A newly identified flavoprotein disulfide reductase Har protects *Streptococcus pneumoniae* against hypothiocyanous acid

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Hypothiocyanous acid (HOSCN) is an antimicrobial oxidant produced from hydrogen peroxide and thiocyanate anions by heme peroxidases in secretory fluids such as in the human respiratory tract. Some respiratory tract pathogens display tolerance to this oxidant, which suggests that there might be therapeutic value in targeting HOSCN defense mechanisms. However, surprisingly little is known about how bacteria protect themselves from HOSCN. We hypothesized that tolerant pathogens have a flavoprotein disulfide reductase that uses NAD(P)H to directly reduce HOSCN, similar to thioredoxin reductase in mammalian cells. Here, we report the discovery of a previously uncharacterized flavoprotein disulfide reductase with HOSCN reductase activity, which we term Har (hypothiocyanous acid reductase), in *Streptococcus pneumoniae*, a bacterium previously found to be tolerant of HOSCN.

*S. pneumoniae* generates large amounts of hydrogen peroxide that can be converted to HOSCN in the respiratory tract. Using deletion mutants, we demonstrate that the HOSCN reductase is dispensable for growth of *S. pneumoniae* in the presence of lactoperoxidase and thiocyanate. However, bacterial growth in the HOSCN-generating system was completely crippled when deletion of HOSCN reductase activity was combined with disruption of GSH import or recycling. Our findings identify a new bacterial HOSCN reductase and demonstrate a role for this protein in combination with GSH utilization to protect *S. pneumoniae* from HOSCN.

*Streptococcus pneumoniae* is a Gram-positive bacterium that causes pneumonia, otitis media, sepsis, and meningitis (1, 2). This bacterium generates hydrogen peroxide (H$_2$O$_2$) during metabolism (3), but it does not have H$_2$O$_2$-degrading enzymes (4). Research has been conducted to examine how *S. pneumoniae* survives H$_2$O$_2$ (5–7); however, in the host, this H$_2$O$_2$ will be consumed by host peroxidases. The heme peroxidase enzyme lactoperoxidase (LPO) is present in saliva, nasal, and airway lining fluid (8–12). This enzyme, and myeloperoxidase (MPO) and eosinophil peroxidase, present in inflammatory environments, catalyze the reaction of H$_2$O$_2$ and thiocyanate (SCN$^-$) to generate hypothiocyanous acid (HOSCN) (13–15). In the lung and nasal airway fluid, the concentration of SCN$^-$ ranges from 30 to 800 μM, whereas in the saliva, the concentration can reach up to 3 mM (8, 16–19). Thus, HOSCN will be a major product generated at these sites. How *S. pneumoniae* survives this microbicidal secondary oxidant has been largely overlooked.

We recently reported that *S. pneumoniae* is relatively tolerant of HOSCN, when compared with another respiratory microbe *Pseudomonas aeruginosa* (20). This raises the question as to what mechanisms are responsible for HOSCN tolerance. We first investigated the contribution of the low molecular weight thiol GSH in *S. pneumoniae* (21). The genes required for the biosynthesis of GSH are absent in *S. pneumoniae*, so the bacteria rely on its import from their environment (22–24). The ability to import and recycle GSH was shown to be important for the ability of *S. pneumoniae* to cope with HOSCN; however, even when they lacked GSH, the bacteria could still survive HOSCN exposure for up to 1 h. Also, they were able to grow, albeit more slowly, in the presence of LPO, where in the presence of SCN$^-$, bacterially produced H$_2$O$_2$ is converted to HOSCN (21). These findings suggested the presence of additional HOSCN protective mechanisms in *S. pneumoniae*.

HOSCN is known to be well tolerated by mammalian cells, owing to the presence of thioredoxin reductase (TrxR), a flavoprotein disulfide reductase (FDR) that can directly reduce HOSCN using NADPH (25). Interestingly, an enzyme that consumes NAD(P)H in the presence of HOSCN has also been reported for streptococcal species present in human oral cavities (26). The HOSCN oxidoreductase activity was correlated with the ability of oral streptococci to recover from exposure to HOSCN in *vitro* (26). Because SCN$^-$ levels reach low millimolar levels (17, 18) and LPO is an abundant peroxidase in saliva (10–12), the ability of oral streptococci to tolerate HOSCN is vital for their survival in this niche. The aim of the present study was to identify enzymes in *S. pneumoniae* with HOSCN reductase activity and to determine if they have any role in protecting this serious human pathogen from HOSCN.
**HOSCN reductase in S. pneumoniae**

**Results**

**Evidence for reduction of HOSCN by an FDR in S. pneumoniae**

To investigate whether *S. pneumoniae* has an enzyme that uses NADPH to reduce HOSCN, we added NADPH to bacterial lysates and observed its consumption by the loss of absorbance at 340 nm upon the addition of HOSCN (Fig. 1A). NADH was also oxidized when HOSCN was added to lysates (Fig. 1B), and NAD(P)H consumption was not observed when either lystate or HOSCN was omitted from the reaction mixture (Fig. 1, A and B). NADH was consumed 20-fold faster than NADPH and while its consumption was significantly higher in the presence of HOSCN, it was also rapidly oxidized in the absence of HOSCN (Fig. 1B). NADH consumption in the absence of oxidant is possibly because of the oxygen-reducing NADH oxidase present in *S. pneumoniae* (27).

To confirm that HOSCN reduction was occurring in this system, we monitored the rate of HOSCN decline in *S. pneumoniae* lysates (Fig. 1, C and D). There was no significant difference between the rates of HOSCN and NADPH consumption measured concurrently on the same lysates (0.11 ± 0.03 and 0.09 ± 0.01 μM/min/mg, mean ± SD; n = 4; paired two-tailed t test). As a control, no appreciable loss of HOSCN was observed over the same period in the absence of the bacterial lysate or NADPH (Fig. 1, C and D). Lysates consumed HOSCN slightly more rapidly in the presence of NADPH (0.15 ± 0.02 versus 0.09 ± 0.01, mean ± SD, p < 0.01, unpaired two-tailed t test).

Varying the concentrations of HOSCN and NADPH at fixed concentrations of either NADPH (200 μM) or HOSCN (100 μM), respectively, produced Michaelis–Menten saturation curves consistent with an enzymatic reduction of HOSCN by NADPH (Fig. 1, E and F). The apparent $K_m$ values for HOSCN and NADPH added to lysates were 5 (2.5–9.0, 95% confidence interval) and 107 (76–151) μM and the $V_{max}$ 0.12 (0.1–0.14) and 0.13 (0.12–0.15) μM/min/mg, respectively. When no NADPH was added, HOSCN was still consumed at approximately 15% of the maximal measured rate, which is likely facilitated by endogenous NAD(P)H present in the lysates.

HOSCN oxidizes GSH to GSSG, which is reduced by glutathione reductase (GR) at the expense of NADPH (21, 22). To test whether the observed NADPH consumption in response to HOSCN in *S. pneumoniae* lysates was mediated by GSH, we used ΔgstT and Δgor mutants of *S. pneumoniae*, which lack GSH or GR, respectively (22). NADPH utilization still occurred in lysates from the mutant strains, indeed to a greater extent than in WT, indicating that it was independent of GSH oxidation and recycling by the GR system (Fig. 2A). To rule out that the observed HOSCN reductase activity involved another low molecular weight thiol such as coenzyme A, we passed lysates through a centrifugal filter with a molecular weight cutoff (MWCO) of 3 kDa and demonstrated that NADPH consumption in response to HOSCN still occurred in the retentate (Fig. 2B). Next, we tested the involvement of TrxR in reducing HOSCN in bacterial lysates as mammalian TrxR is known to have HOSCN reductase activity (25). The TrxR inhibitor auranofin did not affect HOSCN reductase activity in *S. pneumoniae* lysates when added at 20 μM (Fig. 2B), despite having previously been shown to inhibit recombinant bacterial TrxR at a 25-fold lower concentration (28). Similarly, the activity was not inhibited by 500 μM of 1-chloro-2,4-dinitrobenzene (Fig. 2B), a thiol-alkylating reagent that reacts preferentially with TrxR rather than with GSH and fully inhibits human TrxR at 100 μM (29).

We hypothesized that the observed HOSCN reductase activity in *S. pneumoniae* was due to direct reduction by another member of the FDR/pyridine nucleotide-disulfide reductase family. FDRs, such as GR or TrxR, shuttle electrons from NADPH to a tightly bound flavin adenine dinucleotide and then to a redox-active disulfide (Fig. 3A). One of the two resulting reduced thiols then reacts with an oxidized substrate, for example, GSSG or oxidized thioredoxin, to form a mixed disulfide. Subsequent reaction with the adjacent active site thiol results in regeneration of the active site disulfide and release of the reduced substrate (Fig. 3A). HOSCN reacts with thiols to produce sulfenyl thiocyanate derivatives (30, 31). We propose that in the case of a HOSCN reductase, a sulfenyl thiocyanate intermediate is formed, which is then attacked by the other active site thiol resulting in the release of SCN⁻ (Fig. 3A). *S. pneumoniae* lysates lost their ability to consume NADPH in response to HOSCN when they were boiled or passed over Cibacron Blue beads, an affinity absorbent for proteins with a dinucleotide fold (32) (Fig. 3B). These results suggested that HOSCN reduction was due to a heat-sensitive enzyme with a dinucleotide-binding site. To further probe the involvement of an FDR-type reduction mechanism, any potential thiol-containing FDR substrates were alkylated with iodoacetamide (IAM), rendering them unavailable for oxidation by HOSCN. For this, *S. pneumoniae* lysates were incubated with IAM prior to monitoring reductase activity (Fig. 3B). Only a partial inhibition by IAM (25%) was observed when it was incubated with *S. pneumoniae* lysates for 10 min prior to the addition of NADPH and HOSCN, and this effect was not significant. In contrast, the alkylating reagent significantly inhibited the reductase activity when it was coincubated with the lysate in the presence of NADPH to generate the IAM-reactive di–thiol state in the active site (Fig. 3, A and B). These results are consistent with the direct reduction of HOSCN by an FDR (Fig. 3A).

Given that *S. pneumoniae* copes with H₂O₂ generated during their metabolism, we tested whether *S. pneumoniae* lysates can also use NADPH to reduce H₂O₂. NADPH consumption in *S. pneumoniae* lysates was not observed in response to H₂O₂ (Fig. 3B). MPO can use H₂O₂ not only to produce HOSCN but also to oxidize chloride to the potent bactericidal hypochlorous acid (HOCl) (14, 33). HOCl rapidly oxidizes NADPH directly and could therefore not be tested as a possible substrate for the bacterial HOSCN-reducing enzyme. Ammonia chlorammine, a less reactive secondary oxidant derived from HOCl (34, 35), did not appear to act as a substrate as the rate of NADPH oxidation was not enhanced in the presence of *S. pneumoniae* lysates (Supporting information, Fig. S1).
Figure 1. Enzymatic reduction of HOSCN by *Streptococcus pneumoniae* lysates in the presence of NADPH. *Streptococcus pneumoniae* D39 cells were lysed by sonication and consumption of 200 μM NADPH or NADH by bacterial lysates (26–36 μg/ml final protein concentration) was measured in 100 mM phosphate (pH 7.4) and 1 mM EDTA by monitoring the loss of absorbance at 340 nm for 5 min following the addition of 100 μM HOSCN (closed circles) or buffer control (open circles). A, representative absorbance traces for the consumption of NADPH are shown. B, NADPH/NADH consumption was expressed as the amount consumed (ΔA/6220 M⁻¹ cm⁻¹/1 cm) per min relative to the protein content as determined by Bradford. Each symbol represents a separate experiment using lysates obtained from independent cultures and the bar the mean ± SD; * denotes a significant difference (p < 0.01) between consumption rates in the absence (“−,” open circles) and presence (“+,” closed circles) of HOSCN for each respective dinucleotide and # (p < 0.001) between the consumption rates in the presence of HOSCN and NADH versus NADPH as determined by one-way ANOVA with Tukey’s multiple comparisons test. C, HOSCN under conditions described in (A) was quantified by measuring TNB oxidation by the reaction mixture either immediately (0 min) or 20 min after mixing. Symbols represent the mean ± SD of four independent experiments. D, consumption of HOSCN in the absence (“−,” open circles) or presence (“+,” closed circles) of NADPH or NADH was measured as in (C) and expressed as the amount consumed per minute relative to the protein content. Each symbol represents a separate experiment using lysates obtained from independent cultures and the bar the mean ± SD; * denotes a significant difference (p < 0.05) between consumption rates in the absence and presence of each respective dinucleotide and # (p < 0.01) between the consumption rate in the presence of NADPH versus NADH as determined by one-way ANOVA with Tukey’s multiple comparisons test. E, consumption of NADPH (200 μM) or (F) HOSCN (100 μM) by *S. pneumoniae* lysates in the presence of increasing concentrations of HOSCN or NADPH, respectively. Symbols in E and F represent the mean ± SD of at least five independent experiments. A Michaelis–Menten curve fit was applied to derive apparent Kₘ and Vₘₐₓ values ± 95% confidence interval (CI). F, the point for 0 μM added NADPH was excluded from the curve fit.
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**Figure 2. HOSCN reductase activity in Streptococcus pneumoniae is not carried out by glutathione reductase or thioredoxin reductase.** A, reductase activity in lysates from *S. pneumoniae* WT, GSH transporter substrate-binding protein, and glutathione reductase mutants (ΔgshT and Δgor) was measured as described for Figure 1, A and B. B, lysates from WT *S. pneumoniae* were passed through a MWCO 3 kDa centrifugal filter before measuring the reductase activity of the filter retentate. Auranofin (20 μM) and DNCEB (500 μM) were preincubated with WT lysates for 5 min in the presence of NADPH before measuring the reductase activity. Reductase activity in each case was expressed relative to that obtained for untreated lysate. Each symbol represents a separate experiment using lysates obtained from independent cultures, and the bar is the mean ± SD. A significant difference compared with WT in (A) and to the 100% control in (B) was determined by one-way ANOVA with Dunnett’s multiple comparison test and is indicated by *p < 0.05.

**Identification of a candidate HOSCN reductase in S. pneumoniae by LC–MS and confirmation by gene deletion**

To enrich the HOSCN reductase for identification by LC–MS, we performed pull-down experiments on *S. pneumoniae* lysates using the Cibacon agarose beads. Attempts at eluting the activity off the beads using high salt, NADPH, and/or flavin adenine dinucleotide were unsuccessful. Instead, we directly loaded the beads onto an SDS-PAGE gel and following Coomassie staining, excised the protein bands for in-gel tryptic digestion and LC–MS/MS analysis (Fig. S2). Numerous proteins were identified when searching the peptide spectra against the *S. pneumoniae* proteome using database search engines (Supporting information, Protein hits.xlsx). Protein hits were manually filtered for enzymes with oxidoreductase activity, provided they had a high confidence score and the predicted molecular weight was consistent with their position on the SDS-PAGE gel (Table S1). Our IAM results suggested that a reductase activity was involved in the reduction of HOSCN. Of the shortlisted oxidoreductases, only an uncharacterized class I pyridine nucleotide reductase, GR and TrxR, belong to the family of FDRs, the latter two of which we had already ruled out using inhibitors and mutant bacteria. The class I pyridine nucleotide reductase that appeared in band 8 (37–50 kDa) is encoded by gene SPD_1415 in *S. pneumoniae* strain D39. In the *S. pneumoniae* TIGR4 and R6 strains, this gene is annotated as SP1588 and spr1442, respectively. To confirm that the enzyme encoded by *S. pneumoniae* strain D39 gene SPD_1415 is responsible for the HOSCN reductase measured in lysates, we targeted the gene for deletion. This was done by replacing the gene with a spectinomycin resistance gene using overlap extension PCR (Fig. S3A) (36). Genetic replacement was confirmed by restriction digest (Fig. S3B) and sequencing (Fig. S4). Lysates of the constructed *S. pneumoniae* mutant strain no longer consumed NADPH in response to HOSCN confirming that a HOSCN reductase is encoded by this gene (Fig. 4A). We therefore named the gene har and the protein it encodes Har (hypothiocyanous acid reductase).

**The HOSCN reductase in S. pneumoniae combines with GSH to confer tolerance to HOSCN**

To investigate the contribution of our newly identified HOSCN reductase to the ability of *S. pneumoniae* to resist HOSCN, we grew the WT and Δhar strains in the presence of LPO and SCN−, where LPO uses bacterial H2O2 and SCN− to produce HOSCN. The growth of both strains was retarded to a small degree in the presence of the HOSCN-generating system (Fig. 4, B and C); however, the har deletion did not make the bacteria more sensitive to HOSCN.

We have previously shown that the ability of *S. pneumoniae* to import and recycle GSH is important for HOSCN tolerance (21). To test whether the GSH antioxidant system and the HOSCN reductase combine to protect *S. pneumoniae*, we generated double deletion mutants of har with the gene gshT that enables GSH import or with gor that is responsible for recycling GSSG. The absence of HOSCN reductase activity in the double deletion mutants was confirmed using the NADPH consumption assay (Fig. 4A). Bacterial growth in the presence of the HOSCN-generating system was completely inhibited in both double deletion mutants (Fig. 4, D and E). In contrast, while growth was significantly decreased in single gor and gshT deletion mutants when exposed to this system, they eventually reached the same absorbance as untreated cells (21). Catalase protected the double deletion mutants from the LPO system by removing H2O2 and preventing HOSCN generation but not completely (Fig. 4, D and E). In the absence of LPO, catalase had no significant effect on the growth of any of the strains (data not shown), confirming that H2O2 alone does not affect bacterial growth. Collectively, these data highlight the importance of both HOSCN reductase activity and GSH utilization for *S. pneumoniae* to grow in the presence of a HOSCN-generating system.

**Discussion**

We have identified a reductase enzyme in *S. pneumoniae* that combines with GSH utilization to protect these bacteria from HOSCN. When both components were compromised, *S. pneumoniae* growth was completely inhibited in the presence of LPO and SCN−, where H2O2 generated during the normal metabolism of these bacteria was converted to HOSCN. The LPO-catalyzed production of HOSCN in epithelial lining fluids has long been considered an integral part of the innate immune response (8, 31, 37). In addition to bacteria such as *S. pneumoniae*, sources for H2O2 include the epithelial cell dual oxidase (38) and the neutrophil NADPH oxidase (39). When these white blood cells infiltrate a site of infection, they also provide another HOSCN-producing enzyme, MPO (14). As such, combined pharmacological
inhibition of HOSCN reductase activity and GSH import provides a powerful opportunity to disrupt *S. pneumoniae* colonization and infection.

HOSCN is well tolerated by host epithelial cells, at least in part owing to the HOSCN-metabolizing action of mammalian TrxR (25). In contrast, the Gram-negative bacteria *Escherichia coli* and *P. aeruginosa* are sensitive to killing by HOSCN (25, 31, 37), their lysates do not consume NADPH in response to HOSCN, and the TrxR from *E. coli* does not reduce HOSCN (25). Collectively, these studies suggested that HOSCN is selectively toxic for bacteria. However, only a few studies have investigated HOSCN tolerance and defense mechanisms of bacteria that successfully colonize niches in the host where HOSCN is continuously produced, such as oral cavities and the nasopharynx. The ability of bacterial FDRs to act as HOSCN reductases has been largely overlooked. Initial insight came from a study on *H. sapiens*-producing oral streptococci, where some species were shown to have an uncharacterized HOSCN oxidoreductase activity that correlated with their ability to survive HOSCN (26). We noted that *S. pneumoniae* survives HOSCN exposure much better than the opportunistic and HOSCN reductase-negative respiratory pathogen *P. aeruginosa* (20), which unlike *S. pneumoniae*, does not colonize and rarely causes disease in healthy people. We have now identified and characterized a HOSCN reductase in *S. pneumoniae* that contributes to the ability of this deadly pathogen to tolerate HOSCN.

The previous observation that lysates from some oral streptococcal species consume NADPH in the presence of HOSCN under similar conditions to those reported here suggests that HOSCN reductases exist in other bacteria (26). An amino acid sequence alignment revealed that species that exhibited HOSCN reductase activity, that is, *Streptococcus sanguinis*, *Streptococcus mitis*, and *Streptococcus salivarius*, had Har homologs that share 82 to 99% sequence identity with the *S. pneumoniae* enzyme. Furthermore, the *E. coli* enzyme RclA, thought to function as a copper reductase protecting *E. coli* against a combined HOCl and intracellular copper stress (40, 41), has recently been shown to have HOSCN reductase activity (42). RclA has high sequence homology with Har (47% sequence identity, 64% positives) and reduces HOSCN with an apparent $K_m$ value of 2 μM HOSCN, which is...
similar to that found in the present study of 5 μM for lysates of *S. pneumoniae*. In addition, SCN⁻ is reported to be generated during the reaction cycle of RclA (42), which supports the mechanism we propose in Figure 3A. RclA is known to be conserved among bacteria that colonize epithelial surfaces (40), so it is conceivable that HOSCN reductase enzymes provide bacteria with the means to colonize this niche. Future work is needed to determine whether these enzymes protect other human pathogens from HOSCN.

While HOSCN is the sole product of LPO activity in epithelial lining fluids, the neutrophil-derived enzyme MPO, while preferentially oxidizing SCN⁻, can also oxidize chloride.
to produce HOCl (14, 33). At average SCN− and chloride airway fluid concentrations of 400 μM and 100 mM, respectively (8, 19), MPO will use the available H2O2 to produce HOSCN and HOCl at a ratio of 3:2 (14). Because of the reaction of HOCl with NADPH, we have been unable to obtain experimental evidence for the HOCl reductase activity of Har. Furthermore, HOCl reacts rapidly with a myriad of other biological targets (43), including with SCN− to form HOSCN (44). It is unlikely that sufficient amounts of HOCl enter bacteria to react directly with Har. However, HOCl reacts with amines and amino acids to form chloramines, which like HOSCN are more selective for thiol groups and are more likely to be substrates for intracellular Har and related bacterial FDRs (45). Glycine and taurine chloramine were poor substrates for RclA compared with HOSCN, and no other amino acid chloramines were reduced by the enzyme (42). We investigated ammonia chloramine, which is derived from the reaction of chloramines/HOCl with ammonia or the breakdown of dichloramines (34, 35), but saw no evidence of reductase activity in S. pneumoniae lysates. Collectively, these studies suggest that this family of FDR enzymes has a high substrate specificity for HOSCN.

While har deletion did not sensitize the bacteria to HOSCN, we have previously observed a significant decrease in growth when GSH import and recycling was interfered with (21). This suggests that GSH can compensate for the lack of the HOSCN-metabolizing capability by reacting with HOSCN to form GSSG, followed by GR-mediated recycling of GSSG back to GSH as described before (21). However, when both the HOSCN reductase and either GSH import or recycling was blocked, we observed complete suppression of S. pneumoniae growth in the LPO system. Even high concentrations of catalase to remove H2O2 failed to completely protect the double deletion mutants suggesting that only small amounts of HOSCN are required to inhibit growth. This finding provides insight into the exquisite HOSCN sensitivity achieved by the dual targeting strategy. Catalases and other peroxidases are likely going to compete with LPO for the H2O2 in vivo; therefore, our results highlight that there may be therapeutic value in targeting both components of the S. pneumoniae HOSCN defense in order to maximally sensitize these bacteria to this oxidant.

Although deletion of the SPD_1415/har alone was not associated with an increased sensitivity to a HOSCN-generating system, it is conceivable that the HOSCN reductase becomes more important in protecting S. pneumoniae from HOSCN stress in situations where host GSH is limiting. Under physiological conditions, GSH is present in very high concentrations in the alveolar epithelial lining fluid (46), but it can become oxidized during airway inflammation and infection (47, 48). While S. pneumoniae can still assimilate GSH through importing its oxidized form GSSG, the relative uptake efficiency is unknown. Isothermal titration calorimetry studies using a Streptococcus mutans homolog of the substrate-binding protein GshT responsible for GSH utilization suggest that GSSG is a 25-fold less preferred ligand compared with GSH (49). A shift in GSH/GSSG ratio in the epithelial lining fluid during S. pneumoniae infection may thus determine the efficiency of host–GSH acquisition and consequently the relative importance of the HOSCN reductase in S. pneumoniae HOSCN defense.

Further support for the HOSCN reductase being a redundant protective mechanism comes from the fact that we found its activity to be significantly higher in the ΔgshT and Δgor strains compared to WT. This finding suggests that the reductase enzyme may be upregulated when GSH utilization is compromised. Alternatively, increased reductase activity in the GSH mutants could point to higher NADPH/NADH levels as previously observed by deleting GshT in Streptococcus pneumoniae (50). Increased HOSCN reductase activity in the S. pneumoniae Δgor mutant might also explain why we previously found this strain to survive equally well as the WT following exposure to reagent HOSCN in Hank’s buffered saline, despite its inability to recycle oxidized GSH (21). Collectively, these studies highlight the interconnectedness of the bacterial HOSCN defense systems, a better understanding of which might hold the key to successfully crippling S. pneumoniae’s HOSCN tolerance.

In conclusion, the present study is the first to identify a HOSCN reductase in the human pathogen S. pneumoniae and to investigate its role in protecting the bacteria against this oxidant. Future work should focus on understanding whether the HOSCN reductase contributes to the bacteria’s virulence using animal models and endeavor to find ways to inhibit the ability S. pneumoniae to protect itself from HOSCN.

**Experimental procedures**

**Materials**

LPO from bovine milk (ε412 = 112,000 M−1 cm−1 (51)), Hank’s balanced salt solution and PBS for cell culture, Cibacron Blue 3GA agarose (type 3000-CL), 1-chloro-2,4-dinitrobenzene, sodium thiocyanate, NADH, EDTA, IAM, EC-oxyrase, calcium chloride dihydrate, spectinomycin dihydrate, Vivaspin 500 (MWCO = 3 kDa), and Amicon Ultra 0.5 centrifugal filter units (MWCO = 10 kDa) were purchased from Sigma–Aldrich (Merck). Competence stimulating peptide-1 was purchased from AnaSpec. Bovine serum albumin was from Gibco (Thermo Fisher Scientific). Auranofin was from Santa Cruz Biotechnology. H2O2 (30%) (ε290 = 43.6 M−1 cm−1 (52)) was from LabServ. 2-Nitro-5-thiobenzoate (TNB) was prepared from 5,5’-dithiobis-(2-nitrobenzoic acid) (Sigma–Aldrich) through alkaline hydrolysis as described (53). NADPH was purchased from Carbosynth and trypsin (sequencing grade) from Promega. HOSCN was generated and quantified as described (21), kept on ice, and used within 30 min of quantification.

**Media**

Brain heart infusion (BHI) medium (Oxoid; Thermo Fisher Scientific) and Bacto Todd Hewitt medium supplemented with 1.5% yeast extract (THY; BD) were made from dehydrated culture media according to the manufacturers’ instructions. Columbian sheep blood agar medium was made from...
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defibrinated sheep’s blood (Fort Richard Laboratories) and Difco Columbia Blood Agar Base (BD) following the manufacturer’s instructions, and spectinomycin was added to give a final concentration of 150 μg/ml. All media were sterilized by autoclaving, and plates were poured using aseptic technique, cooled, sealed, and stored at 4 °C for up to 10 weeks.

Bacterial strains and culture

All bacterial strains were stored under standard conditions and maintained on Columbian or tryptic soy broth sheep blood agar plates (Fort Richard Laboratories). S. pneumoniae strain D39, serotype 2 (NCTC 7466) mutant strains ΔgshT (unable to import GSH) and Δgor (unable to recycle oxidized GSH) were generated as described previously (22). All strains were statically grown overnight at 37 °C with 5% CO₂ in BHI media, then diluted in fresh BHI, and grown to an absorbance of 0.4 to 0.7 at 620 nm before experiments.

HOSCN reductase activity in bacterial lysates

Bacteria were pelleted by centrifugation at 10,000g for 50 min at 4 °C, washed once with PBS, then resuspended in 2 to 5 ml of 100 mM phosphate (pH 7.4), and 1 mM EDTA. S. pneumoniae was lysed by pulse sonication on ice for 10 min. Bacterial debris was removed by centrifugation at 10,000g for 10 min at 4 °C. The consumption of NADPH or NADH (200 μM) by bacterial lysates (200 μl) after the addition of HOSCN (100 μM) or buffer was measured in 100 mM phosphate (pH 7.4) containing 1 mM EDTA (reductase buffer) by monitoring the loss of absorbance at 340 nm over 5 min in a 1 ml cuvette using an UV-visible spectrophotometer (Agilent 8453). The protein concentration in bacterial lysates was determined by Bradford assay (54). Reductase activity was defined as the rate of NAD(P)H consumption and was calculated using ε₃₄₀ = 6220 M⁻¹ cm⁻¹ for NAD(P)H (55) and the following formula:

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\text{Reductase activity (μmol/min/mg)} = \Delta \text{Abs}_{340}/ \Delta t / 6220 \text{ M}^{-1} \text{ cm}^{-1} \ast 1 \text{ cm} / c_{\text{protein}}
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Reductase activity in S. pneumoniae lysates was also measured in the presence of increasing concentrations of NADPH at a fixed HOSCN concentration (100 μM) and in the presence of increasing concentrations of HOSCN at a fixed concentration of NADPH (200 μM) as well as after the addition of 100 μM H₂O₂ instead of HOSCN. S. pneumoniae lysates were boiled for 5 min at 95 °C or buffer-exchanged using MWCO centrifugal filters (3 kDa Vivaspin or 10 kDa Amicon, at least three additions of the original lysate volume of reductase buffer) before being added to the assay. Cibacron Blue 3GA agarose beads (500 μl) were pelleted at 10,000g and washed with reductase buffer before adding lysate and incubating for 30 to 60 min at 4 °C with end-over-end rotation. Beads were removed by centrifugation, and the supernatant was added to the NADPH consumption assay. For inhibitor studies, auranofin (20 μM), 1-chloro-2,4-dinitrobenzene (500 μM), and IAM (20 mM) were incubated with the lysate and NADPH (200 μM) in reductase buffer. After 5 min, HOSCN (100 μM) was added, and the NADPH consumption was measured. IAM (20 mM) was also incubated with lysates in the absence of NADPH, then NADPH (200 μM) and HOSCN (100 μM) were added, and the NADPH consumption measured straight away.

HOSCN consumption in bacterial lysates

HOSCN was monitored using its ability to oxidize yellow TNB to the colorless dimer 5,5′-dithiobis-(2-nitrobenzoic acid) (56). S. pneumoniae lysates were mixed with reductase buffer, NADPH (200 μM), and HOSCN (100 μM) in a total of 1 ml. Straight away and after 20 min, 30 μl of the reaction mixture was added to 970 μl of 15 μM TNB. The absorbance change at 412 nm was used to measure the HOSCN concentration present in the mixture using ε₄₁₂ = 14,100 M⁻¹ cm⁻¹ for TNB and accounting for the fact that 1 mol HOSCN causes the loss of 2 mol of TNB. The difference in HOSCN concentrations measured at 0 and 20 min was used to calculate the rate of HOSCN consumption relative to the amount of protein present in the lysate. HOSCN was also monitored in response to varying amounts of NADPH (0–500 μM NADPH).

LC–MS on Cibacron Blue 3GA agarose-bound S. pneumoniae proteins

Ammonium sulphate (65%) was added to S. pneumoniae lysate (5 ml) and incubated at 4 °C for 30 min with end-over-end rotation followed by centrifugation at 10,000g for 10 min at 4 °C. The supernatant was removed, the pellet was resuspended in 1 ml of 100 mM phosphate (pH 7.4), 1 mM EDTA, and dialyzed using centrifugal filter units (MWCO = 10 kDa). The sample was added to 500 μl washed Cibacron Blue 3GA agarose beads and incubated for 30 min at 4 °C with end-over-end rotation. At each step of the purification procedure, a sample was removed, mixed with 5× reducing SDS-PAGE sample buffer, heated at 95 °C for 5 min, and separated on a TGX 4 to 15% stain-free gel (Bio-Rad), which was stained with Coomassie Blue R250. Bands were excised and cut into smaller pieces and then washed by adding alternately 80% acetonitrile in water and 100 mM ammonium bicarbonate. When gel pieces were destained, they were dehydrated in 80% acetonitrile, then reduced with 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 45 °C, dehydrated again, and alkylated with 20 mM IAM for 15 min in the dark. After washing, 0.5 μg trypsin in 50 mM bicarbonate buffer was added and incubated at 37 °C overnight. The supernatant was removed, combined with the eluate of a subsequent elution with 80% acetonitrile, dried in a vacuum evaporator, and resuspended in 0.1% formic acid in water. Samples were separated by a Jupiter 4u Proteo 90 Å column (150 mm × 2 mm; Phenomenex) coupled in line to a Velo Pro ion trap mass spectrometer (Thermo Fisher Scientific). Collision-induced dissociation MS/MS spectra in positive-ion mode were acquired for the ten most abundant precursors with a signal intensity of greater than 500, as described previously (57). Fragment spectra were searched
against protein sequences of the unreviewed TrEMBL proteome of *S. pneumoniae* deposited on UniProt using Proteome Discoverer 2.5 (Thermo Fisher Scientific) using Sequest HT and MS Amanda 2.0 search engines using methionine sulf-oxide and carbamidomethyl-cysteines as dynamic modifications. Protein hits for each band can be found in the supporting information.

**Generation of *S. pneumoniae* deletion mutants**

Deletion mutants (*Δhar*, *ΔgshT-Δhar*, and *Δgor-Δhar*) were generated by allelic replacement with a spectinomycin resistance marker using splicing overlap extension (SOE) PCR based on a previously described method (36). Primers used for the generation of *S. pneumoniae* *Δhar* mutant are listed in Table 1. Briefly, primers PP196/PP197 were used to amplify the spectinomycin resistance marker (*aad*), and PP198/PP194 and PP195/PP199 were used to generate overlapping PCR fragments of the 5′ and 3′ flanking regions of *har*, respectively. The 5′ and 3′ fragments were then assembled either side of the resistance gene by SOE PCR of the overlapping fragments and amplified to one construct using PP198/PP199 (Fig. S3A). A NotI restriction site was incorporated into the spectinomycin resistance cassette. The resulting SOE DNA product was amplified and transformed into WT, *Δgor*, and *ΔgshT* *S. pneumoniae* D39 strains.

*S. pneumoniae* D39 WT, *ΔgshT*, and *Δgor* were transformed using the protocol (for transforming *S. pneumoniae* with transposon DNA) as described (58), with alterations. Briefly, *S. pneumoniae* starter cultures with bacteria prepared for competence induction were generated and frozen at −80 °C. When needed, these cultures were supplemented with glycine in THY, pH 6.8, and grown to an absorbance of 0.03 to 0.1 at 600 nm at 37 °C with 5% CO₂. The volume of culture needed to get a total of 1 ml of absorbance of 0.03 at 600 nm was centrifuged at 12,000 g for 5 min. The supernatant was replaced with THY, pH 7.8, supplemented with 0.2% bovine serum albumin, and 1 mM CaCl₂. Bacterial competence was stimulated using 0.23 μM competence stimulating peptide-1 and incubated for 15 min. Har mutant construct DNA (1 μg) was then added and incubated at 37 °C with 5% CO₂ for 45 min. The sample was centrifuged for 8 min at 3000g, suspended in 1/10th volume of the supernatant, and plated onto blood agar plates containing 150 μg/ml spectinomycin. Plates were incubated overnight at 37 °C with 5% CO₂.

Spectinomycin-resistant strains *Δhar*, *ΔgshT-Δhar*, and *Δgor-Δhar* were confirmed by PCR and sequencing. Genomic DNA was isolated from candidate strains using a DNeasy Blood & Tissue Kit (Qiagen), following the protocol for Gram-positive bacteria and subjected to PCR using primers PP198 and PP199. PCR of WT clones produced a 2920 bp product, whereas strains with *har* replacement produced a 2415 bp product. Clones were also digested with NotI to confirm the presence of the spectinomycin gene (which carries a 3′-end NotI site unique to the construct) (Fig. S3B). Sequencing along the 5′ and 3′ arms through the upstream (*SPD_1414*) and downstream (*SPD_1416*) genes flanking *har* revealed no *har* sequence and confirmed complete replacement of *har* with spectinomycin resistance (*aad*) (Fig. S4).

**Bacterial growth in the presence of the LPO/SCN⁻ system**

*S. pneumoniae* WT, *Δhar*, *ΔgshT-Δhar*, and *Δgor-Δhar* strains (starting absorbance at 620 nm = 0.1) were grown for 6 h at 37 °C with 5% CO₂ in the presence or the absence of an enzymatic HOSCN-generating system using LPO (50 nM), SCN⁻ (800 μM), and bacterially produced H₂O₂ as described before (21). Additional treatments containing catalase (100 μg/ml), that is, bacteria + SCN⁻ + CAT and bacteria + SCN⁻ + LPO + CAT, were included to rule out any bacterial growth inhibition because of H₂O₂.

**Statistical analyses**

Graphs were plotted, and the statistical analyses stated in the figure legends were performed using GraphPad Prism (version 8.2.1; GraphPad Software, Inc). A *p* value of <0.05 was considered significant.

**Data availability**

Raw mass spectrometry and sequencing data will be available upon request.

**Supporting information**—This article contains supporting information.

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HOSCN reductase in S. pneumoniae

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Abbreviations—The abbreviations used are: BHL, brain heart infusion; CAT, catalase; FDR, flavoprotein disulfide reductase; GSH, glutathione; GR, glutathione reductase; GSSG, glutathione disulfide; H₂O₂, hydrogen peroxide; Har, hypothiocyanous acid reductase; HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; IAM, iodoacetamide; LPO, lactoperoxidase; MPO, myeloperoxidase; MWCO, molecular weight cutoff; SCN⁻, thiocyanate; SOE, splicing overlap extension; THY, Todd Hewitt medium supplemented with 1.5% yeast extract; TNB, 2-nitro-5-thiobenzoate; TrxR, thioredoxin reductase activity is accompanied by a decrease in NADPH oxidase activity, J. Biol. Chem. 270, 21937–21949

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