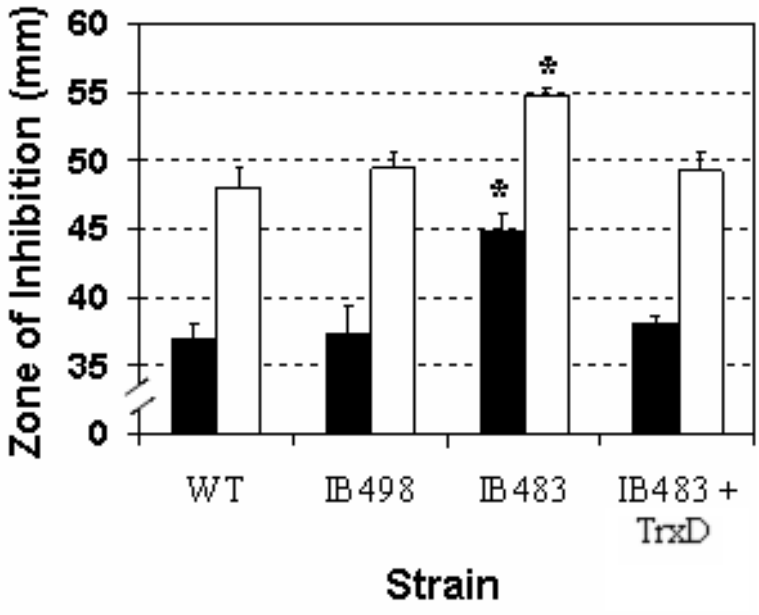
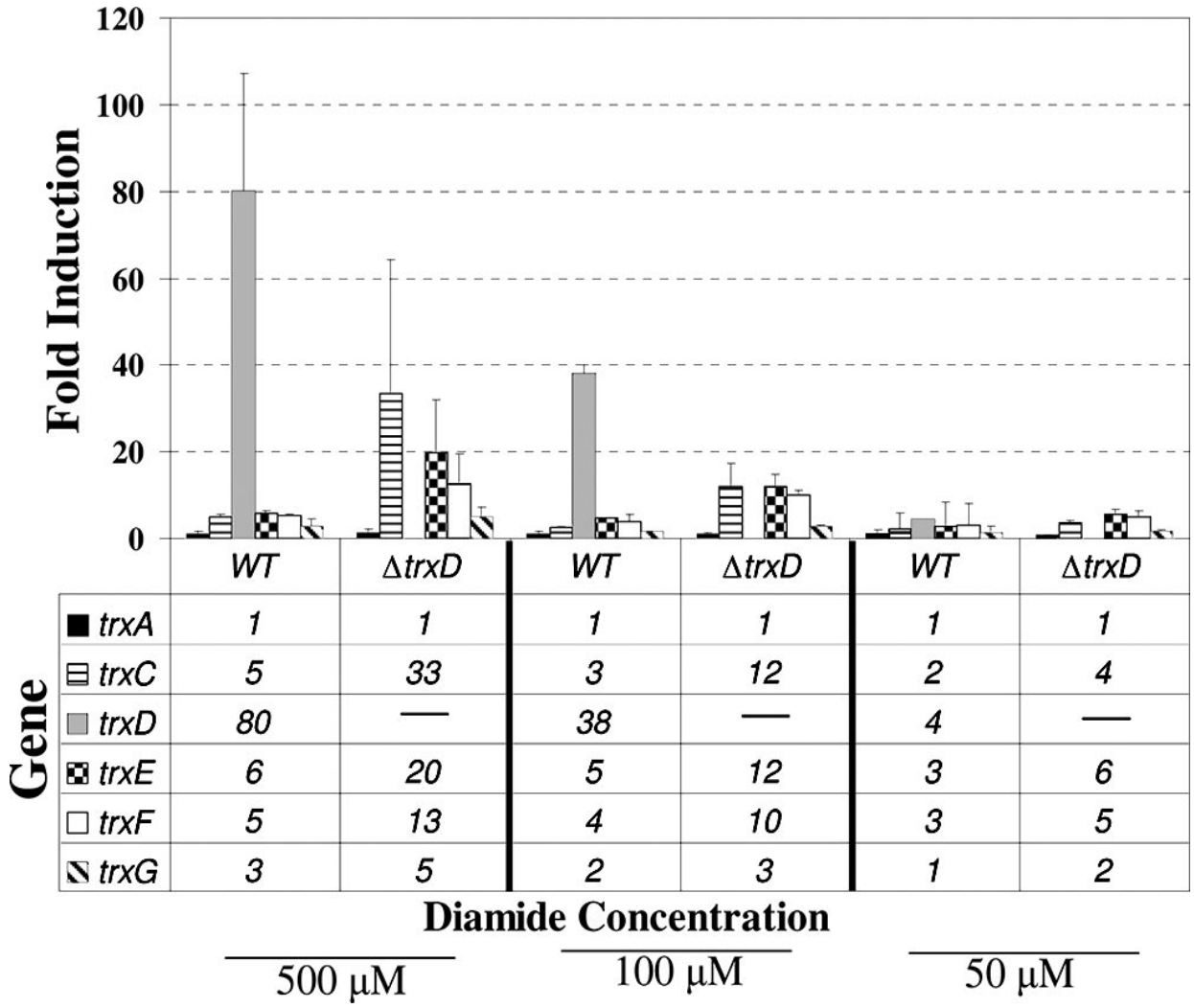

Zone of Inhibition (mm)
60
55
50
45
40
35
0
*
*
WT
IB498
IB483
IB483 + TrxD
Strain

Figure 2.8. Transcriptional analysis of *trx* genes. The parental strain (wild type [WT], *B. fragilis* strain 638R) and the isogenic *trxD* mutant (*trxD*, strain IB469) were exposed for 5 min to 500 μM, 100 μM, and 50μ M diamide or maintained under standard anaerobic conditions (0 μM control). For each condition, RNA was isolated and real-time RT-PCR was performed in triplicate. The sigma-54 modulation protein gene was used as a standard, and the results are expressed as fold induction relative to levels under the control condition. The values are means of fold induction, compared to the 0 μM control, from two independent experiments. The error bars indicate standard deviations.


Fold Induction
120
100
80
60
40
20
0
Gene
WT
ΔtrxD
WT
ΔtrxD
WT
ΔtrxD
trxA 1 1 1 1 1 1
trxC 5 33 3 12 2 4
trxD 80 — 38 — 4 —
trxE 6 20 5 12 3 6
trxF 5 13 4 10 3 5
trxG 3 5 2 3 1 2
Diamide Concentration
500 μM
100 μM
50 μM

the genome sequence of *B. fragilis* revealed that the Trx system was unexpectedly complex with six *trx* genes and at least 11 additional genes for proteins in the Trx superfamily of thiol-disulfide interchange proteins. Our phylogenetic analysis showed that the six bona fide *B. fragilis* Trxs did not arise from recent gene duplication events but had divergent lineages and fell into three distinct classes (Figure 2.1). The diversity among the *B. fragilis* Trx protein sequences suggests some level of functional specialization and this was born out by their differential regulation and phenotypic analyses. In one clear example, TrxA appeared to be essential (Table 2.4) and it was highly expressed during all stress conditions tested (Figure 2.4) suggesting that it plays a specific and crucial role in growth and during stress. Evidence for this is that the *trxA* gene constitutively expressed on a plasmid was able to rescue a *trxA* chromosomal deletion mutant but neither *trxC, trxD, trxE, trxF* nor *trxG* could substitute (Table 2.4). The role of TrxA is not known but we cannot rule out that it may function in DNA replication. One scenario is based on the chromosomal location of *trxA* which is just 62-bp downstream of *dnaE* coding the alpha subunit of DNA polymerase III (Figure 2.3), although it is transcribed independently of *dnaE*. There is some precedent for this type of a role for Trx which is required for the processivity of T7 DNA polymerase but the active site cysteines are not required for this activity (62). Alternatively TrxA could function in its traditional role to reduce ribonucleotide reductase (RNR) but this is generally not required for anaerobic RNRs.

In a previous study we showed that the *B. fragilis* Trx system is dependent on a single thioredoxin reductase, TrxB, and since there is no glutathione system, the deletion of *trxB* completely disrupts cellular redox homeostasis resulting in sensitivity to

oxidative stress (119). This study did not provide any insight into the role of individual Trxs but it did suggest that some Trxs should have specific roles in the OSR. In order to determine these roles, mutant strains harboring single and multiple *trx* deletions were compared with complemented strains in several oxidative stress assays. When comparing the phenotypes observed after the deletion of *trxD* in both the wild type and Δ*trxC* Δ*trxE* Δ*trxF* Δ*trxG* backgrounds (Figure 2.6A and 2.7), and the subsequent complementation of *trxD* on a plasmid (Figure 2.6B and 2.7), we were able to demonstrate the importance of TrxD in protection against diamide induced disulfide stress, a specific subset of oxidative stress. It is possible that TrxD is the preferred electron donor for the repair of inadvertent disulfides due to its transcriptional regulation or it may be a partner in a specific disulfide repair pathway. Future studies to identify TrxD protein partners will be able to provide insight into this.

Results from the current study suggest that all of the Trxs have some role in the OSR as they are induced by aerobic conditions. Further, there may be significant overlap in the Trx stress activities since the cell needed to be depleted of at least four of the six Trxs before there was any effect on growth in the oxidized media (Figure 2.5). This suggests that these proteins can compensate for one another and this was supported by observations on the regulation of their expression. For example, we observed substantial increases in diamide induced expression of four *trx* genes in the Δ*trxD* mutant compared to wild type. However, we should point out that these studies did not directly address the actual levels of Trx proteins produced and there could be forms of post-transcriptional or post-translational regulation that contribute to the overall control of redox homeostasis. In this regard there was the observation of a putative

small RNA species associated with *trxD* transcription and this could potentially be involved in some post transcriptional regulation.

Overall there are many roles that Trx proteins may play during oxidative stress such as providing reducing power for methionine sulfoxide reductase and peroxidases. *B. fragilis* induces 5 peroxidases in the presence of oxygen but only one of these, AhpC, has a known specific reductant (137). Another possible role for the Trxs may be during emergence from oxidative stress. Although *B. fragilis* is an obligate anaerobe, previous studies have shown that expression of an aerobic class Ia RNR is induced in response to aerobic exposure and mutants lacking this RNR have an impaired recovery response following exposure to air (131). There also is the potential need for a class Ia aerobic RNR during growth of *B. fragilis* in the presence of low (nanomolar) concentrations of oxygen (9). Thus there may be several opportunities for some of the Trx proteins to act as reductants for the aerobic RNR.

In other organisms such as *E. coli,* different components of the cellular redox systems show some specificity yet there is significant redundancy as well (17, 111, 112, 135, 158). The glutathione/glutaredoxin and TrxB/Trx systems share the ability to reduce many overlapping cytoplasmic substrates, including RNR (5, 88). However, *E. coli* also demonstrates significant specificity with some substrates, like the membrane associated reducing protein DsbD which requires Trx1 and methionine sulfoxide reductase is optimally reduced by Trx1 (135). Interestingly, the roles of the glutathione system may be tasked by the expanded Trx system in *B. fragilis*, since it lacks an alternative (32, 119).

It should be noted that the OSR in *B. fragilis* is not limited to *trx* genes alone. The OxyR regulon has been shown to be vital for dealing with oxidative stress in *B. fragilis* and our data in Figure 2.5 indicate that the Trx and OxyR systems have an additive effect on resistance to oxidative stress (114, 137). Previous work suggests that the Trx system acts independently on different oxidative stresses than the OxyR regulon (119, 137). Consistent with this, OxyR does not appear to control any of the *trx* genes, including *trxB,* indicating there is separation of the control of thiol metabolism from the peroxide response, which is similar to what occurs in *Bacillus subtilis* (52). However there likely is an important link between Trx and OxyR in *B. fragilis.* This is suggested by previous studies in *E. coli* that have shown a deficit in the reducing power of the cytoplasm can delay the deactivation of OxyR, enhancing the stress response (158). Therefore, if we are able to show in future experiments that a depletion of Trx in *B. fragilis* can likewise delay the modulation of OxyR activity; such data would help to provide evidence of a Trx-controlled OxyR pathway.

The continued examination of *trx* genes in *B. fragilis* illustrates the complexity of this system in this species when compared to other organisms. The evolutionary benefits of acquiring and maintaining the wide array of *trx* genes may offer a partial explanation as to why this obligate anaerobe is able to endure temporary environmental exposures of atmospheric oxygen. Understanding the coordinate regulation of this system and other aspects of the OSR will be necessary to determine how *B. fragilis* is able to adapt to niches outside it normal intestinal environment.

CHAPTER THREE: THE THIOREDOXIN TRXD DISPLAYS SPECIFIC REGULATION AND ACTIVITY IN *BACTEROIDES FRAGILIS*

3.1 Summary

Bacteroides fragilis is a Gram-negative obligate anaerobic bacillus that inhabits the human intestinal tract and has a remarkable ability to tolerate atmospheric concentrations of oxygen. This tolerance to oxidative environments is primarily due to the induction of an oxidative stress response (OSR). The OSR is a coordinated regulation of several sets of genes which react to the introduction of *B. fragilis* to an oxidative stress condition. Previous studies have shown the importance of the thioredoxin (Trx) system to the ability of *B. fragilis* to manage oxidative stress. Experiments utilizing mutant strains lacking the different *trx* genes found that the six *trx* genes had some ability to compensate for each other in growth and survival during the OSR. However, studies reported in this chapter indicated that the Trx proteins also have individual roles in *B. fragilis.* Specifically, TrxD was shown to be important during disulfide stress and thus the current study investigated the roles and regulation of this Trx. The results demonstrate that TrxD is able to interact preferentially with specific proteins that are induced during the OSR.

3.2 Introduction

The toxicity of oxygen to cells is complex, and if not controlled, results in physiological damage to the organism. In this regard, every phylum has evolved multifaceted protective mechanisms to contend with oxidative stress, the production of oxidative radicals, and the cellular damage that occurs when exposed to these stressors (115). Anaerobic organisms generally are not as well equipped as facultative or aerobic

species but some have evolved effective mechanisms to survive in their environments. For example, *Bacteroides fragilis,* a commensal anaerobe found in the human intestinal tract, is unable to multiply at atmospheric O_2 levels (21% O_2) yet it is highly resistant to oxidative stress and will survive prolonged periods in fully aerobic environments. This resistance to oxidative stress is mediated by an oxidative stress response (OSR), involving a wide array of genes activated during exposure to air or other oxidative stressors. The genes and proteins known to be involved in the OSR has continued to expand as more have been discovered, and while the function of some have been deduced, many of their contributions to aerotolerance have yet to be identified (53, 113-115, 137). One set of components which have been shown to be induced by oxidative stress in *B. fragilis*, and may play a crucial role in both survival and the OSR, is the thioredoxin family of proteins (137).

Found in all phylogenetic branches, thioredoxins (Trxs) are small redox active proteins (~12 kDa) that contain a highly conserved Cys-X-X-Cys motif at their active site which allows for the catalysis of thiol-disulfide reactions (3, 119). The flavin adenine dinucleotide-dependent Trx reductase mediates the reduction of Trxs by converting oxidized Trx to the free thiol form (3). While initially discovered to have a role in DNA synthesis and in the reduction of intracellular disulfide bonds, Trxs also have been shown to be involved in defense against oxidative stress (57). This defense involves regeneration of oxidatively damaged proteins, modulation of the activity of oxidative stressing agents, and the ability to serve as electron donors for detoxification enzymes important during the OSR (18, 34, 90, 93, 94).

Genome analysis of *B. fragilis* has shown the presence of one Trx reductase genes (*trxB*) and six Trx gene homologs (*trxA*, *trxC*, *trxD*, *trxE*, *trxF*, and *trxG*). This large number of Trx genes is unusual when compared to most other prokaryotes which usually have no more than three (49, 63, 69, 71, 110, 129). Previous research has shown that the TrxB/Trx system is the major thiol/disulfide redox system in *B. fragilis* and is essential for survival in vitro and in vivo (119). Furthermore, TrxB was shown to play a role in the aerotolerance of *B. fragilis*, and was required for abscess formation in an animal model (119). Another study showed that the *B. fragilis* Trx system demonstrated considerable redundancy in which there was a substantial capacity of *trx* genes to compensate for one another under stress conditions (109). This previous study demonstrated a role of TrxD in protection against diamide-induced disulfide stress, a specific subset of oxidative stress. Additionally, while this study did not provide any insight into the roles of individual Trxs, it did suggest that some Trxs such as TrxD may have specific roles in the OSR.

Evidence from previous experiments showed that TrxD has a major role in managing thiol oxidation and suggested that TrxD may be the preferred electron donor in repair pathways of specific proteins (109). By identifying the TrxD promoter region and several of the TrxD specific protein partners, the current study has provided important information regarding TrxD and will be valuable for future investigations into the *B. fragilis* Trx system.

3.3 Materials and Methods

3.3.1 Strains and Growth Conditions

The *B. fragilis* strains used in this study are listed in Table 3.1. The *trx* gene homologs in strain 638R correspond to the following genes in the genome sequence http://www.sanger.ac.uk/Projects/B_fragilis/: *trxA*, BF0642; *trxC,* BF2694; *trxD*, BF2237; *trxEF*, BF3015, BF3016; *trxG,* BF1279. Strains were grown anaerobically in brain heart infusion (BHI) broth supplemented with hemin and cysteine (BHIS) for routine cultures as described previously (100) unless otherwise indicated. Rifampicin (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.

The *E. coli* strains used for routine DNA manipulations and experimentation with *B. fragilis* genes are listed in Table 3.1. All cultures were grown in Luria-Bertani (LB) medium with appropriate antibiotics as described unless otherwise indicated.

The *S.* cerevisiae strain CY306 used for the yeast two-hybrid system is listed in Table 3.1. For routine growth, cultures were grown in Yeast Extract-Peptone-Dextrose (YPD) medium with 350 µg/ml gentamicin added and grown at 30°C. For the two-hybrid system experiments, yeast were plated onto agar plates containing yeast nitrogen base without amino acids (YNB) (Sigma, St. Louis, MO, USA), 2% glucose, 20 mM 3-aminotriazole (3AT), and supplemented with yeast synthetic drop out media (Sigma, St. Louis, MO, USA) lacking either leucine and tryptophan (2DO) or leucine, tryptophan, and histidine (3DO).

Table 3.1. List of strains used in this study. The relevant phenotypes are listed in the center column.

Strain or Plasmid	Phenotype and/or genotype[a]	Reference or source
B. fragilis		
638R	Clinical isolate, Rif^r	(107)
ADB77	Strain 638R *ΔthyA*, Rif^r, Tp^r	(8)
IB469	Strain 638R *ΔtrxD*, Rif^r	(109)
IB503	Strain 638R containing pFD697 inserted into *bglA,* Erm^r	This Study
E. coli		
DH10B	F$^-$ *mcrA* (*mrr-hsdRMS-mcrBC*) Φ80*lacZ*M15 *lacX74 deoR recA1 endA1 araD139* (*ara leu*)*7697 galU galK rpsL nupG tonA*	Invitrogen
RO36	*Δ(ara–leu*)7697 *araD139 ΔlacX74 galE galK rpsL phoR Δ*(*phoA)PvuII ΔmalF3 thi ΔtrxA ΔtrxC grxA::kan nrdH*::*spc*/pBAD18-*trxC*	(97)
HB101::RK231	Kan^r Tet^r; HB101 containing RK231	(48)
S. cerevisiae		
CY306	MATα ; ura3-52 his3-200 ade2-100 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS::UAS$_{GAL}$1-TATA$_{GAL1}$-HIS3 URA::UAS$_{GAL4}$ 17mers (x3)-TATACYC1-LacZ trx1::KanMX4 trx2::KanMX4, Gent^r	(149)
Plasmids		
pFD340	*Bacteroides-E. coli* expression shuttle vector, (Amp^r), Erm^r	(132)
pFD516	*Bacteroides* suicide vector, derived by deletion of *Bacteroides* replicon pBI143 in pFD288, (Sp^r), Erm^r	(133)
pFD697	*Bacteroides* suicide vector, containing promoterless *xylB* and fragment from *B. fragilis bglA,* (Sp^r), Erm^r	(137)
pGADT7	Prey plasmid for yeast two-hybrid system, (Amp^r)*, LEU2*	Clontech
pGBKT7	Bait plasmid for yeast two-hybrid system, (Kan^r)*, TRP1*	Clontech

[a]Erm^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance; Tp^r, trimethoprim Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance; Gent^r, gentamicin resistance. For *Bacteroides-E. coli* vectors and yeast two-hybrid vectors, parentheses indicate antibiotic resistance expression in *E. coli*.

3.3.2 Construction of *trxD* β-xylosidase (xylB) Transcriptional Fusions

SphI-SalI restriction enzyme fragments containing the full intergenic sequence upstream of TrxD (Fragment #1, 282-bp), a second fragment which contained the region immediately upstream of the hypothetical promoter sequence of TrxD (Fragment #2, 171-bp), and a third fragment immediately downstream of the hypothetical promoter sequence of TrxD (Fragment #3, 98-bp) were individually cloned into the SphI-SalI restriction sites of pFD697. The plasmid pFD697 also contained a 570-bp fragment from *B. fragilis bglA* as a target for integration into the *B. fragilis* chromosome and a 1.2-kb EcoRI fragment from pXA1 containing the promoterless xylosidase/arabinosidase (*xylB*) bifunctional reporter gene (154) cloned into the unique EcoRI site of the construct (137). Sequence analysis was used to confirm the correct sequence of the constructs, pFD1149, pFD1150, and pFD1151, containing the trxD_Fragment1::xylB, trxD_Fragment2::xylB, and trxD_Fragment3::xylB transcriptional fusions, respectively. The plasmids were mobilized from *E. coli* DH10B into *B. fragilis* strains by triparental matings, and they integrated into the *bglA* gene. *B. fragilis* strain IB503, with empty pFD697 integrated into *bglA,* was used as the negative control.

3.3.3 β-xylosidase Activity Assay

The β-xylosidase assays in the IB101 wild type background were carried out utilizing p-nitrophenyl-β-D-xylopyranoside (PNPX) as described previously (117) except that a whole cell assay was used. Briefly, 5 ml of culture with optical density at A_{550} between 0.5 and 1.0 was centrifuged at 3000 x g for 5 min and the pellet resuspended in 1.35 ml phosphate buffer (1 M Na_2HPO_4, 1 M NaH_2PO_4, pH 6.8) with 450 µl of this added to three microfuge tubes. Next, 50 µl of CTAB solution (0.05%

hexadecyltrimethylammonium bromide in 50 mM sodium phosphate buffer, pH 6.8) was added to each tube, mixed gently, and incubated at 37°C for 15min. Ten microliters of PNPX solution (100mM PNPX in DMSO) then was added, gently mixed, and incubated at 37°C for 15 min. Then, 50 µl of a 2% sodium carbonate solution (2% sodium carbonate in ddH_2O, filter sterilized) was added to each tube and mixed well. Cells then were removed by centrifugation and optical density was measured at A_{405}. The cells used in this assay were obtained from mid-log-phase anaerobic cultures of *B. fragilis* in BHIS without cysteine supplementation. The cultures were treated with 500 µM diamide for 1h or exposure to atmospheric oxygen for 1h before the assays were performed. Control cultures kept in anaerobic conditions were also collected, and experiments were run in triplicate. Activity was determined using the following equation:

$$\text{Activity} = \frac{A_{405}}{T \times V \times A_{550}} \text{ X } 1000$$

Where T = time of assay (15 min), V = the volume of cells used in assay calculated to the original volume, and A = light absorbance in nanometers (nm) at the subscripted value.

3.3.4 5' Rapid Amplification of cDNA Ends (5'-RACE)

The Invitrogen 5'-RACE system (Carlsbad, CA, USA) was utilized to obtain the 5' end of the *trxD* mRNA sequence. Briefly, the *trxD* mRNA from total *B. fragilis* RNA was annealed to the first strand gene specific primer (GSP1), specific to a *trxD* region, approximately 400-bp downstream of the transcriptional start site and copied into cDNA

with SuperScript™ II RT. The RNA was then degraded and cDNA purified with GlassMAX™ Spin Cartridge. The purified cDNA was then tailed with dCTP and TdT and the dC-tailed cDNA was PCR amplified using the Invitrogen Abridged Anchor Primer and the second gene specific primer (GSP2), specific to a *trxD* region located upstream of the GSP1 annealing site. The resulting RACE products were agarose gel purified then ligated into the pGEM®-T Easy vector from Promega (Madison, WI, USA) and electroporated into *E. coli* DH10B for cloning and subsequent sequence analysis. The primers used are listed in Table 3.2. A second set of GSP1 and GSP2 primers were used in independent 5'-RACE experiments to confirm the first results.

3.3.5 Trx Overexpression Constructs

Plasmids constitutively expressing specific *trx* genes were constructed by PCR amplification of promoterless *trx* genes using primers listed in Table 3.2. The *trx* gene fragments were cloned into the BamHI and SstI sites of the *Bacteroides-E. coli* shuttle expression vector pFD340 (132) in the same orientation as the IS4351 constitutive promoter. The new constructs; pFD340-*trxA*, pFD340-*trxC*, pFD340-*trxD*, pFD340-*trxE*, pFD340-*trxF*, pFD340-*trxG,* and pFD340-EC*trxC* were mobilized into *B. fragilis* strains via triparental mating. Transconjugants were selected on BHIS containing 20 µg/ml rifampicin, 100 µg/ml gentamicin, and 10 µg/ml erythromycin. The primers used for these plasmid overexpression constructs are listed in Table 3.2. For the constitutive expression of *B. fragilis trx* genes in the *E. coli* ribonucleotide reductase rescue bioassay, the *trx* genes were digested at the SphI site directly upstream of the IS4351 promoter, and the SstI site directly downstream of the *trx* gene. This fragment

Table 3.2. Primer list for generation of PCR products for assays performed.

sequence	forward (5')	reverse (3')
*trxD*_Fragment1	gatc**gcatgc**agtccttgttattttggaagc	gatc**gtcgac**atttttacttttttagtgatt
*trxD*_Fragment2	gatc**gcatgc**cgggacaaggaagaattgctaatataatag	gatc**gtcgac**atttttacttttttagtgatt
*trxD*_Fragment3	gatc**gcatgc**caacatcaaagaaataaataag	gatc**gtcgac**atttttacttttttagtgatt
*trxD*_GSP1_1	taacaaaacttcttctatcaatgc	n/a
*trxD*_GSP2_1	ctgggttttattcatagttcctaat	Abridged Anchor Primer (Invitrogen)
*trxD*_GSP1_2	catacttggttttcctcccatcgg	n/a
*trxD*_GSP2_2	caggttagggattgtacgaatggc	Abridged Anchor Primer (Invitrogen)
*trxA*_pFD340	cagt**ggatcc**gcataacagtgatagatact	cagt**gagctc**cagtcccataaacaaatat
*trxC*_pFD340	tgca**ggatcc**tggaaaaaagaaatcatcc	tgca**gtcgac**gcgtcaggaatcattgtttc
*trxD*_pFD340	tgca**ggatcc**tcctatctttgcaacatcaa	tgca**gtcgac**attaaatgattgatccggt
*trxE*_pFD340	cagt**ggatcc**tatttgccccctgtaaacga	cagt**gagctc**atggccaagctactaatgataa
*trxF*_pFD340	cagt**ggatcc**cattagtagctttggccatg	cagt**gagctc**tccgttgagaaaaccatgcc
*trxG*_pFD340	tgca**ggatcc**gcattgtttcggagtgcagt	acgt**gagctc**gcagtgcttgtcttgataatccg
Ec*trxC*_pFD340	cagt**ggatcc**tactcatcccgaggttagtt	cagt**gagctc**aagagattcgttcagcca
TrxD_C45S_pET26b	catg**catatg**aaagtcattgatttaacaaaagaaagcttc	catg**ctcgag**taacaaaacttcttctatcaatgcttttaa
*trxD*_pGADT7	ctgact**catatg**aaagtcattgatttaaca	ctga**ggatcc**ttataacaaaacttcttctatcaat
*tps*_pGBKT7	ctga**ggatcc**ccgcaacaacaaatttcaaaggac	ctga**ctgcag**aaagacaggctttatacccgcat
*asnB*_pGBKT7	ctga**ggatcc**ccaaagaattaaaaagactaagc	ctga**ctgcag**gtccggttaatactcattaa

All restriction endonuclease sites are in boldface.

containing both the promoter and the *trx* gene was then cloned into pFD516 and electroporated into the cloning host strain *E. coli* strain DH10B. These were selected on LB containing 50 µg/ml spectinomycin and verified by plasmid size and *Sph*I/*Sst*I digest fragment size on 1% agarose gel. These constructs were designated pFD516-*trxA,* pFD516-*trxC*, pFD516-*trxD*, pFD516-*trxE*, pFD516-*trxF*, pFD516-*trxG*, and pFD516-EC*trxC*, respectively for the *trx* genes expressed.

3.3.6 Aerobic Ribonucleotide Reductase (NrdAB) Bioassay

The pFD516 *trx* constructs were electroporated into the *E. coli* strain RO36, selected on LB containing appropriate antibiotics, and verified by agarose gel electrophoresis. Medium was supplemented with 0.2% L-arabinose or 0.2% D-glucose to induce or repress, respectively, expression of *E. coli trxC* under the control of the P_{BAD} promoter (110), and antibiotic selection was maintained for all markers either on plasmids or on the chromosome, at the following concentrations: 200 µg/ml ampicillin; 10 µg/ml chloramphenicol; 40 µg/ml kanamycin; 15 µg/ml tetracycline; and 50 µg/ml spectinomycin (where appropriate).

Overnight cultures of each strain were adjusted to an A_{550} 0.5 and diluted 1:20 in fresh LB. Ten µl was spotted in duplicate on LB media containing 0.2% L-arabinose or 0.2% D-glucose. After 24 h aerobic incubation at 37°C, growth was observed. The plates supplemented with arabinose were used as controls (97). A strain growing on glucose-supplemented media indicated that the *B. fragilis* Trx was able to rescue NrdAB function when the *E. coli trxC* was repressed.

3.3.7 Generation of the TrxD_C45S Mutant

A cysteine to serine point mutation at TrxD amino acid residue 45 was constructed by using the QuickChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). Briefly, a 350 base pair fragment containing the wild type *trxD* PCR product from the 638R chromosome was inserted into the his-tag, overexpression plasmid, pET26b, at the NdeI/XhoI restriction sites and electroporated into *E. coli*. The generation of the point mutation was then performed as described in the QuickChange™ protocol from Stratagene. The plasmid was harvested from *E. coli* and the *trxD* sequence verified by nucleotide sequence analysis.

3.3.8 Thioredoxin Affinity Chromatography

The protocol for isolating Trx protein partners was essentially done as previously described (54, 78, 79). TrxD_C45S was induced by addition of 1 mM IPTG and cell extracts prepared by French press. The cell extract was centrifuged at 9000 x g at 4°C for 30min to remove cell debris and then was passed through a charged nickel column as described by the manufacturer (Novagen, WI, USA). Flow through was collected and then the TrxD_C45S protein was eluted from the column into a series of 1.5 ml tubes. Samples of eluate were ran on a 12% SDS-PAGE to verify protein purification, Bradford assays were used to determine protein concentration, and the fraction containing TrxD_C45S was dialyzed. *B. fragilis (ΔtrxD)* cell-free protein extract was prepared by French press with cells suspended in 25 mM Hepes-NaOH (pH 7.0), 15 mM $CaCl_2$, 5 mM $MgCl_2$, 15% v/v glycerol and 1mM PMSF (Buffer A). Aliquots containing approximately 2.8 mg of protein were then flash frozen and stored at -80°C until needed. An aliquot containing approximately 1.2 mg of pure TrxD_C45S protein

was added to the his-bind beads and incubated at 4°C under gentle agitation for 1 h. The beads were allowed to settle on ice, the supernatant was discarded, and the beads then were washed twice in 1 ml Buffer A. Approximately 2.8 mg *B. fragilis (ΔtrxD)* protein extract was added to the beads and incubated overnight (16h) at 4°C with gentle agitation. The beads were allowed to settle on ice again and the supernatant was discarded. The beads were washed four times in 1 ml 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl (Buffer B). They then were washed three times in 0.5 ml Buffer B containing 60 mM imidazole to remove weakly bound contaminants, and then three more times in 1 ml Buffer B without supplements. Finally, the TrxD_C45S-target protein complexes were released by addition of 120 µl Buffer B containing 1 M imidazole and incubated at 4°C with gentle agitation for 1h. The beads were settled by a short centrifugation at 2000 x *g* for 10s and the supernatant was frozen and stored at -20°C until analyzed. This elution procedure was repeated twice and the eluates were collected. Aliquots of 25 µg of the eluates were precipitated from samples and 100 µl of 0.15% deoxycholic acid was added to a total volume of 1ml and allowed to incubate at room temperature for 10 min. Next, 100 µl of 72% tricholoracetic acid was added and the samples were incubated on ice for 10 min. Then the samples were centrifuged for 10 min and then washed three times with 1 ml of 70% ethanol, making sure to mix well each wash to remove the acid. The samples were allowed to dry inverted for 10 min under a laboratory hood and then spun for 10 min in the Vacufuge™ (Eppendorf, Hamburg, Germany). The samples then were treated with 20 µl of 1x SDS sample buffer (with β-mercaptoethanol), vortexed and spun down briefly and then incubated for 30 min at 37°C. Before loading into the 12% SDS-PAGE gel, the samples were heated to 95°C for 5 min, vortexed, and spun down.

After electrophoresis, the gel was stained with Comassie blue and the visualized bands were excised and placed into 500 µl tubes. Protein identification by tandem mass spectrophotometry was performed by the U.C. Davis Genome Center Core Facility (Sacramento, CA, USA).

3.3.9 Generation of Competent Yeast Cells

Yeast strain CY306 (149) was plated on YPD media and incubated for 3 days. Five milliliters of YPD liquid medium were inoculated with one colony and incubated overnight standing at 30°C. The overnight culture was used to inoculate 100 mL of YPD liquid to an OD_{600} of 0.1 and incubated at 30°C until OD_{600} of 0.6. Next, the culture was centrifuged at 3000rpm for 5min at room temperature and the pellet was suspended in 10 mL of Solution A (10 mM bicine, 1 M sorbitol, 3% v/v ethylene-glycol, pH 8.35 with KOH, autoclaved), washed, suspended into 2 mL of Solution A, separated into 200 µL aliquots, and allowed to slowly freeze by first placing at -20°C for 20min, then placed into -80°C for storage.

3.3.10 Transformation of Competent Yeast Cells

A 200 µl aliquot of competent cells was thawed and placed on ice while 1 µg each of the pGADT7 prey plasmid and the appropriate pGBKT7 bait plasmid were added to 50 µg of denatured salmon sperm. The yeast cells were added and the mixture incubated with rotation for 5 min at 37°C. One milliliter of Solution B (200 mM bicine, 40% polyethylene-glycol, pH 8.35 with KOH, autoclaved) was added and mixed by gentle inversion. After 1 h incubation standing at 30°C, the cells were centrifuged and washed with 800 µL of Solution C (10 mM bicine, 150 mM NaCl, pH 8.35 with KOH,

autoclaved) then suspended in 200 µL of Solution C and spread on appropriate selective media.

3.3.11 Yeast Two-Hybrid System

All experiments were performed in the yeast reporter strain CY306 (149) using the plasmids pGADT7 and pGBKT7 (Table 3.1). The pGADT7 plasmid is a yeast expression vector that constitutively expresses the protein of interest (prey) fused to the GAL4 activation domain (GAL4-AD). The pGBKT7 plasmid is also a yeast expression vector that constitutively expresses the protein of interest (bait) fused to the GAL4 DNA binding domain (GAL4-BD). Prey PCR product encoding *trxD*_C45S allele was cloned into the NdeI-BamHI sites of pGADT7 (Clontech, Mountain View, CA, USA). Bait PCR products encoding AsnB, Tps, TrxB, and TrxX genes were cloned into the BamHI-PstI sites of pGBKT7 (Clontech, Mountain View, CA, USA). After co-transformation of the yeast cells with the appropriate prey and bait plasmids, the yeast were plated in duplicate on agar plates containing the double drop-out (2DO) and triple drop-out (3DO) media containing 20 mM 3AT. After plating on each media, the yeast were allowed to incubate for 4 days at 30°C before the presence of yeast colonies was determined. The assays were independently repeated three times.

3.4 Results

3.4.1 Identification of the *trxD* Promoter Region

Previous experimental data demonstrated positive regulation of TrxD during oxidative stress conditions, which was particularly upregulated during exposure to the thiol-specific oxidant, diamide (109, 119). To determine the promoter region of TrxD,

Figure 3.1. Organization of *trxD* on the *B. fragilis* chromosome. The β-xylosidase analysis was performed utilizing three different PCR primer products containing varying length regions of the 5' end of the region immediately upstream of *trxD.* Fragment lengths and location relative to the *trxD* gene are shown.

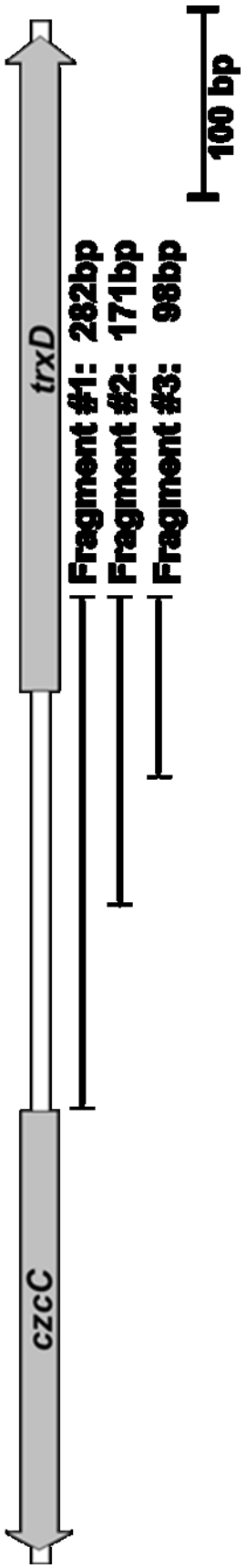
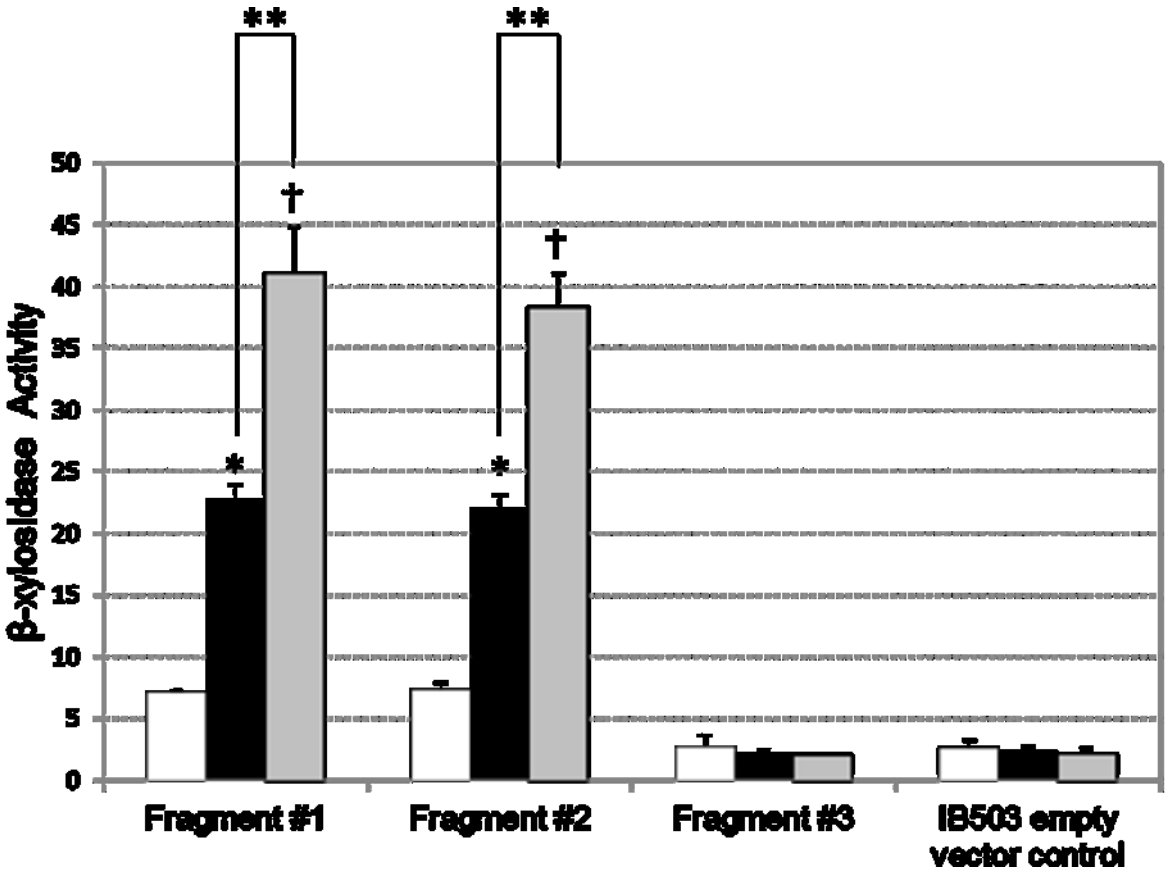

100 bp
trxD
Fragment #1: 282bp
Fragment #2: 171bp
Fragment #3: 98bp
czcC

Figure 3.2. Analysis of the *trxD*::*xylB* transcriptional fusions in 638R wild type strain grown under different oxidative stress conditions. 638R was grown to mid-log phase and then incubated anaerobically (open bars), shaken in air for 1 h (black bars), or challenged with 500 μM diamide for 1 h (gray bars). *****, †, $P < 0.001$ compared to empty vector control and **, $P < 0.001$ comparing diamide to aerobic challenge by Student's t-Test. Error bars represent standard deviation.

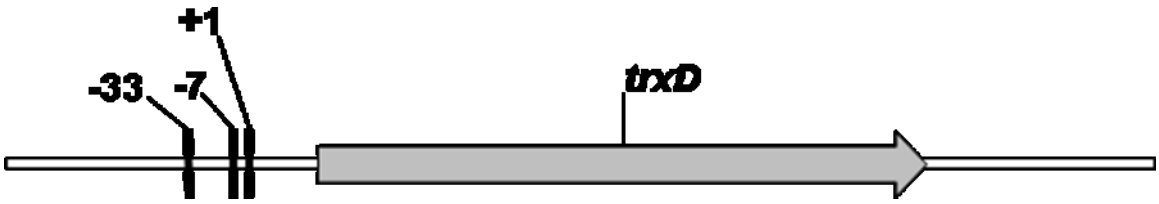

β-xylosidase Activity
50
45
40
35
30
25
20
15
10
5
0
**
**
*
*
†
†
Fragment #1
Fragment #2
Fragment #3
IB503 empty vector control

transcriptional fusions with the region upstream of *trxD* and the *xylB* gene were constructed (Figure 3.1). As seen in Figure 3.2, both fragments encompassing the region containing a *B. fragilis* consensus promoter sequence (-7: TAnnTTTG, -33: TTTG) (10) upstream of *trxD* had significantly increased β-xylosidase activity compared to the empty vector control when cultures were induced by oxidative stress as well as low level constitutive expression in the anaerobic control condition. Furthermore, the diamide stress induced significantly greater β-xylosidase activity compared to the aerobic stress in the bacteria containing these two fragments. Additionally, Fragment #3, which was immediately downstream of the *B. fragilis* -7 consensus sequence, was not significantly different from the control strain, and showed no significant induction of β-xylosidase activity in the two stress conditions nor the low level constitutive expression in the anaerobic control condition seen with the other two fragments.

3.4.2 Mapping of the *trxD* Transcription Start Site

The area containing the *trxD* promoter region on the *B. fragilis* chromosome was mapped using the 5'-RACE technique to determine the location of the +1 start site for transcription of the gene. After several independent experiments were completed using RNA from anaerobic *B. fragilis* cultures with two distinct primer sets for the 5'-RACE, 10 out of 14 (approximately 72%) of the successful sequencing analyses indicated the start site for the trxD transcript was an adenine 40-bp upstream from the TrxD initiation codon and 5-bp downstream from the 3' end of the *B. fragilis* -7 consensus promoter sequence (Figure 3.3). The four 5' ends not matching this location also differed from one another and were likely due to either error in the sequencing or premature transcriptional termination.

Figure 3.3. Organization of the *trxD* promoter region. The locations of the promoter region and the +1 transcriptional start site are shown as determined by β-xylosidase fusion assays and 5' RACE studies, respectively. The -33 and -7 promoter regions (underlined), +1 transcriptional start site (bold), the start of each *xylB* fusion fragment (left bracket, fragment number under bracket), and inverted repeats (dotted arrows) are shown.




```
          .         .         .         .         .         .

  1   [AGTCCTTGTTATTTTGGAAGCAAAAGTACAACTCCCCCATGAAGCAAACCGTTTGAAAGC
      1

          .         .         .         .         .         .

 61   AATTATAAGATACTCAGATAAGATTAGAAAGTTATTAGAAAGCAGAAAAAA[CGGGACAAG
                                                          2

          .         .         .         .         .         .

          ------>                 <------                 --
121   GAAGAATTGCTAATATAATAGGAGAAATAGCTATCTGACAATAAGATCTATCTTCCTATC

          .         .         .         .         .         .

          --->      <-----
181   TTTG[CAACATCAAAGAAATAAATAAGTAATCACTAAAAAAGTAAAAATATGAAAGTCATT
        3                                                 M  K  V  I
                                                         trxD
```

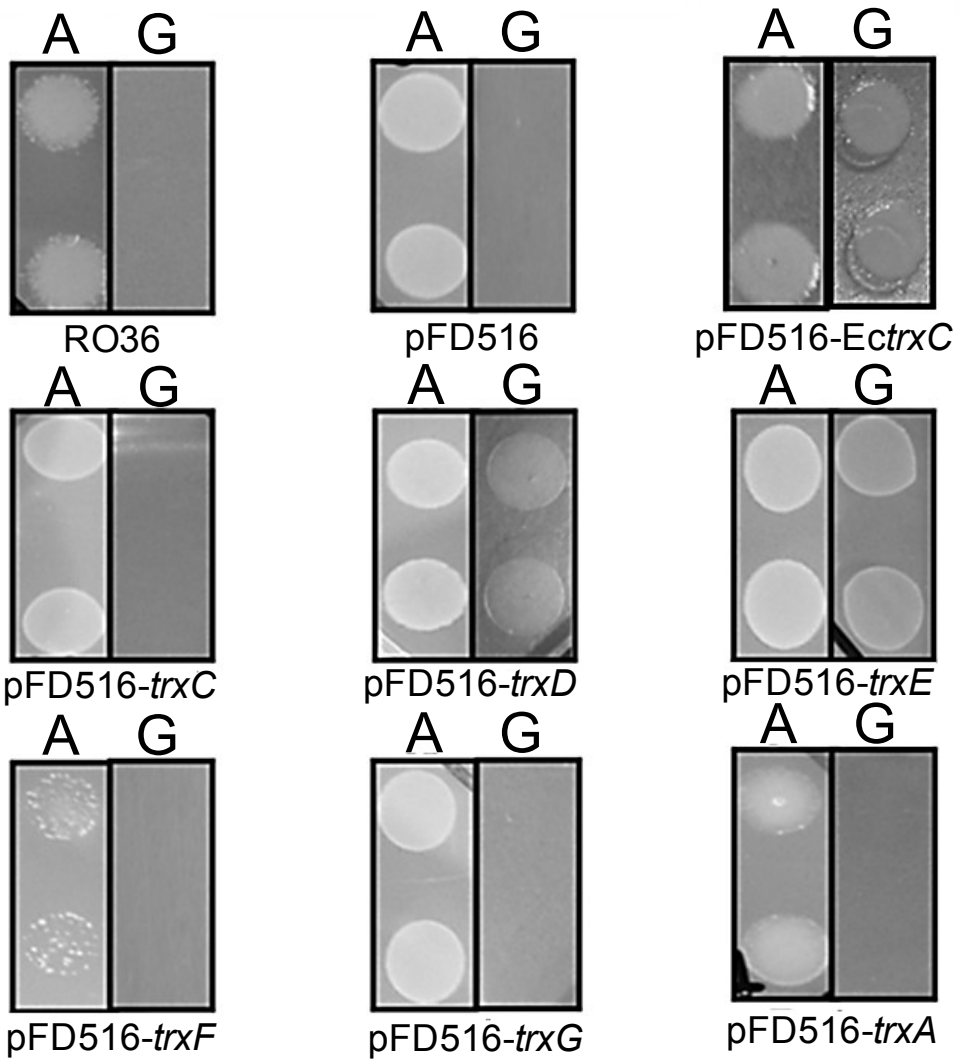
3.4.3 Rescue of *E. coli* NrdAB Activity by *B. fragilis* Trxs

The class I ribonucleotide reductase, NrdAB, requires reduced Trx for activity and heterologous complementation of this Trx function can be used to determine specificity of Trx target interactions. In order to address the question of whether Trxs in *B. fragilis* possess differential target specificity, we tested each for the ability to compensate for native *E. coli* Trx in the *E. coli* RO36 background. The RO36 strain lacks functional chromosomal glutaredoxin (*grx*) or *trx* genes capable of NrdAB complementation, but contains a functional *E. coli trxC* on plasmid pBAD39-*trxC* under control of an arabinose-inducible promoter (97). Strain RO36 derivatives containing the *B. fragilis trx* genes cloned onto a plasmid under the control of the IS4351 promoter, were grown overnight in the presence of 0.1% arabinose and then each strain was replica plated on media containing either 0.1% arabinose (control) or 0.1% glucose as described above. As seen in Figure 3.4, only strains with the *B. fragilis trxD* and *trxE* plasmids, as well as the pFD516 derivative carrying the native *E. coli trxC,* were capable of growth on the glucose-supplemented media. Furthermore, the strain with only *trxF* showed a growth defect on the arabinose-supplemented media, with only sparse colony growth unlike the confluent spots seen with every other strain on the control media plates.

3.4.4 Capture of TrxD Target Proteins

Determining differential protein interactions of *B. fragilis* Trxs in an *E. coli* background is useful; however the identification of *B. fragilis* protein partners that specifically interact with TrxD requires that the work be performed in *B. fragilis.* To

Figure 3.4. Complementation of *E. coli* strain RO36 by *B. fragilis* Trx proteins. Strains grown on arabinose media allow for production of *E. coli* TrxC in trans via arabinose inducible promoter on pBAD18-*trxC*. Strains grown on glucose require activity of *B. fragilis* Trx from pFD516 constructs for reduction/turnover of NrdAB for synthesis of ribonucleotides. Empty vector pFD516 was used as the negative control.


A G
RO36
A G
pFD516
A G
pFD516-Ec*trxC*
A G
pFD516-*trxC*
A G
pFD516-*trxD*
A G
pFD516-*trxE*
A G
pFD516-*trxF*
A G
pFD516-*trxG*
A G
pFD516-*trxA*

efficiently capture proteins which interact with TrxD, the protein-protein interaction between TrxD and its target must be frozen in the mixed disulfide intermediate state in order to be sufficiently stable to be immobilized by Trx affinity chromatography (54). Thus it was first necessary to create a point mutation in the *trxD* gene substituting the cysteine at residue 45 with a serine. The first step of the normal Trx-catalyzed reduction pathway between a Trx and its target involves the cysteinyl residue nearest the N-terminus in the CXXC motif acting as the primary nucleophile that attacks the disulfide of the oxidized substrate. The second cysteine in the motif serves to cleave the mixed disulfide intermediate, allowing the release of oxidized Trx and reduced target protein (13). This mutation prevents the second reactive cysteine in the active site from attacking the mixed disulfide formed when the Trx interacts with its target protein. The *trxD*_C45S allele was expressed on a polyhistidine tag vector in *E. coli* and column purified. The purified protein was then incubated with cell lysate from the *ΔtrxD* mutant (IB469) to ensure that the only TrxD protein in the reaction mixture would be the TrxD_C45S.

After incubation of the reaction mixture, the proteins were eluted and electrophoresed on an SDS-PAGE gel (Figure 3.5). The bands extracted from this gel for mass spectrophotometry analysis (labeled 1 through 9, Figure 3.5) were proteins tightly bound to TrxD_C45S presumably in a mixed disulfide state. Of the many interesting proteins determined to have had strong interaction with TrxD_C45S in the assay (Table 3.3), two proteins, AsnB and Tps, were found to be in high concentrations based on the mass spectrophotometry results, and both are known to be upregulated at the transcriptional level during oxidative stress (137). The gene encoding AsnB was

Figure 3.5. SDS-PAGE gel of *B. fragilis* proteins captured by Trx affinity chromatography. Numbers on the right represent bands excised for sequence analysis. A) molecular weight standards; B) unbound protein flow through; C) bound proteins eluted with imidazole.

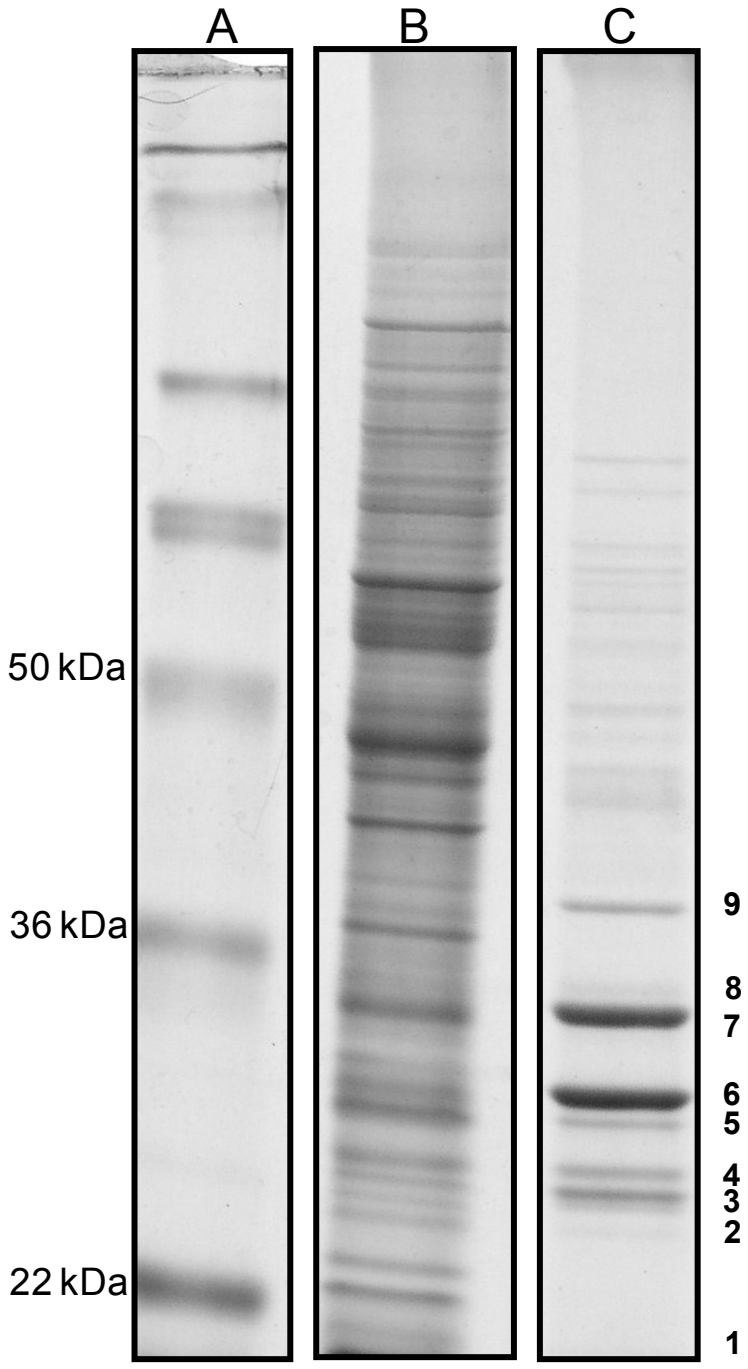

A
B
C
50 kDa
36 kDa
22 kDa
9
8
7
6
5
4
3
2
1

Table 3.3. Proteins captured by Trx affinity chromatography. List represents proteins that were shown through relative spectrum counts to be highly abundant within the sample bands analyzed with tandem mass spectrophotometry.

Protein	Size
Thiol peroxidase (YP_212393)	18 kDa
Peptidly-prolyl cis-trans isomerase (YP_101046)	21 kDa
Putative Rubrerythrin-like protein (YP_212632)	21 kDa
Putative Sugar Isomerase (YP_097455)	22 kDa
Thioredoxin peroxidase scavengase (YP_211984)	24 kDa
Putative 3'-5' exonuclease (YP_210640)	25 kDa
Succinate dehydrogenase/fumarate reductase iron-sulfur subunit (YP_101824)	27 kDa
Ketoisovalerate oxidoreductase (YP_098928)	28 kDa
Fructose-bisphosphate aldolase (YP_212751)	36 kDa
Tyrosine type site-specific recombinase (YP_101755)	37 kDa
L-Asparaginase II (YP_101492)	38 kDa
Branched-chain amino acid aminotransferase (YP_213361)	38 kDa
Putative L-Asparaginase I (YP_212298)	39 kDa
Putative 3-dehydroquinate synthase (YP_210375)	39 kDa
Bifunctional methionine sulfoxide reductase (YP_212985)	41 kDa
Putative Threonyl-tRNA synthetase (YP_211332)	74 kDa

Accession numbers are given in parenthesis.

found to be a homolog to an asparaginase gene, which encodes an enzyme responsible for hydrolyzing asparagine to aspartic acid. Thiol peroxidase scavengase (Tps) was known in *B. fragilis* to be an atypical 2-cysteine peroxidase regulated by oxidative stress in an OxyR-independent manner (136). These two proteins were chosen for further analysis to verify their interaction with TrxD in the affinity capture assay.

3.4.5 Yeast Two-Hybrid System to Confirm TrxD/Protein Interactions

A yeast two-hybrid system was utilized to verify proteins detected in the TrxD affinity chromatography experiment were interacting with TrxD. This system used the CY306 yeast knockout strain lacking functional *trx1* and *trx2* thioredoxin genes. This strain allowed the pGADT7 plasmid (prey) harboring the allele for TrxD_C45S to be the only source of thioredoxin present in the system to interact with the proteins encoded for on the pGBKT7 plasmid (bait). The yeast were co-transformed with both the prey containing the TrxD_C45S allele and the bait containing *asnB, tps, trxB*, *trxX*, or empty vector control. Since TrxB is the reductase for the Trx system in *B. fragilis,* its interaction with TrxD was used as a positive control. TrxX is a putative Trx homolog in *B. fragilis* lacking the conserved CXXC motif, precluding it from performing standard Trx enzymatic activities, rendering it incapable of forming an intermolecular disulfide bond, and thus served as a negative control. Confirmation of protein-protein interaction was determined by successful growth on 3DO-supplemented media lacking histidine. This interaction between the bait and prey proteins is necessary for growth on this media as the 3AT supplement added blocks sustainable autotrophic histidine production unless *his3* is overexpressed due to the interaction of the GAL4-AD (prey) and -BD (bait) fusion proteins at the *his3* GAL promoter.

Table 3.4. Yeast two-hybrid system in Trx depleted CY306 strain. Successful two-hybrid system interaction detected by growth on 3DO-supplemented media lacking histidine. Growth in three individual experimental replications represented by the + symbol.

Bait Plasmid GAL4-BD Fusion Protein Expressed	Media Supplement 2DO	3DO
Empty Vector Control	+	-
trxX	+	-
trxB	+	+
asnB	+	+
tps	+	+

As shown in Table 3.4, yeast cultures harboring the bait plasmids successfully grew on the 2DO-supplement plates containing histidine. However, only the positive control bait plasmid (coding for *trxB*) and the two experimental bait plasmids (coding for *asnB* and *tps*) were capable of growing on the 3DO-supplemented media, indicating that these three proteins were capable of interacting with TrxD_C45S fusion protein encoded for by the prey plasmid.

3.5 Discussion

Research in our lab has demonstrated that the TrxB/Trx system in *B. fragilis* is important for growth and survival during oxidative stress conditions (109, 119). TrxB also has been shown to be necessary for the ability of the bacterium to cause abscesses in a mouse model (119). Additionally, our lab has demonstrated that the six Trx homologs present within the *B. fragilis* genome are differentially regulated during the OSR, have compensatory roles when specific Trxs are functionally absent in knockout strains, and individually may have important specific roles within the cell (109). Research in another lab has demonstrated a specific role for the periplasmic TrxC (also known as TrxP) and have suggested its importance in the maintenance of membrane protein DoxDA (130), part of the terminal quinol:oxygen oxidoreductase complex (92, 108, 124, 130). In our previous studies, TrxA appeared to be essential for *B. fragilis* survival as no chromosomal mutant could be created without functional *trxA* supplied *in trans* on an expression vector. TrxD was also shown to be important in *B. fragilis* upon exposure to the thiol-specific oxidant diamide, suggesting its role as a crucial reducing enzyme of disulfide bonds formed during oxidative stress (109). The goal of the current study was to provide a better understanding of specific Trx function; an area of study

that is significantly lacking in anaerobic bacteria. The research focused primarily on TrxD and provided initial information regarding its regulation and protein interactions.

In order to understand the regulation of TrxD, it is first necessary to document the structure of the TrxD promoter region. Using the nested deletion products of the upstream region in transcriptional fusions (Figure 3.1) it was shown that *trxD* promoter activity was abolished in the construct lacking the region containing sequences which closely matched the *B. fragilis* consensus promoter (Fragment #3, Figure 3.2) (10). The promoter region mapped to an area in the intergenic region between the divergently transcribed *czcC* and *trxD* genes and ended 45-bp upstream of the translational start codon of *trxD.* Once the promoter region was determined, 5'-RACE analysis was performed in order to identify the +1 transcriptional start site for *trxD.* After several rounds of 5' RACE analysis utilizing two distinct primer pairs, the +1 site was mapped to an adenine residue 40-bp upstream from the TrxD start codon and adjacent to the -7 consensus (Figure 3.3). Additionally, an inverted repeat was located upstream of *trxD* in between the Fragment #2 and #3 forward primers, and an additional inverted repeat was seen flanking the Fragment #3 forward primer (Figure 3.3). These inverted repeats could indicate the location of a *trxD* transcriptional regulator binding site. This information will prove valuable in future experiments when attempts to determine potential transcriptional regulators involved in controlling *trxD* transcription are performed. The regulators identified will then be subjected to subsequent CHIP-chip analysis and other methods for confirming DNA-protein interactions.

One known function of Trxs in other bacterial species is the ability to reduce aerobic ribonucleotide reductase, NrdAB, for the reduction of ribonucleotides to

deoxyribonucleotides which is critical to the ability of bacteria to synthesize DNA aerobically (75). In *E. coli*, NrdAB can be reduced by either of its two native Trxs (Trx1 and Trx2) or by glutaredoxin 1 (but not glutaredoxin 2 or 3) and is the only ribonucleotide reductase functional during aerobic growth (47). It is interesting that even though the enzymatic mechanism of all three glutaredoxins in *E. coli* is identical, there exists a hierarchy of these reducing enzymes for certain protein partners, like NrdAB. While no glutaredoxin genes are present in its genome, such target specificity potentially exists for the *B. fragilis* Trxs as well, and protein target specificity would help to explain the large number of *trx* genes in the chromosome. In order to determine if particular Trxs in *B. fragilis* have the ability to be involved in specific reduction pathways, we investigated their capacity to compensate for native Trx in the turnover of the NrdAB in an *E. coli* mutant background. As seen in Figure 3.4, only the strains harboring plasmids producing TrxD and TrxE from *B. fragilis* (and the control plasmid containing *E. coli* TrxC) were able to effectively reduce NrdAB and permit bacterial growth on the inhibitory glucose-supplemented media. This discovery is interesting since *B. fragilis* contains NrdAB homologs which are used during the OSR. It is also noteworthy that not all Trxs in *B. fragilis* were active in this assay, suggesting specific roles of TrxD and TrxE for the reduction of NrdAB homologs during the OSR. Also, given both the apparent necessity of TrxA for the survival of *B. fragilis* as well as its high transcription level in both aerobic and anaerobic conditions, as demonstrated in our previous investigations (109), it is interesting that this Trx was not capable of reducing the *E. coli* NrdAB. This suggests that the high concentration of this protein in the cell does not necessarily translate to an ability to act as a general reducing enzyme for

every disulfide bond formed within the cytoplasm, and that certain proteins may require specific Trxs for optimum enzyme activity. Also interesting is that TrxF appeared to have an inhibitory effect on the ability of the RO36 grown on the permissive arabinose media to fully grow compared to wild type (Figure 3.4). This could be due to either an enhanced ability of TrxF to interact with NrdAB (but a decreased ability to reduce the enzyme) or perhaps an interaction between *B. fragilis* TrxF and *E. coli* TrxC, which lowers the available pool of TrxC for proper NrdAB turnover. An interaction between TrxF and *E. coli* TrxC is possible, given that *trxF* is the second gene in a bicistronic thioredoxin operon with *trxE* on the *B. fragilis* chromosome, and TrxE was the only other *B. fragilis* Trx capable of NrdAB rescue in the bioassay. Further research into the roles of TrxE and TrxF in *B. fragilis* may confirm a functional interaction between these two proteins and demonstrate a unique regulation compared to other Trxs in the organism. Taken together, these findings suggest that Trxs in *B. fragilis* are sufficiently different to allow for specific interactions with target proteins.

To explore the potential of TrxD to target specific proteins, we constructed a mutant of TrxD (TrxD_C45S) in which a point mutation substituted a serine for the second cysteine in the Trx motif, allowing for the capture of TrxD protein partners in a mixed disulfide intermediate by using Trx affinity chromatography. We selected two of the proteins identified by the affinity chromatography, the asparaginase AsnB and the Thiol peroxidase scavengase Tps for further analysis due to both being found in high concentrations in the MS spectrum data (137) and shown to be induced in *B. fragilis* during oxidative stress from previous microarray data (7.3-fold and 6.1-fold, respectively). Further confirmation of the interaction of these two proteins with TrxD

performed by a yeast two-hybrid system (Table 3.4) validated the Trx affinity chromatography data as well as previous experiments using an *E. coli* two-hybrid system that suggested AsnB and Tps interact with TrxD (data not shown). Taken as a whole, these data support the hypothesis that TrxD is the primary reducing enzyme for these two proteins in *B. fragilis*.

AsnB has been shown in anaerobic bacteria to be important for catalyzing the hydrolysis of asparagine to aspartate which can be subsequently converted to fumarate for production of succinate in the generation of terminal electron acceptors (65). In *B. fragilis,* AsnB plays a role in a pathway involving fumarate reductase (FRD), which has been shown to be important in the growth of *B. fragilis* by acting in energy metabolism, the biosynthesis of succinate, and as the terminal component of the anaerobic respiratory chain coupling NADH oxidation to ATP generation (7). Thus the role of TrxD in this pathway by interacting with AsnB could be very important for growth and survival as the regulation of energy metabolism would be of paramount importance during a stress response.

The *B. fragilis* Tps protein is related to the *E. coli* p20 scavengase, a protein that displays antioxidant activities through scavenging H_2O_2 by utilizing a redox-active cysteine residue to reduce the peroxide substrate, and is known to be reduced by the Trx system (6, 20, 136, 151, 160). This protein has been identified in various pathogenic bacteria and is a part of several interesting systems in different species. For example, p20 was originally identified in *Vibrio cholerae* as TagD, part of the toxin-coregulated (TCP)-encoding gene cluster which has been shown to be a major colonization determinant in this species (60). In *Streptococcus,* p20 has been

hypothesized to be a part of a gene cluster encoding adhesins (33, 43, 72, 128). In *B. fragilis,* Tps is divergently transcribed from a putative membrane protein designated oxygen-induced membrane protein (Oim) and is coordinately regulated with Tps by oxygen induction. A previous study in our lab showed that Tps is important in *B. fragilis* for managing oxygen radicals during the OSR in an OxyR-independent manner (136). The reduction of this enzyme by TrxD specifically is interesting and may indicate the existence of an oxidative stress pathway through which TrxD acts as the main reducing enzyme for a subset of OSR redox proteins as neither of these proteins are OxyR-dependent.

The list of proteins captured by the TrxD affinity chromatography (Table 3.3) contains several other interesting genes that appear to be targeted for reduction primarily by TrxD. One of these, the bifunctional methionine sulfoxide reductase, is interesting because in many bacteria methionine sulfide reductase (Msr) activity involves two enzymes, MsrA and MsrB, which have complementary stereospecificities that respectively reduce S- and R-stereoisomers of methionine sulfoxide (19). These enzymes are important because methionines are highly susceptible to oxidation, and the generation of methionine sulfoxides can sterically alter their resident protein by modifying or eliminating their activity. In some pathogenic bacteria MsrA and MsrB are fused to form the bifunctional methionine sulfoxide reductase enzyme, known as MsrBA. The reason for this fusion is typically assumed to allow for the maintenance of protein function necessary for bacterial survival under highly oxidizing conditions. If further research confirms specific targeting of MsrAB by TrxD for enzyme activity, this

will help to solidify the position of TrxD as an important component of OSR, given the imperative of reducing methionine sulfoxides within cellular proteins.

The current study provides an initial look into the specific roles of Trxs in *B. fragilis.* The ability of these proteins to have both redundancy and specificity has been seen previously (4, 109), but with such an abundant catalog of Trx genes in *B. fragilis* it is likely that the redundancy masks many specific roles for individual Trxs as partial overlapping of function is highly probable. We have shown that TrxD is capable of interacting specifically with certain proteins shown to be upregulated during the OSR and that these proteins are involved in important survival pathways in *B. fragilis.* Continued efforts to determine the individual roles for each of the six Trxs in *B. fragilis* will prove valuable toward understanding the evolutionary advantage of having such a large repertoire of these proteins and their role in protecting *B. fragilis* during oxidative stress.

CHAPTER FOUR: GENERAL SUMMARY

The goal of these current studies was to investigate the importance of the Trx proteins in *B. fragilis* during the OSR. These investigations were motivated by earlier data showing that elements of the TrxB/Trx system were critical for proper growth of the organism as well as its ability to survive aerobic stress and induce the formation of abscesses (119). One of the most interesting aspects of this system in *B. fragilis* is the extraordinary number of individual *trx* homologs within the genome. Six distinct *trx* genes falling into two divergent classes is an unusually large system for most bacterial species and suggested both specific and generalized or redundant functions for Trx proteins in *B. fragilis* (Figures 2.1, 2.2, and 2.3).

Early data showed that the phenotypes for individual *trx* null mutants, except for *trxA* which appeared to be an essential gene (Table 2.4), were limited in differences of sensitivity to oxidative stress versus wild type (Figure 2.6). Upon further mutational analysis it was discovered that there was significant evidence for compensatory ability in the Trx system (Figure 2.8). However, TrxD was also shown to be specifically important in the defense against disulfide bond formation during inhibition assays with the thiol-specific oxidant diamide (Figure 2.7). The potential importance of TrxD was further illustrated by experiments demonstrating that it along with TrxE were the only two *B. fragilis* Trxs able to reduce the aerobic RNR NrdAB in *E. coli* (Figure 3.4). TrxD was also shown to be the primary Trx partner of AsnB and Tps; two proteins known to be induced several fold each during the OSR and have functions that would advantageous to regulate during oxidative stress (energy metabolism and peroxide detoxification, respectively). Additional studies will also be performed to confirm the

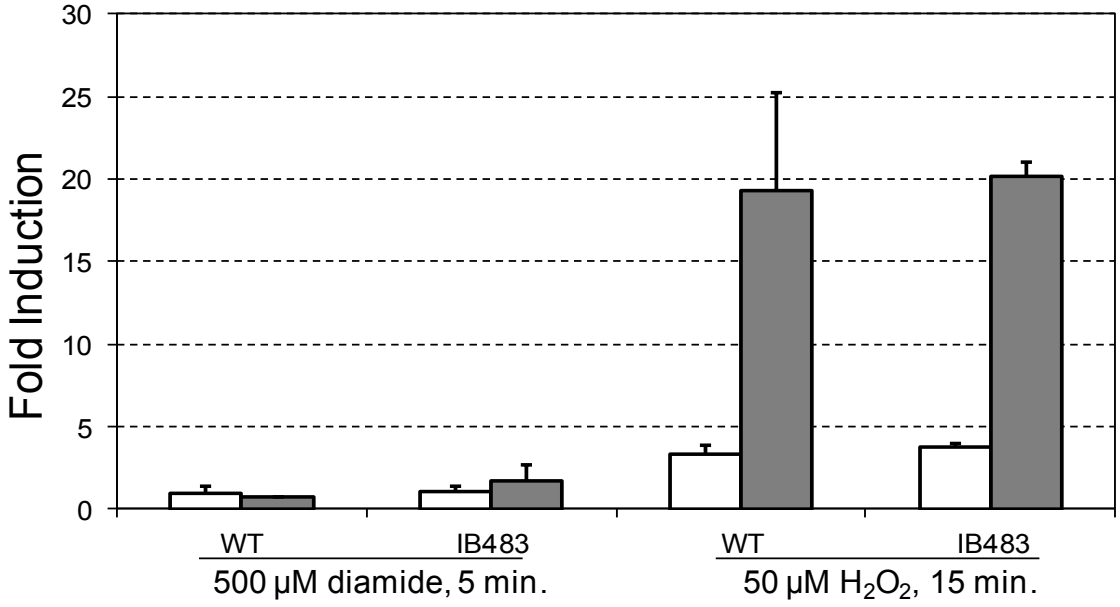
interaction of TrxD with other protein partners identified from the mass spectrophotometry data in order to understand the level of specificity TrxD has with its enzymatic activity.

Further investigation into TrxD will involve the identification of regulatory proteins that modify *trxD* expression. DNA affinity chromatography assays (152) have given us preliminary results indicating that the small, basic, heat-stable DNA binding protein HU is interacting with a region of the chromosome upstream of *trxD,* proximal to the *trxD* promoter region. This protein functions by complexing with DNA to aid in preventing denaturation under damaging conditions but also can be involved in changing DNA architecture to stimulate transcription (96, 139). Interestingly, HU is known to play a role in the regulation of a number of oxidative stress genes in *E. coli*, such as *dps, katG,* and *sodB* (96) which suggests that a role in the regulation of *trxD* in *B. fragilis* is highly possible. Recently, HU has been shown to be important in stimulating the transcription of surface polysaccharide synthesis genes in the anaerobe *Porphymonas gingivalis,* which also belongs to the order Bacteriodales (2). The modulation of these genes is often seen during stress responses like the OSR and further supports the possibility that HU is involved in the regulation of TrxD, and perhaps other genes important in the OxyR-independent response. Further analysis of the interaction between HU and the *trxD* promoter region will confirm these preliminary results and will aid in understanding TrxD regulation specifically, as well as systems of OxyR-independent OSR gene regulation in general.

The role of Trxs in the OSR is predicted to be through OxyR-independent mechanisms (137). However, in other organisms it has been shown that regulation of

OxyR is dependent upon its reduction by glutaredoxins (Grxs) (158) which serve similar and often overlapping functions to Trxs, but *grx* genes are not present in the *B. fragilis* genome (119). In order to investigate if Trxs were responsible for reducing OxyR, Real Time RT-PCR was performed on the quintuple mutant IB483 (Δ*trxC* Δ*trxD::cfxA* Δ*trxE* Δ*trxF* Δ*trxG*) to determine if the regulation of two genes, *tpx* and *katB,* known to be OxyR-dependent (113, 137), were altered in the mutant. The data from this experiment seem to indicate that neither the thioredoxin peroxidase gene *tpx* nor the catalase gene *katB,* were significantly different in their induction in the IB483 background as compared to wild type (Figure 4.1). One major issue with this experiment is that the levels at which these OxyR-dependent gene transcripts were expressed were only looked at for one time point. It is potentially likely that the effect the Trx system would have on the redox status of OxyR, and subsequently the transcription of OxyR-dependent genes, would be seen at time points after the oxidative stress was applied, as the OxyR is being reduced back to its inactive state. Repeating this experiment with multiple time points after diamide exposure may reveal significant differences between these two strains. A second issue is that while the data from this experiment show that the relative levels of OxyR-dependent gene transcripts do not differ significantly between the IB101 and IB483, it does not show the absolute levels of the transcripts, which could be significantly different between these two strains. This issue can be resolved by utilizing absolute quantification Real Time RT-PCR which will provide a quantitative number of each gene transcript. Another issue with this experiment stems from the lack of a fully Trx deplete *B. fragilis* as *trxA* was still functional in the IB483 mutant and is highly expressed in all conditions (Figure 2.4). Thus, TrxA may be the primary reducing

Figure 4.1. Transcriptional analysis of the induction of OxyR-dependent genes in *B. fragilis* IB483 (*ΔtrxC*, *ΔtrxD*::*cfxA*, *ΔtrxE*, *ΔtrxF*, *ΔtrxG*). The parental strain (wild type [WT], *B. fragilis* strain 638R) and IB483 were exposed for 5 min to 500 μM, 15 min to 50 μM H_2O_2, or maintained under standard anaerobic conditions (0 μM control). For each condition, RNA was isolated and real-time RT-PCR was performed in triplicate. The sigma-54 modulation protein gene was used as a standard, and the results are expressed as fold induction relative to levels under the control condition. The values are means of fold induction, compared to the 0 μM control, from two independent experiments. Open bars represent *tpx* induction and gray bars represent *katB* induction. The error bars indicate standard deviations.


30
25
20
15
10
5
0
Fold Induction
WT
IB483
WT
IB483
500 µM diamide, 5 min.
50 µM H_2O_2, 15 min.

enzyme of OxyR or perhaps it is compensating for the specific Trx primarily responsible for the activity absent in the IB483 strain. Further investigations into the role of TrxA will require the use of an inducible *trxA* system, allowing for both the growth of the organism while downregulating TrxA production in experimental conditions to lower, but still viable, levels. However, unpublished microarray data from our lab has shown that in the *trxB* mutant *B. fragilis* strain IB370, these OxyR-dependent genes were likewise unchanged in their regulation compared to wild type.

Previous data has shown the importance of TrxB in the ability to cause abscesses in animal models (119). This result corroborates other research identifying the TrxB/Trx system as important in the virulence and survival of pathogenic bacteria, yeasts, and protozoa (11, 89, 155). These data suggest that perhaps one, or several, of the Trxs in *B. fragilis* are significant to the potential of this organism to induce abscesses. Potentially, this could be due to the general reducing capacity of Trxs conferring survival benefits during the initial aerobic exposure in the peritoneum, or that they are required for optimum reduction of enzymes important in survival or virulence pathways involved in the abscess formation. Unpublished data from our lab utilizing the quintuple *trx* mutant IB483 showed only a modest decrease in the ability of the strain to induce abscesses in the mouse model. Again, this may be due to the fact that TrxA is still present within the IB483 strain or that perhaps TrxB is responsible for reducing another important member of the Trx family of proteins present within the *B. fragilis* genome.

Given the extensiveness of the Trx system in *B. fragilis* it is interesting to postulate the evolutionary benefit that having so many individual Trxs confers to the

organism. As seen in many other bacteria, the overlapping roles of similar reducing *fragilis* Trxs is the periplasmic TrxC (TrxP), shown to be important in maintenance of membrane protein DoxDA (130). Furthermore, the constantly high transcription, and inability to create a null mutant, displays the potentially large importance of TrxA, which may be involved in DNA replication (109). Also, TrxD, whose gene was most highly induced during diamide stress in *B. fragilis,* has been shown from these current studies to display specificity for AsnB, Tps, and potentially many more OSR related proteins, suggesting the importance of its reducing capability of specific proteins during oxidative stress. Further research into the Trx proteins will provide valuable insight into their roles in *B. fragilis* during the OSR as well as help define the protein interactions involved in the OSR in general. Understanding these concepts will be useful for combatting the potentially life-threatening conditions that can occur due to *B. fragilis* opportunistic infections.

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