Broad specificity AhpC-like peroxiredoxin and its thioredoxin reductant in the sparse antioxidant defense system of *Treponema pallidum*

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Little is known about the mechanisms by which *Treponema pallidum* (*Tp*), the causative agent of syphilis, copes with oxidative stress as it establishes persistent infection within its obligate human host. The *Tp* genomic sequence indicates that the bacterium's antioxidant defenses do not include glutathione and are limited to just a few proteins, with only one, TP0509, offering direct defense against peroxides. Although this *Tp* peroxiredoxin (Prx) closely resembles AhpC-like Prxs, *Tp* lacks AhpF, the typical reductant for such enzymes. Functionally, *Tp*AhpC resembles largely eukaryotic, nonAhpC typical 2-Cys Prx proteins in using thioredoxin (Trx, TP0919) as an efficient electron donor and exhibiting broad specificity toward hydroperoxide substrates. Unlike many of the eukaryotic Prxs, however, *Tp*AhpC is relatively resistant to inactivation during turnover with hydroperoxide substrates. As is often observed in typical 2-Cys Prxs, *Tp*AhpC undergoes redox-sensitive oligomer formation. Quantitative immunoblotting revealed that *Tp*Trx and *Tp*AhpC are present at very high levels (over 100 and 300 μM, respectively) in treponemes infecting rabbit testes; their redox potentials, at -242 ± 1 and -192 ± 2 mV, respectively, are consistent with the role of *Tp*Trx as the cellular reductant of *Tp*AhpC. Transcriptional analysis of select antioxidant genes confirmed the presence of high mRNA levels for *ahpC* and *trx* which diminish greatly when spirochetes replicate under in vitro growth conditions. Thus, *T. pallidum* has evolved an extraordinarily robust, broad-spectrum AhpC as its sole mechanism for peroxide defense to combat this significant threat to treponemal growth and survival during infection.

alkyl hydroperoxide reductase | peroxidases | syphilis | syphilis spirochete | oxidative stress

Despite the availability of effective antimicrobial therapy for more than six decades, syphilis, a sexually transmitted infection caused by the spirochetal pathogen *Treponema pallidum*, remains a public health problem of global dimensions (1). Following inoculation, usually in the genital area, *T. pallidum* disseminates to diverse organs where it can establish persistent, even lifelong, infection (2, 3). Investigation of the mechanisms the syphilis spirochete employs to establish and maintain infection traditionally have centered on the characterization of surface-exposed molecules which function at the host-pathogen interface (2–4). *T. pallidum*, an extracellular organism with an exceptionally limited biosynthetic capacity (5, 6), also must acquire essential nutrients from every microenvironment in which it takes up residence, while fending off measures by the host to disrupt bacterial homeostasis (7–9).

T. pallidum is classified as a microaerophile because of its limited oxygen tolerance in vitro (10, 11); however, like all bacterial pathogens, the bacterium must cope with endogenous and exogenous sources of oxidative stress as it disseminates throughout the human body. In other bacteria, such as *Escherichia coli*, incomplete reduction of oxygen by the aerobic respiratory chain is one endogenous source of reactive oxygen species (ROS), some of which can react with iron (Fenton chemistry) and produce protein- and DNA-damaging molecules (12). In lacking the ability to perform oxidative phosphorylation and maintaining minuscule intracellular concentrations of iron (Fe) (5, 6), *T. pallidum* inherently limits the production of such oxidants. On the other hand, upon colonization of genital mucosal sites prior to dissemination, spirochetes likely encounter ROS produced both by commensal flora to ward-off noncommensal intruders (13, 14) as well as by macrophages and neutrophils to combat the microbes (8, 15, 16). Despite these obstacles, *T. pallidum* readily disseminates hematogenously and survives in well oxygenated tissues and chronically inflamed sites (2, 3), suggesting that it possesses robust defenses against oxidative stress (11, 17) a more thorough examination of which would enhance our understanding of syphilis pathogenesis.

T. pallidum encodes some antioxidant proteins common to other pathogenic bacteria but is surprisingly deficient in other systems which support oxidant defense and redox homeostasis in many organisms (Table 1; *SI Results*) (6, 11, 17). Two of the principal ROS bacteria must protect themselves against are superoxide (O_2^{-}) and peroxides (12). While *T. pallidum* lacks an ortholog for superoxide dismutase (SOD), the classical enzyme that detoxifies O_2^{-} (12, 18), it does encode a superoxide reductase (SOR, TP0823) and its putative reductant, rubredoxin (TP0991) (19, 20). *T. pallidum* lacks catalase and contains no homolog for glutathione peroxidase; instead, the spirochete encodes only one known peroxide scavenging enzyme, an alkyl hydroperoxide reductase C (AhpC; TP0509), which belongs to a widespread family of cysteine-based peroxiredoxins with activity against hydrogen peroxide (H_2O_2), organic peroxides, and peroxynitrite (21). Among peroxiredoxins, the AhpC-type enzymes have been best characterized in *Salmonella typhimurium*, *Streptococcus mutans*, and *E. coli* (21–23). These closely related bacterial enzymes (>50% amino acid sequence identity) are recycled by a dedicated flavoprotein disulfide reductase, alkyl hydroperoxide reductase F (AhpF) (21). While AhpF acts as a highly efficient electron donor to AhpC in these organisms, thioredoxin (Trx) can substitute as a less efficient reductant and is apparently the natural, physiological reductant of most nonAhpC peroxiredoxins (21). Remarkably, *T. pallidum* lacks AhpF altogether (6), suggesting that the

Table 1. Redox-relevant proteins encoded within the genomes of *Treponema* and *Borrelia* spirochetes

Protein family	Treponema pallidum	Treponema denticola	Borrelia burgdorferi
Peroxiredoxin	TP0509 (AhpC group)	TDE0011 (Prx6 group)	No
AhpF	No	No	No
TrxR	TP0814	TDE0743	BB0515
NADH oxidase	TP0921	TDE0096	No
CoA disulfide reductase	No	TDE0153 (with rhodanese)	BB0728
Thioredoxin	TP0919 TP0100	TDE0744 TDE0238 (with Se)	BB0061
Catalase	No	No	No
BtuE (Gpx homologue)	No	TDE1729 (with Se)	No
Ohr	No	No	No
Dps/NapA	TP1038	No	BB0690
Superoxide dismutase	No	No	BB0153
Superoxide reductase	TP0823	TDE1754	No
Rubredoxin	TP0991	TDE1052	No
Glutaredoxin	No	No	No
Glutathione reductase	No	No	No
γ-Glutamate-cysteine ligase	No	No	No
OxyR	No	No	No
SoxR	No	No	No

AhpC from this organism relies on a single Trx (TP0919) present in the cytoplasm (11, 21). Without catalase or other peroxide-metabolizing enzymes, including other peroxiredoxin homologs, *T. pallidum* is likely to be highly dependent on AhpC and its reducing partner for protection against H_2O_2 and other hydroperoxides. Moreover, given the absence of other typical cellular thiol reductants such as glutaredoxins and glutathione in *T. pallidum* (Table 1 and *SI Results*), one might expect the single Trx protein to be of exceptional importance in maintaining the proper thiol-disulfide homeostasis in this organism (6).

We report herein that the Trx and AhpC proteins of *T. pallidum* together constitute a very active and robust peroxidase system with broad specificity toward primary and tertiary hydroperoxide substrates. Moreover, these two enzymes are highly abundant in treponemes from inflamed rabbit testes but markedly downregulated in spirochetes grown in an in vitro epithelial cell cocultivation system replete with exogenous antioxidants. Our findings suggest that peroxides are a major form of oxidative stress en-

countered by *T. pallidum* during infection and also provide an explanation for the paradoxical observation that *T. pallidum* has limited oxygen tolerance in vitro but flourishes in blood and highly oxygenated tissues within the human host (2, 3).

Results and Discussion

Bioinformatic Analysis of Peroxiredoxins and Associated Redox Proteins Within the *T. pallidum* Genome. Prx proteins are highly abundant and widely distributed in biology; few organisms lack identifiable Prx(s) in their genomes, and many organisms express multiple Prx family members (Table S1). In spirochetes, however, the situation is quite unusual; species of *Borrelia*, *Spirochaeta*, and *Brachyspira* lack Prx orthologs, while *T. pallidum* and *Treponema denticola* have single Prx proteins encoded in their genomes which belong to separate Prx classes and are only 27.0% identical in amino acid sequence (Table S1). The *T. pallidum* Prx (TP0509) under investigation herein is best classified as an AhpC-like protein (within the broader category of typical 2-Cys Prxs) (Fig. 1); in addition to the peroxide-reactive (peroxidatic) cysteine at the active site, AhpC-like proteins, as well as those within the broader typical 2-Cys Prx group, also conserve a second (resolving) cysteine which participates in catalysis by forming a disulfide bond with the oxidized, sulfenic acid form of the peroxidatic cysteine. The *T. denticola* Prx (TDE0011), on the other hand, falls into the Prx6-like group, proteins which typically conserve only the peroxidatic cysteine (24, 25).

The typical 2-Cys Prx group, which includes the *T. pallidum* Prx, can be subdivided into (*i*) the widespread group represented by human Prx1-like proteins, and (*ii*) the predominantly bacterial AhpC proteins (Fig. 1 and *SI Results*). Based on sequence alignments and conservation of key residues, the *T. pallidum* Prx groups with the AhpC proteins (*Tp*AhpC) (Fig. 1). Earlier analyses suggested that all bacterial genomes with readily identified *ahpC* genes (≥50% amino acid sequence identity compared to *S. typhimurium* AhpC) invariably encode an *ahpF* orthologue immediately downstream of *ahpC* (26) (the name "AhpC" has been used for proteins linked with AhpD or reduced by other Trx-like proteins, but, using our recent bioinformatic analyses, we are not defining these as "canonical" AhpC proteins; *SI Results*). In the case of *Tp*AhpC, which exhibits 51.1% amino acid sequence identity with *S. typhimurium* AhpC (*St*AhpC), no *ahpF* or other putative reductase gene is found near the *ahpC*. As shown in Fig. 1, *Tp*AhpC is 46.6% identical with another structurally characterized representative of the AhpC class, *Amphibacillus xylanus* AhpC, which also is expressed from an *ahpC* gene proximal to an *ahpF* orthologue (27).
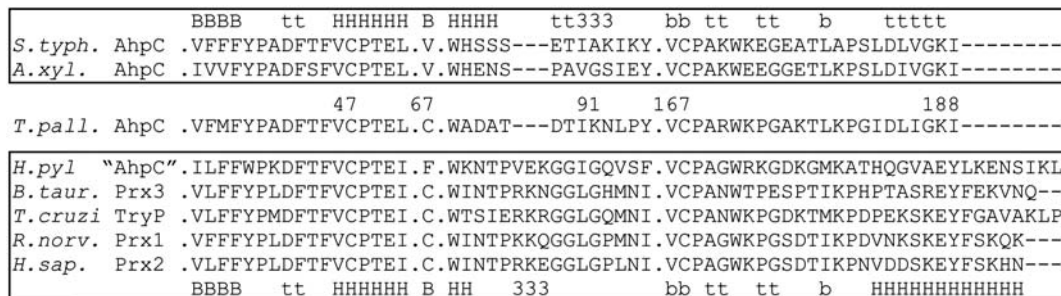

Fig. 1. Alignment of fragments within representative typical 2-Cys peroxiredoxins (Prxs). Shown in the rectangle above the *T. pallidum* AhpC (*Tp*AhpC) sequence are select sequence regions of *S. typhimurium* AhpC (1yep, 51.1% identity with *Tp*AhpC) and *Amphibacillus xylanus* AhpC (1we0, 46.6% identity) and their corresponding secondary structure elements (B = beta strand, H = α helix, 3 = 3/10 helix, t = hydrogen-bonded turn, and b = beta bridge). In the rectangle below the *T. pallidum* sequence are representative typical 2-Cys Prx proteins of known structure from a wide variety of organisms that group separately from AhpC-like proteins in multiple sequence alignments, including proteins from *Helicobacter pylori* (1zof, 33.0% identity with *Tp*AhpC), *Bos taurus* (1zye, 33.5%), *Trypanosoma cruzi* (1uul, 38.4%), *Rattus norvegicus* (1qq2, 37.0%) and *Homo sapiens* (1qmv, 38.4%). Shown are sequence segments (separated by a dot), from (*A*) regions around the peroxidatic cysteine (Cys 47 in *Tp*AhpC), (*B*) the additional (third) cysteine (Cys67) present in *Tp*AhpC but not in the two other AhpC proteins, (*C*) the Prx1-linked GG(i/l/v)G motif, and (*D*) the C terminus including the resolving cysteine (Cys167 in *Tp*AhpC).

The most likely alternative source of electrons for *Tp*AhpC is thioredoxin (Trx); the single *T. pallidum* Trx (*Tp*Trx; TP0919) is similar to both *E. coli* Trx proteins and an *Arabidopsis thaliana* Trxh1 (amino acid identities of 29.4%, 25.9%, and 26.2% with *E. coli* Trx1, *E. coli* Trx2 and *A. thaliana* Trxh1, respectively; Fig. S1*A*). The *T. pallidum* genome contains one additional Trx-related protein, TP0100, which is similar to *Bacillus subtilis* ResA (28) and about twice the size of canonical Trx proteins (17.5% identity between the TP0919 and TP0100). TP0100 is periplasmic, however (29), and could not serve as an electron donor to cytosolic AhpC. The thioredoxin reductase (TrxR) protein required to reduce Trx, TP0814, is 37.4% identical in amino acid sequence with the well-characterized *E. coli* TrxR, with the highest degree of conservation around the functionally important regions (Fig. S1*B*) (30).

Based on these bioinformatic analyses, the two proteins studied herein, *Tp*AhpC and *Tp*Trx, along with the TrxR protein, appear to be the only protein-mediated peroxide defense and redox-buffering system present in *T. pallidum* (Table 1). Given that the biosynthetic machinery for glutathione (e.g., γ-glutamate cysteine ligase in Table 1) is lacking in *Treponema* and *Borrelia* species (6, 31, 32), the possibility exists that these organisms, like some others, use the low molecular weight thiol coenzyme A (CoASH) as their primary "redox buffer" (33). However, the lack of a specialized CoA disulfide reductase (CoADR) in *T. pallidum*, unlike in *Borrelia burgdorferi*, suggests that CoASH is not a major thiol in *T. pallidum* (Table 1 and SI Results) (34, 35). Thus, the presence or nature of any low molecular weight thiol in *T. pallidum* remains to be resolved.

TpAhpC Peroxidase Activity Is Supported By Reductive Turnover with TpTrx and E. coli Trx1, but Not with S. typhimurium AhpF, and Is Higher in Non-His-Tagged Protein. Despite the sequence similarity between *Tp*AhpC and AhpF-dependent AhpC in many bacteria, the absence of an AhpF ortholog in *T. pallidum* suggests *Tp*AhpC might have diverged from other AhpC proteins to the point that it can no longer use AhpF as an electron donor. Indeed, *Tp*AhpC showed substantial peroxidase activity in an NADPH-dependent reductase system containing *Tp*Trx, but none with NADH and *S. typhimurium* AhpF as the electron-donating system (SI Experimental Procedures describes assays used).

Our initial characterization of the enzymatic activity of *Tp*AhpC employed His-tagged protein and included determination of $V_{max,app}$ and $K_{m,app}$ values using varying concentrations of four hydroperoxide substrates and a single concentration (10 μM) of either *E. coli* Trx1 (*Ec*Trx1) or *Tp*Trx. In comparative assays using both Trx proteins, the K_m for H_2O_2 was identical and the maximal rate (V_{max}) was actually about twofold higher with the *Ec*Trx1 enzyme (Table S2). Given these results, *Ec*Trx1 was used as the reductant for most experiments in place of *Tp*Trx primarily due to the large amounts of pure protein available to us. Using this assay system (including 10 μM *Ec*Trx1), His-tagged *Tp*AhpC was shown to exhibit a $V_{max,app}$ of about 1 μM s^{-1} (μM enzyme)$^{-1}$ with all four hydroperoxide substrates tested [H_2O_2, ethyl hydroperoxide, cumene hydroperoxide (CHP), and *t*-butyl hydroperoxide (*t*BHP)], and K_m values that ranged from 3.5 to 10 μM for the first three substrates and about 100 μM for *t*BHP (Table S3). These results yielded $V_{max,app}/K_{m,app}$ values of around 1 or 2 × 10^5 M^{-1} s^{-1} for reactivity of *Tp*AhpC toward all four hydroperoxide substrates in the presence of 10 μM *E. coli* Trx. Activity measured in the presence of increasing amounts of Trx (as high as 80 μM) increased proportionately with Trx concentration, suggesting a nonsaturable interaction of Trx with His-tagged *Tp*AhpC.

In order to determine whether or not the His tag was impairing activity in the recombinant protein, a non-His-tagged version of the protein was expressed and purified. Retesting of H_2O_2, CHP, and *t*BHP with 10 μM *Ec*Trx1 and varying concentrations of per-

oxides demonstrated an increase (by 3 to 13-fold) in the catalytic efficiency ($V_{max,app}/K_{m,app}$) of the enzyme with each substrate (Table S3). The nontagged protein exhibited both a higher $V_{max,app}$ (by 2- to 4-fold) and a lower $K_{m,app}$ (by 1.2- to 6-fold) toward all three substrates relative to the tagged protein (Table S3). In contrast to results obtained with the His-tagged enzyme, the *Ec*Trx1 reductant also interacted with the non-His-tagged *Tp*AhpC in a saturable manner, allowing for the true kinetic parameters for the bisubstrate enzyme to be determined. Further biochemical characterization, therefore, employed the non-His-tagged version of the protein (below).

TpAhpC Is a Broad Specificity Peroxiredoxin. For detailed analysis of the substrate specificity of *Tp*AhpC, a sensitive, fluorescence-based assay was used in which prereduced *Ec*Trx1 (10 to 100 μM), *Tp*AhpC (500 nM), and various concentrations of hydroperoxide substrates were mixed rapidly and monitored for fluorescence decreases reflecting oxidation of *Ec*Trx1; computed rates of reaction were fit globally to the rate equation for a ping pong reaction (SI Experimental Procedures) to yield true, bisubstrate turnover numbers (k_{cat}) and K_m values for both the reducing and oxidizing substrates (datasets and fits using CHP and *t*BHP substrates are shown in Fig. 2). A summary of the overall results for all three of the hydroperoxide substrates studied in detail is provided in Table 2. Several features are notable; first, the catalytic efficiency with hydroperoxide substrates (k_{cat}/K_m^{ROOH}) varies over a relatively narrow range of sevenfold for substrates as distinct as
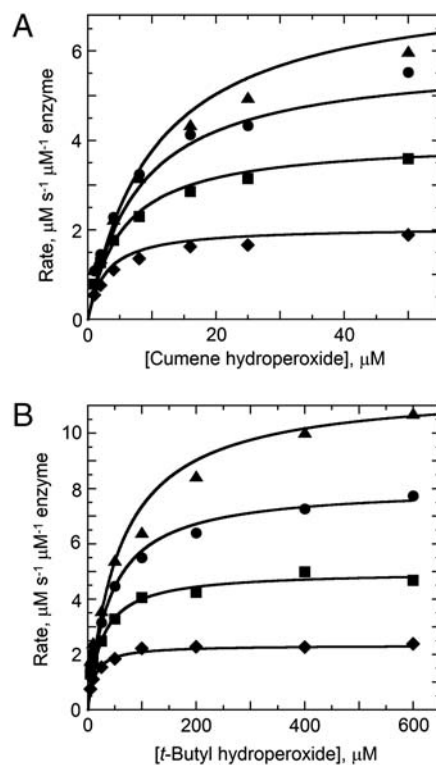

Fig. 2. Activities of *Treponema pallidum* AhpC with tertiary hydroperoxide substrates. Peroxidase activity was measured by mixing *E. coli* thioredoxin (Trx, prereduced by dithiothreitol) and AhpC (0.5 μM) in 50 mM potassium phosphate pH 7.0, 0.5 mM EDTA and 100 mM ammonium sulfate with various concentrations of peroxide substrate in a stopped-flow spectrophotometer at 25 °C. The decrease in Trx fluorescence due to oxidation was monitored using excitation at 280 nm and emission >320 nm. Fixed concentrations of Trx assayed over a range of cumene hydroperoxide (*A*) and *t*-butyl hydroperoxide (*B*) concentrations were 10 μM (diamonds), 25 μM (squares), 50 μM (circles), and 100 μM (triangles). Results using H_2O_2 as substrate closely matched those with cumene hydroperoxide (*A*).

Table 2. Kinetic parameters for reaction of *Tp*AhpC with various peroxide substrates

Peroxide substrate	k_{cat}, s^{-1}	K_m^{ROOH}, µM	K_m^{Trx}, µM	k_{cat}/K_m^{Trx}, µM^{-1} s^{-1}	k_{cat}/K_m^{ROOH}, µM^{-1} s^{-1}
Hydrogen peroxide	17 ± 2	12 ± 2	62 ± 17	0.27	1.4
Cumene hydroperoxide	9.8 ± 0.6	9.6 ± 1.1	38.8 ± 4.9	0.25	1.0
t-Butyl hydroperoxide	20.9 ± 1.4	104 ± 9	79.4 ± 9.7	0.26	0.20

H_2O_2, CHP and, *t*BHP, with a value of 1.4×10^6 M^{-1} s^{-1} for the best substrate (H_2O_2). In fact, the most complex and bulky hydroperoxide, CHP, is very similar to H_2O_2 as a substrate for *Tp*AhpC. Interestingly, the smaller of the tertiary hydroperoxide substrates, *t*BHP, has about a sevenfold higher K_m than CHP but also about a twofold increase in k_{cat}. Not surprisingly, k_{cat}/K_m of *Tp*AhpC with Trx reflecting the reductive half-reaction does not change at all with varying hydroperoxide substrates.

The relatively high reactivity of *Tp*AhpC with multiple substrates, at ~10^6 M^{-1} s^{-1}, supports the view that it must act as a potent antioxidant defense across a range of peroxide substrates, unlike AhpC in *E. coli* or *S. typhimurium*, where the need for broad specificity is less critical given the presence of other Prxs including the organic hydroperoxide defense enzyme Tpx (36). In fact, *St*AhpC, which exhibits a somewhat higher reactivity than *Tp*AhpC with H_2O_2 (k_{cat}/K_m of 3.7×10^7 M^{-1} s^{-1}), is about two orders of magnitude less reactive with *t*BHP and CHP substrates (2.3×10^5 and 4.9×10^5 M^{-1} s^{-1}, respectively) (37). One limitation for the *Tp*AhpC enzyme may be imposed by the need for Trx to interact with many other substrate proteins, unlike the dedicated reductant of *St*AhpC, AhpF. Another implication of the lack of an AhpF homologue in *T. pallidum* is that the electrons for both peroxide defense and thiol-disulfide redox homeostasis must come from NADPH and presumably funnel through the single TrxR and Trx proteins. In *E. coli* and *S. typhimurium*, peroxide defense through AhpC is supported primarily by NADH because of the selectivity of AhpF for this cofactor, while disulfide stress is generally alleviated by NADPH through thioredoxin reductase and glutathione reductase pathways.

Another interesting comparison emerging from these biochemical studies of *Tp*AhpC is the distinction between CHP and *t*-BHP as substrates for this protein (Fig. 2). The difference between these two tertiary hydroperoxides is the presence of a phenyl group in CHP in place of a methyl on *t*BHP; whereas one might expect steric hindrance issues with the bulkier substituent of CHP, the data reflect an opposite trend, with the phenyl group contributing in a positive way to the enzyme-substrate interaction (K_m values of 14 and 104 µM for CHP and *t*BHP, respectively). This suggests the presence of a rather large, hydrophobic pocket for substrate binding in *Tp*AhpC which might potentially also accommodate macromolecular substrates like lipid and protein hydroperoxides in vivo; comparisons of a structural model of *Tp*AhpC with the known structure of *St*AhpC suggest enhanced hydrophobicity in the environment around the putative substrate binding region (Fig. S2).

TpAhpC Is Resistant Toward Substrate-Mediated Inactivation. Because some Prxs, particularly those from eukaryotes, are inactivated during turnover with high levels of substrate (38), the sensitivity of *Tp*AhpC for this type of inactivation was tested with its most potent substrate, H_2O_2. Using a full assay system containing NADPH, *E. coli* TrxR, *Ec*Trx1, *Tp*AhpC, and as much as 10 and 30 mM H_2O_2, a curvature in the loss of 340 nm absorbance over time was observed after multiple turnovers, indicating a very low degree of sensitivity toward inactivation by substrate, slightly less than that observed with the *St*AhpC protein (Fig. S3). Surprisingly, His-tagged *Tp*AhpC under the same conditions exhibited no evidence of inactivation during turnover even at 30 mM H_2O_2 (Fig. S3). Thus, *Tp*AhpC is a very robust enzyme, similar to but even less sensitive than *St*AhpC toward inactivation by peroxide substrates.

The Midpoint Reduction Potentials for TpTrx and TpAhpC Are −242 and −192 mV, Consistent with Their Redox Functions. Knowing the midpoint reduction (redox) potential of redox-active proteins enables one to place them within the hierarchy of known cellular electron donors or acceptors. Measurements of this parameter for *Tp*Trx and *Tp*AhpC were carried out by equilibration of the proteins with *E. coli* glutaredoxin-1 (Grx1) in reduced and oxidized forms, then assessment of the redox status of both proteins following acidification and HPLC analysis (37, 39). Given the known redox potential of Grx1 of −233 mV at pH 7 (39), the redox potential for *Tp*AhpC was computed to be −192 ± 2 mV ($n = 3$, Fig. S4). Like the value previously obtained for *St*AhpC (−178 mV) (37), this is a relatively high redox potential compared with those of many other redox disulfide-containing proteins. The only other redox potentials reported for Prx proteins, to our knowledge, have been for proteins from chloroplast or mitochondria and range from −288 to −325 mV at pH 7 (40, 41), values that are apparently more suited to their specialized function(s) in these organelles. The redox potential for *Tp*Trx, at −242 ± 1 mV ($n = 4$), is higher than that of *Ec*Trx [at −270 mV (42)], perhaps in part due to the alteration of the "WCGPC" motif at the active site (WCGSC for *Tp*Trx; Fig. S1). This is similar to the value of −249 mV obtained for a specialized Trx-like protein, tryparedoxin, from another human pathogen, *Trypanosoma brucei* (43). With redox potentials separated by 50 mV, transfer of electrons from *Tp*Trx to *Tp*AhpC will be thermodynamically favorable even under conditions where the reduced form of *Tp*Trx becomes scarce.

Reduced TpAhpC Forms Stable Octamers or Decamers in Solution Whereas Oxidation Generates a Heterogeneous Mixture of Oligomeric Species. Members of the typical 2-Cys group of Prxs have been shown to form decameric, as well as octameric and dodecameric, complexes in solution through a redox-sensitive interface (called the "A interface"), while maintaining in all states a dimeric protomer form through the "B interface" (which brings the edges of two beta sheets together) (44). When analyzed by sedimentation velocity analytical ultracentrifugation (45), *Tp*AhpC was found to exist in a stable octameric or perhaps decameric form ($M_W \sim 162$ kDa) when fully reduced (Fig. S5). Based on mutational analysis of *St*AhpC (46), decamers are more active than dimers as peroxide scavengers; thus, stabilization of a higher order species for reduced *Tp*AhpC is in line with its apparently critical role in peroxide defense within this organism. At concentrations of 300 µM and higher, a small amount of an additional higher order species was detected in the reduced sample as well (sedimentation coefficient ~14 S versus 8 S for the predominant species; Fig. S5). Following oxidation of *Tp*AhpC, a heterogenous mixture of species was observed which did not fit well to single or two species models and suggested the presence of a range of species ($s = 6$ to 16 S) with overall sedimentation coefficients increasing with concentration (Fig. S5). This suggests that *Tp*AhpC has a greater diversity of oligomeric forms than *St*AhpC, a protein which is decameric when reduced and exhibits a tendency to dissociate into dimers upon oxidation (45).

AhpC and Trx Are Highly Expressed Proteins in T. pallidum. Antisera directed against purified, recombinant *Tp*AhpC and *Tp*Trx was used to probe *T. pallidum* lysates and quantitatively evaluate expression of the native proteins in motile treponemes freshly harvested from rabbit testes based on recombinant protein standards (Fig. 3). Strong bands detected with apparent molecular masses

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of 21,000 Da and 12,000 Da were consistent with those predicted for TpAhpC (20,709 Da) and TpTrx (11,391 Da). Copy numbers calculated per *T. pallidum* cell for TpAhpC and TpTrx were $46,200 \pm 10,400$ and $16,300 \pm 6,800$, respectively; the abundant flagellar protein, FlaA, yielded a value of $15,100 \pm 5,100$ copies per spirochete. Using an estimate of the volume of a single *T. pallidum* cell, the intracellular concentrations of AhpC, Trx, and FlaA in *T. pallidum* were approximately 302, 106, and 99 μM, respectively (Fig. 3). Thus, TpAhpC and TpTrx are very abundant enzymes in the cytoplasm, with Trx concentrations reaching levels above the measured K_m of TpAhpC for EcTrx1 and levels of TpAhpC as much as threefold over that. Such high concentrations of the peroxidase ensure rapid detoxification of any H_2O_2 or macromolecular hydroperoxides generated endogenously, while maintenance of such a large pool of reduced and active TpAhpC would allow for immediate defense against a sudden influx of high levels of peroxides from the environment.

Transcriptional Analysis of Select Antioxidant Genes Underscores the Importance of AhpC and Trx and Reveals That the Corresponding Genes Are Regulated by Changing Growth Conditions.

To put into perspective the contribution of the Trx-AhpC detoxification system to *T. pallidum*'s oxidative stress response, we used quantitative real-time reverse transcription-PCR (qRT-PCR) to survey antioxidant gene expression in the bacterium. The panel of genes chosen for these experiments (Fig. 4) was based on a previous *in silico* analysis of oxidative stress defense pathways encoded in the spirochete's genome (Table 1) (11, 17). As described above, *trxB* (TP0814) encodes a TrxR homologue that is predicted to provide reducing equivalents to Trx and indirectly to AhpC. *sor* (TP0823) encodes superoxide reductase (19, 20), while the *nox* (TP0991) gene product, NADH oxidase, is predicted to detoxify free oxygen by four-electron reduction to form water through the oxidation of two molecules of NADH. Lastly, the *dps* gene product (TP1038) is a dodecameric, ferritin-like protein which binds and sequesters intracellular Fe and may protect DNA from oxidative damage (47). As shown in Fig. 4, *flaA*-normalized values of antioxidant gene expression in treponemes isolated from inflamed rabbit testis (peak orchitis) covered a broad range of transcript levels. The abundance of transcripts for *ahpC* and *trx* was particularly striking and consistent with the high levels

of the corresponding proteins detected by quantitative immunoblotting (Fig. 3).

T. pallidum undergoes limited replication when cultivated with rabbit Sf1Ep cells in the presence of a low partial pressure of oxygen (4%) and antioxidants (e.g., SOD and catalase) (48). We used this in vitro cocultivation system to study the expression of *T. pallidum* genes under conditions in which exogenous sources of oxidative stress are presumably markedly reduced as compared to those encountered by the spirochete within the inflamed rabbit testis. As shown in Fig. 4, transcript levels of three genes (*ahpC, trx,* and *nox*) showed highly significant reductions during in vitro cultivation, while the reduction in a fourth (*sor*) just missed statistical significance. It is noteworthy that the *ahpC* transcript copy number remained well above that of the constitutively expressed *flaA* gene.

Demonstration of decreased mRNA levels for *ahpC* and *trx* genes from treponemes isolated from cocultures with epithelial cells compared with those from the inflamed rabbit testes suggest that the high concentrations of these antioxidant proteins reported in Fig. 3 are regulated and in part a consequence of their inflammatory environment. While this may seem to be of little surprise given the known OxyR and PerR regulons in a variety of bacteria (12), *T. pallidum* lacks any of the known bacterial regulators of antioxidant gene expression, leaving unresolved the mechanism underlying the apparent response observed in the present studies.

Conclusions

The broad substrate specificity of TpAhpC and the antioxidant gene/protein expression data clearly indicate that the Prx-Trx peroxide defense system is a major source of protection against oxidative stress in *T. pallidum*. In support of this conclusion, the spirochete's streamlined metabolism, which lacks the ability to perform oxidative phosphorylation and maintains low intracellular concentrations of Fe (5, 6), would inherently limit the production of superoxide, and by extension, the likelihood that biomolecules (e.g., proteins and DNA) will be damaged by it (11). The functionality of TpAhpC toward a wide range of peroxide substrates (Table 2) suggests that the enzyme is present to combat both H_2O_2 and oxidatively damaged cellular constituents like protein and lipid hydroperoxides [the suggestion that activity with CHP can correlate with activity toward lipid hydroperoxides is supported by work with other peroxiredoxins (36, 49, 50)]. Indeed, in the iron-deficient Lyme disease spirochete,
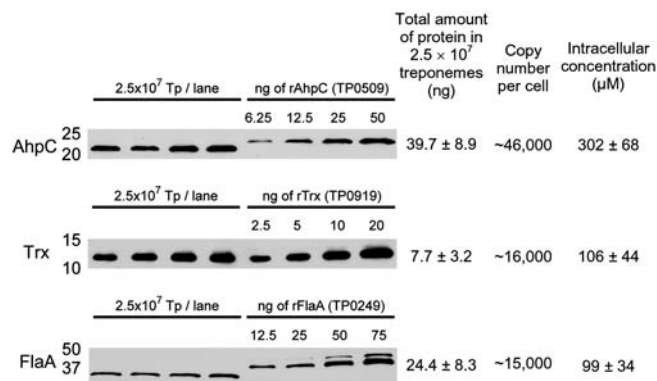

Fig. 3. Immunoblot analysis to evaluate the amounts of peroxiredoxin and thioredoxin proteins expressed in *T. pallidum*. Estimated expression levels of AhpC, Trx, and FlaA in *T. pallidum* harvested from rabbit testes (*left side of gel*) based on purified recombinant protein standards (*right side*) are given as the total amount of native protein (ng) in 2.5×10^7 treponemes (averages ± standard deviations calculated from four samples out of a single harvest). The lower mobility of recombinant AhpC and FlaA reflects the additional His tag on these proteins. Intracellular protein concentrations were calculated based on the estimated volume of *T. pallidum* (2.54×10^{-16} L) (*SI Experimental Procedures*). Concentrations shown for TpAhpC and TpTrx correspond to 6.3 and 1.2 mg/mL in the cell, respectively. Numbers to the *left* of the immunoblots represent molecular weight standards in kilodaltons.
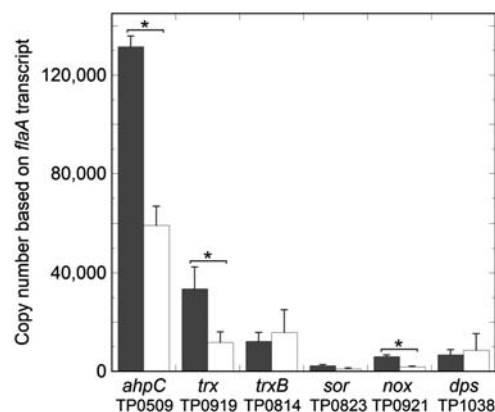

Fig. 4. Quantitative real-time reverse transcription-PCR (qRT-PCR) of antioxidant genes. Shown are the *flaA*-normalized transcript copy numbers (assuming 10,000 copies of *flaA* mRNA) for *T. pallidum* genes involved in oxidative stress protection following harvest from rabbit testes (*black bars*) or subsequent cocultivation with rabbit epithelial cells (*white bars*). Bars represent the means ± SEM for four independent experiments, each analyzed in duplicate. The *asterisk* indicates a significant difference ($p < 0.05$ using the Student t-test) between rabbit-harvested and cocultivated transcript copy numbers.

B. burgdorferi, membrane lipids (e.g., linoleic and linolenic acids) derived from the host have been shown to be the major target of ROS (51). In E. coli, AhpC scavenges basal metabolic levels of endogenous H_2O_2, whereas catalase provides protection from high levels of exogenous H_2O_2 (23). In T. pallidum, the decrease in expression of antioxidant genes in vitro, when compared to the in vivo transcript levels, implies that a substantial amount of peroxide-related stress is exogenous with the in vitro expression values depicting the spirochete's response to ROS of endogenous origin (Fig. 4). Thus, unlike E. coli, but consistent with its genomic parsimony, the syphilis spirochete encodes a single robust AhpC-Trx system which appears to be evolutionarily tailored to protect the bacterium from both endogenous and exogenous sources of peroxide-related stress. The dramatic up-regulation of ahpC and trx in vivo suggests a substantial amount of peroxide-related stress coming from exogenous sources within the testes and also provides a potential explanation to the longstand- ing paradox created by the spirochete's oxygen sensitivity in vitro and its presumed ability to thrive in oxygenated tissues within its obligate human host (10, 52). Ironically, from this insight a new paradox emerges: how does T. pallidum, lacking all known redox regulatory proteins (6), sense and respond to oxidative stress?

Experimental Procedures

Details of materials and methods used are given in *SI Experimental Procedures*. Methods described include bioinformatics, propagation and in vitro cocultivation of T. pallidum, cloning, expression and purification of proteins, enzyme assays, redox potential determination, analytical ultracentrifugation, quantitative immunoblotting, and qRT-PCR.

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