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Original Article

Characterization of the components of the thioredoxin system in *Bacteroides fragilis* and evaluation of its activity during oxidative stress

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ABSTRACT

Objectives: *Bacteroides fragilis* has a pronounced ability to survive prolonged exposure to atmospheric oxygen. The major objective of this study was to biochemically characterize the components of the thioredoxin system in *B. fragilis*. The nitroreductase activity of TrxR was also assayed.
Methods: Components of the thioredoxin system were expressed in *E. coli* and used in a disulfide reductase activity assay. Activity of TrxR was measured with purified recombinant enzyme or with cell extracts after or without exposure to oxygen or hydrogen peroxide, respectively.
Results: Of all six thioredoxins tested, only thioredoxins A, D, and F were reduced by recombinant TrxR and natural TrxR present in *B. fragilis* cell extracts. Exposure to oxygen and hydrogen peroxide increased the activity of TrxR. Further, *B. fragilis* TrxR acts as a nitroreductase with furazolidone or 1-Chloro-2,4-dinitrobenzene as substrates but cannot reduce metronidazole.
Conclusion: TrxR shows an increase in activity under the conditions of oxidative stress and exerts nitroreductase activity.

1. Introduction

The human colon is populated with numerous commensal microorganisms, many of which are anaerobes due to the low redox potential and low levels of oxygen present inside the lower intestinal tract [1]. Among these anaerobic microorganisms, *Bacteroides* spp. are dominant members, involved in various beneficial activities such as protection of the gut epithelia from colonization by pathogens, polysaccharide degradation, energy harvesting, maturation of the systemic immune system, transformation of mutagenic and toxic compounds [2–6]. Additionally, these anaerobes, especially *Bacteroides fragilis*, are opportunistic pathogens that can account for about 50–70% of all anaerobic infections resulting from perforations of the mucosal. This can lead to intra-abdominal infections, the formation of abscesses, bacteremia, and peritonitis [7,8]. Despite the fact that the habitat of *B. fragilis* has an extremely negative redox potential (-450 mV), this bacterium is highly aerotolerant and can survive in presence of atmospheric oxygen for >72 h whereas other anaerobes can survive only < 2 h [9]. The ability to tolerate air provides these anaerobes with an advantage especially during the transmission between hosts or different parts of the host body outside the intestinal lumen where the environment is more oxidizing [10]. To survive, *B. fragilis*, like other anaerobes, had to develop mechanisms to protect itself from damage caused by reactive oxygen species (ROS) like hydrogen peroxide, superoxide anions, and hydroxyl radicals [11]. ROS are also produced during the infection as part of the host defense mechanisms and can cause inactivation of metabolic enzymes, DNA lesions, and destruction of important iron-sulfur clusters all of which might culminate in the death of the microorganism [11–19]. It is known that *B. fragilis* exhibits a coordinated oxidative stress response during exposure to air and H_2O_2 [9,20–23]. Moreover, during such exposure bacteria must cope with thiol oxidation and maintain a thiol-disulfide redox balance in order to prevent oxidation of proteins which can lead to the loss of their respective functions [24–26].

To overcome these issues, eukaryotic and prokaryotic organisms have small and heat-stable oxidoreductases, i.e. thioredoxins, which can reduce disulfides in proteins and non-protein thiols [24,27]. Thioredoxins, in turn, are reduced by thioredoxin reductase (TrxR), an enzyme which is present in the vast majority of the organisms known [28]. Another redox pathway which is based on glutathione and glutathione reductase is present in most organisms but not in many anaerobes, including *Bacteroides* spp [29–32]. In the *B. fragilis* genome one TrxR and six thioredoxins were found (Trx A-F), and thioredoxins, TrxA and TrxB, were discovered to be necessary for survival *in vivo*

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[33,34]. A biochemical characterization of the components of the thioredoxin system in *B. fragilis*, however, is still lacking.

In this study, we expressed the main components of the TrxR system and tested all of the predicted six Trxs for function with TrxR as the reducing enzyme. Measurements were conducted with recombinant TrxR and with extracts of *B. fragilis* either with or without previous exposure to air and hydrogen peroxide. Finally, TrxR was also assayed for nitroreductase activity because nitro compounds have been identified as side substrates of several TrxRases from anaerobic organisms before.

2. Materials and methods

2.1. Chemicals and growth media components

Wilkins-Chalgren anaerobe agar (WC) was purchased from Oxoid (Basingstoke, England) and Brain-heart-infusion broth (BHI) and vitamin K were purchased from Carl Roth (Karlsruhe, Germany). Haemin, metronidazole (MTZ), furazolidone, cytochrome *c*, 1-Chloro-2,4-dinitrobenzene (CDNB), NADPH, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), diphenyleneiodonium chloride (DPI), auranofin and ampicillin were purchased from Sigma-Aldrich (St. Luis, USA). Potassium dihydrogen phosphate (KH_2PO_4), hydrogen peroxide (H_2O_2), Triton X-100 and Anaerocult A were purchased from Merck (Darmstadt, Germany).

2.2. Bacterial strains and growth conditions

BL21-AI™ One Shot™ chemically competent *E. coli* (Invitrogen Waltham, MA, USA) and NEB® 10-beta competent *E. coli* (New England Biolabs Ipswich, MA, USA) cells were grown in LB medium with 20 μg mL^{-1} ampicillin and LB agar plates containing 15 g L^{-1} of agar and 20 μg mL^{-1} of ampicillin. *Bacteroides fragilis* 638R [35] cells were grown and maintained on WC plates in anaerobic jars (Merck, Darmstadt, Germany) at 37 °C using Anaerocult A system (0% O_2 and 18% CO_2). For the preparation of cell extracts, *Bacteroides fragilis* 638R cells were grown in 14 mL sterile, round bottom, two-position vent stopper tubes (Greiner Bio-One) in BHI medium supplemented with 1 mg L^{-1} vitamin K and 5 mg L^{-1} haemin at 37 °C under anaerobic conditions provided inside an anaerobic workstation (BugBox, Baker Ruskinn Technology Ltd).

2.3. Expression of recombinant hexahistidine-tagged TrxR and thioredoxins in E.coli

B. fragilis 638R genomic DNA was used as a template for the amplification of TrxR and Trxs genes. TrxR and Trxs primer sequences are shown in Supplementary Table 1. An NdeI or a BamHI (TrxB only) restriction site was included in the forward primers whereas reverse primers had an XhoI restriction site together with a hexahistidine tag which allowed convenient isolation of proteins. PCR fragments were ligated into pET17b vectors and resulting plasmids were propagated in 10-beta cells according to the manufacturer's instructions. After isolation, plasmids were used for the transformation of BL21-AI cells. Transformed cells were selected using LB agar plates with 20 μg mL^{-1} ampicillin. Recombinant protein expression was performed for 3 h after induction with 0.2% L-arabinose. Harvested cells were disrupted by grinding in a mortar and proteins were purified using Ni-NTA spin columns (Qiagen, Hilden, Germany). Recombinant *B. fragilis* TrxR is being referred to as BfTrxR and thioredoxins as BfTrxA, BfTrxB, BfTrxC, BfTrxD, BfTrxE, and BfTrxF, respectively.

2.4. Disulfide reductase activity assay

The assay was performed as previously described [36] using a combination of TrxR and Trxs. Briefly, the reaction buffer contained 100 mM KH_2PO_4 (pH 6.8), 1 mM DTNB, 0.2 mM NADPH, 5 μg mL^{-1} BfTrxR and 10–100 μg mL^{-1} of BfTrx A-F (10, 20, 40, 80 and 100 μg mL^{-1}). Reduction of DTNB was measured in an PerkinElmer lambda 25 UV/Vis spectrophotometer at $\lambda = 412_{nm}$ ($\Delta\varepsilon_{412} = 13.6$ $mM^{-1}cm^{-1}$) and the respective turnover of DTNB was calculated by applying the Beer-Lambert law ($\Delta E = \varepsilon * c * d$). Measurements were performed over a period of 2 min at room temperature. To demonstrate that the reduction of BfTrxs is specifically catalyzed by BfTrxR, assay was repeated after addition of the TrxR inhibitors DPI (100 μM) or auranofin (10 μM). NADPH usage was measured indirectly at $\lambda = 412_{nm}$ over the period of 2 min at room temperature in the same reaction buffer with a constant amount of BfTrxR and BfTrxD (1 μg mL^{-1} and 10 μg mL^{-1}, respectively) in combination with different concentrations of NADPH in the range of 1–100 μM. Kinetic determinants were calculated using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla California USA).

2.5. Measurements of thioredoxin-reducing activity in B. fragilis cell extracts

B.fragilis cells were grown overnight in anaerobic jars at 37 °C. 5 mL of overnight cultures were centrifuged for 10 min at 3000×*g* and washed once in 1 mL 1xPBS to remove all remaining medium. Pellets were then resuspended in a 1xPBS buffer containing 0.5% Triton X-100 and incubated on ice for 20 min. Insoluble material was removed by centrifugation for 10 min at 17,000×*g* at 4 °C. The supernatant was collected in fresh 1.5 mL tubes and used as cell extract at a concentration of 50 μg of protein mL^{-1} of assay buffer. The thioredoxin-reducing activity of cell extract was measured at $\lambda = 412_{nm}$ by determining the reduction of DNTB in reaction buffer containing 100 mM KH_2PO_4, 0.2 mM NADPH, 1 mM DTNB, 50 μg mL^{-1} of cell extract, and 40 μg mL^{-1} of BfTrx A-F. The assay was also performed using the TrxR inhibitors DPI and auranofin to confirm the reduction of BfTrxA by TrxR isolated from Bf638R cell extracts.

2.6. The thioredoxin-reducing activity of B. fragilis cell extracts after O_2 and H_2O_2 exposure

B.fragilis cells were grown in an anaerobic workstation (BugBox, Baker Ruskinn Technology Ltd) overnight in 5 mL BHI medium using vented tubes. Oxygen-treated cells were transferred into a sterile hood and vent caps were removed for 10 min to allow air to enter the tubes. Vents were then reapplied to the tube in a half-open position and cells were shaken aerobically at 200 rpm for 2 h at 37 °C. Hydrogen peroxide treated cells were incubated anaerobically with 500 μM H_2O_2 for 2 h. In H_2O_2 treated cells, H_2O_2 was added under anaerobic conditions in the anaerobic workstation. Preparation of cell extracts and measurements of thioredoxin activity of cell extracts were performed as described above.

2.7. Nitroreductase activity assay

Reduction of furazolidone (20 μM) and CDNB (250 μM) was measured at $\lambda = 340_{nm}$ ($\Delta\varepsilon_{340} = 6.2 mM^{-1}cm^{-1}$) by determining NADPH oxidation. The reaction buffer contained 100 mM Tris (pH 7.5), 0.2 mM NADPH and 5 μg mL^{-1} BfTrxR. Due to MTZ's high absorbance at $\lambda = 340_{nm}$ reduction of this drug was determined in a modified assay with cytochrome *c* [37]. The MTZ-mediated reduction of cytochrome *c* was measured at λ = 550 nm ($\Delta\varepsilon 550 = 20\ mM^{-1}\ cm^{-1}$). The reduction of the nitro group by BfTrxR leads to the reduction of one molecule of cytochrome *c*. The reduction buffer contained 100 mM Tris (pH 7.5), 0.2 mM NADPH, 50 μM cytochrome *c*, 1 mM MTZ and 5 μg mL^{-1} BfTrxR.

3. Results and discussion

3.1. Expression of recombinant BfTrxR and BfTrxs

Recombinant BfTrxR and all six BfTrxs were expressed in order to confirm their functions in appropriate assays. Furthermore, BfTrxR was assayed for nitroreductase activity as done before with TrxRases from anaerobic protists [38–40]. Recombinant BfTrxR and BfTrxs were successfully produced in *E. coli* BL21-AI upon induction with 0.2% L-arabinose. The presence of hexahistidine tags at the carboxy-terminal position allowed recombinant proteins to be purified via Ni-NTA columns (Supplementary Fig. 1).

Table 1
BfTrxR reduces BfTrxA, BfTrxD and BfTrxF. Disulfide reducing activity of BfTrxR was determined by monitoring reduction of DTNB via BfTrxs at λ = 412. All measurements were performed at least 3 times. ND – not determined.

Bf TrxR				
Thioredoxin Substrate	v_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	Ratio **Trx**:**TrxR** for 50% activity	*K*m NADPH
BfTrxA	7250	80	approx. 26:1	ND
BfTrxD	6150	70	approx. 16:1	3 μM
BfTrxF	1470	16	approx. 26:1	ND

3.2. BfTrxA, BfTrxF, and BfTrxD are reduced by BfTrxR and in Bf cell extracts

Disulfide reducing activity of BfTrxR was confirmed through the reduction of DTNB to 2-nitro-5-thiobenzoic acid (TNB) by BfTrxs. From six tested Trxs, only BfTrxA, BfTrxF, and BfTrxD were confirmed as substrates of BfTrxR, with the highest activity observed with BfTrxA (Table 1). The other three Trxs, i.e. BfTrxB, C, and E, were not reduced by BfTrxR. It is presently unclear, how these three Trxs are reduced for their function.

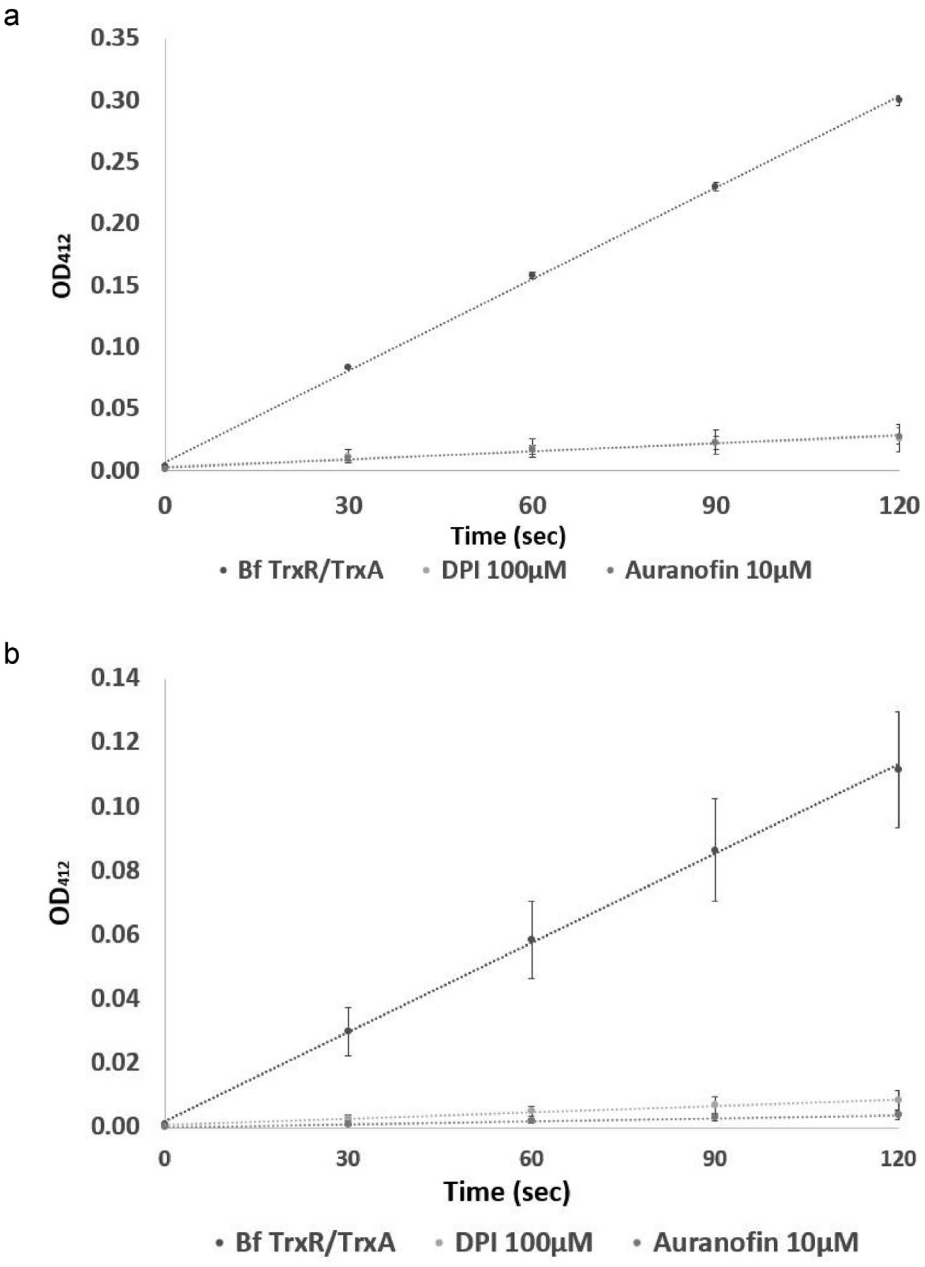

Fig. 1. DPI and auranofin diminishes disulfide reductase activity of purified BfTrxR (a) and TrxR present in Bf638R cell extracts (b). Disulfide reducing activity was measured either in presence or absence of DPI and auranofin at λ = 412. All experiments were performed four times with error bars indicating standard deviations. DPI - diphenyleneiodonium chloride.

Activity of TrxR in Bf cells was confirmed by using recombinant thioredoxins and cell extracts for measurements. Specific reduction of BfTrxs by TrxR was confirmed by using DPI [41] and auranofin [42] as inhibitors in cell extracts and with purified BfTrxR. Indeed, these inhibitors almost completely inhibited reduction of BfTrxs (Fig. 1). Reduction of thioredoxins by Bf cell extract could also be measured (Table 2). Importantly, only BfTrxA, BfTrxF, and BfTrxD were reduced by Bf cell extracts which mirrored the results as obtained with recombinant BfTrxR.

3.3. Exposure to air and H_2O_2 increases the activity of TrxR in Bf cell extracts

The TrxR system plays an important role during oxidative stress as Trxs deliver the reducing power needed for the repair of the inactivated proteins and for detoxifying reactions [43–47]. Previously, it was shown that expression of thioredoxins in *B. fragilis* was induced under conditions of oxidative stress [33]. In order to confirm that this upregulation of expression does result in an upregulation of TrxR activity, we exposed Bf cells to air and H_2O_2 for 2 h. The exposure to air and H_2O_2 treatment under anaerobic conditions both substantially increased BfTrxR activity in cell extracts as compared to untreated controls (Fig. 2). The extent of upregulation was identical when BfTrxA and BfTrxD were used, providing further evidence that *in vivo* both Trxs are reduced by the same enzyme, i.e. BfTrxR. These results clearly demonstrate that the thioredoxin system in *B. fragilis* readily reacts to oxidative stress by increasing TrxR activity.

Table 2
TrxR from Bf638R cell extracts reduces BfTrxA, BfTrxD and BfTrxF. Disulfide reducing ability of TrxR from Bf638R cell extracts was determined by monitoring reduction of DTNB via BfTrxs at λ = 412. Values are expressed with standard deviations from at least three independent experiments.

TrxR activity in Bf638R cell extracts (nmol min^{-1} mg $protein^{-1}$)		
BfTrxA	BfTrxD	TBfrxF
58 ± 21	43 ± 17	21 ± 5

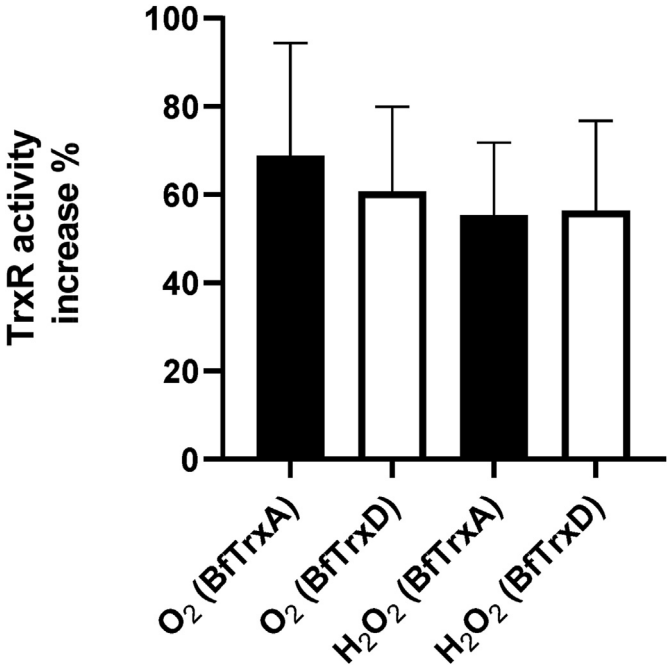

Fig. 2. TrxR activity in Bf638R cell extracts under the conditions of oxidative stress. Values are presented as % increase in activity of TrxR as compared to control after 2 h of exposure to oxygen or hydrogen peroxide. All experiments were performed using 4 biological replicate for each condition. Error bars indicate standard deviations.

Table 3
BfTrxR exerts nitroreductase activity when CDNB and furazolidone are used as substrates but does not reduce metronidazole. All measurements were performed at least 3 times.

Nitroreductase activity of Bf TrxR		
Nitro compound	v_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})
CDNB (250 μM)	660	22
Furazolidone (20 μM)	590	20
Metronidazole (1 mM)	–	–

3.4. BfTrxR exhibits nitroreductase activity in vitro

In order to test if TrxR in *B. fragilis* can act as a nitroreductase similarly to TrxRases in anaerobic protists we measured reduction of metronidazole, furazolidone and CDNB by BfTrxR (Table 3). Importantly, BfTrxR could reduce CDNB and furazolidone but not MTZ, arguing against a role of Bf TrxR in the activation of MTZ as described for TrxRases in *Entamoeba histolytica* [39], *Trichomonas vaginalis* [38], and *Giardia lamblia* [40].

4. Conclusion

Bacteroides spp. are obligate anaerobes but display an astounding resistance to oxidative stress. To survive oxygen exposure outside the gastrointestinal tract, and ROS produced by host immune cells inside abscesses, *B. fragilis* harnesses highly robust protective mechanisms. In the present study, we confirmed that BfTrxR activity in *B. fragilis* cell extracts is increased during/after the exposure to oxidative stress. Interestingly, BfTrxR only uses BfTrxA, F, and D as substrates but not the other three thioredoxins described [33]. Further, it was shown that BfTrxR can act as a nitroreductase but, in contrast to TrxRases from anaerobic protist parasites [38–40], cannot reduce metronidazole. This is of importance because metronidazole and other 5-nitroimidazoles drugs have to be transformed into their toxin intermediates in order to exert toxicity [48]. Our results argue against any involvement of *B. fragilis* TrxR in metronidazole activation.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2021.102507.

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