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Pneumococcal Gene Complex Involved in Resistance to Extracellular Oxidative Stress

Vahid Farshchi Andisi,[a] Cecilia A. Hinojosa,[c] Anne de Jong,[b] Oscar P. Kuipers,[b] Carlos J. Orihuela,[c] and Jetta J. E. Bijlsma[a]

Laboratory of Molecular Bacteriology, Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands[a]; Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, Rijksuniversiteit Groningen, Groningen, The Netherlands[b]; and Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA[c]

***Streptococcus pneumoniae* is a Gram-positive bacterium which is a member of the normal human nasopharyngeal flora but can also cause serious disease such as pneumonia, bacteremia, and meningitis. Throughout its life cycle, *S. pneumoniae* is exposed to significant oxidative stress derived from endogenously produced hydrogen peroxide (H_2O_2) and from the host through the oxidative burst. How *S. pneumoniae*, an aerotolerant anaerobic bacterium that lacks catalase, protects itself against hydrogen peroxide stress is still unclear. Bioinformatic analysis of its genome identified a hypothetical open reading frame belonging to the thiol-specific antioxidant (TlpA/TSA) family, located in an operon consisting of three open reading frames. For all four strains tested, deletion of the gene resulted in an approximately 10-fold reduction in survival when strains were exposed to external peroxide stress. However, no role for this gene in survival of internal superoxide stress was observed. Mutagenesis and complementation analysis demonstrated that all three genes are necessary and sufficient for protection against oxidative stress. Interestingly, in a competitive index mouse pneumonia model, deletion of the operon had no impact shortly after infection but was detrimental during the later stages of disease. Thus, we have identified a gene complex involved in the protection of *S. pneumoniae* against external oxidative stress, which plays an important role during invasive disease.**

Streptococcus pneumoniae is a Gram-positive, catalase-negative bacterium which is classified as a facultative anaerobe. It colonizes the nasopharynx as do other pathogens such as *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Neisseria meningitides*. *S. pneumoniae* can spread from the upper respiratory tract to other parts of the human body, which leads to serious disease, such as pneumonia, otitis media, meningitis, and bacteremia. People susceptible to pneumococcal disease are young children, the elderly, and immunocompromised patients, especially in developing countries. For instance, pneumococcal septicemia is a major cause of infant death in developing countries. It causes about 25% of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year (4, 24).

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$), are a consequence of the use of oxygen (O_2) in fundamental enzymatic reactions and biochemical pathways. Accumulation of ROS has harmful effects on all living organisms; for instance, $O_2^{\bullet -}$ and H_2O_2 damage proteins through oxidation, whereas $OH^{\bullet}$ targets DNA, leading to lesions and mutations, which also may be fatal (22, 23, 63). *S. pneumoniae* encounters external and internal oxidative stress during its life cycle. Major exogenous sources of ROS for *S. pneumoniae* are neutrophils, macrophages, and other lactic acid bacteria in the nasopharynx. Neutrophils and macrophages release a diversity of ROS such as H_2O_2, $OH^{\bullet}$, and $O_2^{\bullet -}$ through the oxidative burst. Hydrogen peroxide is moderately stable and in the presence of metal ions can be converted via the Fenton reaction to $OH^{\bullet}$, which is the only ROS that can directly harm most molecules and can be involved in the production of other ROS (23).

In addition to these exogenous sources, the enzyme pyruvate oxidase (SpxB) internally generates significant amounts of hydrogen peroxide up to 2 mM (46, 58), which may also be produced under *in vivo*-like conditions such as biofilms (31). This concentration is higher than that generated by many other species (46, 48) and is sufficient to kill or inhibit other nasopharyngeal flora members such as *H. influenzae* and *N. meningitides* (47) although it does not seem to interfere with *Staphylococcus aureus* colonization (33). Furthermore, these amounts of H_2O_2 have a cytotoxic effect both *in vivo* and *in vitro* on human epithelial (12, 17) and endothelial (6) cells. The action of SpxB is thought to play a central role in metabolism (48) and influences competence via an as yet unknown mechanism (2). The gene itself is regulated by SpxR (52) and plays an important role in the virulence of pneumococci (41, 53, 58). Thus, a major source of both endogenous and external ROS for *S. pneumoniae* in the nasopharynx is the action of SpxB, and the levels of H_2O_2 produced are sufficient for the generation of $OH^{\bullet}$ via the Fenton reaction in the absence of exogenous H_2O_2 (48, 49).

S. pneumoniae lacks canonical proteins identified in other bacteria that protect against oxidative stress, such as catalase, and homologues of global response regulators, such as OxyR, PerR, and SoxRS (5, 48), and seems to lack an adaptive response to oxidative stress (48). Interestingly, SpxB also plays an important role in resistance to H_2O_2 (48). Other proteins implicated in the defense against oxidative stress in *S. pneumoniae* are the manganese-dependent superoxide dismutase (SodA) (62), an AhpD homologue (44), the putative transcriptional regulator Rgg

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Editor: J. N. Weiser
Address correspondence to Jetta J. E. Bijlsma, j.j.e.bijlsma@umcg.nl.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
S. pneumoniae strains		
G54	Serotype 19F strain	11a
670	Serotype 6B isolate	16a
D39	Serotype 2 strain	26
R6	Unencapsulated serotype 2 lab strain	20a
TIGR4	Serotype 4 strain	60a
D39 *nisRK*	D39 Δ*bgaA*::*nisRK*, Tm^r	41; this study
R6 *nisRK*	R6 Δ*bgaA*::*nisRK*, Tm^r	This study
TIGR4 *nisRK*	TIGR4 Δ*bgaA*::*nisRK*, Tm^r	This study
G54 Δ*tlpA*	Sp^r	This study
670 Δ*tlpA*	Sp^r	This study
TIGR4 Δ*tlpA*	Sp^r	This study
D39 Δ*tlpA nisRK*	D39 Δ*tlpA* Δ*bgaA*::*nisRK*, Tm^r Sp^r	This study
R6 Δ*tlpA nisRK*	R6 Δ*tlpA* Δ*bgaA*::*nisRK*, Tm^r Sp^r	This study
D39 Δ*tlpA nisRK* pNZ8048	D39 Δ*tlpA* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
D39 Δ*tlpA nisRK* pNZ8048::*tlpA*	D39 Δ*tlpA* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
D39 Δ*spd0571-0573 nisRK*	D39 Δ*spd0571-0573* Δ*bgaA*::*nisRK* Tm^r Sp^r	This study
D39 Δ*spd0571-0573 nisRK* pNZ8048::*spd0571-0573*	D39 Δ*spd0571-0573* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
D39 Δ*spd0571-0573 nisRK* pNZ8048::*spd0571-0572*	D39 Δ*spd0571-0573* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
D39 Δ*spd0571-0573 nisRK* pNZ8048::*spd0572-0573*	D39 Δ*spd0571-0573* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
D39 Δ*spd0571-0573 nisRK* pNZ8048::*spd0573*	D39 Δ*spd0571-0573* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
TIGR4 Δ*sp0658-0660 nisRK*	TIGR4 Δ*sp0658-0660* Δ*bgaA*::*nisRK*, Tm^r Sp^r	This study
TIGR4 Δ*sp0658-0660 nisRK* pNZ8048	TIGR4 Δ*sp0658-0660* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
TIGR4 Δ*sp0658-0660 nisRK* pNZ8048::*spd0571-0573*	TIGR4 Δ*sp0658-0660* Δ*bgaA*::*nisRK*, Tm^r Sp^r Cm^r	This study
L. lactis NZ9000	MG1363 Δ*pepN*::*nisRK*	27a
E. coli strains		
DH5α		
BL21(DE3)		New England Biolabs
Plasmids		
pNZ8048	Cm^r; nisin-inducible P*nisA*	11
pNZ8048E	Cm^r; nisin-inducible P*nisA*; *ermB*	11
pNZ8048::*tlpA*	Cm^r; *tlpA* nisin-inducible P*nisA*	This study
pNZ8048::*spd0571-0573*	Cm^r; *spd0571-0573*; nisin-inducible P*nisA*	This study
pNZ8048::*spd0571-0572*	Cm^r; *spd0571-0572*; nisin-inducible P*nisA*	This study
pNZ8048::*spd0572-0573*	Cm^r; *spd0572-0573*; nisin-inducible P*nisA*	This study
pNZ8048::*spd0573*	Cm^r; *spd0573*; nisin-inducible P*nisA*	This study
pET-26b(+)	Km^r	Novagen
pET-26b(+)::*tlpA*	Km^r	This study
pOri28	Em^r Sp^r	26

[a] Em^r, Erythromycin resistance; Tm^r, Trimethoprim resistance; Sp^r, spectinomycin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

(5), the pneumococcal manganese transporter PsaBCA that includes the pneumococcal surface antigen A (PsaA) (36, 61), the serine protease HtrA (21), the MerR/NmlR family transcription factor (51), and the ClpP protease (42, 54). Additionally the PsaD protein has been suggested to be a thiol peroxidase (39). However, how *S. pneumoniae* protects itself against oxidative stress, in particular of extracellular origin, is still not entirely clear.

To elucidate additional mechanisms, we performed a bioinformatic analysis of the genomes of *S. pneumoniae*, *N. meningitides*, and *H. influenzae*, which identified a hypothetical open reading frame (ORF) belonging to the thiol-specific antioxidant (TlpA/TSA) branch of the thioredoxin super family (32). Thioredoxins and thioredoxin-like proteins can protect bacteria against oxidative stress (23). Therefore, we studied the role of this protein in oxidative stress survival. We demonstrated that the ORF and the operon in which it is located play an important role in the protection of *S. pneumoniae* against external peroxide stress and the establishment of long-term infection and disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in the present study are listed in Table 1 and were stored in 15% glycerol at −80°C. *S. pneumoniae* was routinely grown as standing cultures in brain heart infusion (BHI) broth (Oxoid) at 37°C, *Lactococcus lactis* NZ9000 was grown standing in M17 broth (Oxoid) (60) containing 0.5% glucose (GM17) at 30°C, and *Escherichia coli* was grown in LB broth in shaken cultures at 37°C. *L. lactis* and *S. pneumoniae* were grown, respectively, on GM17 and GM17 agar plates with 3% defibrinated sheep blood (Oxoid), whereas *E. coli* was cultivated on LB agar. When necessary, chloramphenicol, spectinomycin, and trimethoprim were added to the growth medium in the following concentrations: 2.5, 150, and 15 μg/ml, respectively, for *S. pneumoniae*; 4 μg/ml chloramphenicol for *L. lactis*; and 20 μg/ml kanamycin for *E. coli*. For *in vitro* complementation analyses, all strains were exposed to nisin (3 ng/ml) during early log phase and at least 2 h before the application of oxidative stress.

Bioinformatic comparison of the genomes of respiratory pathogens. The sequences of the predicted proteins in the genome of *S. pneumoniae* R6 derived from the NCBI GenBank database (http://www.ncbi

TABLE 2 Primers used in this study

Primer	Nucleotide sequence (5′ to 3′)	Restriction site
SPD571for	GCTCTAGAGTTTATTACGAACTCTGGCC	XbaI
SP571rev	CCGGATCCAGAAGGATAGATCCCTGCC	BamHI
SPD573for	GGGAATTCCAGCCTAGGAGGCGTCTTATG	NcoI
SPD573rev	ATCCCATGGGCATCATAGGTGACATGGAC	EcoRI
SPD570for	AAGAGTCGTGGACTTATTTC	
571rev	TGTATTCAAATATATCCTCCTCACCCCATAAAGTTCCTCCTATC	
574for	AATTGAAAAAATGGTGGAAACACTCGCTTATTTACTAGATTATG	
574rev	ACTGGCTAAAGACTTTAAAC	
ctrldtlpAfor	CAATGCCAGTATGCAGAGTT	
ctrltlpArev	GTAATCCTCAGCCACCACAA	
ctrl571-573for	GTCGCAGTAGCCTACTATTG	
ctrl571-573rev	GGCTTGTTTAACACGTTCTC	
tlpA.for	CATGCCATGGAAAAATGGCAAACATGTG	NcoI
tlpA.rev	CCCTCTAGAGCCTCCTAGGATAATTC	XbaI
operon.for	CCCCCATGGGTCATATTTTCTTTTTTC	NcoI
operon.rev	CCCTCTAGATTAATCAACATAATCTAGTA	XbaI
572SQ	CATATGTCAGGCAAGTCCGTGACTAG	NdeI
572SC	CCCTCGAGGGCTAATTCCTTCAAAGT	XhoI
specfor	GTGAGGAGGATATATTTGAATACA	
specrev	AGTGTTTCCACCATTTTTTCAATT	
658F	TGGCAGGGATTCTATCCTTC	
658R	CTAGAAGCCTGAGCACCATC	
571F	CTTGGTTCTGTTTGGAAATG	
571R	CGAACCTGCTCCAAGAACAC	
572F	CAAACTTTGAAGGAATTATC	
572R	TTCGTATCAATAGAACATAG	

.nlm.nih.gov/GenBank/) were sequentially compared to the predicted protein sequences in the genomes of *S. pneumoniae* TIGR4, *Neisseria meningitidis* MC58, *Neisseria meningitidis* Z2491, and *Haemophilus influenzae* Rd KW20 using BlastP. Proteins were considered homologous when the E value was $<10^{-13}$. Subsequently, the list of R6 proteins with a homologue in the other genomes was compared to the sequences of the predicted proteins in the genome of *L. lactis* IL1403 using BlastP, and a protein was considered nonhomologous when the E value was $>10^{-19}$.

Generation of mutants in *S. pneumoniae*. Strain R6 *nisRK* was generated by the introduction of the *nisRK* genes into the *bgaA* locus of R6 as described by Kloosterman et al. (27). Deletion of *spd0572* was accomplished using primers SPD571for/SPD571rev and SPD573for/SPD573rev using plasmid pOri28 as described by Kloosterman et al. (26). The *spd0571-0573* genes were deleted from the D39 genome by allelic replacement mutagenesis (45). Briefly, primers SPD570for/571rev and 574for/574rev (Table 2), respectively, were used to generate 5′ and 3′ flanking fragments of *tlpA* and the operon *spd0571-0573*, respectively. These fragments were fused to a spectinomycin resistance gene amplified with primers specfor and specrev (3). Subsequently the PCR fragments were transformed into various *S. pneumoniae* strains. Generation of the correct deletion mutant of *tlpA* and the operon *spd0571-0573* was verified by PCR using the primers ctrldtlpAfor/ctrltlpArev and ctrl571-573for/ctrl571-573rev, which are located outside the original deletion region; additionally the *tlpA* mutant was verified by Western blotting.

Isolation of RNA and RT-PCR. RNA of *S. pneumoniae* D39 was extracted as described before (14). Briefly, cells were harvested in mid-exponential phase, immediately frozen in liquid nitrogen, and stored at −80°C. RNA extractions were performed as described previously (13); to remove any possible DNA contamination, DNase I treatment was performed with TURBO DNA-free reagent (Ambion, Austin, TX), and the quality of the RNA was assessed using an Agilent RNA Nano Chip (Agilent Technologies, CA). A TaqMan reverse transcription-PCR (RT-PCR) kit (Life technologies) was used for the generation of cDNA from 100 ng of RNA. Control reactions were performed without reverse transcriptase to confirm the absence of contaminating genomic DNA. The resulting cDNA was used as a template for PCR.

Construction of pNZ8048 derivatives containing *tlpA*, the operon, and other derivatives. For complementation analysis, a pNZ8048 (11) derivative was constructed that contained the *tlpA* gene or the *spd0571-0573* operon under the control of *nisA*, a nisin-inducible promoter. Primers tlpAF and tlpaR or primers operonF and operonR, which contained NcoI and XbaI restriction sites, were used to amplify the *tlpA* gene and the *spd0571-0573* operon from the chromosome of strain D39. The resulting PCR products were introduced into the pNZ8048 plasmid using the restriction sites mentioned above, resulting in pNZ8048::*tlpA* and pNZ8048::*spd0571-0573*, respectively. Subcloning of the latter plasmid using AvrII/XbaI, NcoI/BseYI, and NcoI/AvrII unique restriction sites in the operon and the plasmid resulted in pNZ8048::*spd0571-0572*, pNZ8048::*spd0572-0573*, and pNZ8048::*spd0573*, respectively. The plasmids were transferred to *S. pneumoniae* and *L. lactis* for complementation studies.

Paraquat sensitivity assays. Bacteria were grown until mid-log phase, and 200 μl of the cultures was added to 200 μl of medium with 60 mM paraquat (Supleco) or without paraquat (61). Both samples were incubated at 37°C for 2 h. The percent survival was calculated by dividing the number of CFU of the cultures after exposure to paraquat by the number of CFU of the control without paraquat.

Hydrogen peroxide sensitivity assays. The hydrogen peroxide sensitivity assay was performed essentially as described by Pericone et al. (48). Briefly, bacteria were grown in BHI broth until mid-log phase (optical density at 600 nm [OD_{600}] of 0.2 to 0.3), and 100-μl aliquots of each culture were added to 100 μl of medium or 100 μl of medium containing 40 mM H_2O_2 (Merck), resulting in an exposure to 20 mM H_2O_2. The bacterial cultures were incubated at 37°C for 30 min. The reaction was stopped by the addition of 200 U of bovine liver catalase (Sigma); after serial dilutions from each well were prepared, aliquots were spotted onto blood agar plates. The percentage of survival of hydrogen stress was determined and calculated as described above.

***In vitro* competitive growth assay.** TIGR4 and the operon mutant, which contained a spectinomycin resistance cassette, were diluted 1:100 in a 1:1 ratio; as a control, the mutant was also diluted separately to 1:100 in BHI broth. Directly after cultures were mixed, CFU counts were determined at the start of and during exponential (OD_{600} of ~0.2) and stationary (OD_{600} of ~ 0.5) phases and 24 h later. All serial dilutions were plated on blood agar plates; in addition, the mixed cultures were also plated on plates with spectinomycin to determine the number of mutant bacteria. CFU counts were determined after overnight incubation. To calculate the ratio wild type to mutant, the number of spectinomycin-resistant colonies was subtracted from the total number of colonies. The number of CFU of the mutant derived from the mixed culture plated on spectinomycin plates corresponded with the number of mutant CFU derived from the individual culture plated on blood agar. This indicated that there were no negative effects of the spectinomycin and the coculturing with the wild type. Furthermore, the ratio of the wild type to mutant did not change more than 10-fold over the course of 24 h.

Competitive index assay in a mouse pneumonia model. Experiments with female BALB/c mice 6 weeks of age were performed at the University of Texas Health Science Center at San Antonio, Texas, under Institutional Animal Care and Use Committee protocol 09022x-34. To minimize distress, mice were anesthetized with 2.5% vaporized isoflurane during all experimental procedures including inoculation and collection of nasal lavage and prior to euthanasia. Mice were infected with either TIGR4 and TIGR4 Δ*spd0571-0573* (Sp^r) at a ratio of 1:1 or TIGR4 and TIGR4 Δ*spd0571-0573 nisRK* complemented with pNZ8048E or pNZ8048::*spd0571-0573* at a ratio of 1:1. In the latter experiments mice received drinking water supplemented with nisin (2 μg/liter) to drive the expression of the operon from the *nisA* promoter on the plasmid. For intranasal challenge, mice were held up manually, and the left nostril was inoculated with 2.0×10^6 CFU in 25 μl of phosphate-buffered saline (PBS) in a dropwise fashion using a pipette. Immediately afterwards, mice were hung over their cage by their incisors on a wire until they awoke and removed themselves, typically within 20 to 30 s. For determination of bacterial titers in the nasopharynx, total numbers of pneumococci in nasal lavage fluids were quantified. Briefly, using the same protocol as challenge, 10 μl of PBS was inoculated into the left nostril, followed by its aspiration after 5 to 10 s. Typically, 2 to 4 μl was recovered per mouse. For determination of bacterial titers in the blood, 2 to 5 μl of blood was collected from the tail vein. Bacterial titers in nasal lavage fluid and blood samples were determined by serial dilution, plating on blood agar plates, and extrapolation from bacterial counts following overnight incubation. For determination of bacterial titers in the lungs, mice were sacrificed, and the lungs were excised, weighed, and homogenized. Bacterial titers were determined per gram of homogenized tissue. All dilutions were plated in replicate using plates with the appropriate antimicrobial to discriminate between strains. Statistical analysis of bacterial counts was done using a nonparametric Mann-Whitney rank sum test using SigmaStat statistical analysis software (Aspire Software, Ashburn, VA).

Production and purification of the TlpA protein and generation of anti-TlpA antibodies. To avoid problems associated with the purification of proteins covalently attached to the membrane such as lipoproteins, *tlpA* without its signal sequence and its stop codon was amplified from the D39 genome using primers 572SQ and 572SC. The PCR product was cloned into the pET-26b(+) expression vector (Novagen, Inc.) using the NdeI and XhoI restriction sites. The resulting plasmid pET-26b::*tlpA* was subsequently transformed into *E. coli* strain BL21(DE3) for high-level TlpA production. Expression of *tlpA* including a C-terminal His_6 tag, which is provided by the pET26b(+) vector, was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and its production was confirmed by Western blot analysis using an anti-His tag antibody (Zymed-Invitrogen). To purify the protein, a 1-liter culture of *E. coli* strain BL21(DE3) was induced with 1 mM IPTG; after 3 h the cells were harvested by centrifugation and resuspended in binding buffer (20 mM sodium phosphate buffer, 300 mM NaCl, 10% [vol/vol] glycerol, 5 mM imidazole, 3 mM dithiothreitol [DTT], pH 7.4). Cells were disrupted with a bead beater (Bertin) using three cycles of 30 s and after centrifugation at 14,000 rpm for 5 min; the clarified supernatant fraction was applied to a 5-ml His-Trap HP column (GE Healthcare). After the unbound proteins were washed off the column with binding buffer, the His-tagged TlpA protein was eluted from the column using elution buffer (20 mM sodium phosphate buffer, 300 mM NaCl, 10% [vol/vol] glycerol, 3 mM DTT, pH 7.4) with a gradient of increasing imidazole concentrations (up to 500 mM imidazole). The purity of the eluted protein fraction was determined using SDS-PAGE and Coomassie staining, and 1.14 mg of purified protein was used for the generation of a polyclonal antibody (Eurogentec, Seraing, Belgium).

Fractionation of *S. pneumoniae* cells. Fractionation of S. *pneumoniae* cells was performed essentially as described previously (38). *S. pneumoniae* was grown to the desired OD in BHI broth, after which the bacteria were spun down for 10 min at 7,000 rpm. The supernatant was precipitated using 50% trichloroacetic acid (TCA), and the cell pellet was taken up in 1/20 volume of lysis buffer (100 mM Tris-HCl, pH 8.0, 20% sucrose, 20 mM $MgCl_2$) to which fresh lysozyme (5 mg/ml), mutanolysin (200 U/ml), and EDTA-free protease inhibitor (Roche) were added, and the mixture was incubated for 30 min at 37°C. The protoplasts were spun down at 3,000 × *g* for 10 min at 4°C, resuspended in 0.5 ml of sucrose buffer (20% sucrose, 10 mM Tris-HCl, pH 8.0), and disrupted by the addition of 9.5 ml of 100 mM Tris-HCl with EDTA-free protease inhibitor and 1 mM EDTA; undisrupted protoplasts were removed by centrifugation (4,000 × *g* for 10 min at 4°C). The membrane and cytoplasm fractions were separated by centrifugation at 100,000 × *g* for 30 min at 4°C. Samples were taken up in loading buffer, incubated for 5 min at 95°C, and used for SDS-PAGE and Western blot analysis.

SDS-PAGE, Western blotting, and analysis of Western blot signals. The presence of various proteins was detected by Western blot analysis. Protein fractions were separated by SDS-PAGE using precast NuPAGE gels from Invitrogen and then semidry blotted (1.25 h at 100 mA per gel) onto a nitrocellulose membrane (Protran; Schleicher and Schuell). Detection of antibodies was carried out with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit; LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). For antibody detection, fluorescence at 800 nm was recorded using the linear range. Signals on the Western blots were quantified as follows: non-gamma-transformed images with nonsaturated signals, as determined with the plot profile function of ImageJ (rsb.info.nih.gov/ij), were analyzed using the gel function of the same program. Comparisons of the amount of signal for each type of sample were performed on the same Western blot using the "gel lane" analysis function of the same program. The only digital manipulation performed on the scans was the switching of lanes to obtain the desired order.

Thioredoxin activity assay. Thioredoxin activity was determined as described by Holmgren (18). Briefly, 2 μg of purified TlpA or 2 μg of thioredoxin from *E. coli* (Sigma) and 100 mM DTT were mixed, and the reaction was started by the addition of 1 mg of insulin from bovine pancreas (Sigma) in 1 ml of 20 mM potassium phosphate buffer, pH 8.0; reactions were monitored for 1 h by measuring the reduction of disulfide bonds in insulin as determined by the precipitation of the protein, which is measured at 650 nm.

Statistical analysis. Statistical significance of the hydrogen peroxide stress survival was determined using the independent sample *t* test using SPSS, version 16.0. For the analysis of the animal model a nonparametric Mann-Whitney rank sum test using SigmaStat statistical analysis software (Aspire Software, Ashburn, VA) comparing the ratios to a value of 1 was performed.

RESULTS

Identification of a thioredoxin family protein, which is involved in protection against oxidative stress. Three important human

TABLE 3 R6 proteins with homologues in TIGR4, *N. meningitidis*, and *H. influenzae* but not in *L. lactis*

TIGR4 locus	D39 locus	R6 locus	Protein no.	R6 annotation
sp0289	*spd0269*	*spr0266*	NP_357860.1	Dihydropteroate synthase
sp0408	*spd0372*	*spr0369*	NP_357963.1	D-Alanine glycine permease
sp0413	*spd0377*	*spr0374*	NP_357968.1	Aspartate kinase
sp0479	*spd0429*	*spr0426*	NP_358020.1	Trk transporter, membrane spanning
sp0480	*spd0430*	*spr0427*	NP_358021.1	Trk transporter, NAD^+ binding
sp0659	*spd0572*	*spr0576*	NP_358170.1	Conserved hypothetical protein
sp0737	*spd0642*	*spr0648*	NP_358242.1	Sodium-dependent transporter
sp0977	*spd0864*	*spr0880*	NP_358474.1	Tellurite resistance
sp1068	*spd0953*	*spr0974*	NP_358568.1	Phosphoenolpyruvate carboxylase
sp1165	*spd1029*	*spr1052*	NP_358646.1	MATE efflux family protein
sp1305	*spd1158*	*spr1181*	NP_358774.1	NADP-specific glutamate
sp1500	*spd1328*	*spr1353*	NP_358946.1	ABC transporter, substrate-binding

pathogens colonize the nasopharynx, cause similar diseases, and are naturally competent. To identify genes that they have in common and that might contribute to virulence and oxidative stress resistance, all predicted open reading frames (ORF) in the *S. pneumoniae* R6 and TIGR4 genomes were compared to those in the *N. meningitidis* MC58 and *H. influenzae* Rd KW20 genomes using BLASTP (http://blast.ncbi.nlm.nih.gov). We then selected those ORFs that did not display significant homology to any predicted protein in the genome of *Lactococcus lactis* IL1403, which is related to *S. pneumoniae* but is not a pathogen and does not colonize the nasopharynx. This resulted in the identification of several ORFs (Table 3). One ORF is involved in folate synthesis and resistance to sulfonamides (*spd0269*, *sulA*) (15), and another (*spd1029*) belongs to the multiantimicrobial extrusion (MATE) family and might also be involved in drug resistance. Four ORFS are or seem to be involved in amino acid metabolism and uptake (*spd0372*, *spd0377*, *spd1158*, and *spd1328*) (27). Two ORFs (*spd0429* and *spd0430*) encode homologues of proteins that form part of the Trk potassium transport system, which has also been implicated in resistance to antimicrobial peptides (43). Another ORF (*spd0864*) is a homologue of *tehB* and is most likely involved in tellurite resistance (30), and one (*spd0953*) is involved in the pyruvate metabolism of *S. pneumoniae*. One ORF, *spd0572*, is predicted to encode a protein belonging to the thioredoxin-like protein family, of which TlpA, ResA, and DsbE are members (32; http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi? uid = cd02966); thus, we named this protein TlpA. Proteins belonging to this family have been shown to play an important role in the response against oxidative stress (7, 9, 19, 32). Interestingly, the ORF was identified in two previous signature-tagged mutagenesis (STM) studies as being required for successful animal passage (10, 28). Therefore, we investigated the role of TlpA during intracellular and extracellular oxidative stress in *S. pneumoniae*.

TlpA is not involved in the defense against intracellular oxidative stress. To determine whether TlpA is involved in the protection against intracellular oxidative stress, the gene was deleted; no obvious differences in growth compared to the wild type were observed under normal conditions. Subsequently, bacteria were exposed to 30 mM paraquat for 1 h. Paraquat is a redox compound which induces the formation of superoxide in the cytoplasm in the presence of oxygen (8). However, the parent and mutant strains were equally sensitive to paraquat (Fig. 1). Use of higher concentrations of paraquat (60 mM and 125 mM) and a longer incubation time (2 h) did not alter this (data not shown). These results suggest that TlpA is not involved in the protection of *S. pneumoniae* against internal oxidative stress.

Tlp A is involved in the defense against extracellular oxidative stress. The effect of deleting *tlpA* on extracellular oxidative stress survival was investigated by exposing the bacteria to 20 mM H_2O_2 for 30 min. Survival of the mutant was reduced 10-fold compared to the wild type in strain D39 and its unencapsulated derivative R6 (Fig. 2); D39 *nisRK* and R6 *nisRK* strains were selected to be able to complement any observed phenotypes (26). Insertion of the *nisRK* genes into the *bgaA* locus did not seem to have a negative impact on resistance to oxidative stress as survival of the D39 *nisRK* and D39 strains were similar (data not shown). The unencapsulated R6 derivative had a survival percentage similar to that of the encapsulated D39, indicating that the capsule may not play an important role in protection against extracellular oxidative stress (Fig. 2). Strains G54 and 670-6B were more resistant to oxidative stress than R6 and D39, as they had an approximately 2-log higher survival percentage (Fig. 2). Interestingly, in all strains, except for TIGR4 which showed a 5-fold reduction (Fig. 2), deletion of the *tlpA* gene led to at least a 1-log reduction in the survival of 20 mM H_2O_2, which was statistically significant (Fig. 2). Thus, TlpA plays an important role in the protection against oxidative stress in S. *pneumoniae*.

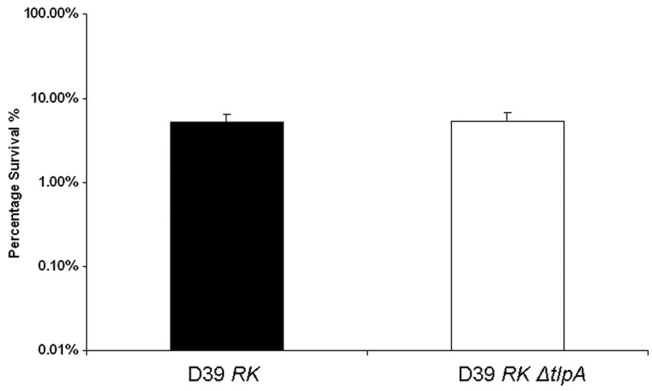


FIG 1 TlpA has no role in protection against intracellular oxidative stress. After growth to mid-log phase, wild-type D39 *nisRK* (■) and D39 *nisRK* Δ*tlpA* (□) were incubated with or without 30 mM paraquat for 2 h, and the percent survival was determined. Error bars indicate standard deviations from the mean. Data are the average of three independent assays.

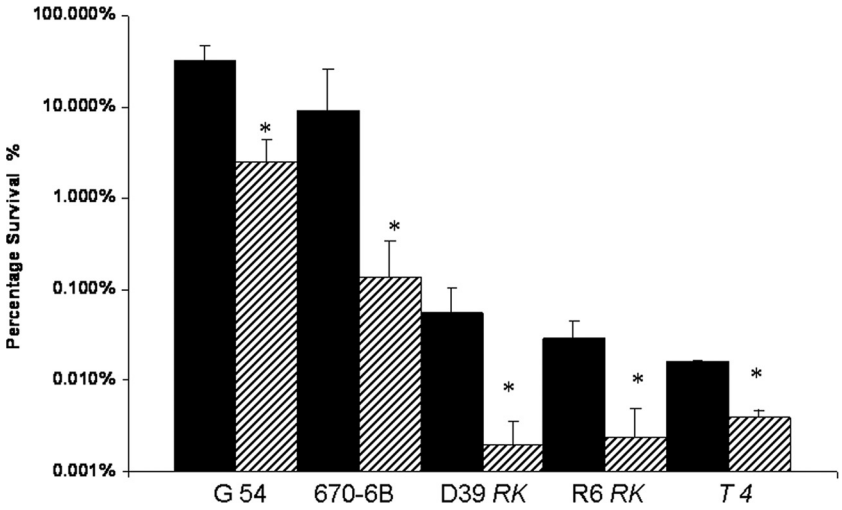


FIG 2 Deletion of *tlpA* impaired hydrogen peroxide resistance of *S. pneumoniae*. Survival of exposure to 20 mM H_2O_2 for 30 min of parent (filled bars) and mutant (striped bars) strains in the background of strains G54, 670-6B, D39 *nisRK* (D39 *RK*), R6 *nisRK* (R6 *RK*), and TIGR4 (T4). The percent survival was calculated by dividing the number of CFU of cultures after exposure to H_2O_2 by the number of CFU of the control cultures without H_2O_2. Data are averages of at least three independent assays. Error bars indicate standard deviations from the mean. *, $P < 0.05$.

TlpA is located in a conserved operon consisting of three genes which are all essential for the protection against oxidative stress. To verify whether sensitivity to oxidative stress was caused only by deletion of the *tlpA* gene, it was cloned into pNZ8048, and the resulting plasmid was transformed into D39 Δ*tlpA nisRK* and used for complementation analysis. For complementation experiments, all strains including controls were exposed to nisin (3 ng/ml) during early logarithmic growth and approximately 2 h before the stress condition was applied. This was done to exclude the possibility that any observed effects in complemented strains were caused by the addition of nisin and to ensure sufficient production of the protein(s). However, expression of the gene failed to increase the survival of the *tlpA* mutant (Fig. 3A). Due to experimental variation, all survival percentages were 10-fold higher in these experiments, but still the difference between the mutant and wild type was 10-fold and statistically significant (Fig. 3A).

Analysis of the genomic region of *tlpA* showed that it is located in the middle of a putative operon (Fig. 4) with ORFS *spd0571*, annotated as *ccdA* with homology to a cytochrome *c*-type biogenesis protein, and *msrAB* (*spd0573*), encoding the methionine sulfoxide reductase A/B protein of *S. pneumoniae* (25). Although there are no orthologs of these genes in *L. lactis*, they were not identified in the bioinformatic analysis for following reasons. In *Neisseria meningitidis*, there is no ortholog of Spd0571; although it has similarity with the N-terminal part of the multidomain protein DsbD, this was not enough to pass the similarity requirements of the BLAST search. For Spd0573, the situation is different. In *S. pneumoniae*, this protein is a polypeptide fusion of the MsrA and the MsrB proteins that occur in many other organisms. Both an MsrA and MsrB protein ortholog are present in *L. lactis*, and their similarity with either the MsrA part or the MsrB part of Spd0573 was high enough to make it seem that an ortholog is also present in *L. lactis*.

S. pneumoniae does not contain cytochromes, strongly suggesting that Spd0571 has another function. MsrAB enzymes catalyze the reduction of oxidized methionines to methionines upon the occurrence of oxidative stress (20). The three genes belong to the core genome of *S. pneumoniae* (40) and are highly conserved (98% identity on the nucleotide level with all *S. pneumoniae* genomes in the public database). Interestingly, despite this high homology, there are some differences in the annotation of the *msrAB* open reading frame in the various strains; in most genomes the *msrAB* gene is larger than that annotated in TIGR4 and D39 (Fig. 4A). Therefore, we analyzed all intergenic regions by hand and with the BPROM software (Softberry, Mt. Kisco, NY); both methods indicated only one possible promoter, which was located in front of *spd0571* in all sequenced strains. Further analysis by RT-PCR showed that the three genes, indeed, form an operon (Fig. 4B and C). Thus, deletion of *tlpA* probably also has a polar effect on *msrAB* expression, which explains why complementation of the mutant was unsuccessful.

The (predicted) function of the proteins encoded by the genes in the operon and their similarity to parts of the *N. meningitidis* PilB and DsbD protein that act together to combat oxidative stress (7) strongly suggested that all three proteins are involved in resistance against H_2O_2. Thus, we deleted the whole operon (*spd0571-0573*) from the genome of D39 *nisRK* (D39 Δ*spd0571-0573 nisRK*) and in parallel cloned this region into pNZ8048 (pNZ8048::*spd0571-0573*) and introduced it into the D39Δ*spd0571-0573 nisRK* strain. Deletion of the operon resulted in a similar sensitivity to oxidative stress as the *tlpA* single mutants (Fig. 3A). Furthermore, induction of expression of the *spd0571-0573* operon resulted in restoration of the resistance to H_2O_2 to wild-type levels in the operon mutant (Fig. 3A). Additionally, overexpression of the three genes in the wild type increased the survival of hydrogen peroxide stress (Fig. 3B). To further confirm that all three genes are necessary for hydrogen peroxide survival, we used unique restriction sites in the genes to subclone the plasmid and generate three derivatives: two lacking either *spd0571* or *spd0573* and one containing only *spd0573*. Western blot analysis of these plasmids in *L. lactis* using an anti-TlpA antiserum that we generated confirmed that TlpA was produced, showing that no mutations interfering with the reading frame had occurred during the

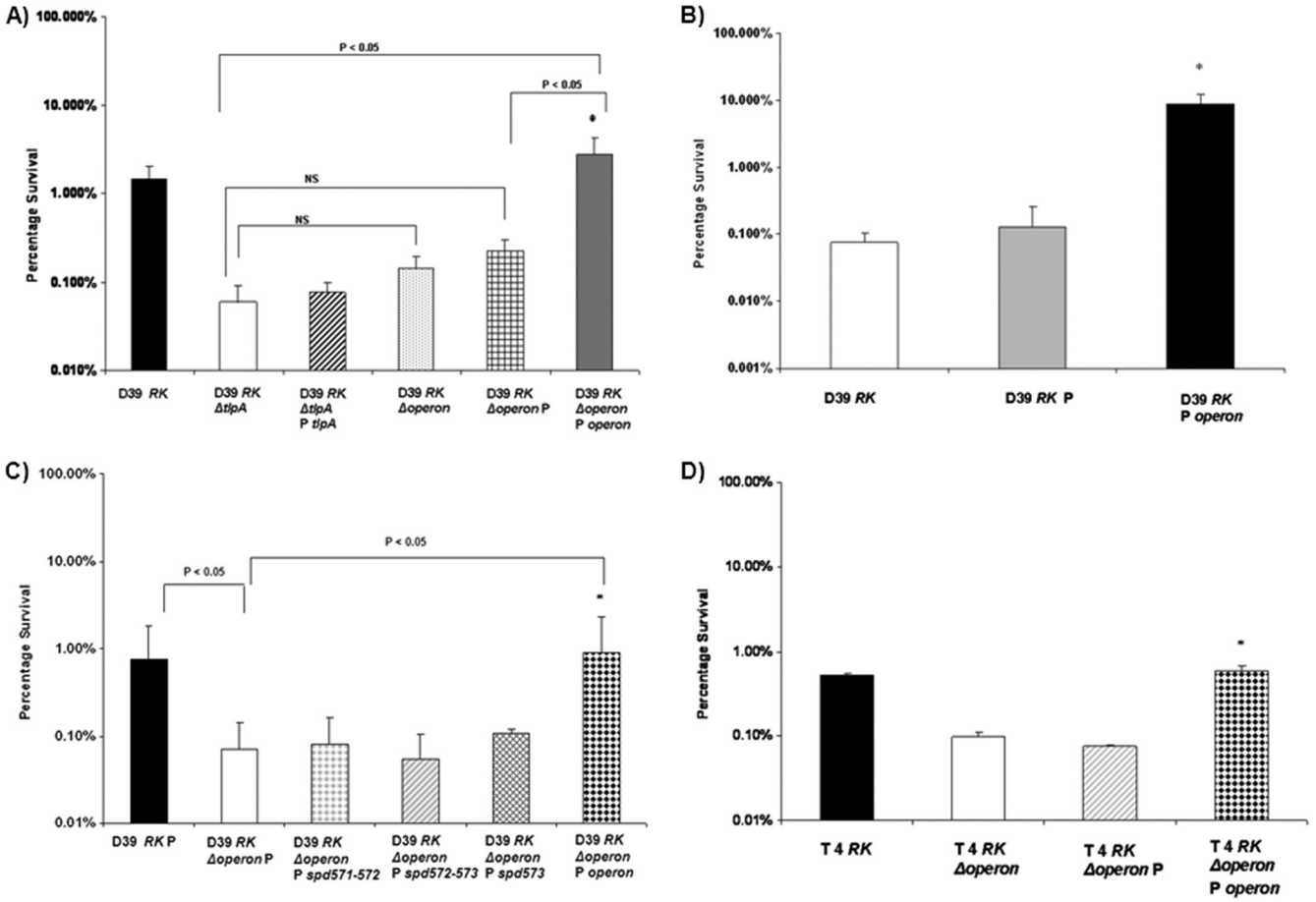
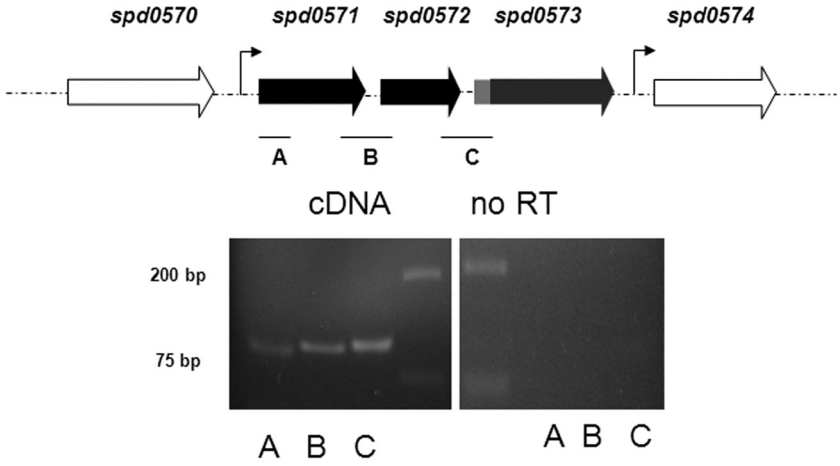


FIG 3 The operon *spd0571-0573* is involved in resistance of *S. pneumoniae* against extracellular oxidative stress. (A) Survival of exposure to 20 mM H_2O_2 of D39 *nisRK* (D39 *RK*), D39 Δ*tlpA nisRK* (D39 *RK* Δ*tlpA*), D39 Δ*tlpA nisRK* pNZ8048::*tlpA* (D39 *RK* Δ*tlpA* P *tlpA*), D39 Δ*spd0571-0573 nisRK* (D39 *RK* Δoperon), D39 Δ*spd0571-0573 nisRK* pNZ8048 (D39 *RK* Δoperon P), and D39 Δ*spd0571-0573 nisRK* pNZ8048:: *spd0571-0573* (D39 *RK* Δoperon P operon). (B) Expression of the operon increased the resistance of *S. pneumoniae* against external oxidative stress. D39 *nisRK* (D39 *RK*) D39 *nisRK* pNZ8048 (D39 *RK* P), and D39 *nisRK* pNZ8048::*spd571-573* (D39 *RK* P operon). (C) All three proteins cooperate with each other to protect the bacteria against oxidative stress. Survival of exposure to 20 mM H_2O_2 of D39 *nisRK* pNZ8048 (D39 *RK* P), D39 *nisRK* Δ*spd0571-0573* pNZ8048 (D39 *RK* Δoperon *P*), D39 *nisRK* Δ*spd0571-0573* pNZ *spd0571-0572* (D39 *RK* Δoperon P *spd0571-0572*), D39 *nisRK* Δ*spd0571-0573* pNZ *spd0572-0573* (D39 *RK* Δoperon P *spd572-573*), D39 *nisRK* Δ*spd0571-0573* pNZ *spd0573* (D39 *RK* Δoperon P *spd573*), D39 Δ*spd0571-0573 nisRK* pNZ8048:: *spd0571-0573* (D39 *RK* Δoperon P operon). All strains were induced during early mid-log phase by nisin (3 ng/ml) before being exposed to H_2O_2. (D) Complementation in TIGR4 strain. TIGR4 *nisRK* (T4 *RK*), TIGR4 Δ*spd0571-0573 nisRK* (T4 *RK* Δoperon), TIGR4 Δ*spd0571-0573 nisRK* pNZ8048 (T4 *RK* Δoperon P), TIGR4 Δ*spd0571-0573 nisRK* pNZ8048::*spd0571-0573* (T4 *RK* Δoperon P operon). Error bars indicate standard deviations from the mean. Data are the average of three independent assays. *, $P < 0.05$.

procedure. The production of TlpA from these plasmids was also significantly higher than in the wild type (data not shown). Neither pNZ8048::*spd0571-0572*, pNZ8048::*spd0572-0573*, nor pNZ8048::*spd0573* was able to restore survival of the operon mutant to wild-type levels upon induction (Fig. 3C). These experiments show that for the protection against oxidative stress, it is not sufficient to (i) express only *msrAB*, (ii) express *tlpA* in combination with *msrAB*, or (iii) express *tlpA* in combination with *spd0571*. Only expression of all three genes is able to protect *S. pneumoniae* against hydrogen peroxide. Therefore, we concluded that all three genes are necessary for resistance to oxidative stress in *S. pneumoniae*.

Expression of the genes is sufficient to increase resistance against oxidative stress. To determine whether *spd0571-0573* are sufficient to protect against extracellular oxidative stress, the genes were heterologously expressed using the nisin-induced controlled expression (NICE) system in the related lactic acid bacterium *L. lactis NZ9000*. This strain is highly sensitive to oxidative stress (Fig. 5); however, induction of expression of the operon in the mid-log growth phase resulted in a dramatic, 1,000-fold higher survival of exposure to 20 mM H_2O_2 which was solely dependent on the presence of the *spd0571-0573* genes on the plasmid (Fig. 5). Even in the absence of the inducer nisin, which results in low expression in *L. lactis* due to the leakiness of the *nisA* promoter (11), survival was still increased 10-fold, strongly suggesting that the level of resistance is correlated with the amounts of protein produced (Fig. 5). Furthermore, the derivatives of the plasmid containing only two genes were also unable to increase survival (data not shown). Thus, the operon is necessary and sufficient to protect bacteria against extracellular oxidative stress.

TlpA is a membrane lipoprotein. As *spd0571-0573* seem specifically involved in the resistance to external oxidative stress and as TlpA is reported to be a lipoprotein (50), we wondered whether the protein is present only in the membrane or whether it is also



Primer pair	Primers	Product size (bp)
A	658F and 658R	100
B	571F and 571R	107
C	572F and 572R	116

FIG 4 The three genes form an operon as determined by RT-PCR. (A) Schematic representation of genome organization and location of primers and expected PCR products. (B) RT-PCR analysis of *spd0571-0573*. Results shown are representative for two independent experiments. (C) Table of primers used and expected product sizes. cDNA was prepared from RNA isolated from D39 harvested at exponential growth and subjected to PCR with the primer pairs as detailed. no RT, cDNA prepared in the absence of reverse transcriptase.

released into the environment. To detect the presence of the protein, polyclonal antibodies were generated against the purified protein. To this end, the *tlpA* gene without signal sequence and including a C-terminal His_6 tag was cloned and overexpressed in *E. coli.* The resulting purified protein was used to generate polyclonal antibodies. Western blot analysis of D39 *nisRK* and its isogenic *tlpA* mutant showed the presence of specific anti-TlpA antibodies in addition to a nonspecific band around 7 kDa (Fig. 6A). Investigation of various fractions of *S. pneumoniae* with this antibody showed that the protein was exclusively present in the membrane (Fig. 6B). To control for accurate separation of the various fractions, cross-reacting antibodies against *S. aureus* TrxA (37) and antibodies against *S. pneumoniae* MgtA were used as controls for cytoplasmic and membrane proteins, respectively (Fig. 6B) (38). Thus, TlpA is a lipoprotein exclusively located in the pneumococcal membrane.

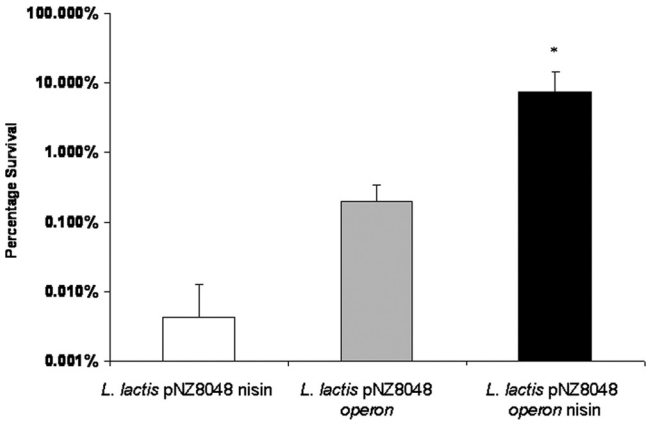


FIG 5 Expression of the operon increased the resistance of *L. lactis* against external oxidative stress. Survival rates of H_2O_2 are shown for *L. lactis* NZ9000 containing pNZ8048 (no insert) induced with nisin (white bar), pNZ with the operon and no nisin induction (gray bar), and pNZ with the operon and with nisin (black bar). Error bars indicate standard deviations from the mean. Data are the average of three independent assays. *, $P < 0.05$.

Extracellular oxidative stress increases the amount of *S. pneumoniae* TlpA. Overexpression of the operon in D39 and *L. lactis* increased the survival of oxidative stress. Thus, we hypothesized that the observed differences in oxidative stress survival between strains might be due to a difference in the amount and/or induction levels of TlpA. The same antiserum was used to compare protein levels under oxidative stress using the linear range of the Odyssey Infrared Imaging System, which can be used to quantify protein levels (64). Samples were equalized based on CFU numbers determined after the oxidative stress procedure and before being loaded on the gel. In addition, the nonspecific signal in the antiserum was also used to control that similar amounts of protein were used and to determine whether observed effects were specific for TlpA. Under normal growth conditions, there was no difference in TlpA levels between D39 and G54 (Fig. 6C). Next, we determined whether extracellular oxidative stress increased the protein levels of TlpA. In strain G54, TlpA levels were increased 2-fold under oxidative stress conditions, whereas there was no increase in strain D39 (Fig. 6C). Furthermore, in the presence of H_2O_2, the levels of TlpA were ~1.5-fold higher in strain G54 than in D39 (Fig. 6D). Thus, extracellular oxidative stress increased the amount of TlpA in G54 but not in D39, which might explain the observed differences in oxidative stress survival in these two strains (Fig. 2).

The gene complex is needed for long-term infection in a mouse pneumonia model. To investigate the role of these genes in

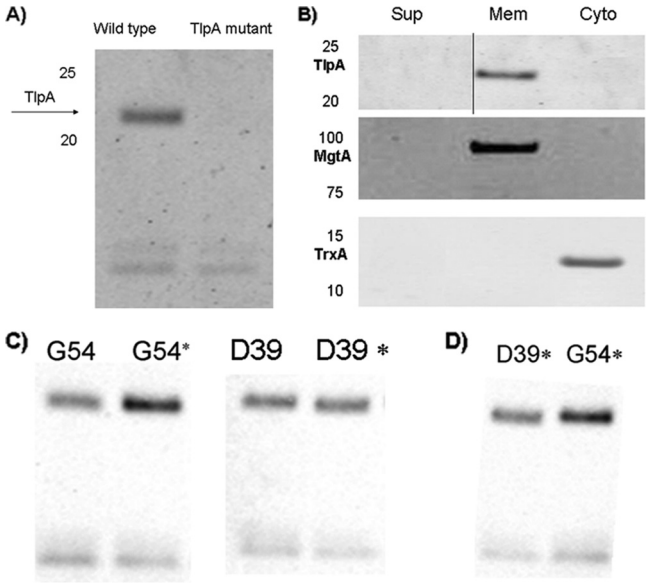


FIG 6 TlpA is a membrane protein, and its amount responds to oxidative stress. (A) Western blot analysis of strains D39 *nisRK* and the *tlpA* mutant determined the specificity of the antiserum and showed the presence of specific anti-TlpA antibodies. (B). Western blot analysis was performed on the supernatant, cytoplasm, and membrane fractions of strain D39 grown in GM17 with the anti-TlpA antibodies. A specific band around 24 kDa was exclusively present in the membrane fraction. As a control for accurate separation, the same fractions were also probed with anti-*S. pneumoniae* MgtA and anti-*S. aureus* TrxA antibodies. They are established as cytoplasmic and membrane proteins, respectively, in Gram-positive bacteria. (C) The amount of TlpA is increased under oxidative stress conditions in strain G54, whereas there was no significant increase in strain D39. When bacteria were grown under aerobic conditions and exposed to oxidative stress, the amount of TlpA was increased 2-fold in strain G54 compared to growth without hydrogen peroxide exposure but not in strain D39. (D) In the presence of H_2O_2, G54 has a 1.5-fold larger amount of TlpA than D39. Cells were exposed to H_2O_2 or mock exposed and harvested after 30 min. Protein samples derived from cells that were exposed to H_2O_2 are indicated by an asterisk. Based on CFU counts, total protein extracts of the same amount of cells were loaded on an SDS-PAGE gel and used for Western blot analysis. Samples were equalized based on CFU numbers determined after the oxidative stress procedure under both conditions before loading. In addition, the nonspecific signal around 7 kDa in the antiserum also indicated that similar amounts of protein were used. Signals of Western blotting were quantified using ImageJ.

pneumococcal infection at various stages of disease over time, the virulence of a TIGR4 operon mutant was assessed in a competitive index pneumonia model starting from nasopharyngeal colonization. To ensure that the results were reproducible, the experiment was repeated, and the results were combined. When strains were grown individually under nonstressed cultures, no differences were observed in growth rate and yield over 24 h *in vitro* (data not shown). Furthermore, during *in vitro* competitive growth the mutant was not outcompeted by the wild type over 24 h, indicating that the function of the operon is not essential and that deletion has no negative influence on fitness under nonstressed conditions. Interestingly, the mutant was initially able to establish infection in the nasopharynx and the lungs and to progress from the lungs to the blood. However, by day 3 the mutant was severely outcompeted in the nasopharynx ($P = 0.008$), with two of the four surviving mice clearing the mutant by day 5 (Fig. 7A). Strikingly, after day 2 the mutant was also outcompeted in the lungs ($P < 0.01$) and blood ($P = 0.01$) (Fig. 7B and C). Thus, these *in vivo* experiments showed that these genes play an important role in the later stages of disease. It is of note that mice that died had high-grade bacteremia with symptoms of bacterial sepsis.

In order to validate the above results, a second set of competition index experiments was performed using mice infected intranasally with TIGR4 Δ*sp0658-0660 nisRK* complemented with pNZ8048E or pNZ8048::*spd0571-0573*. *In vitro* experiments confirmed that the expression of the operon also restored survival to wild-type levels in a TIGR4 Δ*sp0658-0660 nisRK* background (Fig. 3D). These mice received nisin in their drinking water to drive gene expression from the *nisA* promoter of the pNZ8048 plasmid; the strains contain the *nisRK* genes instead of the *bgaA* locus (26) and have the additional burden of plasmid maintenance; thus, the results from these experiments are not directly comparable to those with the wild type and the isogenic mutant. Pneumococci complemented with *spd0571-0573* outcompeted those harboring the empty vector in the nasopharynx at all days tested (Fig. 8A). In the blood of these same mice the complemented mutant was present in greater numbers from day 3 onward (Fig. 8B). Thus, complementation studies confirmed that the operon was required for full virulence. Consequently, these *in vivo* experiments showed that this operon plays an important role in the later stages of disease, presumably because of the ROS released by cells of the immune system recruited to the site of infection at these time points.

DISCUSSION

Throughout the human host, *S. pneumoniae* encounters various sources of extracellular oxidative stress, which presents a major challenge for this facultative anaerobic bacterium. In this study we identified a novel component of the pneumococcal defense strategy against extracellular oxidative stress consisting of *tlpA* and its neighboring genes *spd0571* and *msrAB*. Furthermore, we demonstrated that these three genes play an important role in the establishment of long-term infection. Many of the genes identified in the bioinformatic analysis are involved in virulence, either in the three pathogens used for the comparison or others (16, 27, 29, 43, 59), suggesting that all these genes may be involved in the virulence of *S. pneumoniae*. Some of the genes identified in the bioinformatic analysis are involved in resistance to antibiotics and antimicrobial peptides. The only known niche for these pathogens is the human body, and they are thus constantly exposed to these compounds whereas *L. lactis* is not. In the same line, there may be more or other amino acids or other nutrients available in the human body, which could explain the identification of genes involved in pyruvate and amino acid metabolism in this analysis.

The *tlpA* gene is located in an operon with a *ccdA* homologue and the gene encoding the pneumococcal MsrAB protein (25). Deletion of both *tlpA* alone and the whole operon resulted in a significant decrease in the survival of hydrogen peroxide in several strain backgrounds. This decrease was of similar magnitudes, suggesting that on a functional basis deletion of *tlpA* is equivalent to deletion of the complete operon. Complementation by TlpA alone did not restore the phenotype in the single deletion mutant, indicative of polar effects and strongly suggesting that at least TlpA and MsrAB are needed for protection against oxidative stress. However, expression of *tlpA* and *msrAB* in the three-gene mutant did not increase the survival of oxidative stress, nor did expression of *tlpA* and *spd0571* or of *msrAB* alone. Only expression of all three genes was able to restore the phenotype. Thus, the

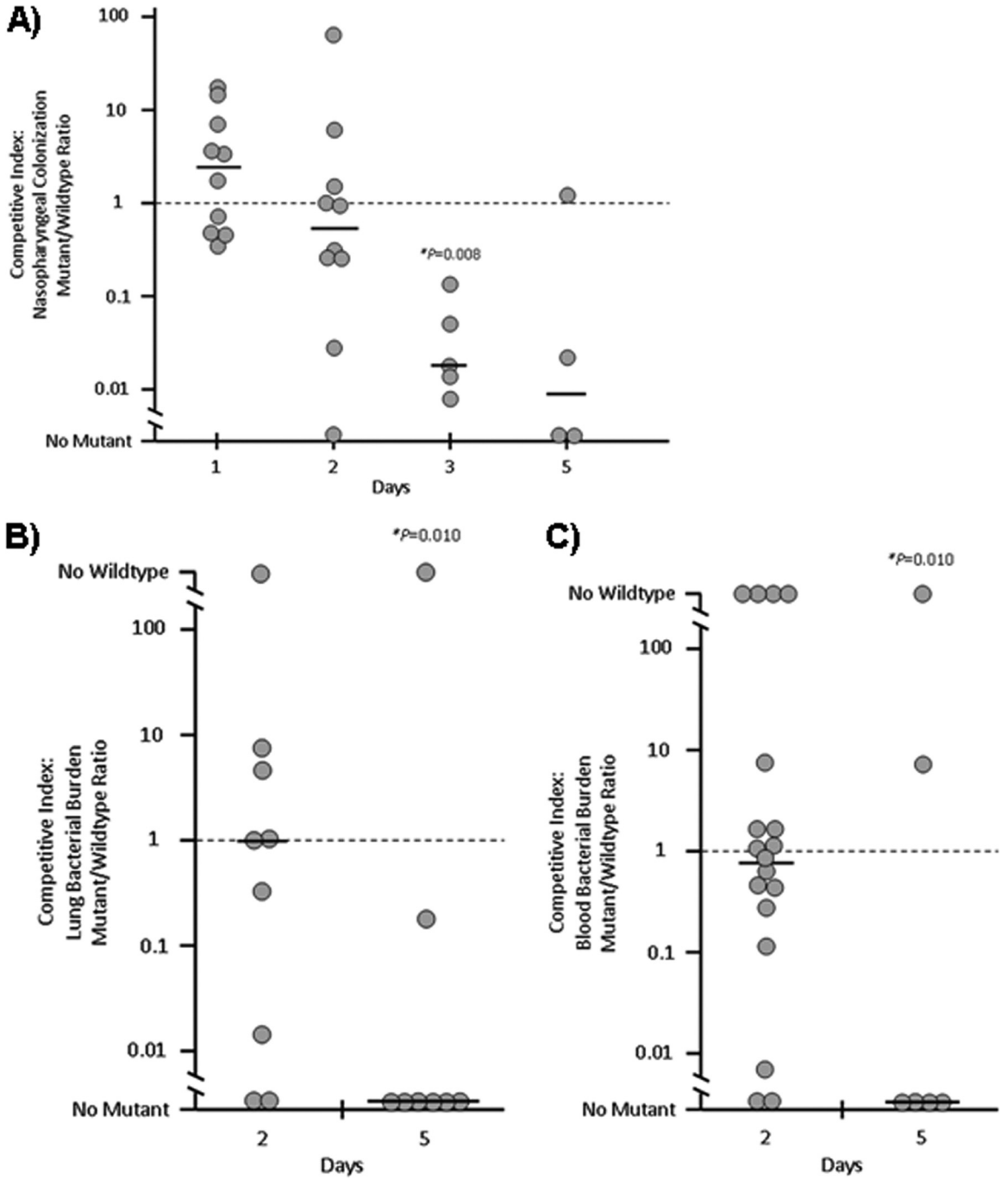


FIG 7 Deletion of the operon reduces virulence of *S. pneumoniae*. The mutant has a survival deficiency in the nasopharynx (A), lungs (B), and blood (C). Mice were infected intranasally with TIGR4 and TIGR4 Δ*sp0658-0660* at a 1:1 ratio; results shown are from two combined experiments. A significant decrease in the amount of the mutant was observed at day 3 in the nasopharynx (A) and at day 5 in the lungs (B) and blood (C). Each circle represents one mouse. The decrease in number of mice over time is a result of mortality. Statistical analysis was done using a nonparametric Mann-Whitney rank sum test testing for a ratio deviating from 1 versus a ratio of 1.

effect of *tlpA* deletion on the survival of the bacteria is not only due to polar effects on the *msrAB* gene. Interestingly, the introduction of the three genes in *L. lactis* dramatically increased the survival of this bacterium against oxidative stress, whereas expression of *tlpA*, *spd0571-spd0572*, or *spd0572-spd0573* had no effect (data not shown). Therefore, we concluded that as in *N. gonorrhoeae* (7), the function of all three proteins is needed for protection against extracellular oxidative stress.

The results of the current study are consistent with Brot et al. concerning the role of this operon in *Neisseria gonorrhoeae* (7). The TlpA protein is homologous (30% identity and 47% positive) to the N-terminal part of the PilB protein. In *N. gonorrhoeae* the

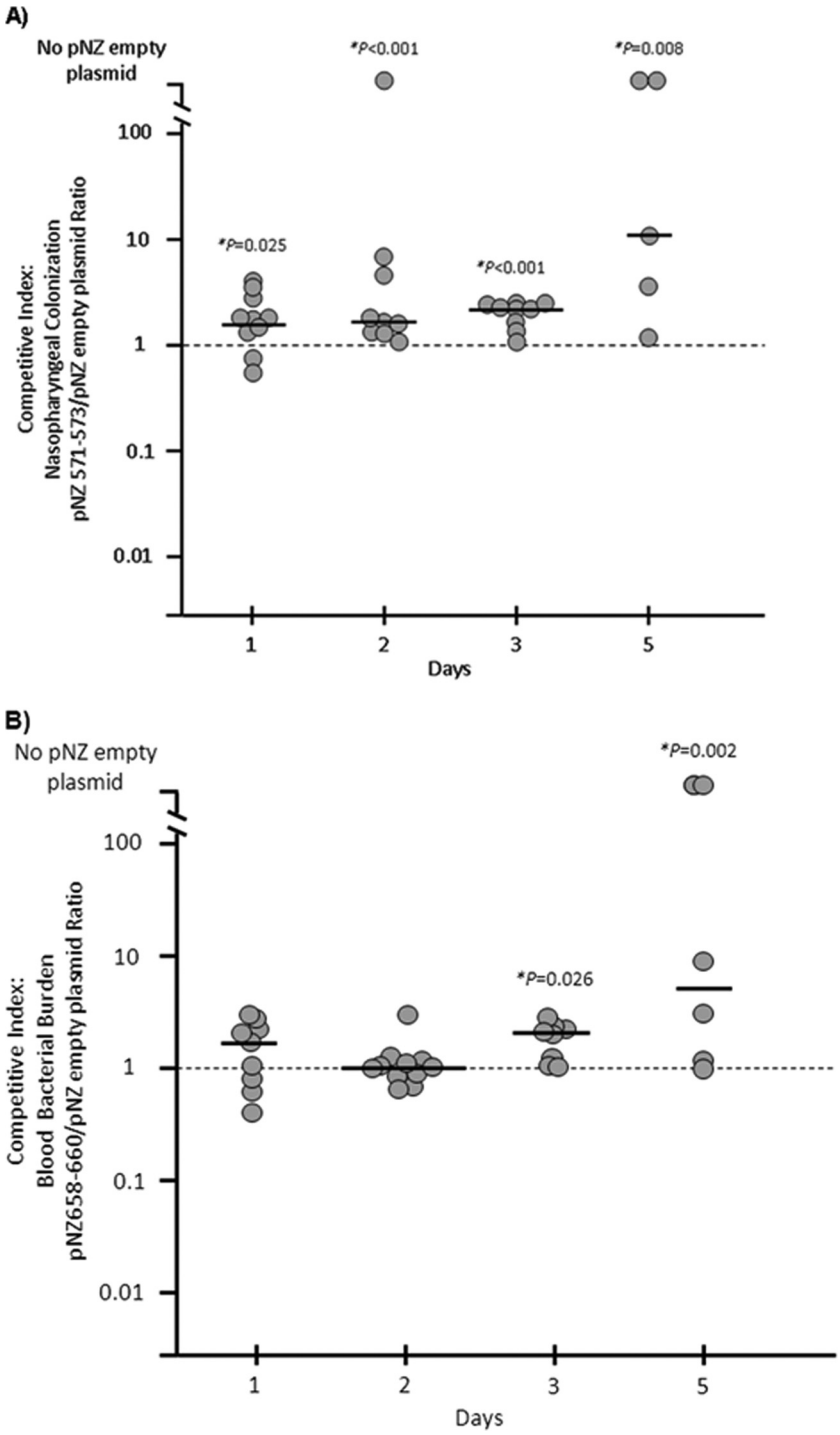


FIG 8 Complementation of the operon mutant enhances virulence of *S. pneumoniae*. Mice were infected intranasally with TIGR4 Δ*sp0658-0660 nisRK* complemented with pNZ8048 or pNZ8048::*spd0571-0573*. The results are compiled from three individual experiments. The complemented mutant outcompeted the empty vector control in the nasopharynx on all days tested (A) and was present in greater numbers in the blood (B) from day 3 onward. Each circle represents one mouse. The decrease in number of mice over time is a result of complete bacterial clearance. Statistical analysis was done using a nonparametric Mann-Whitney rank sum test testing for a ratio deviating from 1 versus a ratio of 1.

N-terminal domain of PilB accepts electrons from DsbD; in *S. pneumoniae* the CcdA protein is homologous (28% identity, 47% positive) to the transmembrane gamma domain of DsbD. Thus, we hypothesize that the complex functions in the following way: TlpA is reduced by an electron derived from CcdA; subsequently TlpA reduces MsrA/B, which can then reduce oxidized methionines. We were unable to find any thioredoxin activity for TlpA (data not shown) using the method of Holmgren (18), which may

be due to an inability to interact with insulin or incorrect folding of the purified protein. CcdA is predicted to be a membrane protein, which would make it possible for the protein to interact with TlpA. Furthermore, in *N. gonorrhoeae* MsrAB has been shown to be located in the outer membrane (57), and Spd0573 is also predicted to be extracellular, strongly suggesting that these three proteins are indeed able to interact in the membrane of *S. pneumoniae*.

Our study underscores the fact that this operon has a similar function in both Gram-positive and Gram-negative bacteria despite the absence of a periplasmic space in *S. pneumoniae*. In addition, overexpression of the operon in *L. lactis* resulted in a dramatic increase in oxidative stress survival despite the presence of an MsrA and an MsrB homologue in the lactococcal genome. This indicates that this is a robust and efficient three-partner system to combat extracellular oxidative stress, independent of the organism or conditions under which it functions. The overexpression experiments in *L. lactis* and wild-type *S. pneumoniae* and the induction of TlpA under oxidative stress conditions in strain G54 indicated that oxidative stress survival is correlated to the amounts of these proteins. Thus, differential regulation of this operon in *S. pneumoniae* may occur.

TlpA is a membrane lipoprotein, and deletion did not have an effect on survival in the presence of paraquat, which causes intracellular oxidative stress. Thus, we hypothesize that the operon is specifically involved in the repair of oxidized methionines in membrane or cell wall proteins and that other mechanisms are functioning inside the cell. The role of the operon during the establishment of infection indicates that damage resulting from extracellular hydrogen peroxide stress to noncytoplasmic proteins, in the form of oxidized methionines, is a major problem for *S. pneumoniae* in the host as it is for *E. coli* (55). At the moment, it is not clear whether this is due to damage of a particular protein or multiple proteins. Many membrane, cell wall, and secreted proteins contain methionines, which makes it hard to address this question.

The role of ROS generated by cells of the immune system in containing pneumococcal infection is not entirely clear. It has been suggested that neutrophils kill pneumococci through the action of serine proteases instead of their oxidative burst (35). On the other hand, pneumolysin induces the production of intracellular ROS in human neutrophils (34), and *S. pneumoniae* seems to modulate the oxidative burst (1). Studies with mice lacking either the $p47^{phox}$ or the $p91^{phox}$ subunits of the NADPH-oxidase involved in ROS generation indicate that ROS generated by nonprofessional phagocytes play a role in the control of *S. pneumoniae* in the host (56). Interestingly, our mutant was able to establish infection but was cleared from the mice at a later stage, perhaps due to the influx of neutrophils and or macrophages at this time point. Both our *in vitro* competition results and those described in the STM study by Lau et al. (28), in combination with the fact that the operon is not essential, demonstrate that this protein complex has no obvious role in the survival of endogenously generated H_2O_2. Furthermore, we did not observe any obvious difference with the wild type when the mutant was grown on plates exposed to ambient air (data not shown). This suggests that it is the oxidative stress generated by the host which impairs the virulence of this mutant and that hydrogen peroxide stress resistance is an important virulence factor. Thus, in conclusion we have identified an operon that is involved in the protection of *S. pneumoniae* against external peroxide stress and therefore plays an important role in virulence.

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