Streptococcus pneumoniae Uses Glutathione to Defend Against Oxidative Stress and Metal Ion Toxicity

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Running title: Glutathione utilisation by S. pneumoniae

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The thiol-containing tripeptide glutathione is an important cellular constituent of many eukaryotic and prokaryotic cells. In addition to its disulfide reductase activity, glutathione is known to protect cells from many forms of physiological stress. This report represents the first investigation into the role of glutathione in the Gram-positive pathogen, *Streptococcus pneumoniae*. We demonstrate that pneumococci import extracellular glutathione using the ABC transporter substrate binding protein GshT. Mutation of *gshT* and the gene encoding glutathione reductase (*gor*) increases pneumococcal sensitivity to the superoxide generating compound paraquat, illustrating the importance of glutathione utilisation in pneumococcal oxidative stress resistance. In addition, the *gshT* and *gor* mutant strains are hyper-sensitive to challenge with the divalent metal ions copper, cadmium and zinc. The importance of glutathione utilisation in pneumococcal colonisation and invasion of the host is demonstrated by the attenuated phenotype of the *gshT* mutant strain in a mouse model of infection.
INTRODUCTION

The accumulation of low molecular weight thiol-containing compounds is a widespread feature of both eukaryotic and prokaryotic cells. The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is the most prevalent intracellular thiol, present in almost all eukaryotes as well as aerobic proteobacteria and some Gram-positive genera (29). Glutathione biosynthesis in bacteria has been shown to be a two-step process catalysed by ATP-dependent enzymes. Glutamic acid is first linked to cysteine by γ-glutamylcysteine synthetase (encoded by gshA) and the product of this reaction is linked to glycine by glutathione synthetase (encoded by gshB) to form GSH (29). In some Gram-positive bacteria these two reactions are completed by a single multi-domain fusion protein (10, 16). The biological roles of glutathione in bacteria are numerous and varied. It has a central role in the maintenance of normal cellular processes via prevention of the formation of aberrant disulfides. This is achieved through the action of glutathione as a protein reductant, which may be direct or via the reduction of glutaredoxin enzymes (36). A consequence of this activity is that glutathione is converted from its reduced form (GSH) to a disulfide-bonded, dimeric form (GSSG). The ratio of GSH:GSSG in the cytoplasm is tightly controlled through the action of glutathione reductase (GOR), which uses reducing equivalents from NADPH. However, the disulfide-reducing activity of GSH is not essential in some organisms due to compensation by the thioredoxin/thioredoxin reductase system (9).

GSH has also been shown to provide protection from different forms of physiological stress, including reactive oxygen and nitrogen species (ROS/RNS), toxic concentrations of metal ions, as well as osmotic and acid stress (reviewed in (27, 29)). ROS and RNS, or the toxic by-products of the reactions of these species with cellular components, may be detoxified via direct interaction with GSH, or via specific enzymes such as glutathione peroxidase, which uses GSH to catalyse the reduction of peroxides (3). Glutathione is able to form metal complexes via non-enzymatic reactions (48) and has been associated with heavy metal tolerance in both eukaryotes (32) and prokaryotes (12, 22, 26). The concentration of GSH in E. coli has been found to increase during osmotic shock and a mutant defective in GSH synthesis shows defective growth in media with high osmolarity (31, 40). The specific
mechanism behind the protection conferred by GSH in this respect is still unclear. The role of GSH in protecting bacterial cells from exposure to acidic conditions may be linked to the effect of GSH on the activity of potassium export channels (8, 35). GSH can also participate in the detoxification of specific toxic compounds including xenobiotics and endogenous electrophiles, such as methylglyoxal, through conjugation catalysed by glutathione S-transferase (GST) or spontaneous reactions and subsequent export or metabolism (48).

Investigations into the role of GSH in bacteria have largely been undertaken using *Escherichia coli* as the model organism. The role of GSH in Gram-positive species has received significantly less attention, a result of the fact that GSH utilisation is significantly less common in members of this group, compared with their Gram-negative counterparts (7). The Gram-positive bacterium *Streptococcus pneumoniae* is a human pathogen of major significance, causing approximately 1 million deaths in children under 5 years annually (33). The pneumococcus is carried asymptomatically in the nasopharynx of a large proportion of the human population, but is capable of invading deeper sites within the body, resulting in diseases such as otitis media, pneumonia, sepsis and meningitis (6). Analysis of published *S. pneumoniae* genome sequences (21) reveals that the genes required for glutathione biosynthesis are not present. However, pneumococci have been shown to take up exogenous glutathione through an as yet unidentified transport system (19, 39). Furthermore, the pneumococcal genome encodes proteins with significant homology to glutathione-dependent proteins (21). The work presented here represents an investigation into the role of glutathione in pneumococcal biology.
MATERIALS AND METHODS

Strains and growth conditions. S. pneumoniae virulent serotype 2 strain D39 was routinely cultured on columbia blood agar base supplemented with 5% (v/v) horse blood at 37°C/5% CO2. Blood agar plates were supplemented with 0.2 μg/ml erythromycin for selection of mutant strains. Growth experiments of pneumococci in liquid culture were performed using a casein-based semi-synthetic medium (C+Y) (20) or a chemically defined medium (11) at 37°C under static conditions. For animal challenge experiments pneumococci were grown in nutrient broth supplemented with 10% (v/v) horse serum.

Construction of mutant strains. The gor and gshT genes were deleted from S. pneumoniae D39 and replaced with an erythromycin resistance cassette by transformation with a linear DNA fragment constructed by overlap-extension PCR (14) using the primers listed in Table 1. Generation of competent S. pneumoniae cells and subsequent transformation was performed as previously described (34).

Measurement of total intracellular glutathione. The total intracellular glutathione concentration of S. pneumoniae strains was determined using the glutathione assay kit from Sigma. Strains were grown in C+Y medium which contains GSH from yeast extract. The GSH concentration of LB medium (which contains an equivalent concentration of yeast extract to C+Y) has been measured at approximately 26 μM (12). Strains were grown to an OD600 of 0.4, washed twice with PBS and re-suspended in a 5% 5-sulfosalicylic acid solution. Cell suspensions were disrupted by sonication and clarified by centrifugation at 13,000 × g for 10 min. The total glutathione concentration of the extracts was then measured according to kit manufacturer’s instructions.

Glutathione reductase assays. Glutathione reductase activity of S. pneumoniae cell extracts was determined using the colorimetric assay described by Smith (41). Pneumococcal strains were grown to an OD600 of 0.4 in C+Y medium, washed once with PBS, and re-suspended in assay buffer (0.1M potassium phosphate buffer pH 7.5, 1mM EDTA). Cell suspensions were disrupted by sonication and clarified by centrifugation at 13,000 × g for 10 min. Extracts were added to assay buffer with 0.1 mM NADPH, 0.2 mM DTNB and 1 mM GSSG and the increase in Absorbance at 412nm was measured using a Spectramax
spectrophotometrically as described by Layne (23).

**Growth inhibition assays.** Pneumococci were grown in C+Y medium to an OD$_{600}$ of 0.4 before being diluted 100-fold into fresh C+Y medium containing the specified stress reagent in a final volume of 200 μl in a 96-well microtiter plate. Plates were incubated at 37°C and OD$_{600}$ readings were recorded at 30 minute intervals using a Spectramax spectrophotometer (Molecular Devices). All stress reagents were purchased from Sigma. Statistical analyses were performed using a Mann-Whitney U test, a $P$ value < 0.05 was taken as statistically significant.

**Murine infection model.** Animal experiments were approved by the University of Adelaide Animal Ethics Committee. Female outbred 4–6 week old CD-1 (Swiss) mice were inoculated intranasally with $5 \times 10^6$ colony-forming units (CFU) of *S. pneumoniae*, as described previously (46). Groups of 15 mice were inoculated for each strain and five randomly selected mice from each group were euthanased by CO$_2$ asphyxiation at 24, 48 and 72 h post infection. Nasal wash, nasopharyngeal tissue, lung and blood samples were collected and processed as previously described (24). Samples were serially diluted and plated onto blood agar plates for enumeration of viable pneumococci. Statistical analyses of log-transformed data were performed using a two-tailed Student’s $t$-test; a $P$ value < 0.05 was taken as statistically significant.
RESULTS

Identification of the S. pneumoniae glutathione transporter. The transport mechanisms responsible for acquisition of extracellular sources of glutathione have been characterised in E. coli and Haemophilus influenzae. Despite the presence of a functional GSH biosynthetic pathway, E. coli expresses an oligopeptide ABC transporter (encoded by the yliABCD operon) to import GSH (45). The obligate human pathogen H. influenzae on the other hand is auxotrophic for GSH and uptake is mediated by the dipeptide ABC transporter DppBCDF primed with the periplasmic-binding protein GbpA (47). The genome of S. pneumoniae encodes numerous ABC transporters, and we hypothesised that one of these is responsible for the acquisition of GSH that has been previously observed (19, 39). AliA, AliB, and AmiA are substrate binding proteins of ABC transporters known to transport oligopeptides in S. pneumoniae (2) and these share significant homology with YliA from E. coli and GbpA from H. influenzae. However, we found that mutation of the genes encoding these proteins did not affect the ability of S. pneumoniae strain D39 to accumulate intracellular GSH (data not shown). An ABC transporter substrate binding protein from Streptococcus mutans has recently been shown to be required for the ability of this bacterium to use GSH as a sole source of sulfur amino acids (43). These authors designated this protein GshT, and demonstrated that gshT transcription was activated by the cysteine synthesis regulator CysR. S. pneumoniae possesses a homologue of gshT (SPD_0150), which we inactivated in strain D39 to investigate its potential role in GSH acquisition. Measurement of intracellular GSH levels revealed that mutation of this gene completely abolishes the accumulation of GSH in pneumococci (Fig. 1A). Pneumococci have been shown to be auxotrophic for cysteine when grown in chemically defined medium (CDM) (11). To determine if pneumococci are able to use GSH as a source of cysteine, and if this is dependent on GSH uptake via GshT as is the case in S. mutans, we conducted growth experiments in CDM. Omitting cysteine from CDM was found to completely abolish growth of S. pneumoniae D39 and gshT strains as expected, however growth of both strains could be completely restored by supplementation with GSH (Fig. 1B). Thus, in contrast to S. mutans, transport of GSH into the cell is not required for pneumococci to use the tripeptide as a source of cysteine.
S. pneumoniae expresses a functional glutathione reductase. Glutathione reductase (GOR) is a key enzyme employed by cells that utilise glutathione to recycle the dimeric oxidised form of the tripeptide (GSSG) to the reduced form (GSH). S. pneumoniae encodes a gene annotated as a glutathione reductase (gor; SPD_0685). To confirm that this gene encodes a functional glutathione reductase, the gene was deleted in strain D39 and the NADPH-dependent GSSG reducing activity of cell extracts was measured. Mutation of gor was found to completely abolish the glutathione reductase activity of pneumococci (Fig. 1C).

Glutathione metabolism is required for pneumococci to defend against oxidative stress. To investigate the physiological role of glutathione utilisation in pneumococci, we initially examined the in vitro growth kinetics of the gshT and gor mutant strains. Given the established importance of glutathione in redox biochemistry and oxidative stress tolerance in many cell types we conducted growth experiments under aerobic conditions. Under these conditions, the gshT mutant strain exhibited a markedly slower growth phenotype compared to wild-type strain D39 (P = 0.01 during exponential phase), but was able to reach a culture density equivalent to wild-type pneumococci (Fig. 2A). The gor mutant strain exhibited growth kinetics nearly identical to wild-type D39 under the same conditions (Fig. 2B). The well characterised disulfide reductase activity of glutathione suggested that GSH utilisation may be required for pneumococcal resistance to the thiol-specific oxidant diamide. However, neither the gshT strain nor the gor strain was found to exhibit a growth phenotype significantly different to wild-type pneumococci in the presence of diamide (data not shown).

Pneumococci are known to produce significant quantities of the reactive oxygen intermediate hydrogen peroxide (H₂O₂) through the action of pyruvate oxidase during aerobic growth (42). Interestingly, pneumococci do not express catalase, the enzyme typically involved in peroxide detoxification by bacteria, but they do encode other putative peroxidases including a thiol peroxidase (PsaD) (30) and a glutathione peroxidase (SPD_0286). The aerobic growth properties of the gshT and gor mutant strains suggest that GSH may not play a large role in pneumococcal resistance to peroxide. This was confirmed by the fact that growth of these mutants in the presence of exogenous H₂O₂, or the organic peroxide tert-Butyl hydroperoxide, was not significantly different to wild-type pneumococci (data not
shown). However, the spectrum of reactive molecules encountered by a cell during conditions of oxidative stress is vast. In addition to peroxides, superoxide (O$_2^-$), produced via reduction of molecular oxygen, is a well characterised reactive oxygen intermediate. To determine if GSH utilisation by pneumococci is required for resistance to superoxide, strains were grown in the presence of the superoxide generating compound paraquat. While both the gshT and gor mutant strains were able to grow in the presence of 0.5 mM paraquat, the growth rate of these strains was slower ($P = 0.047$ for both gshT and gor strains +PQ compared to no stress at exponential phase) and the maximum culture density reached was lower than that of wild-type cells under the same conditions ($P = 0.004$ for both gshT and gor at stationary phase) (Fig. 2A & B).

*S. pneumoniae* uses glutathione to defend against toxic concentrations of divalent metal ions. *E. coli* strains deficient in GSH synthesis have been shown to be less tolerant to challenge with chromium, cadmium, copper, zinc (12), mercury and arsenite (22). The importance of metal ion resistance for pneumococcal physiology and pathogenesis has been well established (17, 37, 38). To determine if glutathione utilisation is required for pneumococcal resistance to metal ions, we tested the ability of both the gshT and gor mutant strains to grow in the presence of a variety of metal ions. Inhibition of glutathione uptake was found to severely inhibit pneumococcal growth in the presence of copper, cadmium and zinc (Fig. 3A, B & C) ($P < 0.01$ for gshT compared to D39 in the presence of each metal ion at stationary phase). Mutation of glutathione reductase also resulted in severe perturbation of pneumococcal growth in the presence of copper and cadmium (Fig. 4A & B), while perturbation by zinc was less severe although still significant (Fig. 4C) ($P < 0.01$ for gor compared to D39 in the presence of each metal ion at stationary phase). Deletion of either gshT or gor did not significantly alter the resistance of pneumococci to cobalt, nickel or chromium (data not shown).

GshT is important for nasopharyngeal colonisation and invasion in a mouse model of pneumococcal infection. To examine the role of glutathione utilisation during pneumococcal infection, the phenotype of *S. pneumoniae* D39, gshT and gor strains in a mouse model of infection was determined. Groups of mice were challenged intranasally with
each strain and the numbers of pneumococci present in various niches (nasal wash, nasal
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tissue, lungs and blood) were determined at 24, 48 and 72 h post-challenge. The number of
gor mutant cells recovered from these niches was found to not differ significantly from the
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number of WT cells recovered at any time point (data not shown). However, the gshT mutant
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strain exhibited a significantly attenuated phenotype (Figure 5). The attenuation was
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particularly prevalent in the nasopharyngeal niche, with lower numbers of gshT mutant cells
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recovered from the nasal wash at 48 and 72 h (Fig. 5C), and from the nasal tissue at 24 and
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72 h (Fig. 5D). The data also illustrate the importance of GshT for pneumococcal
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proliferation in the blood. There was no difference between the number of mutant and wild-
type pneumococci present in the blood of mice at 24 h, but the gshT strain was unable to
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proliferate in this niche as well as wild-type D39, and a statistically significant difference in
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numbers recovered at 72 h was observed (Fig. 5A). A similar trend was observed in the lungs
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of mice, although the difference in bacterial numbers recovered from this niche did not reach
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statistical significance (Fig. 5B).
DISCUSSION

Although the ability of *S. pneumoniae* to import extracellular GSH has been known for many years, the role of glutathione in pneumococcal physiology has not been previously investigated. In the present study, we have shown that the ABC transporter substrate binding protein GshT is essential for intracellular GSH accumulation in *S. pneumoniae*. GshT from *S. pneumoniae* strain D39 shares 53% identity with GshT of *S. mutans*, previously shown to be required for the ability of this organism to use GSH as the sole source of sulfur amino acids (43). These authors demonstrated that *gshT* transcription is regulated by the cysteine synthesis regulator (CysR) in *S. mutans*, and identified putative CysR recognition sequences in the promoter regions of *gshT* orthologs in other streptococci, including *S. pneumoniae*. Interestingly, we found that although pneumococci are able to use GSH as a source of cysteine, this occurs independently of GshT. It should be noted that neither the genome of *S. mutans* nor *S. pneumoniae* appear to encode the enzyme gamma-glutamyl transpeptidase (GGT). The hydrolysis of GSH is thought to be restricted to the activity of GGT due to the fact that the glutamate and cysteine residues are linked via the γ-carboxyl group of glutamate, as opposed to the α-carboxyl peptide linkage common in proteins. This particular peptide linkage prevents GSH from being degraded by most cellular peptidases. GGT has been shown to be essential for the ability of *E. coli* and *Francisella tularensis* to use glutathione as a source of cysteine (1, 44). The mechanism by which pneumococci obtain free cysteine from GSH remains to be elucidated, although our results indicate that it occurs in the extracellular environment.

The importance of glutathione uptake and recycling of reduced GSH from GSSG in protecting pneumococci from oxidative stress is evident from the impaired growth rate displayed by the *gor* and *gshT* mutants in the presence of the superoxide generator paraquat. Oxidative stress is an unavoidable consequence of growth in an aerobic environment, and pneumococci need to detoxify reactive species that may arise from a variety of endogenous reactions, or through interactions with host cells. Pneumococci express a manganese-containing superoxide dismutase (SodA) which confers resistance to paraquat and contributes to virulence in mice (51). Superoxide is able to oxidise solvent-exposed iron-sulfur clusters,
resulting in loss of enzyme activity (15). Superoxide may also oxidise compounds produced during central metabolism, such as an intermediate of the transketolase reaction (4), as well as short-chain sugars (15). Superoxide stress results in an auxotrophy for sulfur amino acids in E. coli, although the specific mechanism by which cysteine synthesis is inhibited is not clear (5). The protective effect of GSH on pneumococci under superoxide stress may either be due to a direct interaction with superoxide itself, or a by-product of its reaction with cellular components. The importance of glutathione utilisation in protection of other lactic acid bacteria from oxidative stress has also been established, however the mechanism by which GSH confers this resistance appears to be different to that observed in pneumococci. Glutathione metabolism protects S. mutans from challenge with the thiol-specific oxidant diamide (39, 50), while GSH accumulation in Lactococcus lactis confers resistance of this organism to H₂O₂ (25). These phenotypes are most likely a result of glutathione’s role in disulfide reductase, and glutathione peroxidase activity, respectively.

In addition to a role in oxidative stress resistance, we also illustrated the importance of glutathione utilisation in pneumococcal resistance to divalent metal ions, specifically cadmium, copper and zinc. The importance of dedicated metal efflux transporters to pneumococcal resistance to the later two metals has been established. Pneumococci encode a copper responsive regulon which includes a P1-type ATPase, copA, required for copper resistance and virulence in mice (38). Zinc resistance has been demonstrated to be conferred by the cation efflux system protein CzcD (17). The importance of GSH to pneumococcal metal ion resistance likely occurs at concentrations that overwhelm the dedicated metal efflux systems. The basis of this protection is likely a result of the fact that GSH is able to form metal complexes via non-enzymatic reactions. The sulfhydryl group of the cysteine moiety of GSH, in particular, has a high affinity for metals (48). While the pneumococcal gor and gshT mutant strains exhibited similar sensitivities to copper and cadmium stress, we observed that the strain lacking GOR activity was significantly less sensitive to zinc stress compared with the strain deficient in GSH uptake. This may be explained by the fact that Zn(II)-GSSG complexes have been shown to occur, albeit under in vitro conditions (18). These authors demonstrate that the affinity of GSSG for zinc is lower than that of GSH, thus our
observation that the pneumococcal gor mutant is still more sensitive to zinc compared to wild-type pneumococci might be expected.

It is interesting to note that there is considerable overlap in the mechanisms by which metal ions and superoxide intoxicate cells. Divalent metal ions can displace iron from iron-sulfur clusters, inhibiting enzyme activity (28, 49) and also interfere with cysteine synthesis (13). Thus, it might be expected that the effects of GSH on pneumococcal biology outlined in this report are achieved via protection of similar cellular processes. The attenuation of the gshT mutant observed in a murine model of infection suggests that this protection is important for both pneumococcal colonisation of a host and development of invasive disease. The fact that GOR appears to be dispensable in this respect may reflect the fact that reduced GSH is likely to be the predominant form of the tripeptide in a host environment, thus pneumococci may be able to bypass the need for GOR by importing GSH and exporting GSSG and glutathione-metal conjugates. We cannot discount the possibility, however, that glutathione has additional roles in pneumococci to those characterised here. The pneumococcal gor mutant exhibits growth kinetics similar to WT under standard culture conditions, while the gshT mutant exhibits a slower growth phenotype. Thus, glutathione might carry out roles in pneumococci that are independent of GOR.

ACKNOWLEDGEMENTS

This work was supported by Program Grant 565526 and Project Grant 627142 from the National Health and Medical Research Council of Australia (NHMRC). J.C.P. is a NHMRC Australia Fellow. C.T. is a Garnett Passe and Rodney Williams Memorial Foundation Fellow.
REFERENCES


Table 1: List of primers used in this study

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FIGURE LEGENDS

FIG. 1. Characterisation of *S. pneumoniae* D39gshT and D39gor mutant strains. (A) Total intracellular glutathione levels of D39 and D39gshT strains. (B) Growth of D39 and D39gshT strains after 12 hours in a chemically defined medium (CDM) (white bars); in CDM with cysteine omitted (grey bars); and in CDM lacking cysteine supplemented with 3.2 mM glutathione (black bars). (C) Glutathione reductase activity of cell extracts of D39 and D39gor strains. Bars represent the mean values of triplicate cultures and Y-error bars represent the standard deviation from the mean.

FIG. 2. Growth of *S. pneumoniae* D39, and either D39gshT (A) or D39gor (B), with or without addition of 0.5 mM paraquat (PQ). Curves represent the mean of triplicate cultures and Y-error bars represent the standard deviation from the mean.

FIG. 3. Growth of *S. pneumoniae* D39 and D39gshT with or without addition of (A) 250 μM copper sulfate (Cu), (B) 10 μM cadmium chloride (Cd), and (C) 100 μM zinc sulfate (Zn). Curves represent the mean of triplicate cultures and Y-error bars represent the standard deviation from the mean.

FIG. 4. Growth of *S. pneumoniae* D39 and D39gor with or without addition of (A) 250 μM copper sulfate (Cu), (B) 10 μM cadmium chloride (Cd), and (C) 100 μM zinc sulfate (Zn). Curves represent the mean of triplicate cultures and Y-error bars represent the standard deviation from the mean.

FIG. 5. The number of *S. pneumoniae* D39 and D39gshT cells recovered from (A) blood, (B) lungs, (C) nasal wash, and (D) nasal tissue of mice 24, 48 and 72 hours following intranasal challenge. *P < 0.05, **P < 0.01, ***P < 0.001 compared to WT. The horizontal dotted line denotes the limit of detection. NB: two mice challenged with WT succumbed to infection prior to 72 h, and so only three mice from this group could be processed at this time point.