

A Periplasmic Thioredoxin-Like Protein Plays a Role in Defense against Oxidative Stress in *Neisseria gonorrhoeae*[∇]

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Thioredoxin-like proteins of the TlpA/ResE/CcmG subfamily are known to face the periplasm in gram-negative bacteria. Using the *tlpA* gene of *Bradyrhizobium japonicum* as a query, we identified a locus (NGO1923) in *Neisseria gonorrhoeae* that encodes a thioredoxin-like protein (NG_TlpA). Bioinformatics analysis indicated that the predicted NG_TlpA protein contained a cleavable signal peptide at the N terminus, and secondary structure analysis identified a thioredoxin fold with a helical insertion (~25 residues), similar to that found in *B. japonicum* TlpA but absent in cytoplasmic thioredoxins. Biochemical characterization of a recombinant form of NG_TlpA revealed a standard redox potential (E^0) of -206 mV. This property and the observation that the oxidized form of the protein exhibited greater thermal stability than the reduced species indicated that NG_TlpA is a reducing thioredoxin and not an oxidizing thiol-disulfide oxidoreductase like DsbA. The thioredoxin activity of NG_TlpA was confirmed in an insulin disulfide reduction assay. A *tlpA* mutant of *N. gonorrhoeae* strain 1291 was found to be highly sensitive to oxidative killing by paraquat and hydrogen peroxide, indicating an antioxidant role for the NG_TlpA in this bacterium. The *tlpA* mutant also exhibited reduced intracellular survival in human primary cervical epithelial cells.

Thioredoxins are small proteins with a conserved secondary structure and a CXXC active site motif (11, 17). Using NADPH-dependent thioredoxin reductase as an electron donor, thioredoxins can catalyze reduction of cysteine disulfides in proteins. Thus, in the cytoplasm thioredoxins have a role in the catalytic cycle of redox enzymes, such as ribonucleotide reductase, and they also have a role in preventing protein disulfide bridge formation in the cytoplasm (27). The extracytoplasmic compartments of the gram-negative bacterial cell, including the periplasm, represent a more oxidizing environment than the cytoplasm. Over the last decade a sophisticated system for disulfide bond formation and protein folding in the periplasm of gram-negative gammaproteobacteria has been described using *Escherichia coli* as a model: the Dsb system involves thioredoxin-related proteins that are involved in oxidation-reduction of thiols/disulfides. Oxidation of protein thiols is catalyzed by DsbA, and recycling of this protein involves its reoxidation by membrane-bound DsbB, which is connected to the respiratory chain (9, 13). Reductive reactions are also an integral part of protein folding processes in the periplasm, and in this case the protein disulfide isomerase DsbC catalyzes the removal of nonnative disulfide bonds from proteins and enables them to refold in the correct conformation. Reducing power for DsbC is provided by the transmembrane protein

DsbD, which uses cytoplasmic thioredoxin as an electron donor (34).

In addition to its role as an electron donor for the action of DsbC, DsbD can also provide reducing power for the membrane-bound, thioredoxin-like protein CcmG (also known as DsbE), which faces the periplasm and is required for the reduction of cysteine residues in apo-cytochromes *c*, which form thioether linkages to heme groups in a reaction catalyzed by CcmF and CcmH (15, 26, 34). Thus, a concept is emerging in which DsbD plays a role as a “redox hub” for the translocation of reducing power to periplasmic “thioredoxin-like” proteins. In addition to CcmG, in gram-negative bacteria a number of thioredoxin-like proteins have been identified that are periplasmic or are anchored to the cytoplasmic membrane but face the periplasm. An example is the thioredoxin-like protein A (TlpA) of the nitrogen-fixing soil bacterium *Bradyrhizobium japonicum*. Mutational analysis indicates that this protein is essential for the biogenesis and maturation of cytochrome oxidase of the *aa*₃ type (18). Although bioinformatics analysis of bacterial genomes identifies proteins related to CcmG and TlpA (the TlpA/ResE/CcmG family), the function of these thioredoxin-like proteins has not been fully explored.

Neisseria gonorrhoeae is a gram-negative betaproteobacterium that is predicted from bioinformatics analysis to possess a Dsb system for protein disulfide bond formation. Genes encoding homologues to DsbB, DsbC, and DsbD can be identified within the genome, and two DsbA proteins are predicted by genomic analysis (31). The presence of a CcmG homologue is also predicted by the presence of several species of *c*-type cytochrome (36). *N. gonorrhoeae* is a mucosal pathogen that is adapted to its human host. One of these interactions in females is the invasion and survival of this bacterium within cervical

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epithelial cells (5). Indeed, analysis of mutants of *N. gonorrhoeae* has demonstrated that defense against oxidative and nitrosative stress is important for its intracellular survival (29). In this study, we characterized a novel periplasmic thioredoxin-like protein (TlpA) of *N. gonorrhoeae*. This reveals a new role for a periplasmic thioredoxin-like protein in defense against oxidative stress and for survival of *N. gonorrhoeae* within cervical epithelial cells.

MATERIALS AND METHODS

Materials. Except when stated otherwise, all chemicals were purchased from Sigma (Castle Hill, NSW, Australia). Restriction enzymes were from New England BioLabs (Arundel, QLD, Australia).

Bacterial strains and culture conditions. *N. gonorrhoeae* strain 1291 (supplied by Michael Apicella, University of Iowa) was grown on brain heart infusion agar (BHI; Acumedia) supplemented with 10% Levithal's base and 1% IsoVitalEx (Difco) at 37°C in 5% CO₂. Growth in liquid culture was conducted in supplemented BHI broth (Oxoid) at 37°C under aerobic conditions at 170 rpm. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C; for growth on solid medium LB broth was supplemented with 1.5% agar. Unless stated otherwise, ampicillin, kanamycin, and tetracycline were used at final concentrations of 100 µg · ml⁻¹, 50 µg · ml⁻¹, and 5 µg · ml⁻¹, respectively.

Cloning of *tlpA*. The *tlpA* coding sequence was amplified by PCR from *N. gonorrhoeae* strain 1291, using the primers TlpAFN (5'-TAACCATGGACGAACTGGCCGGATGGAAA-3') and TlpAR_IN (5'-TAACCATGGACGAACTGGCCGGATGGAAA-3'). These primers were designed to omit the region encoding the N-terminal signal sequence to the periplasm and contained restriction sites for NcoI (underlined in the forward primer) or PstI (underlined in the reverse primer). The resulting PCR product was digested with NcoI and PstI restriction enzymes and cloned into the NcoI and PstI sites of the pPROEX HTa expression vector (Invitrogen), which contains an N-terminal His₆ tag used for purification.

Expression and purification of TlpA. For expression of NG_TlpA, *E. coli* BL21 cells harboring pPROEX::*tlpA* were grown in LB medium supplemented with ampicillin. When cells reached an A_{600} of 0.3, the temperature was lowered from 37°C to 30°C, 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and the induction phase was conducted for 6 h. The cells were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C. Pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mg ml⁻¹ lysozyme) and incubated for 15 min on ice before sonication. Lysed cells were then centrifuged at 18,000 × *g* for 50 min at 4°C. The cleared lysate was filtered and loaded onto a Ni-nitrilotriacetic acid column (HisTrap HP 5 ml; GE Healthcare) equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM imidazole). After an exhaustive washing with buffer A, the retained proteins were eluted with a gradient going from 0% to 100% of buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 500 mM imidazole) in 3 column volumes. The fractions corresponding to purified NG_TlpA were pooled, and the preparation was dialyzed overnight against 50 mM HEPES (pH 7.0)-100 mM NaCl. NG_TlpA was more than 99% pure as determined by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The concentration of purified NG_TlpA (molecular weight of 18,097.5 g · mol⁻¹) was determined by measuring A_{280} values and using a calculated molar absorption coefficient ($\epsilon_{280\text{ nm}} = 38,180\text{ M}^{-1} \cdot \text{cm}^{-1}$), which was calculated from the amino acid composition of the recombinant NG_TlpA using the method of Gill and von Hippel (7).

Thermal unfolding of TlpA. The temperature-induced unfolding curves of the reduced and oxidized forms of NG_TlpA were monitored by the change in the UV circular dichroism signal. The complete oxidation of NG_TlpA was ensured by incubation of the purified protein with copper phenanthroline (1.7 mM) for 1 h at 4°C. After removal of the oxidant using a PD10 column (GE Healthcare) equilibrated with 100 mM phosphoric acid-NaOH and 1 mM EDTA, pH 7.0, half of the preparation was used to prepare the reduced NG_TlpA sample by addition of 0.75 mM dithiothreitol (DTT).

UV circular dichroism spectra (from 250 to 190 nm) of 10 µM protein solutions were recorded at 25 and 95°C on a Jasco J-715 spectropolarimeter. The wavelength with the largest change in signal was determined from the differential spectrum at 25 and 95°C. Thermal unfolding was then monitored at 220 nm from 25 to 90°C with a heating rate of 1°C · min⁻¹. All measurements were carried out in 100 mM phosphoric acid-NaOH with 1 mM EDTA, pH 7.0 (in the presence of 0.75 mM DTT for reduced NG_TlpA), using a 1-mm quartz cuvette. Transi-

tions were normalized by assuming a linear dependence of the spectroscopic signal of the native and unfolded states on temperature.

Determination of the redox properties of TlpA. The redox equilibrium of NG_TlpA with glutathione (GSH) was determined using the method described by Wunderlich and Glockshuber (37). Briefly, 0.95 to 1.5 µM protein was incubated for 16 h at 25°C in 100 mM phosphoric acid-NaOH with 0.1 mM EDTA, pH 7.0, containing 1 mM oxidized GSH (GSH disulfide [GSSG]) and increasing concentrations of reduced GSH. The redox equilibrium of DsbA, a periplasmic disulfide isomerase from *E. coli*, was also determined as a positive control. The redox state of NG_TlpA and DsbA was followed by fluorescence emission at 333 nm and 322 nm, respectively (excitation at 280 nm). Measurements were performed in triplicate, and experiments were repeated with three independent enzyme preparations. The equilibrium constants (K_{eq}) were determined by fitting the original fluorescence data according to equation 1:

$$F_m = F_{\text{ox}} + ([\text{GSH}]^2/[\text{GSSG}]) \times (F_{\text{red}} - F_{\text{ox}})/(K_{\text{eq}} + [\text{GSH}]^2/[\text{GSSG}]) \quad (1)$$

where F_m is the measured fluorescence, and F_{ox} and F_{red} are the fluorescence intensities of the oxidized and reduced proteins, respectively (21).

The standard redox potentials of NG_TlpA and DsbA were calculated from the Nernst equation using the GSH standard potential ($E^{0'}_{\text{GSH/GSSG}} = -240\text{ mV}$): $E^{0'}(\text{pH of } 7) = E^{0'}_{\text{GSH/GSSG}} - (RT/nF) \ln(K_{\text{eq}})$, where n is the number of electrons transferred in the reaction, F is Faraday's constant, and R is the gas constant. The equilibrium constant K_{eq} was calculated by fitting the data according to equation 2:

$$R = ([\text{GSH}]^2/[\text{GSSG}])/(K_{\text{eq}} + [\text{GSH}]^2/[\text{GSSG}]) \quad (2)$$

where R is the fraction of reduced protein at equilibrium measured using the specific NG_TlpA or DsbA fluorescence (8).

Thioredoxin activity assay. A thioredoxin activity assay was carried out essentially as described by Holmgren (10). Briefly, enzymatic reactions were conducted in 20 mM potassium phosphate, pH 8, at 25°C and were followed by measuring the reduction of disulfide bonds in insulin, as indicated by precipitation of this protein with the attendant increase in optical density at 650 nm. Reactions were started by the addition of 2 µg of purified NG_TlpA, previously reduced with 100 mM DTT, to 1 mg of insulin from bovine pancreas (Sigma) in 1 ml of 20 mM potassium phosphate buffer, pH 8.0, and reactions were monitored for 1 h. Thioredoxin from *E. coli* (Sigma) was treated as NG_TlpA and used as a reference. Experiments were performed in triplicate and repeated using independent enzyme preparations.

Chromosomal inactivation of *tlpA* and marker rescue. The *tlpA* gene was amplified by PCR from *N. gonorrhoeae* strain 1291, using the primers TlpAF (5'-TCATCCGTTTGGCGGTTTATCGGA-3') and TlpAR_OUT (5'-ATGATGCTCATCGAGTCGTCCTT-3'), and the resulting PCR product was cloned into pGem-T Easy (Promega) according to the manufacturer's instructions. The *tlpA* knockout was constructed by digesting pGEM::*tlpA* with RsrII, which cut the plasmid once in the coding region of *tlpA* (see Fig. 4A). The restriction enzyme was then heat inactivated for 20 min at 65°C, and the ends of the linearized plasmids were filled with T4 DNA polymerase. pUC4Kan (Pharmacia), a plasmid containing a kanamycin resistance cassette, was digested with HincII, and the fragment containing the kanamycin resistance gene was isolated and ligated to the linearized plasmid. The plasmid, designated pGEM::*tlpA*::*kan*, for the *tlpA*::*kan* mutant, was linearized with NotI prior to transformation. *N. gonorrhoeae* strain 1291 was transformed as described previously (12), and recombinant strains were selected by growth on BHI agar containing kanamycin (50 µg · ml⁻¹ and 100 µg · ml⁻¹). The presence of the inactive allele was confirmed by Southern hybridization analysis as previously described by Tseng and coworkers (35) using BglII restriction endonuclease-digested genomic DNA of *N. gonorrhoeae* 1291 and digoxigenin-labeled *tlpA* probes (data not shown). The *tlpA*⁺ strain (*tlpA*^{*}) was constructed by marker exchange: the pGEM::*tlpA* plasmid was linearized with NotI and transformed into *N. gonorrhoeae* 1291 *tlpA*::*kan* mutant strain as described previously. Recombinant strains were screened by the absence of growth on BHI agar containing kanamycin. The *N. gonorrhoeae* 1291 *tlpA*^{*} strain was confirmed by PCR amplification with TlpAF and TlpAR_OUT (see Fig. 4A).

Western blotting. Antibodies were raised against NG_TlpA by immunization of rabbits essentially as described by Power and coworkers (24). Western blotting was performed essentially as previously described (6) and developed using anti-TlpA serum (1:100,000) and anti-rabbit immunoglobulin G conjugated to alkaline phosphatase secondary antibody (1:5,000; Sigma-Aldrich) with BCIP/NBT

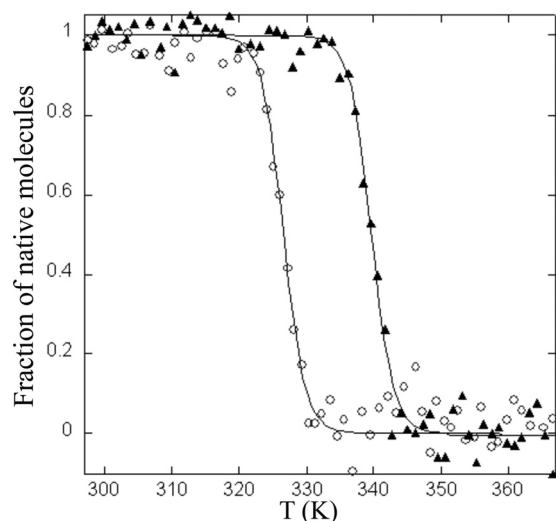


FIG. 1. Thermal unfolding of NG_TlpA measured in 100 mM phosphoric acid–NaOH–1 mM EDTA, pH 7.0. Reduced NG_TlpA was measured in the presence of 0.75 mM DTT. ▲, oxidized NG_TlpA; ○, reduced NG_TlpA; T, temperature.

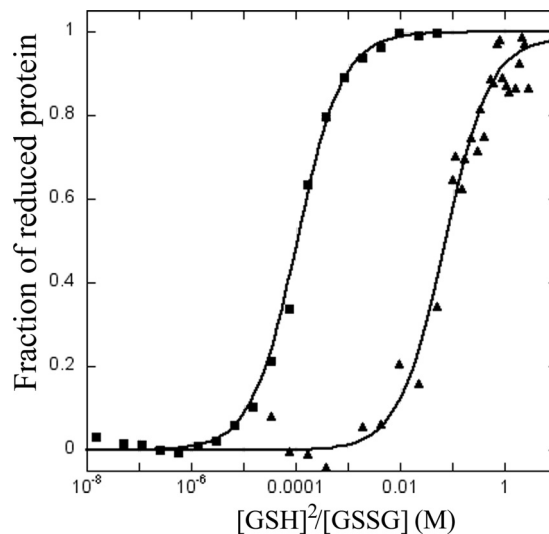


FIG. 2. Redox equilibrium of NG_TlpA (▲) and DsbA (■) with GSH. The relative amount of reduced protein at equilibrium (R) was determined by the redox-state-dependent fluorescence of NG_TlpA and DsbA at 333 nm and 322 nm, respectively (excitation at 280 nm), as described in Materials and Methods. The solid lines correspond to fits of the normalized data according to the overall TlpA/DsbA/GSH equilibrium.

(5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) as chromogenic substrates.

Survival assays. Oxidative killing assays using paraquat and hydrogen peroxide were carried out as previously described (35). Briefly, approximately 10^6 *N. gonorrhoeae* cells (wild type, *tlpA::kan* mutant, or *tlpA**) were added to a solution of BHI broth to a final volume of 100 μ l. Experiments were initiated by the addition of paraquat at a final concentration of 1 mM or of hydrogen peroxide at a final concentration of 40 mM. The initial number of cells and the number of viable cells remaining after 15, 30, 45, or 60 min of treatment were determined by direct colony counts.

Cervical cell invasion and survival assays. Assays of the survival of *N. gonorrhoeae* in primary human cervical epithelial (pex) cells were performed as described in a previous publication (23). Briefly, pex cells were challenged with gonococci at a multiplicity of infection of 100 for 90 min. Gentamicin was then omitted from (association assays) or added to (invasion and intracellular survival assays) infected pex cell monolayers to kill extracellular cell-associated bacteria. The pex cell monolayers were subsequently lysed, or they were subjected to a second incubation in antibiotic-free medium before cell lysis (intracellular survival assays). For all assays, serial dilutions of the cell lysates were plated to enumerate viable CFU.

RESULTS

Identification and in silico analysis of a periplasmic thioredoxin in *N. gonorrhoeae*. Using *B. japonicum* TlpA (GenBank accession number NP_009593) as a query in BLASTP searches, locus NGO1923 was identified in the *N. gonorrhoeae* strain FA1090 as a gene encoding a thioredoxin-like protein (NG_TlpA) with 28% amino acid sequence identity to *B. japonicum* TlpA. The NGO1923 open reading frame is 614 nucleotides in length, and the predicted NG_TlpA protein has a calculated relative molecular mass of 17,595 Da. The TlpA protein of *B. japonicum* has been shown to consist of an N-terminal membrane-spanning helix that anchors a periplasmic-facing water-soluble domain to the cytoplasmic membrane (3). In contrast, NG_TlpA contains a signal peptidase I cleavage site typical of gram-negative bacteria, with a cleavage site between amino acids 22 and 23 in the TlpA sequence, as predicted using SignalP, version 3.0 (1). This is consistent with a periplasmic location for NG_TlpA.

Protein folding analysis using Phyre (16) shows that NG_TlpA incorporates a thioredoxin fold with a helical insertion (~25 residues), similar to that found in *B. japonicum* TlpA but absent in thioredoxin (3, 14). NG_TlpA also contains the Cys-X-X-Cys motif characteristic of thiol-disulfide oxidoreductases of the thioredoxin superfamily (20). There is a correlation between the dipeptide sequence between the cysteine residues and the redox potential of thioredoxin-like proteins (21, 25). In NG_TlpA this is Gly-Pro, similar to that of a thioredoxin and suggesting that the $E^{0'}$ of the thiol/disulfide couple is probably around -200 mV. According to the analysis of Wunderlich and Glockshuber (37), this would be consistent with the idea that NG_TlpA acts as an electron donor.

Biochemical characterization of neisserial TlpA. *N. gonorrhoeae* TlpA was overexpressed in *E. coli* as a recombinant protein in which the 22 amino acid residues at the N terminus were deleted and replaced with a six-histidine tag. The recombinant NG_TlpA was purified as described in the Materials and Methods section. Thioredoxins operate as disulfide reductases, and as a consequence the oxidized form of this protein is more stable toward thermal denaturation. Figure 1 shows the thermal denaturation curves for reduced and oxidized forms of NG_TlpA. The oxidized form of NG_TlpA was denatured at 339 K while the reduced species was denatured at 326 K, consistent with NG_TlpA's being a reducing thioredoxin. Sequence analysis predicted that the $E^{0'}$ of the thiol/disulfide couple of NG_TlpA would be around -200 mV. We investigated this prediction using redox potentiometry. Figure 2 shows the change in the redox state of NG_TlpA, as indicated by Trp fluorescence, as a function of ambient redox potential defined by various ratios of GSH and GSSG. The equilibrium constant (K_{eq}) for the thiol-disulfide exchange reaction with GSH (Fig. 2) for NG_TlpA is $0.75 \pm 0.1 \times 10^{-1}$ M, which

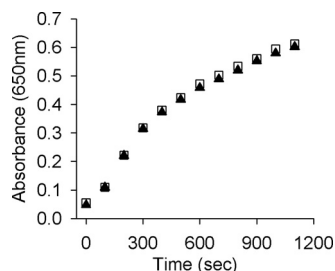


FIG. 3. Insulin reduction assay using purified recombinant NG_TlpA protein of *N. gonorrhoeae* 1291 (▲) and recombinant thioredoxin of *E. coli* (□). Insulin reduction was indicated by the increase in optical density at 650 nm.

converts into a standard redox potential (E^0) of -206 mV. Figure 2 also shows a redox titration using *E. coli* DsbA. The E^0 measured for this oxidizing thioredoxin-like protein is in good agreement with the previously published value (-122.8 mV versus -119 mV) (38) and confirms that neisserial TlpA is, indeed, a thioredoxin that is likely to have a role as a disulfide reductase. This was confirmed using the insulin reduction assay in which purified NG_TlpA protein exhibited disulfide reductase activity, which is typical of a thioredoxin (Fig. 3).

Characterization of a *tlpA* mutant of *N. gonorrhoeae* strain 1291. In view of the importance of proteins exhibiting thiol-disulfide oxidoreductase activity in the periplasm, we investigated the potential function of NG_TlpA by mutational analysis. A *tlpA* mutant was constructed in *N. gonorrhoeae* strain 1291 by disruption of the *tlpA* gene with a promoterless kanamycin antibiotic resistance cassette (Fig. 4A). In addition, a *tlpA** strain was constructed to act as a control for second-site suppressor mutations. In this strain the *tlpA::kan* allele was replaced by wild-type *tlpA* by homologous recombination. The phenotypes of the *tlpA::kan* mutant and *tlpA** strains were confirmed by probing the gonococcal cells with TlpA antibody, generated using recombinant NG_TlpA protein (Fig. 4B). Investigations in *B. japonicum* have revealed that TlpA was re-

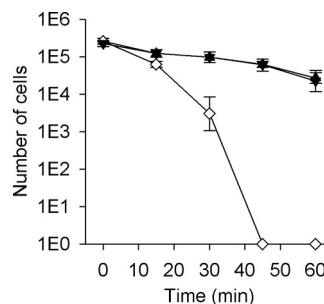


FIG. 5. Paraquat killing assay of *N. gonorrhoeae* 1291 (wild-type; ▲), 1291 *tlpA::kan* (*tlpA* mutant; ◇), and 1291 *tlpA** (knock-in mutant; ▼). In this assay, cells were grown on BHI agar and exposed to 1 mM paraquat. Experiments were performed in triplicate and repeated on several occasions. Error bars indicate ± 1 standard deviation of the mean.

quired for the assembly of the Cu_A center of the aa_3 -type cytochrome oxidase (18). Since pathogenic *Neisseria* cells contain a single oxidase (28), cytochrome *cbb_3*, which lacks a Cu_A center, it seemed unlikely that NG_TlpA would have a role in the biogenesis of the neisserial cytochrome oxidase. As expected, both wild-type and mutant strains stained positive for the cytochrome oxidase test (data not shown).

Thioredoxins and related proteins with thiol-disulfide oxidoreductase activity have a number of important roles within bacterial cells including maintenance of a reducing environment of the cytoplasm and the protection of cells against peroxide stress (4, 11, 33). As a consequence we tested whether NG_TlpA might have a role in protection against oxidative stress in *N. gonorrhoeae* as indicated by sensitivity to paraquat, a generator of superoxide ions, or hydrogen peroxide. Figure 5 shows that the *tlpA::kan* mutant was sensitive to killing by paraquat while the wild-type and *tlpA** strains were more resistant, and a significant fraction of the population survived (about 20% of the population) even at 45 min of incubation with paraquat. Figure 6 shows that the *tlpA::kan* mutant was

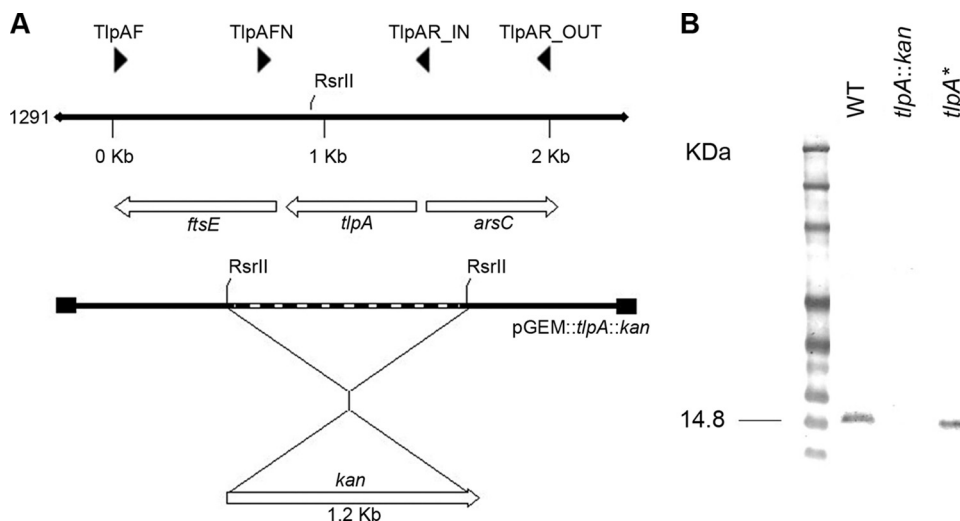


FIG. 4. (A) Organization of *N. gonorrhoeae* 1291 chromosomal region containing the *tlpA* gene and strategy for insertional inactivation of *tlpA* using a kanamycin cassette. (B) Western blot of soluble extracts from *N. gonorrhoeae* 1291 (WT), 1291 *tlpA::kan*, and 1291 *tlpA** strains probed using anti-TlpA antibody.

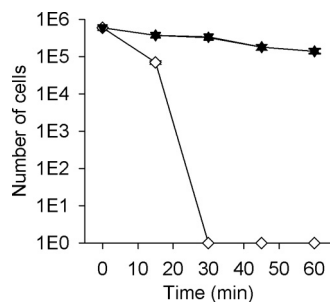


FIG. 6. Hydrogen peroxide killing assay of *N. gonorrhoeae* 1291 (wild-type; ▲), 1291 *tlpA::kan* (*tlpA* mutant; ◇), and 1291 *tlpA** (knock-in mutant; ▼). In this assay, cells were grown on BHI agar and exposed to 40 mM hydrogen peroxide. The *N. gonorrhoeae* 1291 *kat::kan* mutant strain was used as a control (data not shown) (35). Experiments were performed in triplicate and repeated on several occasions. Error bars indicate ± 1 standard deviation of the mean.

highly sensitive to killing by hydrogen peroxide while the wild-type and *tlpA** strains were resistant. The data obtained from these oxidative killing assays suggest that NG_TlpA contributes to defense against oxidative stress in *N. gonorrhoeae*.

A *tlpA* mutant exhibits reduced colonization of cervical epithelial cells. To determine whether TlpA might be physiologically relevant for successful gonococcal infection, the ability of *N. gonorrhoeae* 1291 and 1291 *tlpA::kan* to associate with, invade, and survive within pex cells was tested. The pex cells were challenged with either the wild-type or mutant strain, and infection was allowed to progress for up to 5 h. There was no significant difference observed in the ability of the *tlpA::kan* strain to associate with pex cells in comparison to the wild type (Fig. 7). However, the *tlpA::kan* strain showed reduced ability to survive and/or replicate within pex cells compared with the wild-type strain (Fig. 7). This deficiency was corrected in the *tlpA** strain.

DISCUSSION

The importance of protein disulfide reduction reactions in the periplasm of gram-negative bacteria is now well established with respect to protein disulfide isomerization and protein folding as well as *c*-type cytochrome maturation. However, it is increasingly clear that there are additional thioredoxin-like proteins which operate in the periplasm that are distinct from DsbC/DsbG and CcmG. NG_TlpA is one example, and our biochemical analysis clearly indicates that the thermodynamic and catalytic properties of this protein are consistent with its operating as a disulfide reductase. As for the DsbC/DsbG and CcmG proteins, the source of electrons for NG_TlpA seems likely to be DsbD, consistent with the latter's role as a redox hub for transfer of thiol reducing power from cytoplasmic thioredoxin to periplasmic-facing thioredoxins (34).

Although we can be confident that TlpA operates as a thioredoxin, the nature of the biomolecules to which NG_TlpA donates electrons has not been established. A clue about the potential role for NG_TlpA comes from our observation that the *tlpA::kan* mutant is sensitive to oxidative killing by hydrogen peroxide and superoxide. One possibility is that NG_TlpA is associated with the maintenance of specific cysteine residues of some periplasmic proteins in a reduced state. Oxidizing

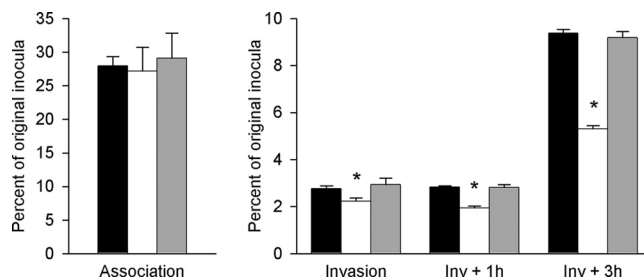


FIG. 7. TlpA mutant gonococci are impaired in their ability to invade and survive within primary cervical cells: association, invasion (Inv), and survival assays were performed as described in the text, where association corresponds to a 90-min infection and invasion refers to a 90-min infection plus gentamicin treatment. Survival (Inv + 1 h or 3 h) refers to the number of gonococci that survive within pex cells at 1 h or 3 h following gentamicin treatment. Values depicted were determined as a function of the original inoculum and the number of CFU formed with subsequent plating of the pex cell lysates. Data given were obtained from three trials performed in triplicate. *P* values were determined using a Kruskal-Wallis *k*-sample analysis of variance calculated for the *tlpA::kan* mutant (white) or knock-in mutant (gray) gonococci upon comparison to the wild-type bacteria (black), as outlined in the text (*, $P < 0.001$).

agents such as hydrogen peroxide can cause oxidation of cysteine thiols that can react with small-molecule thiols or cysteine thiols in proteins to form mixed disulfides or disulfide bonds between proteins. Such possibilities have been suggested for the periplasmic thioredoxin from *Chlamydia pneumoniae*, DsbH (19), although it should be noted that DsbH does not appear to be closely related to NG_TlpA or the TlpA/ResE/CcmG family of thioredoxin-like proteins. Bardwell and co-workers have suggested that a generally more reducing periplasm may represent an adaptation to an intracellular environment, as in the case of *Chlamydia*. Methionine and cysteine are the amino acids that are most sensitive to oxidation by hydrogen peroxide; the former is oxidized to methionine sulfoxide while the latter can form a cysteine sulfenic acid, which in turn can react with a thiol to form a disulfide. *N. gonorrhoeae* possesses a highly unusual methionine sulfoxide reductase (MsrAB) that is located in the outer membrane rather than the cytoplasm, as in most other bacteria (32). Although there is in vitro biochemical evidence that the thioredoxin domain of MsrAB interacts directly with DsbD (2), another possibility is that periplasmic TlpA mediates electron flow between these inner and outer membrane proteins. In this context it is interesting that a *pilB* mutant of gonococcus is also sensitive to killing by hydrogen peroxide (32).

N. gonorrhoeae grows extracellularly in the human host, but it can also invade and grow within cervical epithelial cells (5). The potential importance of NG_TlpA to pex cell infection was illustrated by the reduced survival of the *tlpA::kan* mutant compared to wild-type and *tlpA** strains. However, the effect of the *tlpA* mutation was modest compared to that of other mutations that affect defense against oxidative and nitrosative stress and that have been analyzed using this same (pex cell) model system (22, 30). The host factors (e.g., hydrogen peroxide or nitric oxide production) contributing to the impaired ability of the TlpA strain to survive during the course of pex cell challenge will require further analysis in the future. How-

ever, our data may collectively reflect a redundancy in periplasmic oxidative stress defense systems in *N. gonorrhoeae*.

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