Free Radical Biology and Medicine 97 (2016) 1–13

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Functional thioredoxin reductase from pathogenic and free-living *Leptospira* spp.

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ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form
9 May 2016
Accepted 9 May 2016
Available online 10 May 2016

Keywords:
Leptospira
Thioredoxin reductase
Peroxiredoxin
Redox metabolism

ABSTRACT

Low molecular mass thiols and antioxidant enzymes have essential functions to detoxify reactive oxygen and nitrogen species maintaining cellular redox balance. The metabolic pathways for redox homeostasis in pathogenic *(Leptospira interrogans)* and free-living (*Leptospira biflexa*) leptospires species were not functionally characterized. We performed biochemical studies on recombinantly produced proteins to in depth analyze kinetic and structural properties of thioredoxin reductase (*Lin*TrxR) and thioredoxin (*Lin*Trx) from *L. interrogans*, and two TrxRs (*Lbi*TrxR1 and *Lbi*TrxR2) from *L. biflexa*. All the TrxRs were characterized as homodimeric flavoproteins, with *Lin*TrxR and *Lbi*TrxR1 catalyzing the NADPH dependent reduction of *Lin*Trx and DTNB. The thioredoxin system from *L. interrogans* was able to use glutathione disulfide, lipoamide disulfide, cystine and bis-γ-glutamyl cysteine and homologous peroxiredoxin as substrates. Classic TrxR activity of *Lin*TrxR2 had not been evidenced *in vitro*, but recombinant *Escherichia coli* cells overexpressing *Lbi*TrxR2 showed high tolerance to oxidative stress. The enzymatic systems herein characterized could play a key role for the maintenance of redox homeostasis and the function of defense mechanisms against reactive oxidant species in *Leptospira* spp. Our results contribute to the general knowledge about redox biochemistry in these bacteria, positioning TrxR as a critical molecular target for the development of new anti-leptospiral drugs.

1. Introduction

The genus *Leptospira* includes saprophytic and pathogenic species, being classified into serovars according to their serological characteristics. The *L. biflexa* serovar Patoc is an example of non-pathogenic and free-living bacterium that can be found in wet environments and surface water. Different is the case of *L. interrogans* serovar Copenhagenii, a human pathogen that can survive in different environments (from the soil and water to the mammalian host's tissues) and is one of the etiologic agents of leptospirosis (the predominant in human disease) [1]. Genomic analysis provides evidence that show *L. interrogans* as the result of many evolutive events, including horizontal gene transfer, operating modifications in bacteria in the environment, thus producing the conversion of saprophytic *L. biflexa* into pathogenic *L. interrogans* [2,3].

Bacteria are exposed to both reactive oxygen (ROS) and reactive nitrogen (RNS) species from the environment, and/or from those generated by aerobic metabolism [4]. Detoxification of these dangerous species is carried out by enzymatic and non enzymatic systems from which the available information is scarce. The thioredoxin (Trx) system plays a critical role for the accurate maintenance of the intracellular redox homeostasis in many organisms [5]. The system is composed by Trx and Trx reductase (TrxR), which working together catalyze the transfer of reduction equivalents from NADPH to different intracellular oxidant substrates [6]. Trx is a redox protein ubiquitously found in all kingdoms (from archaebacteria to humans) that plays different functions; mainly: (i) to protect proteins from oxidative inactivation,

Abbreviations: AhpC, bacterial 2-Cys peroxiredoxin; bis-γ-GC, bis-γ-glutamylcysteine; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSSG, glutathione disulfide; L-TrxR, low molecular mass thioredoxin reductase; H-TrxR, high molecular mass thioredoxin reductase; MBQ, 2-methyl-benzoquinone; Trx, thioredoxin; TrxR, thioredoxin reductase; *t*-BuOOH, *tert*-butyl hydroperoxide

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http://dx.doi.org/10.1016/j.freeradbiomed.2016.05.008
0891-5849/

(ii) to participate in the defense against oxidative stressing conditions produced by host immune system during infection, (iii) to mediate programmed cell death, and (iv) to act as a growth factor.

Trxs have a canonical CGPC catalytic motif, whose cysteine residues are involved in disulfide reduction of other oxidized proteins. Cysteine residues in Trx are ultimately maintained reduced by TrxR at the expense of NADPH [5]. TrxR is an antioxidant enzyme that belongs to the flavoenzyme oxidoreductases family [6]. Two distinct types of TrxRs have been described, both of them are dimeric proteins differing in their amino acid sequences and catalytic mechanisms. The first group includes low molecular mass TrxR (L-TrxR, 35 kDa per subunit), found in bacteria, plants and fungi [7]. L-TrxRs have a catalytic cycle that involves electrons transfer from NADPH to flavin and subsequently to the redox active disulfide, generating a dithiol [8,9]. The other group comprises high molecular mass TrxR (H-TrxR, 55 kDa per subunit), mainly present in insects and mammals. H-TrxRs have an N-terminal redox-active disulfide, which receives electrons from NADPH-reduced flavin to transfer them to the C-terminal active site in the opposite subunit, where a selenylsulfide bridge is reduced to selenothiol. Thus, the enzyme can later reduce Trx and other substrates [10].

Like any other infectious agent, *L. interrogans* must evade the oxidative and nitrosactive stress generated by host cells, including macrophages [1]. The functionality of different proteins involved in the resistance against oxidative stress has been already evidenced; for example, catalase (a hemeprotein that catalyze the decomposition of H_2O_2 to H_2O and O_2) [11,12] and a typical 2-Cys peroxiredoxin (AhpC) (a thiol-peroxidase, which are defined by their ability to reduce H_2O_2, and other organic hydroperoxides) [13]. On the other hand, the saprophytic species *L. biflexa* is more susceptible to exogenous hydrogen peroxide *in vitro* than *L. interrogans* [14], indicating that some antioxidant proteins in the pathogenic species could play an important role in the infection. The redox metabolic pathways for these organisms have been poorly studied. Herein, we report the molecular cloning, recombinant expression and functional characterization of the TRX system from *L. interrogans* and *L. biflexa.* The comparative analysis between these two species provides a novel perspective on *Leptospira* evolution, environmental persistence and causation of disease. Since antibiotics and vaccines that have been used in the management of leptospirosis have a variable efficacy [15,16], our results contribute to identify drug targets for the rational design of new therapeutic compounds.

2. Materials and methods

2.1. Materials

Bacteriological media were purchased in Britania Laboratories and BD Biosciences (Argentina). All other reagents and chemicals were of the highest quality commercially available.

2.2. Bacteria and plasmids

Escherichia coli Top 10 cells (Invitrogen) and *E. coli* BL21 (DE3) were utilized in routine plasmid construction and expression experiments. The vector pGEM-T Easy (Promega) was selected for cloning and sequencing purposes. The expression vector was pET28c (Novagen). Genomic DNA from *L. interrogans* and *L. biflexa* was obtained using Wizard® Genomic DNA Purification Kit (Promega). DNA manipulation, *E. coli* cultures, and transformations were performed according to standard protocols [17].

2.3. Molecular cloning of trxA and trxB genes

Based on available information about *L. interrogans* serovar Copenhageni and *L. biflexa* serovar Patoc (http://meta.microbesonline.org/) genome sequences, the genes *lintrxA, lintrxB, lbitrxB* and *lbitrxB-1* were amplified by PCR using genomic DNA and specific primer pairs (Supplementary data - Table 1). Each PCR was performed under the following conditions: 95 °C for 10 min; 30 cycles of 95 °C for 1 min, 55–65 °C for 1 min 72 °C for 1 min, and 72 °C for 10 min. The PCR products were subsequently purified and ligated into pGEM-T-Easy vector (Promega), its fidelity and identity was confirmed by complete DNA sequencing (Macrogen, South Korea).

The constructions obtained were digested with appropriate restriction enzymes (Supplementary data - Table 1), being the DNA fragments purified by gel extraction after agarose gel electrophoresis. The purified ORF was ligated to pET28c vector (Novagen) using T4 DNA ligase (Fermentas) for 16 h at 4 °C. Competent *E. coli* BL21 (DE3) cells were transformed with the construct. Transformed cells were selected in agar plates containing Lysogeny Broth (LB; 10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone, pH 7.4) supplemented with kanamycin (50 μg/ml). Preparation of plasmid DNA and subsequent restriction treatment were performed to check the correctness of the construct.

2.4. Overexpression and purification of recombinant proteins

A single colony of *E. coli* BL21 (DE3) transformed with the appropriate recombinant plasmid was selected. Overnight cultures were diluted 1/100 in fresh Terrific Broth (TB; 12 g/L peptone, 24 g/L yeast extract, 4 ml/L glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, pH 7.0) supplemented with 50 μg/ml kanamycin and grown under identical conditions to exponential phase, OD_{600} of 0.6. The conditions for the expression of the respective recombinant proteins were: 0.25 mM IPTG for 16 h at 25 °C. After, the cells were harvested and stored at −20 °C until the posterior usage.

Purification of each recombinant protein was performed using HiTrap™ chelating HP (GE Healthcare). Briefly, the bacterial pellet was resuspended in binding buffer (20 mM Tris–HCl pH 7.5, 400 mM NaCl and 10 mM imidazole) and disrupted by sonication. The lysate was centrifuged (10,000*g* for 30 min) to remove cell debris. The resultant crude extract was loaded onto column that had been equilibrated with binding buffer. After being washed with 10 bead volumes of the same buffer, the recombinant protein was eluted with elution buffer (20 mM Tris–HCl, pH 7.5, 400 mM NaCl, 300 mM imidazole). Fractions containing pure protein were pooled, concentrated and frozen with 20% (v/v) glycerol at −80 °C. *Lin*AhpC, Trx from *Prunus persica* (*Ppe*Trxh) and Trx from *Entamoeba histolytica (Ehi*Trx8) were purified as previously described [18,19]. *E. coli* Trx (*Eco*Trx) was obtained by expression of the pET32a vector.

2.5. Protein methods

Proteins were analyzed by SDS–PAGE using the Mini-Protean II (Bio-Rad) apparatus. Coomassie Brilliant Blue R-250 was used for protein staining. Protein concentration was measured with BCA (Pierce™ BCA Protein Assay Kit, Thermo Scientific), using bovine serum albumin as standard.

Serum anti-*Lin*TrxR was prepared by rabbit immunization with the purified recombinant proteins according Vaitukaitis et al. [20]. Bacteria protein extracts were prepared resuspending the pellets in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS). Proteins in SDS-PAGE gels were blotted onto nitrocellulose membranes using a Fast Blot, Semi-Dry Electophoretic Transfer apparatus (Biometra GmbH). The membrane was blocked overnight at 4 °C, subsequently incubated at room temperature for 1 h with primary

antibody and then with a HRP-conjugated anti-rabbit secondary antibody. Bands were visualized using the ECL Western blotting detection reagents (Thermo Scientific).

2.6. Flavin determination

Purified TrxRs were boiled in the dark for 10 min and centrifuged to remove the denatured protein. The protein cofactor was visualized by resolving the supernatant at room temperature and in the dark by thin layer chromatography (TLC) on silica sheets 25 TLC ALUMINIUM plates (Merck). The mobile phase was a solution of butanol:acetic acid:water (12:3:5). The chromatogram was analyzed by fluorescence with the *Typhoon* scanner (GE Healthcare). A solution of commercial FAD and FMN was used as a standard. After identification of the flavin, its concentration was quantified spectrophotometrically using the molar extinction coefficient at 450 nm of 11.3 $mM^{-1}\,cm^{-1}$.

2.7. Determination of native molecular mass by gel filtration chromatography

The molecular mass of the recombinant proteins in their native states was determined by gel filtration chromatography in a Superdex 200 h Tricorn column (GE) equilibrated with 50 mM HEPES buffer pH 8.0 containing 100 mM NaCl and 0.1 mM EDTA, at a flow rate of 0.2 ml min^{-1}. The calibration curve was constructed by plotting the logarithm of the molecular mass (log Mr) vs. the distribution coefficient (K_{av}) measured for each molecular mass standard: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) (Gel Filtration Calibration Kit – GE).

2.8. Stress tolerance assays

For the disk inhibition assay, recombinant *E. coli* cells were grown overnight at 37 °C in LB containing kanamycin (50 μg/ml), and then was diluted 1/50 in a fresh medium to OD_{630} of 0.6. These cells were induced for 1 h in presence of 0.1 mM IPTG. Then, 200 μl of cells with normalized OD_{630} were added in fresh top agar [agar 0.65% (w/v)] as a vehicle medium to following pouring into plates with the addition of kanamycin (50 μg/ml). A sterilized filter disk (6 mm in diameter) was placed in the middle of the plate and soaked with either 5 μl of 1 M methyl viologen (MV), 5 μl of 100 mM 2-methyl-benzoquinone (MBQ), 5 μl of 500 mM diamide, 5 μl of 1 M H_2O_2 or 5 μl of 10 mM *t*-BuOOH. The plates were incubated overnight at 37 °C. Results were statistically processed using ANOVA-test with a significant level of 95%.

2.9. Enzymatic assays

All the enzyme assays were performed at 30 °C, in a final volume of 50 μl, and using a Multiskan Ascent one-channel vertical light path filter photometer (Thermo Electron Co). TrxR activity was measured by monitoring the reduction of DTNB at 405 nm [21] in a reaction mixture comprising 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 200 μM NADPH, 1 mM DTNB, 20 μM *Lin*Trx and 3–1000 nM TrxR. Activity was calculated using the molar extinction coefficient of 13.6 $mM^{-1}\,cm^{-1}$ and considering that 1 mol of NADPH yields 2 mol of thionitrobenzoate. Steady-state kinetic analysis was performed using 0.5–20 μM *Lin*Trx, 3–200 μM NADPH and 17 nM TrxR. TrxR inhibition assays were performed using 17 nM TrxR, and different concentrations of each inhibitor: 500–1000 μM $NADP^+$, 1.6–100 μM Zn^{2+}, 1.6–100 μM Co^{2+}, 1.6–100 μM Cu^{2+}, 0.2–10 μM Hg^{2+}, 1.6–100 μM pyocyanin, 0.09–6.25 μM methylene blue, 0.05–3 μM mercurochrome or 1.6–100 μM quercetine. IC_{50} refers to the concentration of the inhibitor giving 50% of the initial activity [22].

Disulfide reductase activity of Trx system was determined by monitoring the oxidation of NADPH at 340 nm in a reaction mixture containing 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 200 μM NADPH, 1 μM TrxR, 0.25–16 μM *Lin*Trx and 15–1000 μM GSSG, 15–1000 μM cystine, 20–200 μM lipoamide or 20–200 μM bis-γ-glutamylcysteine (bis-γ-GC) [23].

The ability of Trx system to reduce *S*-nitrosothiols was evaluated by monitoring the oxidation of NADPH at 340 nm in a reaction mixture containing 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 200 μM NADPH, 1 μM TrxR, 2.5–32 μM *Lin*Trx and *S*-nitrosocysteine (CySNO), *S*-nitrosoglutathione (GSNO) or *S*-nitroso-γ-glutamylcysteine (γ-GCSNO) to final concentration of 0.125–1 mM. S-nitrosothiols were prepared according the methodology described by Arias et al. [19].

Diaforase activity of Trx system was measured by monitoring the oxidation of NADPH at 340 nm in the reaction mixture composed to 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 200 μM NADPH, 17 nM TrxR, 15–1000 μM MBQ, 15–1000 μM potassium ferricyanide, 15–1000 μM 1-chloro-2,4-dinitrobenzene (CDNB), 15–1000 μM sodium selenite or 1.56–100 μM methylene blue (MB).

Hydroperoxide detoxification activity of Trx system was determined by monitoring the oxidation of NADPH at 340 nm, in a reaction mixture containing 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 200 μM NADPH, 1 μM TrxR, 10 μM *Lin*Trx, 0.06-5 μM *Lin*AhpC and 100 μM H_2O_2 or 100 μM *t*-BuOOH as final acceptor. For steady-state kinetic analysis the assay was performed using 0.38–20 μM *Lin*Trx and 0.39–25 μM H_2O_2 or *t*-BuOOH [24].

Kinetic data were plotted as initial $v \cdot [\text{enzyme}]^{-1}$ (min^{-1}) versus substrate concentration (μM). The kinetic constants were acquired by fitting the data with a nonlinear least-squares formula and the Michaelis–Menten equation using the program Origin® 7.0. Kinetic constants were the means of at least three independent sets of data, and they were reproducible within ± 10%.

2.10. Determination of redox potential of LinTrx

Redox potential for *Lin*Trx was determined by following changes in the absorbance at 340 nm and 30 °C. Oxidized *Lin*Trx q(15–30 μM) was mixed with 100 μM NADPH in a total volume of 50 μl of 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, followed by the addition first of 0.5 μM *Lin*TrxR and then an excess of $NADP^+$ (1.2 mM) as described previously [23]. Redox potential was calculated according to the Nernst equation, based in the reactants concentration in the equilibrium. A value of −320 mV was used as redox potential of NADPH at pH 7.0.

2.11. Culture of Leptospira strains

Bacteria were grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium (which is an albumin-tween 80 medium at pH 7.4), and were incubated in static culture for 3–5 days at 28 °C [25,26]. The cell density was recorded at 420 nm in an S-26 spectrophometer (Boeco-Germany). The number of bacteria was estimated according the relation established for Louvel et al. [27] where $OD_{420\ nm}$ of 0.3– 0.4 represent $6–9 \cdot 10^8$ bacteria/ml.

2.12. Drug sensibility, oxidative stress tolerance and peroxide detoxification in vivo

Toxicity of different drugs, on the growth of *L. interrogans* was evaluated using bacteria in exponential phase. The cell density was normalized to $OD_{420\ nm}$ to 0.1 (in 1 ml) and this culture was exposed to 0.78–100 μM of these compounds for 5 days to 28 °C, and

after that, the cell density was determined and estimate the IC_{50} for each drug.

Tolerance to exogenous peroxides was evaluated using bacteria in exponential growth phase. Bacteria were normalized to $OD_{420\ nm}$ of 0.04 in phosphate-buffered saline [PBS (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH 7.4)]. The bacterial suspension was incubated to 28 °C, with 0.19–10 mM H_2O_2 or *t*-BuOOH. As control assay were used bacteria in the absence of oxidant compounds. AlamarBlue® (Thermo Scientific) was used to measure the relative viability.

For measure the ability to detoxify peroxides, bacterial suspensions ($OD_{420\ nm}$∼0.040) were incubated to 28 °C, with 100 μM H_2O_2 or *t*-BuOOH. The reaction was stopped at different times, and then the cultures were harvested at 10,000*g* by 5 min at 4 °C. The supernatants were recovered and kept on ice until the peroxide remnant was determined with the ferrithiocyanate method [28].

Antioxidant protein modulation experiments were performed using exponential *L. interrogans* or *L. biflexa* cells. Bacteria harvested by centrifugation at 10,000*g* for 10 min at 25 °C were washed twice with PBS buffer to remove residual medium components. The cells were incubated in PBS buffer (1 ml at final $OD_{420\ nm}$=0.135) with 1 mM *t*-BuOOH or 1 mM H_2O_2 at 28 °C for 4 h. Negative controls were performed in absence of peroxide. Subsequently, the cells were harvested and disrupted with reducing SDS-PAGE buffer. Protein extracts were analyzed by western blot using anti-*Lin*AhpC, anti-*Lin*TrxR, anti-*Lin*Catalase or anti-lipoprotein 32 (Lp32) as primary antibodies and HRP-conjugated anti-rabbit as secondary antibody. Bands were visualized using the ECL Western blotting detections reagents (Thermo Scientific).

2.13. Measurement of catalase activity by in-gel activity assay

A final volume of 800 μl of bacterial suspension (normalized to $OD_{420\ nm}$∼0.5) was incubated at 28 °C for 15 or 30 min, in absence or presence of *t*-BuOOH (25 or 50 μM). After that, the cultures were harvested at 10,000*g* for 10 min at 4 °C. The cellular pellet was recovered, disrupted using BugBuster® Protein Extraction Reagent (Merck Millipore) and finally centrifuged again at 10,000*g* for 15 min at 4 °C. The protein content of them was measured with BCA (Pierce™ BCA Protein Assay Kit, Thermo Scientific) and finally the activity was detected by native PAGE following the protocol described by Weydert et al. [29], using *Aspergillus niger* catalase (Sigma) as standard.

3. Results

3.1. Recombinant production of LinTrx, LinTrxR, LbiTrxR1 and LbiTrxR2

Nucleotide sequences coding for a putative Trx (*trxrA*, LIC11978) and a TrxR (*trxrB*, LIC11470) were obtained from the *L. interrogans* serovar Copenhagenii genome project (http://aeg.lbi.ic.unicamp.br/world/lic/). Additionally, *L. biflexa* genomic information (http://meta.microbesonline.org/) showed the presence of two genes coding for TrxR (*trxrB*, LEPBI_I2359 and *trxrB*-1, LBF_2230), and only one gene coding for Trx (LBF_2520). These specified genes were amplified by PCR from genomic DNA with primers specifically designed (see Section 2). Then, they were cloned into pGEM-T-Easy and their identities confirmed by DNA sequencing.

The *L. interrogans trxA* gene encodes a protein of 105 amino acids (*Lin*Trx), with a theoretical molecular mass of 11.48 kDa. *Lin*Trx has an additional cysteine residue next to the redox active side (WCGPC) in the primary structure (Supplementary data - Fig. 1), different from that observed in Trxs from other sources

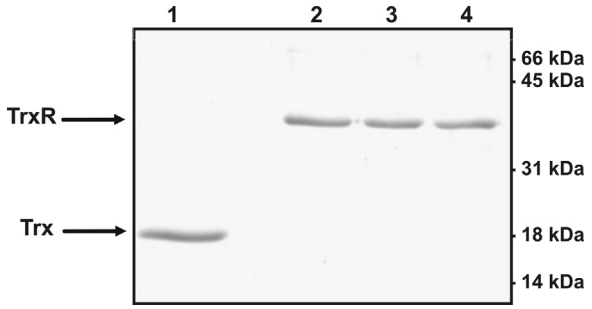

Fig. 1. SDS-PAGE of purified recombinant proteins. Purified recombinant proteins were defined by electrophoretic migration, under reducing and denaturing conditions, and subsequent staining with Coomassie blue. Lane 1: *Lin*Trx (2 μg), lane 2: *Lin*TrxR (1 μg), lane 3: *Lbi*TrxR1 (1 μg), and lane 4: *Lbi*TrxR2 (1 μg).

[30,31]. The *L. interrogans trxrB* and *L. biflexa* genes (*trxrB* and *trxrB-1*) encode for proteins of ∼310 amino acids with theoretical molecular masses ∼34 kDa (*Lin*TrxR and *Lbi*TrxR1 and *Lbi*TrxR2). The primary structure of *Lin*TrxR and *Lbi*TrxR1 reveals the presence of an NADPH binding domain, a flavin binding domain and a catalytic redox active site. The *L. biflexa trxB*-1 gene encodes a protein (*Lbi*TrxR2) with similar characteristics to other TrxRs, except that it lacks some residues in the NADPH binding domain (Supplementary data - Fig. 2), highly conserved between L-TrxRs. This latter feature has only been previously reported for the TrxR from Thermoplasma acidophilum [32]. Each amplified gene was cloned into the pET28c vector and expressed in *E. coli* BL21 (DE3) cells to produce the respective recombinant protein fused to an N-terminal His_6-tag. The analysis by SDS-PAGE (Fig. 1) under reducing conditions showed that the recombinant enzymes were produced and purified to near homogeneity (95% of purity estimated from the gel).

3.2. Characterization of native structure of LinTrx, LinTrxR, LbiTrxR1 and LbiTrxR2

The native molecular mass of the purified proteins was determined by size exclusion chromatography on Superdex 200 column. The purified TrxRs eluted as proteins of ∼70 kDa in gel filtration chromatography (data no shown), suggesting that each enzyme forms a homodimeric structure in its native (active) state. These results are in agreement with previous reports on TrxR from other sources [6]. The purified *Lin*TrxR, *Lbi*TrxR1 and *Lbi*TrxR2 proteins exhibited identical electrophoretic profiles in non-reducing SDS-PAGE, independently of the presence of 10 mM DTT or 10 mM diamide. *Lin*Trx has a monomeric structure (∼14 kDa) similar to other Trxs [5].

The UV–visible absorption spectrum of oxidized *Lin*TrxR, *Lbi*TrxR1 and *Lbi*TrxR2 displayed two peaks of maximal absorption at 375 and 450 nm (Fig. 2). For *Lin*TrxR and *Lbi*TrxR1, under aerobic conditions, the peak at 450 nm decreased after addition of 100 μM NADPH, indicating the reduction of a flavin prosthetic group. These spectral behaviors are characteristic of NAD(P)H-dependent flavoreductases, and more specifically of TrxR [33,34]. Under identical experimental conditions *Lbi*TrxR2 was insensible to the presence of NAD(P)H (no flavin reduction was observed), in agreement with results reported for TrxR from *T.* acidophilum [32].

In most flavoproteins the cofactor is tightly but not covalently bound and this seems to be the case for TrxRs from *Leptospira*, as a bright yellow compound was released after boiling the purified protein for 10 min. The resulting prosthetic group was identified as FAD by TLC (Fig. 3). The free flavin fraction from the samples compared to the standards (FAD or FMN, respectively), indicates that FAD forms the prosthetic group in these enzymes (Fig. 3). These results indicate that *Lin*TrxR and *Lbi*TrxRs have similar

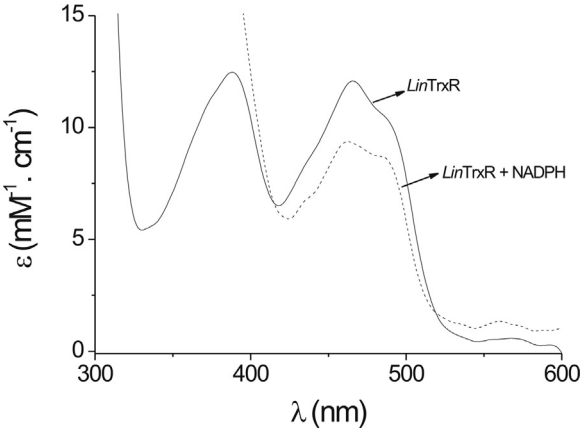

Fig. 2. Absorption UV–visible spectrum of *Lin*TrxR. The assays were performed at 25 °C and pH 7.0. The molar extinction coefficients (ε) were calculated using different *Lin*TrxR concentrations (6, 11 and 22 μM). Oxidized *Lin*TrxR (solid line), and *Lin*TrxR plus 100 μM NADPH (dashed line).

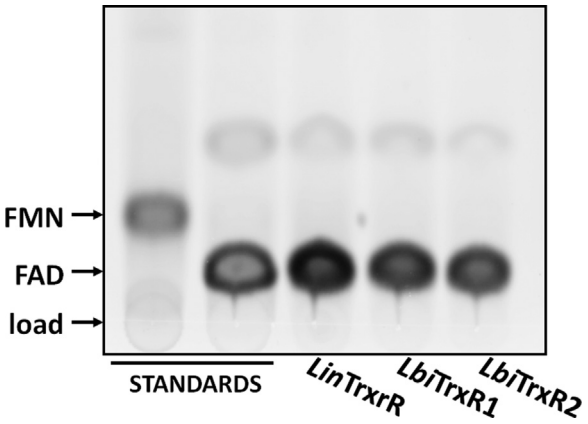

Fig. 3. Determination of flavins by thin layer chromatography. Chromatography was revealed by fluorescence in a Typhoon scanner. The mobile phase was a solution of butanol:acetic acid:water (12:3:5). Commercial FMN and FAD (standard), and supernatants obtained after a heat treatment (to obtain the cofactor of the protein) of purified *Lin*TrxR, *Lbi*TrxR1 and *Lbi*TrxR2 were loaded.

structural properties than other members of L-TrxR family [19,35].

3.3. Kinetic properties of the Trx system from Leptospira species

To demonstrate the functionality of the recombinant proteins, we performed the complete *in vitro* enzymatic characterization. In a first approach we employed DTNB reduction assay (classical assay to measure TrxR activity, [19]) to evaluate the functionality of *Lin*TrxR, *Lbi*TrxR1 and *Lbi*TrxR2. Only *Lin*TrxR and *Lbi*TrxR1 were able to reduce *Lin*Trx specifically using NADPH as electron donor and DTNB final acceptor (Fig. 4). Under these conditions, the enzymatic systems exhibited maximal activity at pH ~ 7.0 (Supplementary data - Fig. 3). However, no activity was detected for any of the enzymes when NADH (in equivalent concentration) was used instead of NADPH (data not shown), a result that is consistent with previous reports concerning the enzymatic behavior of L-TrxRs [8]. Unlike the other tested TrxRs, *Lbi*TrxR2 did not exhibit TrxR activity with this classic *in vitro* assay using NAD(P)H as reducing substrate, Fig. 4. Alternatively, we evaluated the ability of redox proteins, such as [2Fe2S] ferredoxin, [4Fe4S] ferredoxin or flavodoxin (plus ferredoxin-NADP reductase and NADPH), for reducing *Lbi*TrxR2 and no activity was detected (data not shown).

Steady state kinetic assays indicate that the NADPH-dependent

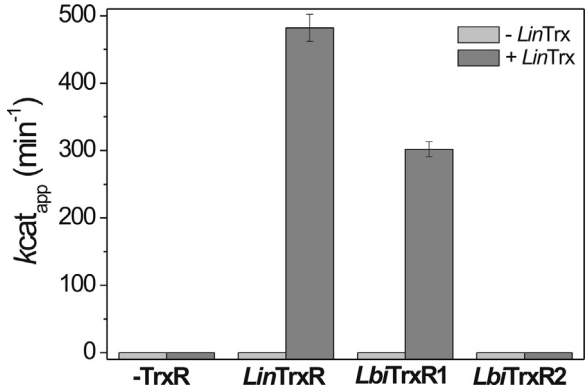

Fig. 4. Functionality of the Trx system. DTNB reduction assays were performed at 30 °C and pH 7.0 in the presence of 200 μM NADPH, with or without 3 μM *Lin*Trx and the respective TrxR. Activity was calculated using the molar extinction coefficient of 13.6 mM^{-1} cm^{-1} and considering that 1 mol of NADPH yields 2 mol of thionitrobenzoate.

reduction of *Lin*Trx followed Michaelis–Menten kinetics (Fig. 5), exhibiting the kinetic parameters detailed in Table 1. Results in Table 1 indicate that *Lin*TrxR and *Lbi*TrxR1 exhibit relatively high values of affinity toward NADPH, and of catalytic efficiency ($k_{cat} \cdot Km^{-1}$) for reducing *Lin*Trx. These values are practically in the same order of magnitude than parameters reported for other L-TrxRs [35]. In addition, these enzymes were specific for *Lin*Trx reduction and no activity was observed when *Ppe*Trxh, *Eco*Trx or *Ehi*Trx8 were evaluated instead *Lin*Trx (data no shown).

Moreover, we evaluated the capacity of *Lin*TrxR and *Lbi*TrxR1 to reduce non-Trx substrates, such as MBQ, $[Fe(CN)_6]^3$, CDNB, SeO_3^{2-} or low molecular mass disulfides (such as GSSG, cysteine or lipoamide). The kinetic data showed that only MBQ and $[Fe(CN)_6]^{3-}$ could be used as electron acceptor substrates for the enzymes (Table 1), the reduction of them following a Michaelis–Menten kinetic (data no shown). The calculated catalytic efficiencies for MBQ and $[Fe(CN)_6]^{3-}$ reduction were between two and three order of magnitude lower than the obtained for Trx-reduction (Table 1). These results show us the low promiscuity of these TrxRs in comparison to other L-TrxRs [36–38].

The inhibitory capacity of $NADP^+$ on TrxR activity of *Lin*TrxR and *Lbi*TrxR1 was evaluated. Firstly, we determined the enzymes activity in presence of different concentrations of NADPH, at fixed concentration of *Lin*Trx and variable concentrations of $NADP^+$. The $NADP^+$ presented a competitive inhibition mechanism respect to NADPH with a K_{ic} of 90 μM for *Lin*TrxR and K_{ic} of 128 μM for *Lbi*TrxR1. Secondly, we evaluated the enzymes activities in presence of different concentrations of *Lin*Trx, at fixed concentration of NADPH, and different $NADP^+$ concentrations. The results indicate that the $NADP^+$ acted as mixed inhibitor regarding *Lin*Trx to both enzymes. The calculated inhibition constants were K_{ic} of 1.7 mM and K_{iu} of 2.2 mM for *Lin*TrxR, being the constants for *Lbi*TrxR1 K_{ic} of 1.1 mM and K_{iu} of 0.9 mM. These results support the idea that both substrates bind to different sites in the enzymes, as it was previously found for TrxR from other sources [39,40]. Additionally, it was evaluated the inhibitory effect of divalent cations on the TrxR reductase activity of *Lin*TrxR and *Lbi*TrxR1. Only Zn^{2+}, Co^{2+}, Cu^{2+} and Hg^{2+} exhibited a significant inhibitory effect (Fig. 6 and Supplementary data - Fig. 4), with the respective IC_{50} values detailed in Table 2. The inhibitory effects of Zn^{2+}, Co^{2+} and Cu^{2+} were partially recovered with EDTA treatment (data no shown). These results support the participation of cysteine residues in the disulfide reductase active site of these enzymes, because the binding of metal ions block the ability of a thiolate group to participate in thiol/disulfide exchange reactions [41], thus inhibiting the TrxR activity.

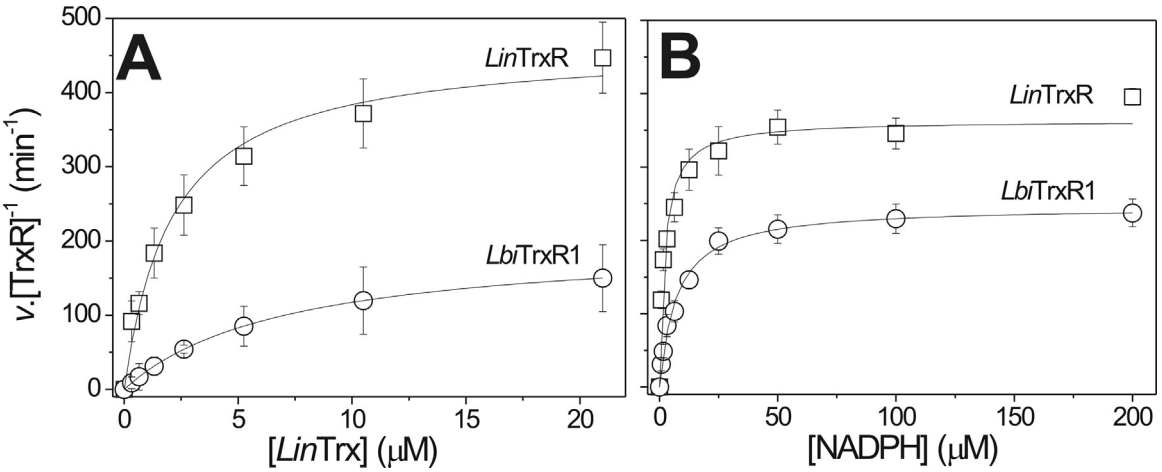

Fig. 5. Kinetic of NADPH-dependent *Lin*Trx reduction by *Lin*TrxR and *Lbi*TrxR1**.** Assays were performed at 30 °C and pH 7.0 in the presence of 5 mM DTNB and 17 nM of each TrxR, and with different concentrations of *Lin*Trx (0.5–20 μM) and 200 μM NADPH (A) or different concentrations of NADPH (3–200 μM) and 20 μM *Lin*Trx (B).

Table 1
Apparent kinetic parameters of *Lin*TrxR or *Lbi*TrxR1 for the reduction of disulfide and non-disulfide substrates at pH 7.0 and 30 °C. N.D.: not determined.

Protein	Co-substrate	Substrate	*K*m (μM)	k_{cat} (min^{-1})	k_{cat}/*K*m ($M^{-1}\,s^{-1}$)
***Lin*TrxR**	*Lin*Trx 14 μM	NADPH	2.2 ± 0.3	363 ± 11	$2.8 \cdot 10^6$
	NADPH 200 μM	*Lin*Trx	2.1 ± 0.3	464 ± 20	$3.7 \cdot 10^6$
		Na_2SeO_3	N.D.	N.D.	N.D.
		CDNB	N.D.	N.D.	N.D.
		DTNB	908 ± 73	12.3 ± 0.3	$2.3 \cdot 10^2$
		MBQ	1369 ± 392	637 ± 149	$7.6 \cdot 10^3$
		$K_3Fe(CN)_6$	369 ± 79	625 ± 101	$2.8 \cdot 10^4$
***Lbi*TrxR1**	*Lin*Trx 14 μM	NADPH	7 ± 1	246 ± 20	$5.9 \cdot 10^5$
	NADPH 200 μM	*Lin*Trx	6.7 ± 0.8	802 ± 74	$1.9 \cdot 10^6$
		Na_2SeO_3	N. D.	N. D.	N.D.
		CDNB	N. D.	N. D.	N.D.
		DTNB	1514 ± 291	29 ± 3	$3.2 \cdot 10^2$
		MBQ	132 ± 21	856 ± 45	$1.1 \cdot 10^5$
		$K_3Fe(CN)_6$	383 ± 97	198 ± 22	$8.6 \cdot 10^3$

3.4. Low molecular mass disulfure reduction by LinTrx and its redox capacity

The disulfide activity measurement for *Lin*Trx was performed following the NADPH oxidation in a reaction containing a kinetic excess of *Lin*TrxR. Under these conditions, reaction (1) occurs fast, and generates enough reduced *Lin*Trx, which then acts on various disulfides using a non-enzymatic redox exchange according to reaction (2).

$$NADPH + Trx - S_2 \leftrightarrow NADP^+ + Trx\,(SH)_2 \quad (1)$$

$$Trx(SH)_2 + RSSR \rightarrow Trx{-}S_2 + 2RSH \quad (2)$$

By this way, the rate law $v = k \cdot [Trx(SH_2)] \cdot [RSSR]$ can be proposed to estimate the second order rate constant (k) for disulfide substrate reduction by *Lin*Trx. The values obtained for the enzyme at pH 7.0 and 30 °C were: 64 $M^{-1}\,s^{-1}$ for lipoamide, 50 $M^{-1}\,s^{-1}$ for cystine, 30 $M^{-1}\,s^{-1}$ for GSSG and 33 $M^{-1}\,s^{-1}$ for bis-γ-GC. The calculated catalytic efficiencies for low molecular mass disulfide reduction by *Lin*Trx are similar to those obtained for Trx from other organisms [42–45].

S-nitrosothiols (SNOs) are produced for host cells with defence purposes during infective process [46]. The reduction of nitrosothiols by *Lin*Trx, suggests that pathogenic bacteria possess enzymatic systems to resist damage generated by these toxic nitrogen agents. *Lin*Trx was neither effective to reduce CySNO nor GSNO, but able to reduce γ-GCSNO ($k = 138\,M^{-1}\,s^{-1}$). The distinctive enzyme specificity for γ-GCSNO could be part of a detoxification system to cope the stress generated by RNS.

To better evaluate the capacity of the redox system present in *L. interrogans* involving TrxR, we determined the redox potential of *Lin*Trx following the approach previously descripted [23]. As shown in Supplementary data - Fig. 5, the reaction between oxidized *Lin*Trx and NADPH, catalyzed by LinTrxR, was found reversible. The $E^{\circ\prime}$ for *Lin*Trx was calculated in -290 ± 5 mV, a value

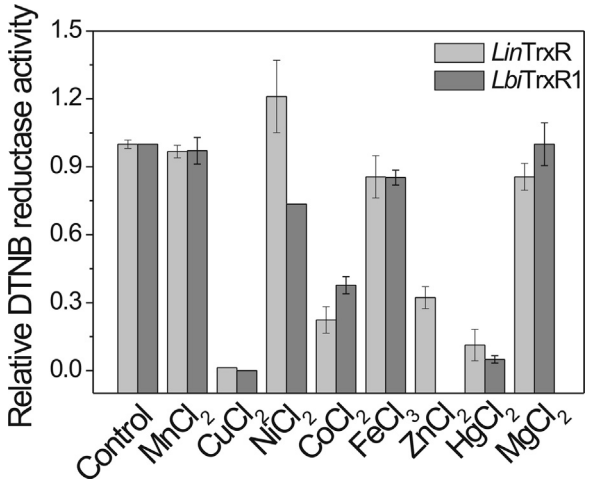

Fig. 6. Inhibitory effect of heavy metal ions on the DTNB reductase activity of *Lin*TrxR and *Lbi*TrxR1. Assays were performed at 30 °C and pH 7.0 in the presence of 5 mM DTNB, 200 μM NADPH and 100 μM of the respective heavy metal ion.

Table 2
Inhibitory effect of heavy metal ions on the activity of TrxR. The assay was performed at 30 °C and pH 7.0.

Enzyme	Me^{2+}	IC_{50} (μM)
***Lin*TrxR**	Zn^{2+}	80 ± 2
	Co^{2+}	100 ± 22
	Cu^{2+}	10.5 ± 0.3
	Hg^{2+}	0.82 ± 0.06
***Lbi*TrxR1**	Zn^{2+}	5 ± 1
	Co^{2}	20 ± 5
	Cu^{2+}	28 ± 1
	Hg^{2+}	0.28 ± 0.04

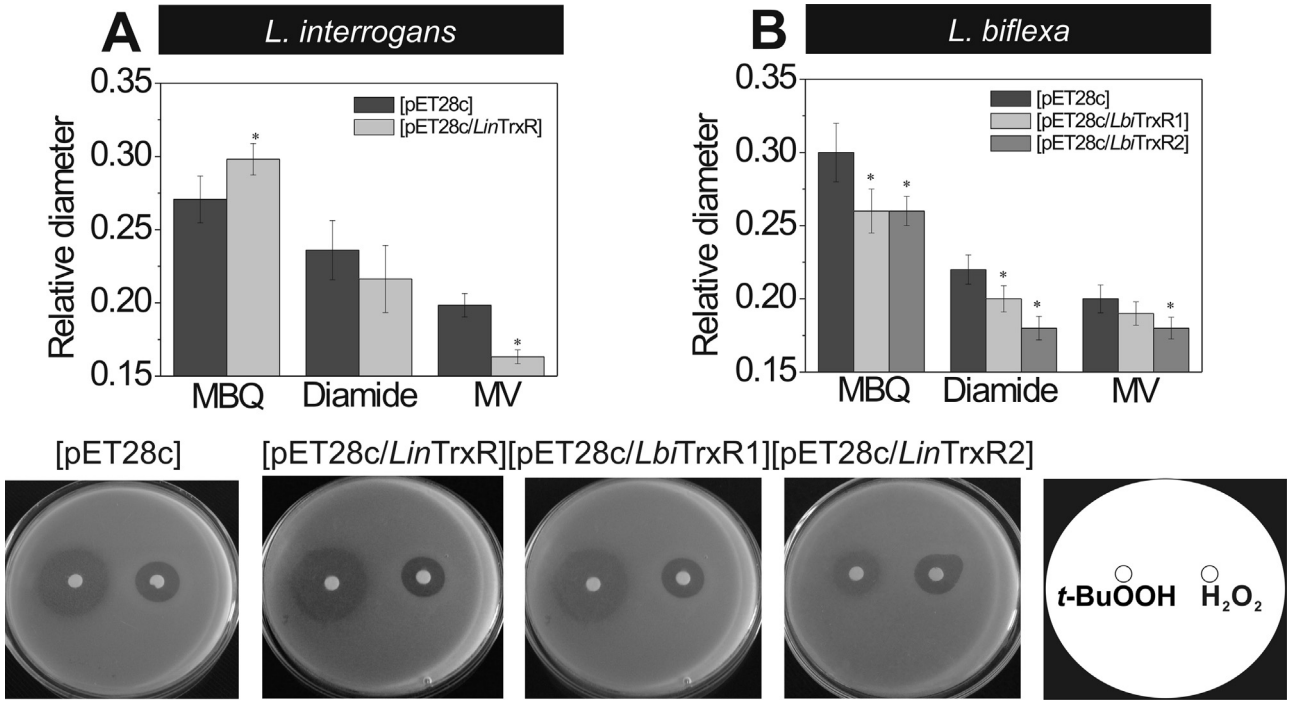

Fig. 7. Disk inhibition assays for growing of transformed *E. coli* in presence of oxidant agents. *E. coli* cells transformed with pET28c plasmid (control assay) or with pET28c/*Lin*TrxR, pET28c/*Lbi*TrxR1 or pET28c/*Lbi*TrxR2 vector, and with an OD_{630} of 0.6 were induced for 1 h in presence of 0.1 mM IPTG. Then, cells with normalized OD_{630} were added in fresh top agar with the addition of kanamycin (50 μg ml^{-1}). Finally, these cells were exposed to 1 M methyl viologen (MV), 0.1 M 2-methyl-benzoquinone (MBQ), 0.5 M diamide, 1 M H_2O_2 or 0.01 M *t*-BuOOH. After the overnight incubation of the plates at 37 °C, the differences between control and *Lin*TrxR transformed cells were reliable repetition in at least three independent experiences. (*): indicates statistically significant difference ($p < 0.05$). Relative diameter = inhibitory ring diameter (cm)/plate diameter (cm).

quite similar to that reported for TRX from different organisms [23]. Also, the $E^{\circ\prime}$ exhibited by *Lin*Trx suggests that this protein could be playing a key role in the redox metabolism of the bacteria, being involved in the interchange of reducing equivalents with different metabolites. In this way, the Trx system could be operative not only working together with *Lin*AhpC ($E_{m\ 7.5} = -217$ mV, [13]) to detoxify hydroperoxides, as previously reported, but also has the appropriate redox potential to interact with other low molecular weight thiol compounds.

3.5. Disk inhibition assays

After evaluating the properties of the recombinant proteins *in vitro*, we performed disk inhibition assays to test the enzymes functionality *in vivo*. Inhibition rings were observed in the presence of different oxidant compounds such as MV, MBQ, diamide, H_2O_2 or *t*-BuOOH. The plates containing *E. coli* cells producing *Lin*TrxR or *Lbi*TrxR1 showed inhibition rings of lower diameter than control plates (*E. coli* cells transformed with pET28c) in the presence of MV (Fig. 7A-B). Instead, no significant difference was observed between the control and cells expressing *Lin*TrxR or *Lbi*TrxR1 when they were grown in the presence of H_2O_2, *t*-BuOOH or diamide (Fig. 7A-B). On the other hand, cells expressing *Lin*TrxR were more sensitive to MBQ (Fig. 7A); which is expected from the fact that MBQ behaved as a substrate of *Lin*TrxR (see Table 1). This result suggests that reduction of this quinone by *Lin*TrxR followed by a redox cycling would turn this antioxidant protein into a pro-oxidant enzyme. Interestingly, *E. coli* cells overexpressing *Lbi*TrxR2 showed a lower inhibition than the control in presence of MBQ or peroxides (Fig. 7), which suggests that *Lbi*TrxR2 could present an antioxidant function *in vivo*.

3.6. Peroxide detoxification and antioxidant activity of the bacterial cell extract

Bacteria contain enzymatic systems to detoxify both endogenous and exogenous peroxides [47], after which we decided to study the peroxidase activity by *in vitro* and *in vivo* assays. Thus, an *in vitro* assay was performed to evaluate the ability of the reduced *Lin*Trx to operate together with *Lin*AhpC in the NADPH-dependent reduction of peroxides (Fig. 8). In this coupled system, the rate of H_2O_2 or *t*-BuOOH reduction was dependent of the *Lin*AhpC concentration (data no shown). Table 3 shows the kinetic parameters for Trx-dependent reduction of H_2O_2 and *t*-BuOOH by *Lin*AhpC, which have an apparent hyperbolic mechanism (Fig. 8). *Lin*AhpC was able to act with homologous reducing systems with catalytic efficiencies comparable to those previously obtained using *E. coli* Trx system [13].

To further evaluate the physiological relevance of the enzymatic Trx system, we performed two additional *in vivo* assays. First, we evaluated how the presence of *t*-BuOOH or H_2O_2 affects the cell viability of *L. interrogans* and *L. biflexa* cultures. Similar IC_{50} values were obtained for both peroxides ($IC_{50\ H_2O_2} = 0.33 \pm 0.05$ mM and $IC_{50\ t\text{-BuOOH}} = 0.42 \pm 0.1$ mM) for *L. interrogans* cells, which suggests that this organism has the same tolerance to both peroxides (Fig. 9A). It has been reported the strong catalase activity from *L. interrogans* [48]; however, this activity was inactivated by *t*-BuOOH (Fig. 9B), in agreement with previous findings in other organisms [49]. Thus, our results support the existence of a redox scenario integrated with a group of proteins (besides catalase) that function together to help the cell to cope with oxidative stress. These redox macromolecules include *Lin*AhpC and other peroxiredoxins identified in the *L. interrogans* genome project: glutathione peroxidase-like protein, LIC12648; glutahione peroxidase-like protein, LIC13442; thiol-peroxidase, LIC12765; and two bacterioferritin comigratory protein, LIC20093 and LIC10732. On the other hand, *L. biflexa* presented higher IC_{50}

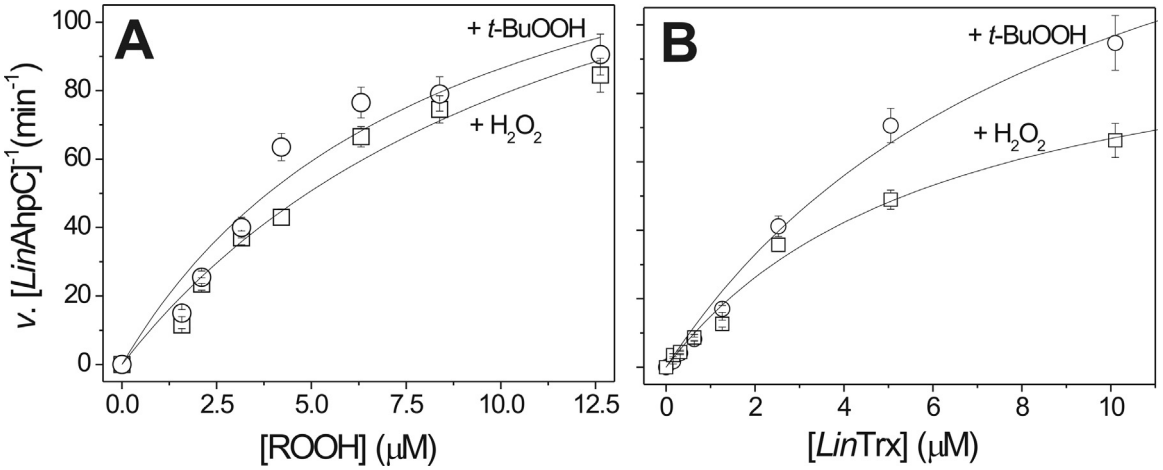

Fig. 8. Trx peroxidase activity of *Lin*AhpC. A) Kinetics of *t*-BuOOH or H_2O_2 reduction by His-tagged *Lin*AhpC. Reactions were performed in the presence of 10 μM *Lin*Trx and 0.39–12.5 μM of H_2O_2 or *t*-BuOOH. B) Kinetics of *Lin*Trx oxidation by His-tagged *Lin*AhpC. Reactions were conducted in the presence of 100 μM of H_2O_2 or *t*-BuOOH, and using 0.38–20 μM *Lin*Trx. Kinetic parameters were determined by monitoring NADPH consumption at 340 nm. All reactions were executed at pH 7.0 and 30 °C.

Table 3
Apparent kinetic parameters for *Lin*AhpC at pH 7.0 and 30 °C.

Protein	Co-substrate	Substrate	*K*m (μM)	k_{cat} (min^{-1})	k_{cat}/Km ($M^{-1}\,s^{-1}$)
***Lin*AhpC**	*Lin*Trx 10 μM	*t*-BuOOH	7 ± 2	157 ± 27	$3.7 \cdot 10^5$
	*Lin*Trx 10 μM	H_2O_2	10 ± 2	159 ± 21	$2.7 \cdot 10^5$
	t-BuOOH 100 μM	*Lin*Trx	9 ± 2	186 ± 24	$3.4 \cdot 10^5$
	H_2O_2 100 μM	*Lin*Trx	6 ± 1	109 ± 13	$3.0 \cdot 10^5$

value for *t*-BuOOH than for H_2O_2 ($IC_{50\ H_2O_2} = 0.07 \pm 0.01$ mM and $IC_{50\ t\text{-BuOOH}} = 3.5 \pm 0.9$ mM) (Fig. 9A). This effect is consistent with the low catalase activity described for this bacterium [50], since the function of this enzyme is critically important for exogenous H_2O_2 consumption [47]. Moreover, we tested the ability of both bacteria to detoxify *t*-BuOOH or H_2O_2, thus determining the residual peroxide at different times using the method of ferrithiocyanate. The result showed that detoxification of H_2O_2 was faster but that of *t*-BuOOH was slower in *L. interrogans* than in *L. Biflexa* (data no shown). The latter supports the importance of catalase activity for pathogenic species, and the possible implication of the enzyme in the virulence of microorganisms [12].

By performing western blot experiments using polyclonal antibodies raised against the recombinant enzyme, we evaluated the abundance of *Lin*TrxR (about 0.019 ± 0.002% of the total protein) in cellular extracts from *L. interrogans*. Signal recognition of a protein band of ~35 kDa molecular mass was visualized, which correlates with the M_r of *Lin*TrxR (Supplementary data - Fig. 6). The α-*Lin*TrxR antiserum was also used to evaluate the presence of *Lbi*TrxR1 and *Lbi*TrxR2 in *L. biflexa* cells. The antibody was only able to detect recombinant *Lbi*TrxR1, but not *Lbi*TrxR2 (data no shown). However, we proved the gene transcription of *Lbi*TrxR2 using RT-PCR. The transcript was checked by agarose gel electrophoresis, as a band of ~900 pb (Supplementary data - Fig. 7), thus suggesting that the enzyme is constitutively translate in *L. biflexa*.

Taking into account the possible involvement of Trx system (together AhpC) in peroxide detoxification *in vivo*, we tested the effect of peroxide exposure on protein modulation by western blot experiment (Fig. 10). As shown, no change in TrxR level of stressed leptopires was observed; however, we noted an increase in signal intensity of AhpC in both bacteria exposed to H_2O_2 or *t*-BuOOH with respect to that in unchallenged ones. Similar effects were observed previously [13]. These results suggest that AhpC (a peroxiredoxin capable of reducing both peroxides) contributes significantly to the antioxidant defense *in vivo*. It is important to note that the Trx system showed to be an efficient reducer of this peroxiredoxin (Table 3). This would strengthen the important role of Trx as an antioxidant system, since it is the only supplier of reducing equivalents to the peroxiredoxin *in vivo* described so far. In addition, it is worth to note the decrease in the level of catalase when *L. interrogans* cells were exposed to *t*-BuOOH (Fig. 10); which might probably be due to the inactivation effect of the peroxide previously described. This result supports the importance of AhpC (in association with the Trx system) in the detoxification of peroxides in this pathogenic bacterium.

In addition to antioxidant enzymes, low molecular mass thiols have an essential function in scavenging ROS and RNS, being important to keep the cellular redox balance. We further analyzed the ability of *Lin*Trx to reduce low molecular thiols by estimating the total content of thiols in the pathogenic and non-pathogenic species under oxidative stress. Cellular suspensions were incubated in the presence or absence of 5 mM H_2O_2, and then the amount of thiols was measured using DTNB. *L. interrogans* exhibited large amount of thiols, both under basal (without H_2O_2, 5 ± 1 pmol/10^7 bacteria) and stress (2.8 ± 0.8 pmol/10^7 bacteria) conditions in comparison with *L. biflexa* (0.4 ± 0.1 pmol/10^7 bacteria and 0.010 ± 0.008 pmol/10^7 bacteria without and with H_2O_2, respectively). These results indicate that *L. interrogans* is better fortified than *L. biflexa* to cope with oxidative stress, which could be consistent with a role of these metabolites in pathogenesis exerted by *L. interrogans* [51].

3.7. Evaluation of inhibitors by in vitro and in vivo assays

Assays on the inhibition of the disulfide reductase activity of purified *Lin*TrxR showed that mercurochrome was the best inhibitor ($IC_{50} = 0.34 \pm 0.09$ μM), followed by methylene blue ($IC_{50} = 0.8 \pm 0.3$ μM), quercetine ($IC_{50} = 17 \pm 4$ μM) and pyocyanin ($IC_{50} = 19 \pm 7$ μM). Similar assays performed with *Lbi*TrxR1 revealed the following behavior for the inhibitors: mercurochrome ($IC_{50} = 0.099 \pm 0.009$ μM); methylene blue ($IC_{50} = 2.8 \pm 0.6$ μM), quercetine ($IC_{50} = 25 \pm 3$ μM) and pyocyanin ($IC_{50} = 9 \pm 1$ μM). Subsequently, we evaluated the different chemicals on *L. interrogans* cultures (Fig. 11), observing that methylene blue and mercurochrome caused growth inhibition ($IC_{50} = 7 \pm 1$ μM and $IC_{50} = 6 \pm 2$ μM, respectively), which is could be associated with results above described for *in vitro* assays performed with recombinant *Lin*Trx. Thus, the *Lin*Trx system could be considered as a promising molecular target for drug design. On the contrary,

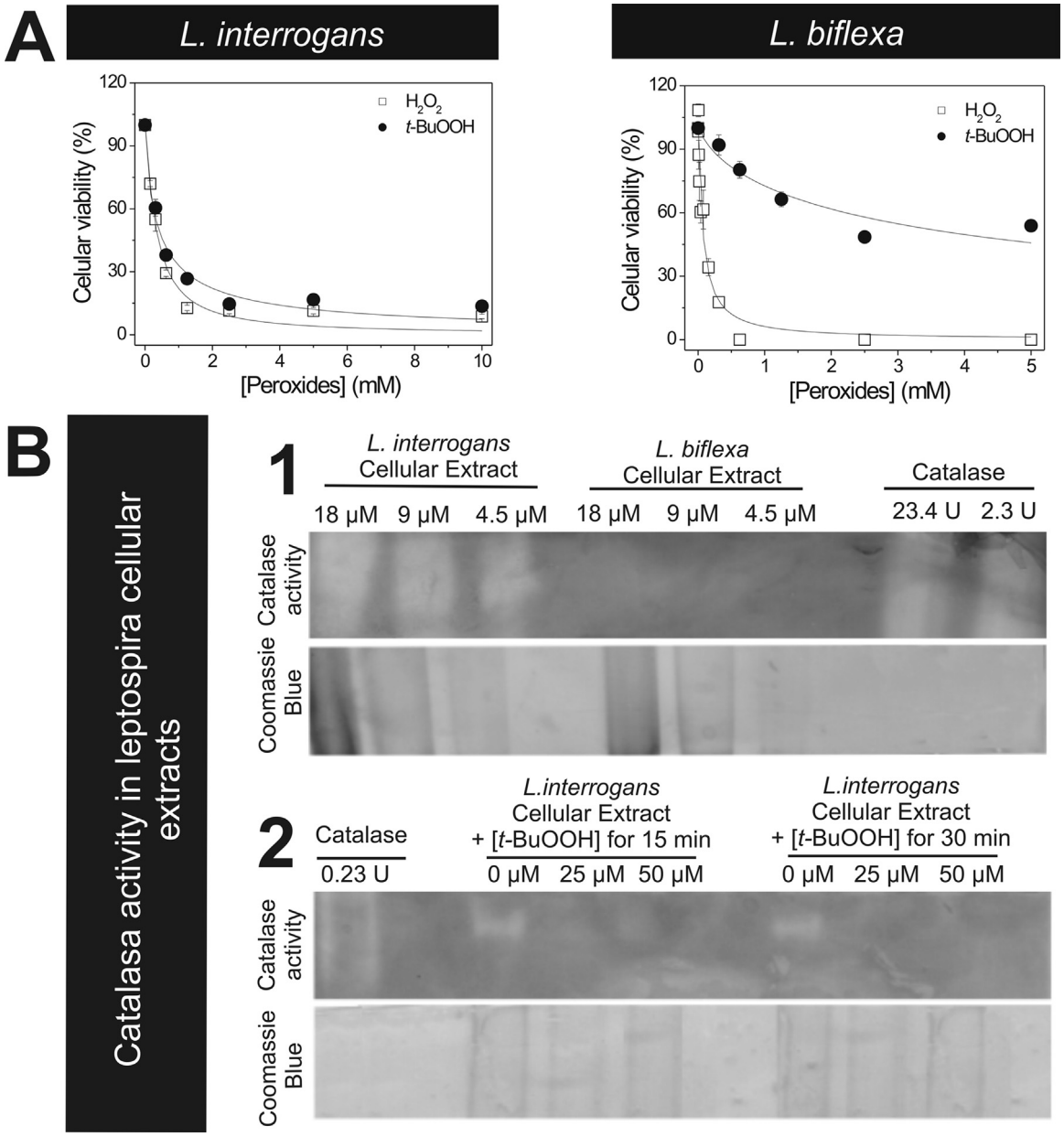

Fig. 9. Cell viability under oxidative stress. A) Bacterial suspension ($OD_{420\ nm}$~0.04) were incubated at 28 °C, with different amounts of H_2O_2 or *t*-BuOOH (0–10 mM) and AlamarBlue® which is a proven indicator for cell viability. B) Catalase activity in gel. 1) Catalase activity was evaluated in *Leptospira spp* cellular extracts, containing 4.5, 9, 18 µg of protein 2) *L. interrogans* bacterial suspensions ($OD_{420\ nm}$ ~0.5) were incubated to 28 °C for 15 min or 30 min, in absence or in presence of *t*-BuOOH (25–50 µM). After that, the cell extracts were prepared using BugBuster® Protein Extraction Reagent (Merck Millipore) and finally, different amount of these were sown on 8% native gels.

pyocianin did not exhibit any effect on cells growth (Fig. 11). Ampicillin, the drug of reference in leptospirosis [52,53], had an $IC_{50}=33\pm6$ µM and was used as experimental control. Interestingly, quercetin stimulated the growth of *L. interrogans*. This effect was observed previously in other pathogenic organisms such as *Clostridium perfringens* or *Bacteroides fragilis* [54] and could be due to the ability of this polyphenol (or metabolic derivatives) to present antioxidant activity over cultures. Similar effect was observed in *Lactobacillus casei* cultures in presence of antioxidants [55].

Methylene blue fulfils the criteria for a BONARIA drug and it has been used in clinical trials for many decades [56]. Since methylene blue proved to be a potent inhibitor of *Lin*TrxR *in vitro*, we further advanced characterizing its mechanism of action (Fig. 12). We could not define it as a classical inhibitor, since it acted as a subversive substrate; meaning that it has the ability to convert an antioxidant disulfide reductase enzyme into a pro-oxidant [57, 58]. Thus, both proteins, *Lin*Trx and *Lib*TrxR, could be a target for the attack of phenothiazine derivatives, and also both proteins could have ability to reduce it. We employed a DTNB reduction reaction to follow the loss of *Lin*TrxR activity, showing that methylene blue can be reduced not only by TrxR but also by Trx, highlighting a synergistic effect of increasing concentrations of Trx in the presence of TrxR (Fig. 12A). They were necessary very high concentrations of *Lin*TrxR (in absence of *Lin*Trx) to reduce methylene blue (Fig. 12B). Methylene blue reduction followed Michaelis-Menten saturation kinetics (Fig. 12A), with values of *K*m for methylene blue of 12 µM, a k_{cat} of 91 min^{-1} and a $k_{cat}\cdot Km^{-1}$~10^5 M^{-1} s^{-1}. Although in our study, the kinetic constants were obtained in the presence of *Lin*Trx, which apparently has the capacity to donate electrons to methylene blue, the calculated values were similar to those reported for other

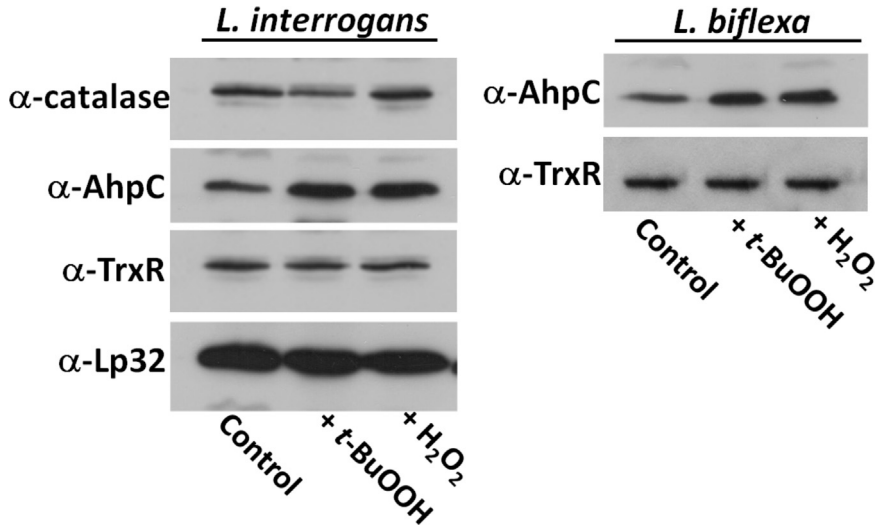

Fig. 10. Western blot analysis of the effect of peroxide exposure on antioxidant protein expression in Leptospira. Bacteria (*L. interrogans* or *L. biflexa*) in exponential phase were washed twice with PBS. The cell pellets were suspended in PBS to have a bacterial suspension with $OD_{420\ nm}=0.135$. Bacterial suspensions (1 ml) were incubated with 1 mM *t*-BuOOH or 1 mM H_2O_2 at 28 °C for 4 h. Negative controls were performed in absence of peroxide. After the incubation, the bacteria were harvested and suspended in SDS-PAGE sample buffer. The Samples were analyzed by western blot experiments. Rabbit anti-*Lin*Catalase antibody (1/1000), anti-*Lin*TrxR (1/1000), anti-*Lin*AhpC (1/1000), anti-Lp32 (1/5000) and HRP-conjugated anti-rabbit antibody (1/10,000; Thermo Scientific) were used (1 h of incubation each), and the signal was detected with ECL (Thermo Scientific).

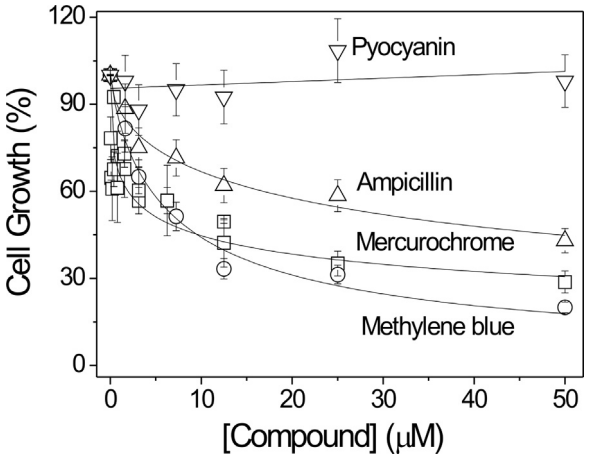

Fig. 11. *L. interrogans* cell growth inhibition by different compounds. Bacteria with a normalized cell density to $O.D_{405\ nm}$~0.1 (in 1 ml) were exposed to 1.6–100 μM of ampicillin, pyocianin, methylene blue, mercurochrome or quercetin, for 5 days to 28 °C. After that, the cell densities were determinate and estimate the IC_{50} for each drug. The methylene blue ($IC_{50}=7\pm1$ μM), mercurochrome ($IC_{50}=6\pm2$ μM) and the ampicillin ($IC_{50}=33\pm6$ μM) cause inhibitory effect on cell culture, while pyocyanin not cause effect.

flavoenzymes, such as *Plasmodium falciparum* GR [31].

4. Discussion

L. interrogans is the causative agent of leptospirosis, a disease with a mortality of about 30%. In humans the severity of the infection varies with the serovar of *Leptospira* involved and its virulence, together with the health and immune status of the patient. Pathogenic leptospiras are invasive, mainly due to their ability to survive and grow in tissues, escaping natural host-defense mechanisms, such as complement system and phagocytic cells, similar to others microbial pathogens [1,14,59,60]. Canale-Parola [61] has previously speculated that pathogenic spirochetes are derived from free-living and commensal forms. Herein, we have performed a comparative functional characterization of the functionally of the enzymes conforming the Trx system from pathogenic and saprophytic species. Our results aim to contribute to the right assignment of structure-function relationships for these enzymes, adding value to the data available from the genome project. Results support the occurrence of functional differences between the antioxidant metabolism in pathogenic and free-living *Leptospira* spp.

The structural and functional properties of TrxRs indicate that the enzymes belong to the L-TrxRs, presenting homodimeric structures. Each monomer has an NADPH binding site (incomplete in *Lbi*TrxR2), a FAD binding site and a redox active site composed by two cysteine residues. The recombinant enzymes, *Lin*TrxR and *Lbi*TrxR1, were able to catalyze the NADPH-dependent reduction of *Lin*Trx. *In vivo*, the reducing equivalents from NADPH (as reducing equivalent donors), can be obtained from tricarboxylic acid cycle and the oxidative pentose phosphate pathway [8,60,62]. Although exhibiting lower catalytic efficiency, we also evidenced that these TrxRs can use other non-physiological substrates such as MBQ and potassium ferricyanide. We found that both enzymes, *Lin*TrxR and *Lbi*TrxR1, were sensitive to the inhibition by heavy metal ions, supporting the involvement of reactive thiols in the activity reducing disulfide substrates. Furthermore, *Lin*TrxR and *Lin*Trx were able to work together with *Lin*AhpC, catalyzing the NADPH-dependent reduction of peroxides. The peroxiredoxin (*Lin*AhpC) exhibited similar kinetic properties using both homologous and heterologous Trx system (using *E. coli* Trx and TRXR) [13]. These results support that this system would operate *in vivo* to eliminate H_2O_2 and lipid hydroperoxides with similar efficiency. However, as we informed previously [13] the peroxiredoxin is inactivated after over-oxidation by elevated concentration of peroxides. The *t*-BuOOH can inhibit most of the catalase-like activity in *L. interrogans*, which highlights the importance of other proteins with peroxidase activity to eliminate organic peroxides and maintain the redox balance.

In the case of *L. biflexa*, the functionality of *Lbi*TrxR2 was evidenced only after disc inhibition assays, where *E. coli* cells overexpressing *Lbi*TrxR2 showed high tolerance to the oxidative stress generated by several oxidant compounds. Analysis of the amino acid sequence of *Lbi*TrxR2 suggests that the lack of activity *in vitro* could be due to neutral and non-polar amino acids replacing the canonical arginine-rich motif. The last motif is conserved between

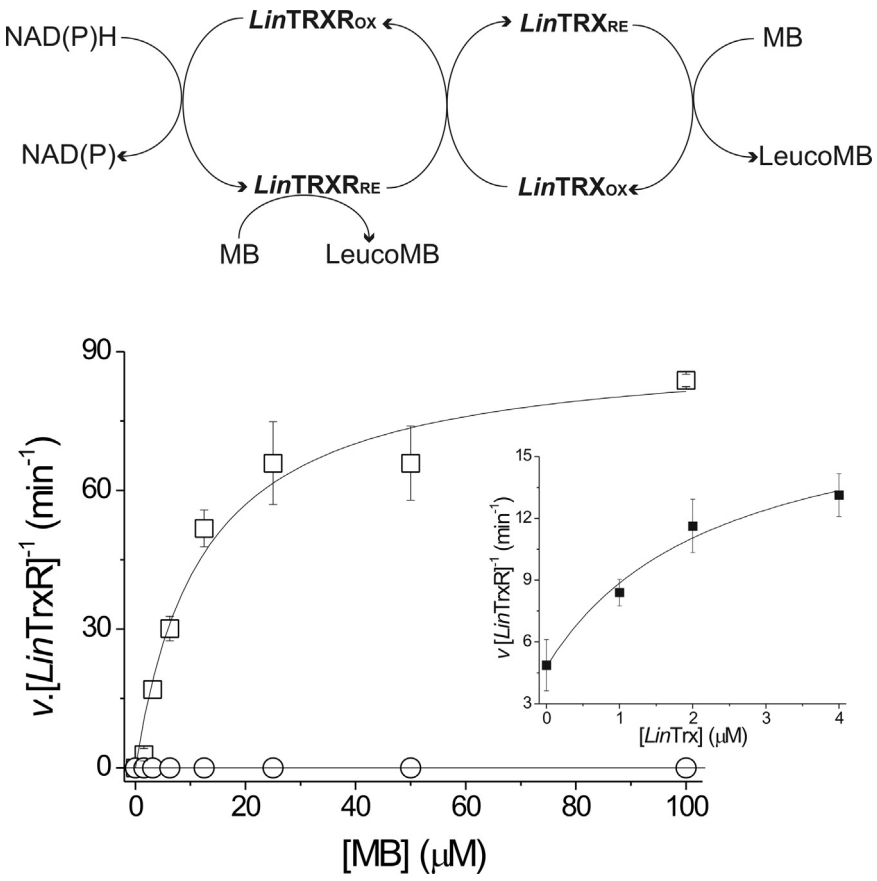

Fig. 12. Kinetics for the reduction of methylene blue by the *Lin*Trx system. The assay was performed at 30 °C and pH: 7.0, with 1.56–100 μM of methylene blue (MB), in absence (○) or in presence of *Lin*Trx (□). Inset: Synergistic effect in reducing MB by *Lin*Trx, it means high MB reduction with highest *Lin*Trx concentration. The reactions were performed in presence of different *Lin*Trx concentrations, 100 μM of MB, 0.2 μM *Lin*TrxR and 200 μM NADPH. The activity was measured by monitoring the oxidation of NADPH at 340 nm.

L-TrxRs and it is implicated in the binding of NADPH [35]. However, the antioxidant activity of *Lbi*TrxR2 against peroxides (observed in *in vivo* assays) was higher than the antioxidant activity of *Lbi*TrxR1. This result supports the view that *Lbi*TrxR2 would be important for the bacterial survival.

*Lin*TrxR was unable to reduce low molecular mass thiols (cystine, GSSG, lipoamide, and bis-γ-GC), while *Lin*Trx was able to reduce them but with a lower catalytic efficiency in comparison with the protein from other organisms. The analysis of these results, together with the fact that *L. interrogans* has a high thiol content (that in addition is poorly affected by the presence of oxidants), open two primary questions: i) is γ-GC the major low molecular mass thiol in *L. interrogans*? and ii) which is the importance of this thiol for the bacterium metabolism? Looking to reach answers, we first analyzed the current information available in the *L. interrogans* genome project (http://aeg.lbi.ic.unicamp.br/world/lic/), searching for gene coding for macromolecules related to the glutathione metabolism. We neither found genes coding for glutathione synthetase nor for glutathione reductase. This is not unusual in nature, as for example the glutathione system is absent in other bacteria, such as *Helicobacter pylori, Mycobacterium tuberculosis, Bacillus subtilis, Bacteroides fragilis, Lactobacillus casei* [8], *Borrelia burgdorferi* [63] and *Treponema pallidum* [64]. Thus, being the glutathione system absent in *L. interrogans*, the Trx system becomes very important for the cellular thiol/disulfide balance. Moreover, there is a gene encoding a γ-glutamylcysteine ligase (LIC11812), pointing out that *Leptospira* might accumulate γ-glutamylcysteine as low molecular mass thiol, which might play as redox cofactor for the putative glutathione peroxidases (LIC12648, LIC13442). In relation to the glutathione dependent redox metabolism, the main difference between *L. interrogans* and *L. biflexa* species, is that the latter has a gene coding for a putative glutathione synthetase (LEPBI_I1912), being possible the presence in this organism of both thiols.

The Trx system from *L. interrogans* would catalyze the reduction of cystine *in vivo*, which would be relevant (because none coding sequence for a putative cystine reductase has been reported) to maintain the intracellular concentration of cysteine. The salvage of the cysteine by the Trx system would become more important in the absence of glutathione. Another possibility for *L. interrogans* might be the importation of glutathione from the host, as previously reported in *Haemophilus influenzae* [65] or *Streptococcus pneumonia* [66]. However, the knowledge about the thiols metabolism is still not complete and is a matter of in depth analysis in our group. These bacteria are also exposed to reactive species of nitrogen, from their endogenous nitric oxide production or during infections [67]. These reactive species cause post-translational thiol-modifications (S-nitrosylation), that in the case of enzymes that require a reduced Cys thiol in the redox active site, could be inhibited. The ability of *Lin*Trx to reduce γ-GCSNO can suppose that the *L. interrogans* Trx system would be involved in protein denitrosylation, preventing nitrosative stress. Then, if γGC is the main low molecular mass thiol in these bacteria, it may be proposed that γ-GC could remove protein-SNO groups via transnitrosylation to produce the γ-GCSNO which will be reduced by the Trx system.

Finally, the relative high abundance of *Lin*TrxR detected by western blot indicates a possible functional relevance of *Lin*TrxR at the cellular level. Thus, the potent inhibitory effect of methylene blue (an effective antimalarial agent [56]) over the Trx system

from *L. interrogans* is indicated by its lower IC_{50} (~0.8 μM). This effect together with its toxicity on bacterial cultures (IC_{50}~7 μM), could be considered as a starting point in the development of new anti-leptospiral drug. It is worth to point out that this is the first report about identification, molecular cloning and functional characterization of the Trx system in *Leptospira* spp. The present work contributes with key information for in depth advance in the biochemical characterization of the redox metabolism in these organisms.

Acknowledgements

We thank to Silvia Fusco (Ministerio de Salud de la Provincia de Santa Fe – Argentina) for providing the strains of *L. interrogans* serovar Copenhagenii and *L. biflexa* serovar Patoc, Dr. Caroline E. Cameron (University of Victoria, Canada) for *L. interrogans* anti-catalase antibodies. This work was supported by grants from UNL (CAI+D'11), PIO-CONICET-YPF 2015-2016 and ANPCyT (PICT-2013-0253, PICT-2014-3256 and PICT-2014-2103). NS is fellows from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas). AAI, SAG and DGA are investigator career members from CONICET.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.05.008.

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