2019

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RESEARCH ARTICLE

Glutathione Synthesis Contributes to Virulence of *Streptococcus agalactiae* in a Murine Model of Sepsis

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ABSTRACT *Streptococcus agalactiae*, a leading cause of sepsis and meningitis in neonates, utilizes multiple virulence factors to survive and thrive within the human host during an infection. Unique among the pathogenic streptococci, *S. agalactiae* uses a bifunctional enzyme encoded by a single gene (*gshAB*) to synthesize glutathione (GSH), a major antioxidant in most aerobic organisms. Since *S. agalactiae* can also import GSH, similar to all other pathogenic streptococcal species, the contribution of GSH synthesis to the pathogenesis of *S. agalactiae* disease is not known. In the present study, *gshAB* deletion mutants were generated in strains representing three of the most prevalent clinical serotypes of *S. agalactiae* and were compared against isogenic wild-type and *gshAB* knock-in strains. When cultured *in vitro* in a chemically defined medium under nonstress conditions, each mutant and its corresponding wild type had comparable growth rates, generation times, and growth yields. However, *gshAB* deletion mutants were found to be more sensitive than wild-type or *gshAB* knock-in strains to killing and growth inhibition by several different reactive oxygen species. Furthermore, deletion of *gshAB* in *S. agalactiae* strain COH1 significantly attenuated virulence compared to the wild-type or *gshAB* knock-in strains in a mouse model of sepsis. Taken together, these data establish that GSH is a virulence factor important for resistance to oxidative stress and that *de novo* GSH synthesis plays a crucial role in *S. agalactiae* pathogenesis and further suggest that the inhibition of GSH synthesis may provide an opportunity for the development of novel therapies targeting *S. agalactiae* disease.

IMPORTANCE Approximately 10 to 30% of women are naturally and asymptomatically colonized by *Streptococcus agalactiae*. However, transmission of *S. agalactiae* from mother to newborn during vaginal birth is a leading cause of neonatal meningitis. Although colonized mothers who are at risk for transmission to the newborn are treated with antibiotics prior to delivery, *S. agalactiae* is becoming increasingly resistant to current antibiotic therapies, and new treatments are needed. This research reveals a critical stress resistance pathway, glutathione synthesis, that is utilized by *S. agalactiae* and contributes to its pathogenesis. Understanding the role of this unique bifunctional glutathione synthesis enzyme in *S. agalactiae* during sepsis may help elucidate why *S. agalactiae* produces such an abundance of glutathione compared to other bacteria.

KEYWORDS *Streptococcus agalactiae*, glutathione, hydrogen peroxide, hypochlorous acid, oxidative stress, virulence

Glutathione (GSH) is an antioxidant that is ubiquitous across all kingdoms of life and is used to protect organisms against reactive oxygen species (ROS) (1). In *Streptococcus agalactiae* (commonly known as group B streptococcus and one of the key causative agents for neonatal meningitis), GSH is maintained in concentrations nearly ten times higher than in other organisms (2), suggesting that *S. agalactiae* relies heavily

Citation Walker EA, Port GC, Caparon MG, Janowiak BE. 2019. Glutathione synthesis contributes to virulence of *Streptococcus agalactiae* in a murine model of sepsis. J Bacteriol 201:e00367-19. https://doi.org/10.1128/JB.00367-19.

Editor Tina M. Henkin, Ohio State University

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Received 30 May 2019
Accepted 10 July 2019
Accepted manuscript posted online 22 July 2019
Published 20 September 2019

on GSH. While other Gram-positive streptococci, such as *Streptococcus mutans* and *Streptococcus pneumoniae*, import GSH from their environment (3, 4), *S. agalactiae* appears to rely primarily on *de novo* synthesis over import (5) and has been demonstrated to produce more GSH on a per-cell basis than any organism tested (2, 3). In addition, under oxidative growth conditions, GSH levels are approximately three times higher than when *S. agalactiae* is grown under reducing conditions (2, 5). Two outstanding questions remain: (i) why does *S. agalactiae* synthesize so much glutathione, and (ii) is glutathione synthesis required for production of a robust infection?

Previously, we demonstrated that *S. agalactiae* synthesizes GSH using a bifunctional enzyme, γ-glutamylcysteine synthetase-GSH synthetase, encoded by a single gene, the glutathione synthesis gene *gshAB* (2). In contrast, humans produce two enzymes, encoded by three genes, to facilitate GSH synthesis (6). In addition to the structural differences of the GSH biosynthetic enzymes, the enzyme's properties and kinetics are significantly different between the *S. agalactiae* and human GSH biosynthesis enzymes (2), giving credence to the possibility that *S. agalactiae* GSH synthesis could be a viable drug target against *S. agalactiae* if it is found that GSH synthesis contributes significantly to virulence.

We hypothesize that *S. agalactiae* synthesizes and maintains large pools of GSH in order to survive and thrive under stressful conditions. Other antioxidant systems in *S. agalactiae* have been shown to be virulence factors. For example, both superoxide dismutase (7) and the polyene pigment β-hemolysin/cytolysin (8) have been shown to contribute to the survival of *S. agalactiae* inside immune cells, as well as toward the production of a robust infection in mice. Simultaneously, GSH synthesis has been shown to be a virulence factor in other bacteria, such as *Escherichia coli* (9) and *Listeria monocytogenes* (10). The presence of GSH, whether synthesized or imported, has been shown to be necessary for protecting most organisms, including other streptococci such as *S. pneumoniae* (4).

To determine whether GSH synthesis could also be a virulence factor in *S. agalactiae*, we set out to characterize the contribution of GSH synthesis to the viability of *S. agalactiae* in a mouse model of sepsis. This study is the first step to address our central hypothesis that *S. agalactiae* synthesizes GSH in response to cellular stresses in order to use GSH to survive and thrive during an infection.

RESULTS

Generation of *gshAB* in-frame deletions in three serotypes of *S. agalactiae*. To begin to determine the role of GSH synthesis in *S. agalactiae*, strains from three of the most prevalent clinical serotypes of *S. agalactiae* in the United States (Ia, III, and V) were used as the parent strains to generate two mutants each, a mutant lacking *gshAB* (Δ*gshAB*) and a knock-in mutant (*gshAB*$^+$) whereby a copy of the *gshAB* gene derived from 2603V/R was restored to the native location in all three deletion strains (Table 1). Strain A909 (serotype Ia, which accounts for ~23% of all U.S. *S. agalactiae* infections) was isolated in the clinic from a septic neonate (11). Strain COH1 (serotype III, which accounts for almost half of all *S. agalactiae* infections) was isolated from human blood in the clinic (12). Strain 2603V/R (serotype V, which causes ~9% of *S. agalactiae* infections) was also isolated from a patient in the clinic (13). Together, these three serotypes account for 80% of all *S. agalactiae* infections.

To determine whether the deletion of *gshAB* resulted in a polar mutation affecting expression of downstream genes, quantitative PCR (qPCR) was performed for *gshAB* and the first downstream gene, *hyp* (SAG_1821 in strain 2603V/R) on all strains generated (wild type [WT], Δ*gshAB*, and *gshAB*$^+$) for all serotypes (Fig. 1A). As predicted, expression of *gshAB* was undetectable in all of the Δ*gshAB* mutants, while the *gshAB* knock-in (*gshAB*$^+$) showed comparable levels of *gshAB* expression to the WT for each serotype (Fig. 1B to D). It is noteworthy that all of the *gshAB*$^+$ knock-in strains utilized the same *gshAB* gene and the upstream putative promoter region from 2603V/R, which contains 3-bp substitutions in the 169 intergenic region and 7 amino acid substitutions in the coding region between the various coding sequences (see Fig. S1 in the

TABLE 1 Bacterial strains and plasmids used in this study

Strain (alternate name) or plasmid	Description[a]	Source or reference
Strains		
COH1 (GCP1418)	Serotype 3, hyperpigmented	29
COH1 Δ*gshAB* (GCP1432)	COH1 with *gshAB* (SAN_1965*) in-frame deletion produced by allelic exchange with pGCP1422	This study
COH1 *gshAB*$^+$ (GCP1437)	GCP1432 with full-length *gshAB* produced using plasmid pGCP1417, WT revertant	This study
2603V/R (GCP1419)	Serotype 5, nonpigmented	29
2603V/R Δ*gshAB* (GCP1429)	2603V/R with *gshAB* (SAG1821*) in-frame deletion produced by allelic exchange with pGCP1422	This study
2603V/R *gshAB*$^+$ (GCP1436)	GCP1429 with full-length *gshAB* produced using plasmid pGCP1417, WT revertant	This study
A909 (GCP1427)	Serotype 1a, nonpigmented	29
A909 Δ*gshAB* (GCP1439)	A909 with *gshAB* (SAK_1841*) in-frame deletion produced by allelic exchange with pGCP1422	This study
A909 *gshAB*$^+$ (GCP1442)	GCP1439 with full-length *gshAB* produced using plasmid pGCP1417, WT revertant	This study
Plasmids[b]		
pGCP213	4.3-kb *E. coli*-streptococcal temperature-sensitive shuttle vector for allelic exchange	31
pGCP1417	pGCP213 with a *gshAB* (SAG1821†) full-length open reading frame for complementation	This study
pGCP1422	pGCP213 with a *gshAB* (SAG1821†) in-frame deletion allele	This study

[a]*, loci are based on the COH1, 2603V/R, or A909 *S. agalactiae* genomes, respectively; †, allelic-exchange constructs for *gshAB* utilized 2603V/R genomic DNA. See Materials and Methods for details.
[b]All plasmids encode an erythromycin-resistant marker.

supplemental material). Both Δ*gshAB* and *gshAB*$^+$ mutants showed comparable expression levels of *hyp* (SAG_1822) to the WT, for all strains, demonstrating that the *gshAB* deletion and reinsertion mutations have no polar effect on the expression of downstream genes (Fig. 1B to D). Lastly, when the expression levels of *gshAB* relative to *recA* were compared between WT strains, there was no significant difference between transcript levels (data not shown).

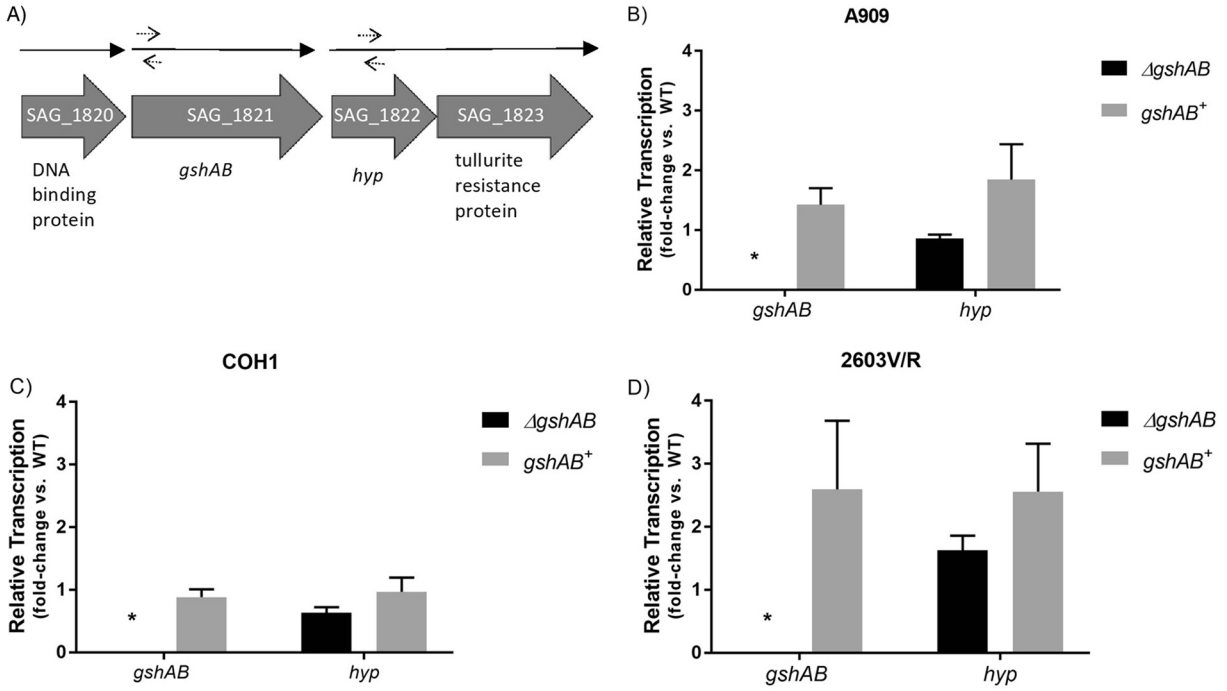

FIG 1 Deletion and reinsertion of *gshAB* only affects *gshAB* and not downstream genes. (A) Position of SAG_1821 *gshAB* in reference to its first downstream gene, SAG_1822 *hyp*, in strain 2603V/R as indicated. An identical genomic arrangement is present in A909 and COH1; therefore, only 2603V/R is shown as a reference. Filled block arrows represent individual open reading frames, dashed arrows indicate forward and reverse qPCR primers, and solid line arrows represent putative operons. *gshAB* and *hyp* are predicted to be encoded in independent operons (thin arrows above the block arrows) separated by 123 bp. Real-time qPCR was used to determine the relative transcript levels of *gshAB* and *hyp* in the Δ*gshAB* and *gshAB*$^+$ mutants compared to their respective WT strain. (B to D) Results for A909 (B), COH1 (C), and 2603V/R (D) show that the *hyp* levels are approximately equivalent to that of the WT (~1-fold change) for both Δ*gshAB* and *gshAB*$^+$ mutants, whereas the *gshAB* levels are approximately equivalent for the *gshAB*$^+$ mutant but were undetectable for the Δ*gshAB* mutant (indicated by an asterisk).

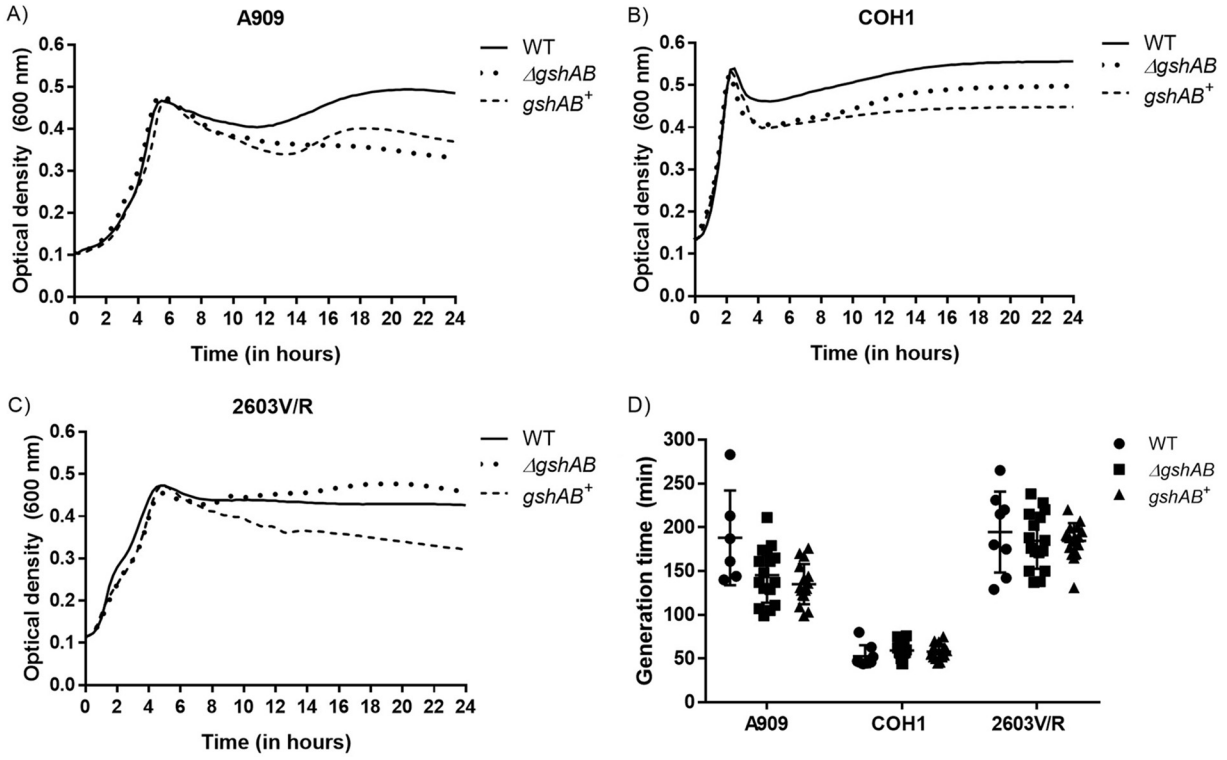

FIG 2 Growth curves and generation times of WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* for strains A909, COH1, and 2603V/R were assayed by using a standard 96-well plate assay. (A to C) A 200-μl portion of GSH-free CD medium was inoculated with one bacterial colony ($n \geq 8$), and the OD_{600} was measured every 15 min for 24 h to produce growth curves. (D) The generation time was determined using the formula for the indirect method of determining generation time [$G = t_{(A\ 0.4)} - t_{(A\ 0.2)}$]. Error bars represent the SEM, and no significant differences were observed between WT, Δ*gshAB*, and *gshAB*$^+$ strains of any of the three serotypes, as determined by a Mann-Whitney rank sum test.

***S. agalactiae* mutants lacking *gshAB* have growth rates, generation times, and growth yields comparable to those of WT and *gshAB*$^+$ *S. agalactiae*.** To determine the impact of GSH synthesis on bacterial growth, the growth curves, generation times, and growth yields of the Δ*gshAB* and *gshAB* knock-in strains were measured and compared to their WT counterparts. Thus, all of the strains and mutants were grown in GSH-free chemically defined (CD), and optical density at 600 nm (OD_{600}) was measured every 15 min. Representative growth curves were produced for each strain, for which the generation time was considered the time, during exponential growth, when the OD_{600} doubled. Lastly, at 24 h postinoculation, each sample was serial diluted and plated to quantify CFU per milliliter for the final growth yield. Representative growth curves showed similar sizes and shapes for each strain of each serotype (Fig. 2A to C). For all three strains (A909, COH1, and 2603V/R) of *S. agalactiae* tested, the generation times of the Δ*gshAB* and *gshAB*$^+$ mutants differed by $<$12.5% compared to the generation time of their WT counterparts in CD medium under nonstress conditions (Fig. 2D). These changes within strains were not significant based on the Mann-Whitney rank sum test ($n = 10$). However, COH1 showed significantly decreased generation times compared to A909 and 2603V/R. Final CFU per milliliter growth yields for each serotype showed no significant difference between the Δ*gshAB* and *gshAB*$^+$ mutants and their WT counterparts (Table 2). These data demonstrate that the absence of GSH synthesis does not affect the growth of any of the three strains when GBS is grown under standard, static growth conditions, suggesting that glutathione synthesis is not required for growth under these conditions.

Both WT and *gshAB*$^+$ *S. agalactiae* strains produce high levels of GSH, whereas the Δ*gshAB* strains are unable to produce GSH. To begin to evaluate the importance of GSH to *S. agalactiae* when grown under static growth conditions, total GSH levels were determined for WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* strains after growth to

TABLE 2 Growth yields of *S. agalactiae* wild-type and mutant strains after growth for 24 h in CD medium

	Avg CFU/ml ± SEM[a]		
Strain	**A909**	**COH1**	**2603V/R**
Wild type	1.00E8 ± 4.62E7	3.71E7 ± 1.47E7	5.83E7 ± 4.71E6
Δ*gshAB* mutant	7.40E7 ± 1.33E7	2.71E8 ± 9.80E7	6.20E7 ± 5.48E6
gshAB$^+$ mutant	6.01E8 ± 1.83E8	1.12E8 ± 5.97E7	7.75E7 ± 1.76E7

[a]$n \geq 4$. Results were not statistically significant between any of the strains for any of the serotypes.

stationary phase in GSH-free CD medium using a standard GSH recycling assay. As expected, mutants lacking *gshAB* were unable to synthesize detectable levels of GSH, whereas all of the *gshAB*$^+$ and WT *S. agalactiae* strains exhibited measurable levels of GSH (Fig. 3). Notably, *S. agalactiae* 2603V/R was observed to produce 10 to 13 times more GSH compared to A909 and COH1 (ca. 350 to 400 nM/mg protein versus ca. 25 to 50 nM/mg protein, respectively) (Fig. 3). Because all of the *gshAB*$^+$ knock-in strains utilized the same *gshAB* gene from 2603V/R and yet only 2603V/R displayed high GSH concentrations, sequence differences in the promoter region and/or coding sequence of *gshAB* do not account for differences in GSH production between strains (Fig. S1). Together, these data not only demonstrate that *gshAB* is required for *de novo* GSH synthesis in *S. agalactiae* but also highlight that an at least 2-fold greater amount of GSH than other bacteria such as *E. coli* (14) is synthesized, *de novo*, by representative strains from the three of the most clinically prevalent serotypes of *S. agalactiae* in the United States.

GSH synthesis in *S. agalactiae* protects against ROS stress under atmospheric conditions. To address the impact of glutathione synthesis on the sensitivity to ROS, *S. agalactiae* was tested for ROS susceptibility on solid media by measuring the zone of growth inhibition surrounding ROS infused discs. Prior to experimentation, the assay was calibrated to use concentrations of hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) that produced a >5-mm zone of inhibition for WT 2603V/R *S. agalactiae* (Fig. S2). Thus, WT and Δ*gshAB S. agalactiae* for all three strains was tested for growth inhibition utilizing two model ROS (HOCl and H_2O_2) and compared to phosphate-buffered saline (PBS) as a nonstressed control (which showed no zone of inhibition [data not shown]) or erythromycin as a non-ROS control for stress (Fig. 4). As shown, all three strains had the same pattern of exhibiting an increase in zones of inhibition for the Δ*gshAB* mutants relative to WT and *gshAB*$^+$ strains for both H_2O_2 and HOCl but no differences in the presence of erythromycin (Fig. 4). However, the increase in the zones of inhibition was not uniform across strains (Fig. 4). In addition, in COH1,

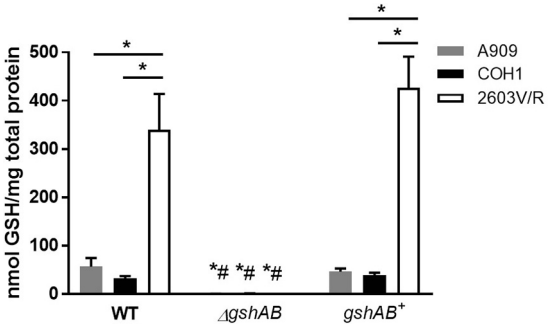

FIG 3 Glutathione production of WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae*. WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* strains were grown overnight at 37°C in GSH-free CD medium prior to GSH analysis. Three serotypes were assayed: A909, COH1, and 2603V/R. A "#" symbol indicates that the Δ*gshAB* mutant was below limit of detection (1 nmol/mg), error bars represent the SEM, and an asterisk indicates a significant difference ($P < 0.05$). To determine the significance between Δ*gshAB* and WT strains and between Δ*gshAB* and *gshAB*$^+$ strains, the limit of detection (1 nmol/mg) was assumed for all Δ*gshAB* values. There was no significant difference determined by the Mann-Whitney rank sum test between WT and *gshAB*$^+$ strains for any of the three serotypes.

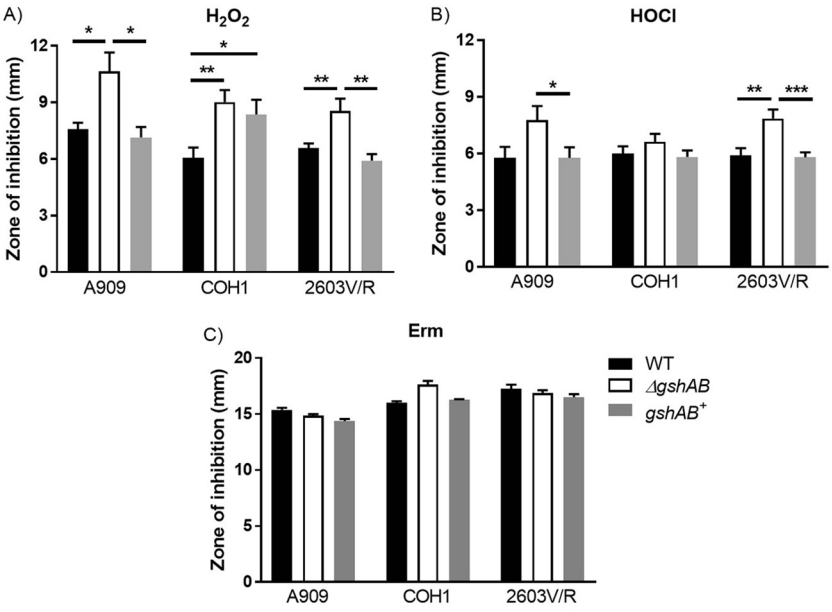

FIG 4 Bacterial growth restriction on solid media in response to H_2O_2, HOCl, and erythromycin. Bacterial growth restriction was assessed between WT, Δ*gshAB*, and *gshAB*$^+$ strains grown in the presence of 750 mM H_2O_2 (A), 589 mM HOCl (B), or 15 μg of erythromycin (C). Error bars represent the SEM with $n \geq 3$, and asterisks indicate a significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between WT and Δ*gshAB* strains, as determined by a Mann-Whitney rank sum test.

the *gshAB*$^+$ mutant subjected to H_2O_2 stress was more similar to the Δ*gshAB* mutant than to the WT. These results suggest that while in some strains GSH exhibits a modest protective role, in other strains the protection is minimal and not significant.

To further quantify whether and how GSH synthesis protects *S. agalactiae* against ROS, the MICs, as well as minimal bactericidal concentrations (MBCs), for H_2O_2 and HOCl in GSH-free (CD) medium (Fig. 5) were determined for the three strains. Differential bacterial inhibition and killing were observed among the strains for both stress conditions. For H_2O_2, only 2603V/R (Fig. 5E) showed a decrease in MIC when GSH synthesis was not present in comparison to both WT and *gshAB*$^+$ strains. However, for HOCl, strains A909 (Fig. 5A) and COH1 (Fig. 5C) showed a decreased MIC when GSH synthesis was removed. For bacterial killing, both A909 (Fig. 5B) and 2603V/R (Fig. 5F) Δ*gshAB* mutants showed increased MBC to H_2O_2, with only 2603V/R (Fig. 5F) showing an increased MBC against the Δ*gshAB* mutant when exposed to HOCl. Together, the MIC and MBC data suggest that different levels of GSH synthesis may provide *S. agalactiae* with different levels of protection against ROS, since the strain that produced the most GSH (2603V/R) was protected under a majority of the stress conditions (Fig. 5E and F).

***S. agalactiae* deficient in GSH synthesis exhibit decreased virulence and bacterial burden in a mouse sepsis model of infection.** To determine whether GSH is a virulence factor for *S. agalactiae*, 8- to 10-week-old C57BL/6 mice ($n \geq 10$) were challenged with ~10^8 CFU of WT, Δ*gshAB*, or *gshAB*$^+$ *S. agalactiae* COH1 by intravenous injection. Strain COHI was chosen for *in vivo* challenge because (i) it is a representative of the most common serotype (III) causing *S. agalactiae* neonatal meningitis (15) and (ii) it produced the least amount of GSH in comparison to A909 and 2603V/R (Fig. 3). Therefore, if GSH synthesis was deemed to be a virulence factor in the COH1 serotype, then the results would likely apply to other serotypes that produce more GSH. Mice were monitored twice daily after infection, and a Kaplan-Meier analysis revealed that GSH-producing *S. agalactiae* COH1 was significantly ($P < 0.0001$) more virulent than GSH-deficient *S. agalactiae* COH1, as determined by the percent survival over the course of 6 days (Fig. 6A). Quantification of the bacterial burden revealed a significantly higher bacterial load in the blood for GSH-producing bacteria at both 4 h postinfection

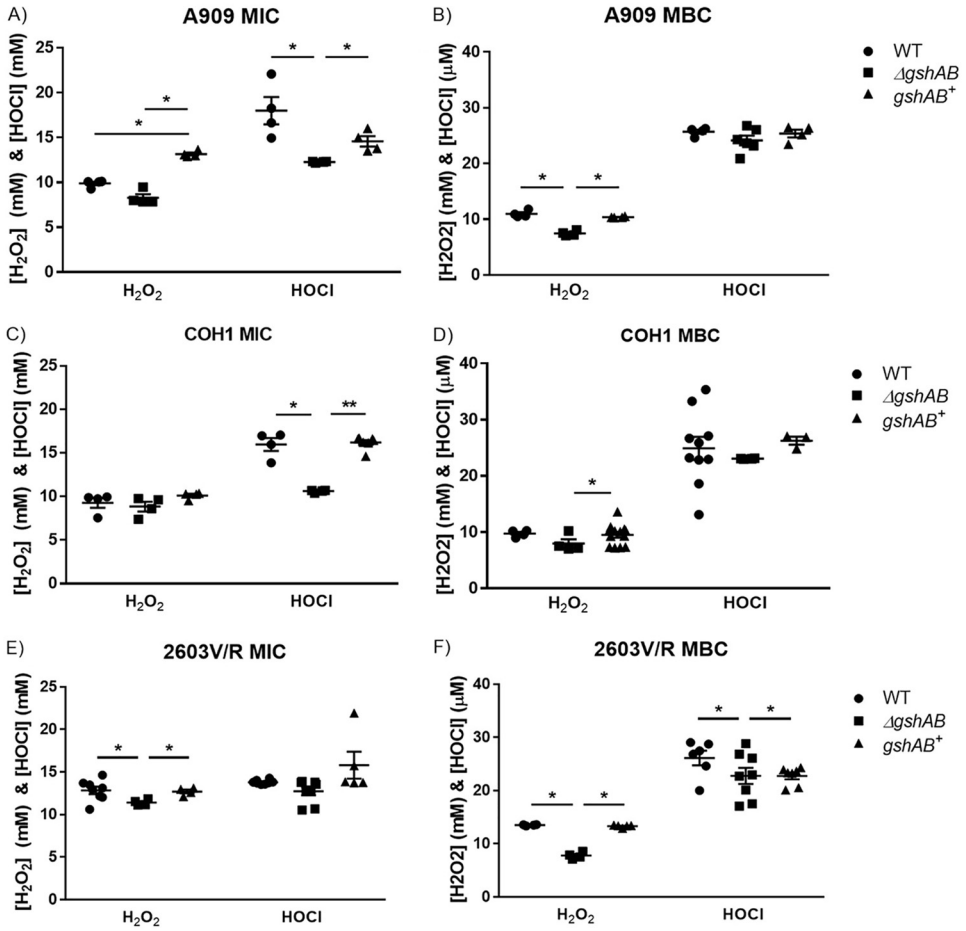

FIG 5 MICs (A, C, and E) and MBCs (B, D, and F) of H_2O_2 and HOCl on *S. agalactiae* serotypes A909 (A and B), COH1 (C and D), and 2603V/R (E and F). Asterisks indicate a significant difference (*, $P < 0.05$; **, $P < 0.01$). WT and *gshAB*$^+$ strains were not significantly different for any condition, except the H_2O_2 MIC for A909. Error bars represent the SEM, and significance was determined by a Mann-Whitney rank sum test ($n \geq 4$).

($P \leq 0.001$) and at the time of death or the end of the experiment (day 6) (Fig. 6B). These results suggest that GSH synthesis is indeed a virulence factor for *S. agalactiae*, since mutants deficient for GSH synthesis were unable to produce a robust septic infection despite the presence of the equivalent starting inoculum.

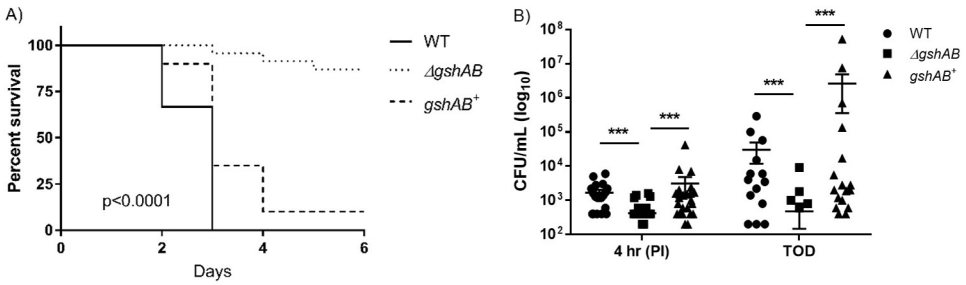

FIG 6 GSH is essential for full virulence of *S. agalactiae* in mice. (A) WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* strains produce significantly different survival profiles depending on the GSH synthesis ability following intravenous challenge of C57BL/6 mice. Murine survival is significantly different ($P < 0.0001$) between WT/*gshAB*$^+$ and Δ*gshAB* strains. (B) Bacterial loads (as measured in blood) are significantly higher at 4 h postinfection and at the time of death (TOD) for bacteria able to synthesize GSH. Significance for survival was determined by using Kaplan-Meier analysis, whereas the significance for the bacterial load was determined by using a Mann-Whitney rank sum test with $n \geq 10$.

DISCUSSION

To begin to evaluate the roles of GSH synthesis in *S. agalactiae*, Δ*gshAB* and *gshAB*$^+$ mutants were generated in three strains of *S. agalactiae* (A909, COH1, and 2603V/R). We found, using qPCR, that the creation of the mutants did not result in any polarity effects (Fig. 1). By comparing responses to oxidative stresses, we observed that *S. agalactiae* lacking *gshAB* was modestly more sensitive to the ROS stress conditions than were WT and *gshAB*$^+$ *S. agalactiae* strains (Fig. 4 and 5). By comparison, *S. agalactiae* lacking *gshAB* was significantly less virulent in a mouse model of sepsis than were WT and *gshAB*$^+$ *S. agalactiae* (Fig. 6). These results strongly suggest that GSH synthesis contributes minimally to ROS stress resistance and significantly toward the virulence of *S. agalactiae*. Since GSH synthesis is not universal among bacterial species, and the *S. agalactiae* GSH synthetic enzyme is distinct from the human biosynthetic enzymes, these results suggest that GSH synthesis could be a viable drug target that would be more selective and specific than the broad-spectrum antibiotics currently used to prevent *S. agalactiae* infections in neonates. Although GSH synthesis has been shown to correlate with the virulence of *L. monocytogenes*, this is the first study to our knowledge to directly correlate GSH synthesis to virulence in *S. agalactiae*.

To determine whether GSH synthesis impacted bacterial growth of *S. agalactiae*, the growth curves, generation times, and growth yields of the Δ*gshAB* and *gshAB*$^+$ mutants were measured and compared to their WT counterparts. Our results suggest that GSH synthesis in *S. agalactiae* is not required for aerobic, nonstressed growth of *S. agalactiae*, since deletion of the *gshAB* gene did not cause a growth defect under nonstress conditions (Fig. 2). However, deletion of the *gshAB* gene did result in more sensitivity to ROS stress in about half of the cases considered (Fig. 4 and 5), suggesting that GSH, at least in part, assists *S. agalactiae* in neutralizing ROS stress. The ability to maintain WT growth rates under nonstress conditions demonstrates that the increased ROS sensitivity seen in the Δ*gshAB* mutants is due to the lack of GSH synthesis and not an off-target, general growth defect. These results are consistent with previous reports from other bacteria, since ROS sensitivity has been observed when GSH synthesis was removed from *E. coli* (9) and *L. monocytogenes* (10), as well as when GSH import was removed from *S. pneumoniae* (4).

We have previously shown that *S. agalactiae* produces robust quantities of GSH compared to other bacteria, including common pathogens (2). Results from the present study suggest that even within *S. agalactiae* strains, there is a large variation in GSH production. Specifically, 2603V/R produces approximately 10 to 13 times greater levels of GSH compared to COH1 (Fig. 3). There are very few, mostly conservative differences in the protein sequence and upstream intergenic sequence between the different strains (Fig. S1), and the same gene sequence from 2603V/R (SAG1821) was utilized to generate the GSH-deficient mutants and the GSH knock-in strains in all three strains. The fact that all three *gshAB*$^+$ mutants restored *gshAB* expression (Fig. 1) and the GSH concentration to levels similar to their WT counterparts (Fig. 3) suggests that differences in promoter regions and/or amino acid substitutions are not responsible for the differences in GSH levels between the strains and that perhaps other undefined regulatory elements are involved. Alternatively, it is possible that this increase in GSH production in 2603V/R could be associated with the lower levels of granadaene, a carotenoid pigment which also has antioxidant capabilities, produced by 2603V/R in comparison to COH1 (16). Because COH1 produces large quantities of granadaene (16), it presumably does not require high quantities of other antioxidants, such as GSH, to combat ROS. This hypothesis has yet to be tested, but it will be a focus for a future study involving mutants lacking the ability to synthesize granadaene.

Although there is a large difference between the amount of GSH produced among *S. agalactiae* strains, even the least-productive strain, COH1, still produces more GSH per cell than any other prokaryotic organism previously reported. For example, *Escherichia coli*, a Gram-negative bacterium, is the model organism for GSH synthesis in bacteria (14), and yet it synthesizes 2-fold less GSH than *S. agalactiae* COH1 (17). It is

important to note that *E. coli* uses the more common pathway of two enzymes for GSH synthesis, whereas *S. agalactiae* uses the less common mechanism of a single bifunctional enzyme (*gshAB*) (2). However, other Gram-negative bacteria, such as *Pasteurella multocida*, synthesize GSH using a bifunctional enzyme, similar to *S. agalactiae*, and yet still produces much smaller quantities (18). Even when *S. agalactiae* is compared to *L. monocytogenes*, another Gram-positive organism with a bifunctional GSH synthesis enzyme, the GSH levels of *S. agalactiae* are much higher (17). These observations indicate that the Gram status and mechanism of synthesis are not predictive of GSH production levels.

When *S. agalactiae* is compared to its human host, whose cells typically produce millimolar levels of GSH, *S. agalactiae* still synthesizes 2- to 10-fold-higher quantities of GSH, depending on the strain of *S. agalactiae* (19). The unique abundance of GSH suggests that GSH synthesis is important for *S. agalactiae* biology and that there may be additional roles for GSH beyond its antioxidant properties in *S. agalactiae*. For example, GSH has been shown to help in the detoxification of metal stresses (4, 20) and chlorine compounds (21), which can be regulated by oxygenation or starvation pathways (21). *S. agalactiae*, along with other streptococci species, is also able to import differing levels of GSH (3). Future studies will further characterize the ability of *S. agalactiae* to import GSH, in conjunction with its ability to synthesize such large quantities of GSH. It is likely that different environmental stimuli promote import versus synthesis and that GSH has more than one role in *S. agalactiae*.

To begin to understand why *S. agalactiae* produces and maintains such elevated levels of GSH, the role of glutathione synthesis was quantified in *S. agalactiae* in response to H_2O_2 and HOCl, two ROS that GSH has been shown to provide cellular protection against in other bacteria (4, 18, 20). As expected, some of the Δ*gshAB* mutants showed growth inhibition using a disk assay in response to H_2O_2 and HOCl but not to a non-ROS control, erythromycin (Fig. 4). Although none of the Δ*gshAB* mutants showed growth inhibition to erythromycin, surprisingly, only the Δ*gshAB* mutants for A909 and 2603V/R showed significant growth inhibition in response to ROS stress between their WT and *gshAB*$^+$ strains, whereas COH1 showed no inhibition for either stress (Fig. 4A and B). Again, this result could be due to the redundant antioxidant systems seen in *S. agalactiae*, particularly the antioxidant granadaene pigment seen in COH1 (22). However, another explanation for the lack of sensitivity of COH1 may be related to the method employed to investigate ROS sensitivity, namely, growth on a brain heart infusion (BHI) agar plate, which may allow for some limited amount of GSH import.

To further quantify the protection against H_2O_2 and HOCl, MIC and MBC values for each ROS were determined for the three strains. For a majority of the cases tested, there was a significant or trending difference between WT/*gshAB*$^+$ and Δ*gshAB* strains, with COH1 being the least susceptible to ROS stresses and 2603V/R being the most susceptible. Therefore, it appears that GSH synthesis levels (Fig. 3) largely correlate with ROS susceptibility, as seen in the disk assay.

It is important to note that the ROS susceptibility assay and the MBC assay utilized bacterial plating on BHI agar plates, whereas the MIC assay did not require plating, and therefore the bacteria were only in contact with CD medium lacking glutathione. Because it is hypothesized that *S. agalactiae* is able to import GSH (3), this import of GSH could have an impact on these results. However, the murine model of sepsis utilized only BHI medium for growth, and a significant difference was seen in murine disease and bacterial infection loads, suggesting that GSH import is not sufficient to rescue *S. agalactiae* lacking GSH synthesis during infection (Fig. 6).

There are multiple factors that may explain the differential effects of HOCl and H_2O_2 in the assays used. First, HOCl is one of the most potent bactericidal substances because it is a strong electrophile capable of oxidizing many important biological molecules (23). Interestingly, HOCl is especially toxic to molecules containing thiols, like GSH (24), thus making GSH an ideal antioxidant for *S. agalactiae* to defend against HOCl. Bacteria possess very few mechanisms capable of defending against chlorine stresses, which

likely explains why neutrophils utilize HOCl as a main source for bacterial killing (25). However, the same features that make HOCl bactericidal also make it harmful to immune cells. During the oxidative burst, immune cells utilize GSH to react with and protect against HOCl, thereby protecting against self-inflicting damage (26). Therefore, it is likely that *S. agalactiae* utilizes GSH in a similar manner to protect against cellular damage caused by the oxidative burst of the immune cells. Our data suggest that GSH synthesis is an important mechanism by which *S. agalactiae* protects itself against HOCl.

Second, the greater effectiveness of HOCl (micromolar) versus H_2O_2 (millimolar) in the MBC assay may be due to the fact that the MIC assay measures the resistance of cells growing in the exponential phase, whereas the MBC assay examines bacteria in the stationary phase. In the presence of HOCl, GSH becomes sacrificial (9), detoxifying the HOCl, but then is nonrecyclable. In other words, in contrast to H_2O_2, HOCl-oxidized GSH cannot effectively be reduced by GSH reductase to reduced GSH, and thus the restoration of GSH levels after HOCl detoxification is dependent on *de novo* synthesis.

A third explanation for the extreme decrease in HOCl required to kill *S. agalactiae* observed in the MBC assay compared to H_2O_2 (Fig. 5) could be due to hydrogen peroxidases. Unlike other streptococcal species which produce GSH peroxidase (27), *S. agalactiae* does not appear to encode such an enzyme and instead likely uses GSH directly to detoxify H_2O_2 and also uses a GSH-independent peroxidase, alkyl hydrogen peroxidase C (28), which can detoxify H_2O_2 but not HOCl. The presence of a GSH-independent peroxidase may allow the GSH-deficient bacteria to survive more efficiently against H_2O_2 than HOCl, which would explain why much lower concentrations of HOCl cause bacterial killing compared to H_2O_2 concentrations.

Our results show that without the *gshAB* gene, *S. agalactiae* COH1 is less virulent than it is with the *gshAB* gene. We hypothesize that the attenuated virulence is due to the lack of synthesis of GSH. However, an alternative hypothesis regarding the enzyme expressed by *gshAB*, γ-glutamylcysteine synthetase-glutathione synthetase, is that the enzyme itself may be required for virulence by interacting with other proteins and/or molecules and not just by synthesizing the end product, GSH. While we have not ruled out the alternative hypothesis, we plan to test that hypothesis in a future study. In the meantime, our results are consistent with what is observed for another Gram-positive pathogen, *Listeria monocytogenes* (10), which has been shown to utilize GSH itself as a virulence factor. It is reasonable to conclude that *S. agalactiae* is also using GSH itself as a virulence factor. In addition, based on other bacteria, it is very likely that GSH may have other roles other than GSH detoxification, such as protection against heavy metals or regulation of virulence factors. Our present study begins to analyze potential mechanisms, and further studies are under way in our lab to evaluate other mechanisms and roles for GSH.

In summary, this study demonstrates that the presence of the GSH synthesis gene contributes to *S. agalactiae* virulence and plays a small role in ROS resistance. Because having the *gshAB* gene allows for virulence in COH1, and COH1 synthesizes the smallest amount of GSH compared to the other strains of *S. agalactiae* tested, GSH synthesis could be a mechanistic target for future treatments of *S. agalactiae*. Thus, future studies will focus on elucidating the cellular regulation of GSH synthesis, as well as the other roles GSH may play in *S. agalactiae*, including immune cell evasion, cell signaling, virulence gene expression, colonization, and GSH transport (import and export).

MATERIALS AND METHODS

Bacterial strains. *S. agalactiae* 2603 V/R (ATCC BAA-611 [29]), A909 (ATCC BAA-1138 [29]), and COH1 (ATCC BAA-1176 [29]) were used as parental strains to generate the Δ*gshAB* and *gshAB*$^+$ strains of *S. agalactiae* used in this study. Construction of the Δ*gshAB* and *gshAB*$^+$ strains of *S. agalactiae* are described below, and the mutant derivatives generated are listed in Table S1 in the supplemental material.

Bacterial growth. To construct the *S. agalactiae* mutants, bacteria were grown in Todd-Hewitt medium (Difco) supplemented with 0.2% yeast extract (Difco) (THY medium) in sealed culture tubes at 37°C under static conditions. Solid medium for construction of the *S. agalactiae* mutants was prepared

by the addition of 1.4% Bacto agar (Difco) (THY agar). When appropriate, erythromycin at 1 μg ml^{-1} was added to the medium during generation of *S. agalactiae* mutant derivatives.

Routine culture of the *S. agalactiae* parent and mutant strains, once constructed, involved creating fresh streak plates for isolation of the bacteria on agar plates composed of BHI broth and 1.5% Bacto agar (Difco), followed by incubation at 37°C under atmospheric conditions. For each experiment, colonies were inoculated from solid medium and grown in CD liquid medium, which is naturally free of GSH (30). Prior to experiments, all cultures were grown in CD medium with loosely capped lids. Cultures were grown overnight (~18 h) at 37°C in a shaking incubator with 200 rpm to ensure cultures received sufficient and constant aeration.

Construction of GSH-deficient *S. agalactiae* and knock-in strains. Routine molecular cloning and plasmid propagation utilized *E. coli* DH5α that was cultured in Luria-Bertani medium at 37°C. When appropriate, erythromycin at 500 μg ml^{-1} was added to the medium, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 μg ml^{-1} was added to solid medium.

Plasmid DNA was isolated via standard techniques and used to transform *E. coli* using standard methods. In-frame deletion and complementation mutants of *gshAB* (NCBI reference sequence NC_004116.1) were generated using the temperature-sensitive shuttle vector pGCP213 (31) to construct the mutagenic plasmids listed in Table S2 in the supplemental material. The *gshAB* deletion allele (lacking amino acids 17 to 733) and complementation allele (restoring complete open reading frame encoding 750 amino acids) were generated by PCR using the oligonucleotide primers (IDT) listed in Table S1 through a process of overlap extension PCR (32) and utilizing genomic DNA isolated from strain 2603V/R by a commercially available kit (Wizard Genomic DNA; Promega) as the template. Modified alleles were inserted directly into pGCP213 at the M13F and M13R universal primer binding sites utilizing the overlap extension PCR cloning method described in detail elsewhere (33). The fidelity of all molecular constructs and mutated chromosomal loci were confirmed by PCR and determination of the DNA sequences (Genewiz) using primers of the appropriate sequences. Plasmid DNA was isolated by standard methods (Hurricane Maxi-Prep [Gerard Biotech], followed by ethanol precipitation) and used to transform *S. agalactiae*, as previously described with minor modifications (34). Transformants appeared after 2 to 4 days at 30°C on THY agar containing erythromycin. Passaging of temperature-sensitive mutagenic plasmids and screening for *S. agalactiae* mutants was performed as previously described with minor modifications (35). After two consecutive days of passaging at 37°C, overnight cultures were diluted at 1:10,000 into fresh THY medium in the absence of erythromycin at 30°C and then passaged daily until the desired mutants were isolated. To isolate mutants, overnight cultures were spread onto THY agar and incubated overnight at 30°C. Colonies were then screened for erythromycin sensitivity by replica plating, and sensitive colonies were screened by colony PCR for the presence of a mutated allele. Of note, all *gshAB*$^+$ knock-in strains were generated with the 2603V/R allele.

RNA transcript levels of GSH-deficient and GSH knock-in *S. agalactiae* verified by qPCR. Transcript abundances of select genes were analyzed by real-time reverse transcription-PCR, referred to here as qPCR. For *gshAB* gene expression analysis, overnight cultured cells were diluted 1:25 or 1:10 into fresh CD medium and harvested at logarithmic phase for mRNA extraction. Select genes, *gshAB* and *hyp* (SAK_1842), were analyzed by qPCR and normalized to *recA* using the primers listed in Table S2. *gshAB* and *hyp* (SAK_1842) transcript is displayed as threshold cycle (ΔC_T), normalized to *recA* for each strain, normalized to the wild type (36). RNA data shown are means and standard errors of the means (SEM) derived from four biological replicates run in duplicate.

Bacterial growth analysis. To determine whether GSH synthesis, or the lack thereof, affects the growth of *S. agalactiae* strains under nonstressed conditions, a growth analysis in CD (GSH-free) medium was performed. Briefly, 200 μl of CD medium was placed into each well of a 96-well microtiter plate. Individual wells were inoculated with a single colony from a BHI plate from each of the bacterial strains ($n = 12$). Growth at 37°C was monitored in a PowerWaveX plate reader (Bio-Tek Instrument, Inc.). During growth, the plate was shaken for 5 s every 15 min for the duration of 24 h, and the OD_{600} was measured every 15 min. Each data point was then plotted, creating a standard bacterial growth curve. The generation time, or doubling time, was determined using the formula for the indirect method of determining generation time [$G = t_{(A\ 0.4)} - t_{(A\ 0.2)}$]; the lag phase and stationary phase were both excluded from analysis. The average generation time for each mutant was then compared to its respective wild type. Growth yields were determined by performing 10-fold serial dilutions from cultures grown for 24 h in CD medium under static conditions and plating onto BHI agar to calculate the CFU per milliliter. Growth curves were produced using a representative biological replicate for each stain, and generation times were produced using a minimum of six biological replicates.

Determination of GSH levels. To compare GSH levels between the three serotypes, as well as to determine whether GSH synthesis was indeed eliminated by removing the *gshAB* gene, the total intracellular GSH levels were measured in WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* A909, COH1, and 2603V/R strains after overnight growth in CD (GSH-free) medium using a routine GSH reductase enzyme recycling assay (37). Quantitation was performed using a standard curve constructed with 0 to 5 nmol of GSH. To normalize the samples per mg of total protein, the protein concentration was analyzed in the unprecipitated sample using a bicinchoninic acid protein concentration kit (Pierce). The GSH levels shown are the means and SEM of $n \geq 3$ biological replicates.

***S. agalactiae* susceptibility to oxidative stresses and erythromycin.** To determine how GSH synthesis, or the lack thereof, protects *S. agalactiae* from either ROS or general susceptibility to antibiotics, a standard antibiotic disk assay (38) using H_2O_2, HOCl, or erythromycin (a common antibiotic used to treat *S. agalactiae* in patients) was performed. First, to determine the dose of ROS to use in the disk assay, a dose-response curve for HOCl and H_2O_2 inhibition of growth on solid medium was

performed using WT 2603V/R *S. agalactiae* spread onto quadrants of a BHI agar plate. One sterile disk (Becton Dickinson) was placed within each quadrant, and 10-μl portions of 2-fold dilutions of HOCl, H_2O_2, or PBS were placed on the disk. The plates were incubated at 37°C under aerobic conditions for 24 h, and the zones of inhibition were recorded. After determining the growth-inhibitory concentrations for 2603V/R, the assay was then used to measure the zones of inhibition for all WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* strains using 589 mM HOCl, 750 mM H_2O_2, 15 μg of erythromycin (Hardy Diagnostics), and PBS (nonstressed control) under aerobic growth conditions. Briefly, overnight cultures grown in CD medium were normalized to an OD_{600} of 0.1 in PBS, and 100 μl of normalized bacteria was spread onto separate quadrants of a BHI quad plate in which there are four distinct partitions made of plastic (Midwest Scientific). The ROS stress embedded on a sterile disk, on an erythromycin antibiotic disk, or on PBS embedded on a sterile disk was placed in the center of each quadrant, and plates were incubated overnight at 37°C under standard atmospheric conditions. The zones of inhibition, in millimeters, were then measured to determine the susceptibility to the ROS, the erythromycin, or the PBS control. The zones of inhibition shown are the mean and SEM for $n \geq 7$ biological replicates.

MIC assays of oxidative stresses to *S. agalactiae*. To determine how GSH synthesis, or the lack thereof, protects *S. agalactiae* from growth inhibition by ROS, MIC assays were performed for two model ROS, hydrogen peroxide (H_2O_2; Sigma-Aldrich) and hypochlorous acid (HOCl; Sigma-Aldrich), in CD (GSH-free) medium. The MICs for H_2O_2 and HOCl were determined by incubating the WT, Δ*gshAB*, and *gshAB*$^+$ *S. agalactiae* COH1 strains with 0 to 68.8 mM H_2O_2 or with 0 to 31.5 mM HOCl. Using a 96-well microtiter plate, each ROS was subjected to 2-fold serial dilution with CD medium for a working volume of 100 μl per well. Separate CD medium was then inoculated with the appropriate bacterial strain by adding 100 μl of a bacterial solution consisting of fresh bacterial colonies derived from a BHI agar plate into CD medium normalized to an OD_{600} of 0.1 for a final volume of 200 μl. Plates were incubated overnight under static aerobic conditions for 18 h at 37°C. After incubation, OD_{600} measurements were recorded on a PowerWaveX plate reader to detect bacterial growth in response to the various concentrations of H_2O_2 and HOCl. The MIC was considered the point at which growth was inhibited ≥99% compared to the nonstressed control. The MICs shown are the mean and SEM for $n \geq 4$ biological replicates.

Minimum bactericidal concentration assays of oxidative stresses to *S. agalactiae*. To determine how GSH synthesis, or the lack thereof, protects *S. agalactiae* from ROS-mediated killing, minimum bactericidal concentration (MBC) assays were performed for the same two model ROS, H_2O_2 and HOCl, in CD (GSH-free) medium. The MBCs for H_2O_2 and HOCl were determined by subjecting *S. agalactiae* that had been previously grown to stationary phase in CD medium with 0 to 68.8 mM H_2O_2 and with 0 to 492.2 μM HOCl in fresh CD medium. Briefly, using a 96-well microtiter plate, each ROS was subject to 2-fold serial dilution with PBS for a working volume of 100 μl per well. Bacteria grown to stationary phase in CD medium were normalized in PBS to an OD_{600} of 0.5. Then, 100 μl of the normalized bacteria was added to various concentrations of H_2O_2 and HOCl. The plate was then incubated for 1 h at 37°C under aerobic conditions. After incubation, each condition was subjected to 10-fold serial dilution and plated on BHI agar. CFU were calculated to determine the MBC for each bacterial strain. The MBC was considered the point at which ≥1 log less viability occurred compared to the control with no stress. The MBCs shown are the mean and SEM for $n \geq 3$ biological replicates.

***In vivo* murine model of sepsis for *S. agalactiae* COH1.** To determine whether GSH synthesis mediated by *gshAB* is a virulence factor in *S. agalactiae*, a murine model of sepsis was utilized (16). The animal protocol was approved by the Saint Louis University IACUC, protocol 2201, which ensured that death was not used as an endpoint. Briefly, 8- to 10-week-old C57BL/6 mice (Jackson Laboratories) were injected in the tail vein with a targeted dose of ~10^7 CFU of logarithmic-phase WT, Δ*gshAB*, or *gshAB*$^+$ *S. agalactiae* COH1 strains. Bacteremia was assessed at 4 h postinfection by submandibular blood collection; samples were serial diluted and plated on BHI plates. Mouse health and survival was monitored twice daily for 6 days, moribund mice were immediately sacrificed, and all remaining mice were sacrificed at day 6. Bacterial loads at the time of death were determined by serially diluting and plating blood from cardiac punctures taken after euthanasia. The survival curves and bacterial loads shown are the mean and SEM for $n \geq 20$ biological replicates.

Statistical analysis. A Mann-Whitney rank sum test was used for all comparisons ($n \geq 3$). In all cases, WT and *gshAB*$^+$ strains were compared to the Δ*gshAB* strain, with WT and *gshAB*$^+$ strains showing no significant differences for most experiments (unless otherwise noted).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00367-19.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

ACKNOWLEDGMENTS

We thank Rachel Treat, Lauren Grant, Julia Nims, Shiragi Patel, Yasmeen Dhindsa, Tom George, Sabina Lin, Michael Flom, Shuran Ma, Ami Modi, Ravi Patel, Kiran Singh, Timothy Baker, Maria Schwabe, Priya Desai, and Amisha Patel (Saint Louis University) for their support and efforts in this study. In addition, we thank Claire Gendrin, Jay Vornhagen, and Lakshmi Rajagopal (University of Washington, Seattle, WA) for providing helpful advice on making *S. agalactiae* competent, and Lloyd Robinson, Warren

Lewis, and Amanda Lewis (Washington University, St. Louis, MO) for providing the A909 *S. agalactiae* strain.

The research in this study was supported by Saint Louis University startup funds to B.E.J., by the Saint Louis University President's Research Fund to B.E.J., by a Monsanto PhD STEM Fellowship to E.A.W., and in part by NIH NIAID R01 AI046433 and AI7075 grants to M.G.C. and a W. M. Keck Fellowship to fund G.C.P.

E.A.W. and G.C.P. contributed to the acquisition, analysis, and interpretation of the data, as well as to the writing of the manuscript. B.E.J. and M.G.C. contributed to the conception of the study, the interpretation of the data, and the writing of the manuscript.

We declare no conflicts of interest.

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