Group A Streptococcus AdcR Regulon Participates in Bacterial Defense against Host-Mediated Zinc Sequestration and Contributes to Virulence

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ABSTRACT Colonization by pathogenic bacteria depends on their ability to overcome host nutritional defenses and acquire nutrients. The human pathogen group A streptococcus (GAS) encounters the host defense factor calprotectin (CP) during infection. CP inhibits GAS growth *in vitro* by imposing zinc (Zn) limitation. However, GAS counterstrategies to combat CP-mediated Zn limitation and the *in vivo* relevance of CP-GAS interactions to bacterial pathogenesis remain unknown. Here, we report that GAS upregulates the AdcR regulon in response to CP-mediated Zn limitation. The AdcR regulon includes genes encoding Zn import (*adcABC*), Zn sparing (*rpsN.2*), and Zn scavenging systems (*adcAll*, *phtD*, and *phtY*). Each gene in the AdcR regulon contributes to GAS Zn acquisition and CP resistance. The $\Delta adcC$ and $\Delta rpsN.2$ mutant strains were the most susceptible to CP, whereas the $\Delta adcA$, $\Delta adcAll$, and $\Delta phtD$ mutant strains displayed less CP sensitivity during growth *in vitro*. However, the $\Delta phtY$ mutant strain did not display an increased CP sensitivity. The varied sensitivity of the mutant strains to CP-mediated Zn limitation suggests distinct roles for individual AdcR regulon genes in GAS Zn acquisition. GAS upregulates the AdcR regulon during necrotizing fasciitis infection in WT mice but not in *S100a9*^{-/-} mice lacking CP. This suggests that CP induces Zn deficiency in the host. Finally, consistent with the *in vitro* results, several of the AdcR regulon genes are critical for GAS virulence in WT mice, whereas they are dispensable for virulence in *S100a9*^{-/-} mice, indicating the direct competition for Zn between CP and proteins encoded by the GAS AdcR regulon during infection.

KEYWORDS Zn acquisition, bacterial pathogenesis, gene regulation, host nutritional immunity, streptococcus

Transition metals such as iron (Fe), manganese (Mn), and zinc (Zn) are critical micronutrients for bacterial growth. Zn is an essential cofactor required for the catalytic activity and structural integrity of various metalloproteins involved in bacterial pathophysiological processes (1–3). Thus, the pathogenic bacteria must acquire Zn during infection for successful survival and proliferation. Consequently, the host deploys nutritional immune factors such as calprotectin (CP) to sequester Zn and inhibit microbial growth (4–8). Bacterial pathogens have evolved counterstrategies to evade host nutritional immune mechanisms and establish successful colonization. This includes metal acquisition by bacterial surface-bound or secreted metal transport/scavenging systems, minimizing cellular metal use by metal-sparing responses, and metal mobilization from intracellular stores (9–14). Consistent with their significant contribu-

Citation Makthal N, Do H, Wendel BM, Olsen RJ, Helmann JD, Musser JM, Kumaraswami M. 2020. Group A streptococcus AdcR regulon participates in bacterial defense against host-mediated zinc sequestration and contributes to virulence. *Infect Immun* 88:e00097-20. <https://doi.org/10.1128/IAI.00097-20>.

Editor Nancy E. Freitag, University of Illinois at Chicago

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Received 17 February 2020

Returned for modification 11 March 2020

Accepted 6 May 2020

Accepted manuscript posted online 11 May 2020

Published 21 July 2020

tion to bacterial survival in the host, the components of bacterial adaptive responses to host-imposed metal limitation are critical virulence determinants and key contributors to bacterial pathogenesis (13, 14).

Streptococcus pyogenes, also known as group A streptococcus (GAS), is a versatile human pathogen. GAS colonizes diverse host anatomic sites and causes an array of disease manifestations ranging from mild pharyngitis and impetigo to life-threatening, severe invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (15–17). GAS encounters CP-mediated Zn limitation during infection (9, 18). CP, a heterodimer composed of proteins S100A8 and S100A9, is produced predominantly by infiltrating neutrophils and participates in host defense against several bacterial pathogens (9, 19–25). CP is a major constituent of the cytosolic protein content of neutrophils (8, 26). When the host encounters the invading pathogen, neutrophils and neutrophil-associated CP are recruited to the site of infection as a component of the first line of host innate defenses (5, 8, 26). Subsequently, CP is released by neutrophils, and the extracellular CP chelates Zn and Mn from bacterial colonization surfaces in a calcium-dependent manner (5, 27–29). CP has two metal-binding sites that contribute to its antimicrobial activity: metal-binding site 1 (S1) sequesters Zn, whereas site 2 (S2) chelates both Zn and Mn (4, 6, 7). The S1 site comprises amino acids H17 and H27 from S100A8 and H91 and H95 from S100A9, whereas the amino acids H83 and H87 from S100A8 and H20 and D30 from S100A9 participate in metal binding at the S2 site (4, 6).

GAS adaptive responses to Zn limitation are coordinated by the Zn-sensing transcription regulator *adhesion competence repressor* (AdcR) (18, 30). AdcR belongs to the multiple antibiotic resistance family of regulators (MarR) and mediates Zn-dependent transcriptional regulation of genes involved in Zn scavenging, sparing, and acquisition during Zn limitation (18, 30, 31). As for other metalloregulators, the Zn-bound AdcR binds to target promoters and downregulates the expression of the AdcR regulon during Zn sufficiency (30). Conversely, under Zn-limiting conditions, the metal-free AdcR dissociates from promoters and relieves the repression of target genes (30). The core AdcR regulon in GAS comprises the *adcA*, *adcB*, *adcC*, *adcAll* (also known as *lmb*, *lsp*, or *lbp*) (32–34), *rpsN.2*, *phtD* (also known as *htpA*) (35, 36), and *phtY* (also known as *slr* and *inIA*) genes (18, 30, 37). The gene products of the core AdcR regulon contribute to bacterial Zn acquisition and GAS virulence (9, 18, 32, 33, 36, 37). The AdcR regulon also contains accessory genes that include *adh* genes encoding alcohol dehydrogenases and *dpp* and *has* operons that encode dipeptide permeases and capsule biosynthesis genes, respectively. However, the role of the accessory AdcR regulon genes in GAS Zn acquisition is unknown. Our investigations in this study are focused on the core AdcR regulon genes. For the purpose of readability, we refer to the core regulon as the AdcR regulon. The AdcR regulon is upregulated during GAS growth under Zn-limiting conditions *in vitro*, suggesting that AdcR regulon genes may participate in GAS Zn acquisition and maintenance of cytosolic Zn homeostasis. However, the contribution of individual genes of the AdcR regulon to GAS defense against CP-mediated Zn limitation *in vitro* and during infection remains unknown.

In this study, we discovered that GAS upregulates the AdcR regulon as its primary line of defense in response to CP-mediated Zn limitation. The AdcR regulon genes are critical for bacterial Zn acquisition and GAS growth *in vitro* in the presence of CP. Interestingly, GAS isogenic mutant strains in which individual genes in the AdcR regulon have been deleted had different levels of sensitivity to CP. These results suggest different roles for each AdcR regulon gene in GAS Zn acquisition. Using a mouse model of necrotizing myositis, we demonstrate that GAS significantly upregulates the AdcR regulon only during infection in wild-type (WT) mice, but not in mice lacking CP, thereby indicating that the host imposes CP-mediated Zn limitation *in vivo*. Finally, we show that genes in the AdcR regulon contribute significantly to GAS virulence in WT mice, whereas these genes are dispensable for bacterial pathogenesis in *S100a9*^{−/−} mice that do not produce CP. These results highlight the complex

FIG 1 Global gene regulatory responses of GAS to CP. (Left) Heat map revealing the alterations in gene expression pattern at the global level in WT GAS grown in the presence of CP. (Right) The regions corresponding to genes from the AdcR regulon are highlighted. The scheme for the color values is shown at the bottom. Red shades in the heat map indicate genes that are upregulated, green shades represent GAS genes that are downregulated, and black represents genes that are not differentially expressed or detected in the presence of CP.

interplay between CP and the GAS AdcR regulon *in vivo* and the contribution of the AdcR regulon to evade and overcome the host nutritional defenses.

RESULTS

GAS upregulates the AdcR regulon during growth in the presence of CP. To determine GAS adaptive responses to CP-mediated metal limitation, we performed comparative transcription profiling of wild-type (WT) GAS grown in the presence or absence of a subinhibitory concentration of CP by RNA-sequencing (RNA-seq). GAS was grown to the mid-exponential phase ($A_{600} \sim 0.8$) in laboratory medium (Todd-Hewitt broth supplemented with yeast extract [THY]) supplemented with or without 125 $\mu\text{g/ml}$ of CP. GAS genes with a ≥ 2 -fold change in transcript levels between the two growth conditions with statistical significance ($P < 0.05$) were considered differentially regulated (see Tables S1 and S2 in the supplemental material). A total of 42 genes were differentially regulated in the presence of CP, 25 of which were upregulated and 17 of which were downregulated (Tables S1 and S2). The AdcR regulon was significantly upregulated in the presence of CP (Fig. 1 and Table S1). Other upregulated genes of known function include genes encoding the iron efflux transporter (*pmtA*), dipeptide uptake system (*dppABCDE*), capsule biosynthesis pathway (*hasABC*), mitogenic factor (*mf4*), and cell envelope protease (*prtS*) (Table S1). GAS genes downregulated in the presence of CP encode putative enzymes of carbohydrate metabolism (*lacABCD.1*), virulence factors (*cfa* and *grab*), a putative regulator of copper homeostasis (*copY*), collagen-binding protein (*cbp*), and several proteins of unknown function (Table S2). CP also sequesters Mn to inhibit microbial growth (4, 6, 28). However, no alterations were observed in the expression of the MtsR regulon that is involved in GAS adaptive responses to Mn limitation (38). This is likely due to the relatively low Mn concentration in THY and upregulation of the MtsR regulon during GAS growth in the unsupplemented THY (38). As a result, supplementation of THY with CP may not impose further Mn limitation on GAS and consequently fails to evoke regulatory responses specific for Mn deficiency. Additional studies using Mn-supplemented THY are required to elucidate GAS adaptive responses to CP-mediated Mn limitation. CP has also been implicated in imposing iron limitation on bacterial pathogens during infection (39–41). However, our results showed that iron efflux transporter *PmtA* is upregulated during GAS growth in the presence of CP (Table S1). Given that the expression of *pmtA* and its orthologs is typically induced by high iron levels, it is likely that GAS may not encounter CP-imposed iron limitation under the tested conditions (42–44). The results from the transcriptome studies presented here demonstrate that GAS upregulates the AdcR regulon during growth *in vitro* in the presence of CP.

GAS upregulates the AdcR regulon in response to CP-mediated Zn limitation. CP has roles beyond metal sequestration *in vivo* (45–47). Thus, we investigated whether

FIG 2 GAS upregulates the AdcR regulon in response to CP-mediated Zn limitation. (A and B) A schematic diagram illustrating WT CP (A) or mutant CP ($\Delta S1/\Delta S2$) (B) used in the transcript analyses studies is shown. The schematics of Zn-binding (site 1 [S1]) and Zn/Mn-binding residues (site 2 [S2]) of the S100A8/S100A9 heterodimer in CP are shown. The metal-binding capacity of CP was disrupted in the $\Delta S1/\Delta S2$ mutant CP (B) by site directed mutagenesis of metal-binding residues. (C) WT GAS cells were grown to the mid-exponential growth phase ($A_{600} \sim 0.8$) in the presence or absence of the indicated concentrations of WT or $\Delta S1/\Delta S2$ CP. GAS grown in the presence of Zn-specific chelator, *N,N,N',N'*-Tetrakis (2-pyridylmethyl)ethylenediamine (TPEN), was used as a positive control. The unsupplemented GAS growth was used as the reference bacterial growth. Transcript levels of the AdcR regulon genes were assessed by qRT-PCR. The fold change in transcript levels relative to the reference is shown. The data are the mean $\pm$ standard deviation for three biological replicates grown on separate occasions.

the derepression of the GAS AdcR regulon occurs specifically in response to CP-mediated Zn limitation. To this end, we generated recombinant $\Delta S1/\Delta S2$ mutant CP in which the metal-binding ligands at sites 1 and 2 were replaced with inactivating alanine substitutions (Fig. 2A and B). The $\Delta S1/\Delta S2$ mutant CP is defective in metal binding (Fig. S1) and impaired in antimicrobial activity against other pathogens (21). GAS was grown in the presence of either WT or $\Delta S1/\Delta S2$ mutant CP to the mid-exponential phase of growth ($A_{600} \sim 0.8$), and changes in the transcript levels of the AdcR-regulated genes were assessed by reverse transcriptase quantitative PCR (qRT-PCR). GAS grown in the presence of the Zn-specific chelator TPEN was used as a positive control, whereas GAS grown in the untreated Zn-replete THY medium was used as the reference (Fig. S1).

As expected, GAS upregulates the expression of the AdcR regulon in response to TPEN-mediated Zn chelation compared to unsupplemented growth (30) (Fig. 2C). Similarly, GAS grown in the presence of WT CP had a drastic increase in the transcript levels of AdcR regulon genes, and the level of upregulation was comparable to that of

GAS grown in the presence of TPEN (Fig. 2C). In contrast, the addition of $\Delta S1/\Delta S2$ mutant CP to GAS growth failed to cause upregulation of the AdcR regulon, indicating that GAS induces the AdcR regulon to counter host-imposed metal limitation (Fig. 2C). CP exerts its antimicrobial activity by sequestration of both Mn and Zn (5, 6). Thus, to determine whether GAS employs the AdcR regulon specifically in response to CP-mediated Zn limitation, we measured transcript levels of AdcR regulon genes in GAS grown in the presence of WT CP supplemented with either excess Zn or Mn. The supplementation of Zn reduced the transcript levels of AdcR regulon genes to levels similar to the untreated control, whereas addition of Mn did not repress the transcription of AdcR regulon genes (Fig. 2C). Collectively, these data indicate that GAS upregulates the AdcR regulon in response to CP-mediated Zn limitation, and the AdcR regulon genes may aid GAS growth under Zn-limiting conditions found in the host.

Individual components of the AdcR regulon contribute to GAS defense against CP-mediated Zn limitation. Previous studies indicated that Zn acquisition by the tripartite importer AdcABC is critical for bacterial defense against CP and GAS survival in the host (9). However, the contribution of other genes in the AdcR regulon to resisting host-mediated Zn limitation and GAS survival remains unknown. Thus, we tested the hypothesis that the AdcR-regulated genes (*adcA*, *adcAll*, *adcC*, *rpsN.2*, *phtD*, and *phtY*) are critical for optimal GAS growth in the presence of CP by comparing the growth characteristics of WT and isogenic mutant strains. We constructed isogenic mutant strains by deleting individual genes in the AdcR regulon ($\Delta adcA$, $\Delta adcC$, $\Delta adcAll$, $\Delta rpsN.2$, $\Delta phtD$, and $\Delta phtY$) (Fig. S2 and Table S4). The *adcAll* and *phtD* genes form an operon (Fig. S2). Thus, to ensure that the in-frame inactivation of *adcAll* or *phtD* has no polar effect on the transcription of the noninactivated gene, we measured the transcript levels of noninactivated genes. The mutant strains had WT-like transcript levels of the noninactivated genes under Zn-limiting growth conditions, suggesting an absence of polar effect on the expression of neighboring genes in the mutant strains (Fig. S3A and B). The GAS genome contains two *pht* genes (*phtD* and *phtY*). The four Pht proteins in pneumococci had been shown to be functionally redundant (48). Thus, in anticipation of functional redundancy between GAS Pht proteins, we also generated a double *pht* mutant ($\Delta phtD/\Delta phtY$).

GAS strains were grown in the presence of increasing concentrations of CP, and their sensitivity to CP-mediated metal limitation was assessed by monitoring bacterial growth. All mutant strains had WT-like growth characteristics in the absence of CP (Fig. 3A and 4A; Fig. S4). In contrast, each mutant strain was defective in growth in the presence of CP relative to the WT, indicating that the mutant strains are more sensitive to CP-mediated metal limitation (Fig. 3A and 4A; Fig. S4). The $\Delta phtY$ mutant was an exception to this observation. The $\Delta phtY$ mutant had WT-like sensitivity to CP-mediated metal limitation and WT-like growth phenotype in the presence of CP (Fig. 4A and Fig. S4). The CP sensitivity of the AdcR regulon mutants varied among different mutant strains. Based on their sensitivity to CP, the mutant strains can be categorized into two groups (Fig. 3A and 4A; Fig. S4). The $\Delta adcC$ and $\Delta rpsN.2$ mutant strains belonging to the first group (group 1) were more sensitive to CP than the second group (group 2), which comprises the $\Delta adcA$, $\Delta adcAll$, $\Delta phtD$, and $\Delta phtD/\Delta phtY$ mutant strains (Fig. 3A and 4A). The $\Delta adcC$ and $\Delta rpsN.2$ mutant strains failed to grow even in the presence of the lowest CP concentration tested (150 $\mu\text{g/ml}$ CP) (Fig. 3A and Fig. S4). On the other hand, the mutant strains from group 2 had a WT-like growth phenotype in the presence of 150 $\mu\text{g/ml}$ CP (Fig. 4A and Fig. S4). However, subtle differences in CP sensitivity were observed among the different group 2 mutant strains. The $\Delta adcA$ and $\Delta adcAll$ mutant strains were more sensitive to CP than the $\Delta phtD$ and $\Delta phtD/\Delta phtY$ mutant strains (Fig. 4A and Fig. S4). The growth of the $\Delta adcA$ mutant was retarded at 150 $\mu\text{g/ml}$ CP, whereas the mutant strain failed to grow at 300 $\mu\text{g/ml}$ CP (Fig. 4A and Fig. S4). Similarly, the $\Delta adcAll$ mutant strain had slower growth at 150 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$ CP but required 325 $\mu\text{g/ml}$ CP for growth inhibition (Fig. 4A). An interesting difference in CP sensitivity was observed between the two *pht* mutant strains. The $\Delta phtD$ mutant strain had increased sensitivity to CP-mediated metal limitation compared to the WT, and

FIG 3 *adcC* and *rpsN.2* genes are critical for GAS defense against CP-mediated Zn limitation. The growth kinetics of GAS strains grown in the presence or absence of CP are shown. (A and B) GAS was inoculated in THY-CP medium (38%) (vol/vol), THY medium, and 62% (vol/vol) CP buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM β -mercaptoethanol, and 3 mM $CaCl_2$ supplemented with the indicated concentrations of either recombinant WT (A) or $\Delta S1/\Delta S2$ mutant CP (B). (C) Bacterial growth was supplemented with 25 μ M $ZnCl_2$. Growth was monitored by absorption at 600 nm in a microplate reader at indicated time points. Three biological replicates were grown on separate occasions, and the mean $\pm$ standard deviation is shown.

growth was inhibited at 325 μ g/ml CP (Fig. 4A). In contrast, the $\Delta phtY$ mutant strain had a WT-like growth phenotype even at the highest CP concentration tested (Fig. 4A). To verify the validity of the $\Delta phtY$ mutant strain, we measured *phtY* transcript levels in a $\Delta phtY$ mutant grown in the presence or absence of the Zn-specific chelator TPEN. The *phtY* transcript levels were undetectable, indicating that the gene is inactivated in the $\Delta phtY$ mutant strain (Fig. S3C). To investigate the possibility that PhtY has a secondary role in Zn acquisition, we assessed the growth phenotype of the $\Delta phtD/\Delta phtY$ mutant in the presence of CP. The inactivation of *phtY* in the $\Delta phtD$ mutant strain did not exacerbate the growth defect of the $\Delta phtD/\Delta phtY$ mutant strain, and growth characteristics of the $\Delta phtD/\Delta phtY$ mutant were comparable to those of the $\Delta phtD$ mutant (Fig. 4A). These results suggest that *phtD* is critical for GAS CP resistance, whereas *phtY* is dispensable for GAS growth *in vitro* under Zn-limiting conditions.

To investigate whether the growth defect of AdcR regulon mutant strains is due to CP-mediated metal limitation, we assessed the growth characteristics of GAS strains in the presence of $\Delta S1/\Delta S2$ mutant CP that is defective in metal binding. All the tested GAS strains had a WT-like growth phenotype even in the presence of the highest inhibitory CP concentration, indicating that the increased sensitivity of the mutant strains is due to CP-mediated metal limitation (Fig. 3B and 4B). Finally, to ensure that the growth inhibition of AdcR regulon mutant strains by WT CP is due to Zn limitation, we compared the ability of WT CP to inhibit GAS growth in THY supplemented with excess Zn. The provision of additional Zn rescued the defective growth of mutant strains in the presence of WT CP to WT-like phenotype (Fig. 3C and 4C), indicating that the AdcR regulon genes participate in GAS defense against CP-mediated Zn limitation. Collectively, these results demonstrate that each gene in the AdcR regulon (except *phtY*) contributes to CP resistance and GAS fitness under host-imposed Zn-limiting growth conditions.

Individual genes in the AdcR regulon contribute to GAS Zn acquisition. Our observation that the AdcR regulon plays a major role in evading host-imposed Zn limitation suggests that these proteins may participate in Zn acquisition and maintenance of optimal intracellular Zn concentration. To test this hypothesis, GAS strains were grown to the mid-exponential phase (A_{600} , 0.8) in THY supplemented with Zn-specific chelator, TPEN. The intracellular metal concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS). Consistent with their role in

FIG 4 *adcA*, *adcAll*, and *phtD* genes participate in GAS defense against CP-mediated Zn limitation. The growth kinetics of GAS strains grown in the presence or absence of CP are shown. (A and B) GAS was inoculated in THY-CP medium (38%) (vol/vol), THY medium, and 62% (vol/vol) CP buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM β -mercaptoethanol, and 3 mM CaCl_2 supplemented with the indicated concentrations of either recombinant WT (A) or $\Delta S1/\Delta S2$ mutant CP (B). (C) Bacterial growth was supplemented with 25 μM ZnCl_2 . Growth was monitored by absorption at 600 nm in a microplate reader at the indicated time points. Three biological replicates were grown on separate occasions, and the mean $\pm$ standard deviation is shown.

Zn acquisition, each mutant strain had significantly reduced intracellular Zn levels compared to WT GAS (Fig. 5). These data show that the AdcR-regulated genes aid in GAS growth under Zn-limiting conditions by participating in Zn acquisition.

GAS upregulates adaptive responses to Zn limitation during infection to combat host-induced metal limitation. To determine whether GAS encounters host-imposed Zn starvation during the course of infection, we assessed the upregulation of the AdcR regulon by measuring the transcript levels of AdcR-regulated genes in the infected lesions. Compared to GAS grown in Zn-replete THY *in vitro*, the lesions from WT mice had significantly higher transcript levels of AdcR-regulated genes (Fig. 6). To investigate whether the upregulation of the AdcR regulon occurs in response to CP-mediated metal limitation during infection, we performed analogous transcript-level analyses in the lesions of *S100a9*^{-/-} mice that are devoid of CP. The transcript levels of AdcR-regulated genes were significantly decreased in the infected lesions from *S100a9*^{-/-} mice compared to that of WT mice (Fig. 6), indicating that GAS encounters less pronounced Zn limitation in *S100a9*^{-/-} mice. However, we note that the transcript levels of AdcR regulon genes in *S100a9*^{-/-} mice were significantly higher than that of GAS grown in Zn-replete medium (Fig. 6), suggesting that additional CP-independent host-mediated Zn sequestration mechanisms may exist. Collectively, these results indicate that GAS encounters CP-mediated Zn limitation during infection and deploys the AdcR regulon to evade host-imposed Zn starvation.

Adaptive responses to Zn limitation are critical for CP resistance and GAS virulence. Based on the results of the *in vitro* studies, we hypothesized that genes in the AdcR regulon participate in GAS defense against CP-mediated Zn limitation during

FIG 5 Intracellular metal content of WT and isogenic mutant GAS strains as assessed by ICP-MS. GAS strains were grown to the mid-exponential growth phase (A_{600} 0.8) in THY broth supplemented with TPEN. (A) The intracellular Zn levels of WT, Δ *adcC*, and Δ *rpsN.2* strains grown in THY supplemented with 30 μ M TPEN are shown. (B) The cytosolic Zn levels of each indicated strain grown in THY supplemented with 32.5 μ M TPEN are shown. Cell pellets were washed twice in PBS containing 1 mM nitrilotriacetic acid, followed by two washes in chelexed PBS, and suspended in chelexed PBS. The Zn content in the clarified cell lysate was analyzed with ICP-MS. The Zn levels were normalized to the total cytosolic protein concentration. The data are the mean $\pm$ standard deviation for two biological replicates. *P* values (*, $P < 0.05$; **, $P < 0.01$) were determined by comparison to the respective WT GAS control and were derived from a two-sample *t* test.

infection and contribute significantly to bacterial virulence during invasive infection. To test this hypothesis, we compared the virulence phenotype of the mutant strains with WT GAS using an intramuscular mouse model of infection that mimics necrotizing myositis. Mice were infected intramuscularly with 1×10^8 CFU of either WT GAS or one of the mutant strains and monitored for near mortality. The mutant strains were significantly attenuated for GAS virulence compared to the WT ($P < 0.05$ when comparing any mutant strain with WT) (Fig. 7A), indicating that the AdcR regulon contributes significantly to GAS pathogenesis. To determine whether the AdcR-regulated genes are engaged in competition against CP-mediated Zn limitation during infection, we assessed the near mortality of *S100a9*^{−/−} mice infected intramuscularly with WT or individual mutant strains. The virulence phenotype of each mutant strain was comparable to WT GAS in *S100a9*^{−/−} mice, and no significant differences among strains were observed (Fig. 7B), indicating that the AdcR regulon genes are dispensable for GAS virulence in the absence of host-imposed Zn limitation. Together, these data demonstrate that the AdcR regulon aids in GAS defense to overcome CP-mediated Zn limitation and contributes significantly to GAS virulence in this model of necrotizing myositis.

DISCUSSION

In this study, we demonstrate that GAS orchestrates a sophisticated gene regulatory response to evade and overcome host-imposed Zn limitation. GAS combats host-imposed Zn sequestration by upregulating the AdcR regulon, which comprises systems for Zn acquisition (*adcABC* and *adcAll*), intracellular Zn sparing (*rpsN.2*), and extracellular Zn scavenging (*adcAll*, *phtD*, and *phtY*). Inactivation of each gene in the AdcR regulon impaired the ability of GAS to outcompete CP for Zn acquisition and resulted in defective GAS growth in the presence of CP. The *phtY* gene is an interesting exception to the general role of the AdcR regulon in GAS defense against host-imposed Zn limitation. GAS induces *phtY* expression significantly during Zn deficiency, and PhtY

FIG 6 GAS employs the AdcR regulon genes to combat CP-mediated Zn limitation during infection. Transcript level analyses of the indicated genes in the intramuscular lesions from *S100a9*^{-/-} or *S100a9*^{+/+} (WT) mice infected with WT GAS. Samples were collected at 24 h postinoculation from the lesions ($n = 5$ per strain) and analyzed in triplicate with qRT-PCR. The transcript levels of each of the genes in WT GAS grown to the mid-exponential growth phase (A_{600} , 0.8) in Zn-replete THY medium were used as a reference, and fold changes in transcript levels relative to the reference are shown. P values (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) were determined from a two-sample t test.

contributes to Zn acquisition. However, the *phtY* gene is dispensable for GAS growth *in vitro* in the presence of CP. Finally, mouse infection studies using WT and *S100A9*-inactivated mice lacking CP showed that GAS encounters CP-mediated Zn limitation during infection. Consistent with our *in vitro* findings, the AdcR-regulated genes participate in GAS defense against CP-mediated Zn limitation during infection and contribute to GAS virulence. However, the AdcR regulon is dispensable for GAS pathogenesis in *S100a9*^{-/-} mice, emphasizing the direct battle between CP and AdcR regulon genes for Zn during GAS infection.

Relatively little is understood regarding the role of CP in host defense against pathogenic streptococci. CP is present at GAS colonization surfaces during invasive infection and inhibits GAS growth *in vitro* (9). However, the *in vivo* contribution of CP to host defense in GAS infection control remained uncharacterized. Similarly, two studies investigating the role of CP in host defense against pneumococci ascribed conflicting roles to CP. One study attributed a protective role for CP against pneumococcal infection due to its participation in neutrophil recruitment rather than metal limitation (20). In contrast, a second study showed that CP sequesters Zn but promotes pneumococcal virulence by mitigating host-imposed Zn toxicity (49). In this regard, the findings from this study demonstrate that CP participates in host defense against GAS infection by imposing Zn limitation and suggest a similar role for CP against other streptococcal pathogens.

The contribution of the AdcR regulon to streptococcal virulence has been extensively characterized (9, 32, 48, 50–60). However, the mechanisms by which the streptococcal Zn acquisition systems confer a survival advantage to the pathogens during infection remained unknown. The results presented here show that the AdcR regulon genes are required for bacterial growth *in vitro* (Fig. 3 and 4) and GAS pathogenesis only in the presence of CP (Fig. 7). These findings ascribe a direct role for the AdcR-regulated genes in bacterial resistance to host-imposed Zn limitation. Thus, it is

FIG 7 Genes in the AdcR regulon are critical for GAS resistance against CP-mediated Zn limitation *in vivo* and contribute significantly to GAS virulence. $S100a9^{+/+}$ (WT) (A) or $S100a9^{-/-}$ (B) mice ($n = 5$ per group) were infected intramuscularly with 1×10^8 CFU of each indicated bacterial strain. The Kaplan-Meier survival curve with P values derived from a log-rank test is shown.

likely that the AdcR regulon in other pathogenic streptococci contributes to bacterial virulence by evading and overcoming host nutritional immune mechanisms.

The components of the AdcR regulon are predicted to contribute to GAS Zn acquisition by distinct mechanisms (18). Thus, the varied susceptibility of individual AdcR regulon mutants to CP-mediated Zn limitation is not surprising. The *adcABC* genes encode the only dedicated Zn-specific GAS importer that is required for extracellular Zn acquisition (Fig. 8). AdcA constitutes the surface-exposed extracellular Zn-binding domain, AdcB is the intramembrane permease, and AdcC is the cytoplasmic ATPase that provides the energy for Zn uptake (Fig. 8). The *rpsN.2* gene is also predicted to play an essential role in GAS survival during Zn limitation, albeit by a mechanism different from AdcC. GAS contains two copies of functionally redundant 30S ribosomal S14 subunit protein; *rpsN* encodes a Zn-containing S14, and *rpsN.2* encodes a Zn-free S14 protein designated S14* (30). During Zn limitation, GAS upregulates the expression of the Zn-free S14* gene, which is predicted to functionally replace its Zn-containing counterpart, *rpsN* (30). Swapping of S14 with S14* that does not require Zn for function is thought to aid in bacterial survival by facilitating continued *de novo* synthesis of functional ribosomes and by sustaining cellular protein synthesis under Zn-limiting growth conditions (61–63) (Fig. 8). Consistent with the essential roles of *adcC* and *rpsN.2* in extracellular Zn acquisition and Zn sparing, respectively, the ΔadcC and $\Delta\text{rpsN.2}$ mutants were the most sensitive to CP (Fig. 3) and attenuated for GAS virulence (Fig. 7). These results suggest that *adcC* and *rpsN.2* constitute the primary line of GAS defense against CP.

The *adcA* and *adcAll* genes encode two proteins that have extracellular Zn-binding domains. AdcA is composed of two Zn-binding domains, a surface-exposed amino-terminal ZnuA-like domain and an inner membrane carboxyl-terminal ZinT-like domain. AdcAll shares significant amino acid sequence homology with the ZnuA-like domain but lacks the ZinT-like domain of AdcA (Fig. 8) (18). The roles of AdcA and AdcAll in streptococcal Zn acquisition have been characterized (18, 32, 33, 51, 57, 58, 64, 65). Characterization of AdcA and AdcAll in pneumococci and *S. agalactiae* suggested that they are functionally redundant but employ distinct Zn acquisition mechanisms (51, 58). AdcA functions independently as a surface-exposed Zn-binding protein, whereas extracellular Zn acquisition by AdcAll is dependent on Pht proteins (56, 58, 64, 65).

FIG 8 A model showing the molecular mechanisms employed by GAS to overcome CP-mediated Zn limitation. GAS encounters Zn limitation imposed by CP during invasive infection. To facilitate growth under Zn-limiting conditions, GAS upregulates the expression of the AdcR regulon that comprises several proteins at the cell surface (AdcABC, AdcAll, PhtD, and PhtY) and in the cytosol (*rpsN.2*). The cell wall- and membrane-localized molecules compete with CP for extracellular Zn, facilitate Zn acquisition, and aid in GAS growth under Zn-limiting conditions. In the cytosol, the Zn-free ribosomal S14* encoded by *rpsN.2* is produced under Zn-limiting conditions. This enables the *de novo* assembly of ribosomes under conditions in which cytosolic Zn levels are insufficient to support the activity of Zn-containing S14 protein. The *de novo* assembly of ribosomes containing S14* facilitates continued protein synthesis and GAS growth during Zn scarcity.

Despite the possible differences in the mechanism of extracellular Zn acquisition, both AdcA and AdcAll require the AdcCB component of the tripartite importer to transport Zn into the cytosol (56). Further studies are required to delineate the molecular mechanisms by which AdcA and AdcAll/Pht acquire extracellular Zn, transport via the AdcCB importer, and contribute to streptococcal Zn uptake. Nevertheless, the findings from this study demonstrated that AdcA or AdcAll contribute to GAS virulence by combating host-imposed Zn limitation (Fig. 7).

The cell wall-anchored PhtD and PhtY proteins contain Zn-binding poly-histidine triad motifs and are speculated to function as extracellular Zn scavengers (Fig. 8) (66). The expression of *pht* genes was upregulated during GAS growth in the presence of CP (Fig. 6), and both Pht proteins contribute to Zn acquisition (Fig. 5B). However, only the $\Delta phtD$ mutant was sensitive to CP-mediated Zn limitation, whereas the $\Delta phtY$ mutant was insensitive to CP *in vitro* (Fig. 4). These observations suggest a more pronounced role for PhtD than PhtY in GAS defense against CP under the tested conditions. In contrast, the 4 pneumococcal Pht proteins are functionally redundant, and each Pht protein contributes to optimal growth and bacterial survival *in vivo* (48). Additional investigations are required to fully elucidate the individual contributions of Pht proteins to GAS Zn acquisition and CP resistance.

Finally, our findings that the AdcR regulon genes were upregulated *in vivo* in *S100a9*^{-/-} mice lacking CP (Fig. 6) suggest that additional CP-independent Zn sequestration mechanisms may exist in the host. However, the literature on CP-independent extracellular Zn withholding mechanisms is sparse. Possible effectors for mediating host-imposed Zn limitation on pathogens include metallothioneins and the Zn importer ZIP14 (67, 68). Metallothioneins are a group of small proteins that binds multiple

Zn atoms with high affinity (69, 70). During the acute stages of bacterial infection, the expression of metallothioneins and ZIP14 is upregulated (68, 71, 72). Interestingly, upregulation of the two effectors coincides with a reduction in plasma Zn levels (72). It was postulated that ZIP14 transports Zn from plasma into hepatocytes, wherein the cytosolic metallothioneins sequester Zn. Collectively, these two host molecules are proposed to impose Zn limitation on pathogens by redistributing Zn from plasma to intracellular storage in liver during infection. However, experimental evidence demonstrating a direct link between Zn sequestration by metallothioneins and ZIP14 and host defense against bacterial infections is lacking.

In conclusion, our results identify the molecular arsenals and potential mechanisms used by a human pathogen to overcome host-imposed Zn limitation and cause disease. Our findings also indicate that several AdcR regulon genes have the desired traits of GAS vaccine candidates, which include their accessibility on the bacterial cell surface, production during infection, and critical contribution to GAS virulence. Thus, the results from this study may assist in developing novel prophylactic strategies toward GAS infection control.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S3. Strain MGAS10870 is a representative of serotype M3 strains that cause invasive infections (73). The whole genome of MGAS10870 has been fully sequenced and has wild-type sequences for all major regulatory genes (73). *Escherichia coli* DH5 α was used as the host for plasmid cloning, whereas *E. coli* BL21(DE3) was used for recombinant protein overexpression. GAS strains were routinely grown in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY; Difco) or Trypticase soy agar containing 5% sheep blood (bovine serum albumin [BSA]; Becton, Dickinson). For growth studies in the presence of CP, GAS strains were grown in THY-CP medium supplemented with recombinant WT or mutant CP. THY-CP medium was prepared by adding 38% (vol/vol) THY to 62% (vol/vol) CP medium (20 mM Tris HCl [pH 7.5], 100 mM NaCl, 10 mM β -mercaptoethanol, and 3 mM CaCl₂). *E. coli* strains DH5 α and BL21(DE3) were grown in lysogeny broth (LB; Teknova). Overnight cultures of GAS were inoculated in fresh medium with an initial absorption of 0.03 at A₆₀₀. Bacterial growth was monitored by measuring the optical density at A₆₀₀ with a microplate reader. Chloramphenicol and ampicillin were added to the cultures to a final concentration of 5 μ g/ml or 80 μ g/ml, respectively, when required.

Overexpression and purification of recombinant human CP. Overexpression and purification of recombinant human WT or mutant calprotectin (CP) were carried out as previously described (9). Overnight cultures of BL21(DE3) containing the coding sequences of *S100a8* or *S100a9* in plasmid pET15b were diluted 1:50 in fresh LB medium and grown at 37°C until the A₆₀₀ reached 0.4 to 0.6. Protein overexpression was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and cells were grown at 37°C for an additional 4 h. Equal amounts of cell pellets (by weight) containing *S100a8* and *S100a9* overexpression plasmids were suspended in buffer A (20 mM Tris HCl [pH 8.0], 0.1 M NaCl, 10 mM β -mercaptoethanol, 1 mM EDTA, and 0.5% Triton X-100) supplemented with a protease inhibitor pellet (Roche). Cells were lysed with a cell lyser (Microfluidics), and inclusion bodies containing S100A8 and S100A9 proteins were fractionated by centrifugation at 21,000 $\times$ g for 30 min. The pellet was resolubilized by suspending it in buffer containing 50 mM Tris HCl (pH 8.0), 100 mM NaCl, 10 mM β -mercaptoethanol, and 4 M guanidine hydrochloride. Refolding of the proteins was achieved by overnight dialysis using a 10-kDa cutoff membrane against the base buffer containing 20 mM HEPES (pH 8.0). After three rounds of dialysis, the final dialyzed sample was centrifuged at 21,000 $\times$ g for 30 min and filtered with a 0.22- μ m syringe filter. The S100A8/S100A9 heterodimer was separated by ion-exchange chromatography using a Mono-Q column (GE Lifesciences) preequilibrated with base buffer (20 mM HEPES [pH 8.0], 10 mM β -mercaptoethanol) and eluted using a salt gradient of 0 to 300 mM NaCl. The sample was further purified by size exclusion chromatography using Superdex 26/600 200 kDa (GE Lifesciences). Finally, the metal-free form of the S100A8/S100A9 heterodimer was prepared with a two-step dialysis as follows: first, against storage buffer containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 10 mM β -mercaptoethanol, and 10 mM EDTA and, second, against chelexed storage buffer without EDTA. The S100A8/S100A9 heterodimer was concentrated using a YM-10 filter to a final concentration of 10 mg/ml, and flash frozen aliquots were stored at -80°C until used.

RNA sequencing. Two biological replicates of WT GAS were grown to the mid-exponential phase of growth (ME phase; A₆₀₀ $\sim$ 0.8) in THY-CP medium supplemented with or without 125 μ g/ml CP. RNA isolation and purification were performed using an RNeasy (Qiagen) minikit according to the manufacturer's protocol. Samples were treated with DNase using the Turbo DNA-free kit (Ambion). The quality and concentration of RNA were analyzed with an Agilent 2100 bioanalyzer. rRNA was removed using a Ribo-Zero treatment kit (Epicenter) according to the manufacturer's protocol and further purified using the MinElute RNA purification kit (Qiagen). The rRNA-depleted sample was then used to synthesize adaptor-tagged cDNA libraries using the ScriptSeq v2 RNA sequencing (RNA-seq) library preparation kit (Epicenter). cDNA libraries were then run on a NextSeq instrument using the Illumina v2 reagent kit (Illumina). Approximately 20 million reads were obtained per sample, and the reads were mapped to the MGAS315 genome (73) using Rockhopper v2.0.3 (74, 75). For RNA-seq analysis, the total number of reads

per gene between the replicates was normalized by reads per kilobase per million mapped reads (RPKM). Using the RPKM values, pairwise comparisons were carried out between the two samples to identify the differentially expressed genes. Genes with a 2-fold difference and *P* value of <0.05 after applying Benjamini-Hochberg correction were considered to be statistically significant.

Creation of isogenic strains in GAS. Isogenic strains containing gene inactivation of the entire coding region of interest were generated as previously described (76). Briefly, a PCR fragment was generated in which the entire coding region of interest was deleted using a two-step PCR process (Fig. S2). In step 1, the PCR fragment was generated by amplifying the 5' or 3' flanking region of the gene of interest using primer pair A-B or C-D. In step 2, primer pair A-D was used to generate a single PCR fragment containing the fusion of the 5' flanking region and the 3' flanking region without the gene of interest and was subsequently cloned into the multi-cloning site of the temperature-sensitive plasmid pJL1005 (77). The resultant plasmids were electroporated into group A streptococci, and colonies with plasmid incorporated into the GAS chromosome were selected for subsequent plasmid curing. DNA sequencing was then performed to confirm that the desired mutations were present and no spurious mutations were introduced. The primers used to generate isoelectric mutant strains are listed in Table S4.

Site-directed mutagenesis of CP. Single-amino acid substitutions within the *S100a8* and *S100a9* coding region in plasmid pET15b were introduced using a quick-change site-directed mutagenesis kit (Agilent). Mutations were verified by DNA sequencing (Applied Biosystems). The primers used to introduce the mutations are listed in Table S4.

Transcript-level analysis by qRT-PCR. GAS strains were grown to the mid-exponential phase ($A_{600} \sim 0.8$) under the indicated growth conditions and incubated with two volumes of RNeasy Protect (Qiagen) for 10 min at room temperature. Cells were harvested by centrifugation, and the cell pellets were snap-frozen in liquid nitrogen. RNA isolation and purification were performed using an RNeasy kit (Qiagen) according to the manufacturer's protocol. The quality of the isolated total RNA was assessed with an Agilent 2100 bioanalyzer. The concentration of RNA was measured using a NanoDrop 8000 instrument (Thermo Fisher Scientific). cDNA was synthesized from 2 μ g of total RNA using superscript III (Invitrogen), and quantitative PCR (qPCR) was performed using SYBR green Q-PCR master mix (GenDEPOT) with an AB1 7500 fast system (Applied Biosystems). The specificity of the reaction was verified with melt curve analysis. Comparison of transcript levels was performed using the threshold cycle (ΔC_T) method of analysis using *tufA* as the endogenous control gene (30, 78). The primers used for reverse transcriptase quantitative PCR (qRT-PCR) are listed in Table S4.

Bacterial growth studies in the presence of CP. A 1:100 dilution of overnight GAS growth was inoculated into THY broth supplemented with CP buffer. The indicated concentrations of CP were added to the starter culture, and growth was monitored by measuring the absorption at 600 nm with a microplate reader. Samples were analyzed in triplicate, and at least two different CP preparations were used.

Metal content analysis by inductively coupled plasma mass spectrometry. The intracellular metal concentration of GAS was determined as described earlier (9, 42, 79). The indicated GAS strains were grown overnight in THY, diluted 1:100 in fresh THY containing TPEN, and grown to the mid-exponential phase of growth ($A_{600} \sim 0.8$). Cells were harvested by centrifugation at $4,000 \times g$ at 4°C for 10 min and washed twice with 2 volumes of sterile phosphate-buffered saline (PBS) containing 1 mM nitrilotriacetic acid. Cells were subsequently washed in sterile chelexed PBS, suspended in 0.4 ml sterile PBS, and lysed by ballistic disintegration (lysing matrix B and Fastprep96 automated homogenizer; MP Biomedicals). Cell lysate was centrifuged at $16,000 \times g$ for 30 min to separate the cytosolic content, and the total protein concentration was measured using the Bradford assay (80). Samples were mixed with a buffer containing 5% HNO_3 and 0.1% Triton X-100, and heated in a sand bath at 95°C for 30 min. Samples were centrifuged, and supernatants were diluted in HNO_3 (2% vol/vol final concentration). Metal levels were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin-Elmer ELAN DRC II) using Ga as an internal standard. The total concentration of metal ions is expressed as micrograms of ion per gram of total protein.

Animal virulence studies. All animal experiments were conducted under a protocol approved by the Houston Methodist Research Institute Institutional Animal Care and Use Committee (approval number AUP-0318-0016). The virulence of the GAS strains was assessed using an intramuscular mouse model of infection. Five 7- to 8-week-old WT C57 (*S100a9*^{+/+}) or *S100a9*^{-/-} mice were inoculated in the right hindlimb with 1×10^8 CFU of each strain and monitored for near mortality. Results were graphically displayed as a Kaplan-Meier survival curve and analyzed using the log-rank test.

Transcript analysis from infected tissue. *In vivo* transcript levels were assayed by collecting limb lesions from four mice per infecting strain 24 h postinfection, and the tissue samples were incubated with RNAlater (Qiagen). Samples were snap-frozen with liquid nitrogen and stored at -80°C until use. RNA was isolated and purified using an RNeasy fibrous tissue minikit (Qiagen) per the manufacturer's protocol. The quality and concentration of RNA were assessed with an Agilent 2100 bioanalyzer and NanoDrop 8000 (Thermo Fisher Scientific), respectively. Superscript III (Invitrogen) was used to prepare cDNAs, and transcript levels were measured with qRT-PCR using SYBR green qPCR master mix (GenDEPOT). Data were analyzed using the ΔC_T method.

Data availability. Transcriptome data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number [GSE142458](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142458).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4 MB.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants 1R01AI146048 and 1R01AI109096 to M.K. and R35GM122461 to J.D.H. and by funds from the Fondren Foundation and National Institutes of Health (grant R21AI139369 to J.M.M.).

N.M., H.D., B.M.W., R.J.O., and M.K. designed and performed the research; N.M., H.D., B.M.W., R.J.O., J.D.H., and M.K. analyzed the data; N.M., J.D.H., J.M.M., and M.K. wrote the manuscript.

We declare no conflict of interest.

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