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Niche-specific contribution to streptococcal virulence of a MalR-regulated carbohydrate binding protein

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Summary

Low G+C Gram-positive bacteria typically contain multiple LacI/GalR regulator family members which often have highly similar amino-terminal DNA binding domains suggesting significant overlap in target DNA sequences. The LacI/GalR family regulator catabolite control protein A (CcpA) is a global regulator of the Group A *Streptococcus* (GAS) transcriptome and contributes to GAS virulence in diverse infection sites. Herein, we studied the role of the maltose repressor (MalR), another LacI/GalR family member, in GAS global gene expression and virulence. MalR inactivation reduced GAS colonization of the mouse oropharynx but did not detrimentally affect invasive infection. The MalR transcriptome was limited to only 25 genes, and a highly conserved MalR DNA-binding sequence was identified. Variation of the MalR binding sequence significantly reduced MalR binding in vitro. In contrast, CcpA bound to the same DNA sequences as MalR but tolerated variation in the promoter sequences with minimal change in binding affinity. Inactivation of *pulA*, a MalR regulated gene which encodes a cell-surface carbohydrate binding protein, significantly reduced GAS human epithelial cell adhesion and mouse oropharyngeal colonization but did not affect GAS invasive disease. These data delineate a molecular mechanism by which hierarchical regulation of carbon source utilization influences bacterial pathogenesis in a site-specific fashion.

Keywords

Streptococcus; maltodextrin; pharyngitis; adhesion

Introduction

Recently there has been a significant increase in understanding the underappreciated but important relationship between basic metabolic processes and virulence in pathogenic bacteria (Alteri *et al.*, 2009, Majerczyk *et al.*, 2010, Eisenreich *et al.*, 2010, Marrero *et al.*,

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2010). A good example of the close ties between microbial metabolism and virulence is the LacI/GalR family of transcriptional regulators of which more than 1000 characterized and hypothetical members are currently known (Swint-Kruse & Matthews, 2009, Weickert & Adhya, 1992). LacI/GalR family regulators control a broad range of bacterial metabolic processes ranging from selective carbon source utilization to nucleotide synthesis to amino acid catabolism (Nguyen & Saier, 1995, Swint-Kruse & Matthews, 2009, Weickert & Adhya, 1992). Moreover, inactivation of members of the LacI/GalR family influences the virulence of several important human bacterial pathogens (Iyer & Camilli, 2007, Eswarappa *et al.*, 2009, Li *et al.*, 2010).

For low G+C Gram-positive pathogens, the best studied LacI/GalR family member is catabolite control protein A (CcpA) (Shelburne *et al.*, 2010, Seidl *et al.*, 2009, Kinkel & McIver, 2008, Varga *et al.*, 2008, Abranches *et al.*, 2008). CcpA is the master regulator of selective carbon source utilization for low G+C Gram-positive bacteria as well as a critical regulator of virulence factor genes in a diverse array of human Gram-positive pathogens (Shelburne *et al.*, 2008a, Seidl *et al.*, 2008, Varga *et al.*, 2008, Abranches *et al.*, 2008, Singh *et al.*, 2008, Sonenshein, 2007, Kietzman & Caparon, 2010). All major low G+C Gram-positive organisms contain more than one LacI/GalR family member, which are characterized by an amino-terminal helix-turn-helix DNA binding domain, the DNA binding “Hinge-Helix”, and a carboxy-terminus domain that interacts with a variety of modulating agents (Weickert & Adhya, 1992, Swint-Kruse & Matthews, 2009). The relatively high degrees of homology in the DNA binding domains and the multiple LacI/GalR family members typically present in the same bacteria suggest that the distinct LacI/GalR regulators may target the same DNA sequences (Francke *et al.*, 2008, Shelburne *et al.*, 2008b). However, the molecular mechanisms underlying same-site DNA-targeting by distinct LacI/GalR regulators and if such targeting affects bacterial pathogenesis remain unknown.

Among pathogenic bacteria, the study of LacI/GalR family members has been relatively prominent in Group A *Streptococcus* (GAS), which causes infections in humans ranging from uncomplicated pharyngeal or skin infections to life-threatening bacteremia, pneumonia, and necrotizing fasciitis (Musser & Shelburne, 2009, Shelburne *et al.*, 2008b, Kinkel & McIver, 2008, Almengor *et al.*, 2007). The two LacI/GalR family members that have been studied in GAS are CcpA and the maltose repressor MalR (Shelburne *et al.*, 2010, Shelburne *et al.*, 2007b, Kietzman & Caparon, 2010, Almengor *et al.*, 2007). CcpA influences the transcript level of several hundred GAS genes and affects GAS virulence at multiple different infection sites (Shelburne *et al.*, 2010, Shelburne *et al.*, 2008a, Kietzman & Caparon, 2010, Kinkel & McIver, 2008). The study of MalR has been much more limited, focusing mainly on the effects of MalR on the catabolism of α -1,4-linked glucose polymers known as maltodextrins (Shelburne *et al.*, 2008b, Shelburne *et al.*, 2007b).

Amino acid residues critical for CcpA binding to target DNA sequences have been elucidated via crystallographic analysis in *Bacillus* species (Schumacher *et al.*, 2011, Schumacher *et al.*, 2007, Schumacher *et al.*, 2004). The amino-terminal DNA binding domain of GAS CcpA is highly homologous to CcpA from *Bacillus* species (86% identical, 97% similar) indicating that GAS CcpA likely functions in a similar fashion to *Bacillus* CcpA (Fig. S1). Similar to GAS CcpA, GAS MalR also contains several of the same amino acid residues previously shown to be critical for DNA binding in *Bacillus* CcpA (Fig. S1) suggesting that GAS CcpA and MalR may employ similar mechanisms to recognize cognate DNA sequences. Herein we report on studies designed to test the hypothesis that MalR has a significantly narrower effect on GAS virulence and a more restricted transcriptome compared to CcpA despite the similarities of the DNA binding domains of the two proteins. Our results provide new insights into the hierarchical control of carbon source utilization in

pathogenic bacteria and further expand our understanding of the links between bacterial metabolic processes and virulence.

Results

Creation of MalR inactivated strains from a pharyngitis parental GAS strain

Previous work on MalR in GAS has used the parental invasive serotype M1 isolate MGAS5005 which contains an inactive control of virulence sensor kinase (CovS) (Shelburne *et al.*, 2008b, Shelburne *et al.*, 2007b, Sumby *et al.*, 2006). It has recently been recognized that GAS strains isolated from pharyngitis tend to have a functional CovS protein, perhaps because an intact CovS imparts an increased ability to adhere to and colonize pharyngeal epithelium (Sumby *et al.*, 2006, Hollands *et al.*, 2010). Thus, to better understand the role of MalR in GAS pharyngeal disease, we replaced the *malR* gene with a spectinomycin resistance cassette in the pharyngitis serotype M1 isolate MGAS2221 (which contains an active CovS protein) to create strain 2221 $\Delta malR$ -1 (see Table 1 for strain details). Similar to our previous work in strain MGAS5005, we were unable to genetically complement strain 2221 $\Delta malR$ -1 *in trans* (Shelburne *et al.*, 2007b). In an effort to ensure that the observed effects of MalR inactivation were not due to the introduction of spurious mutations, we repeated the entire mutant strain creation process to generate strain 2221 $\Delta malR$ -2. The growth curves of the parental and MalR-inactivated strains during growth in a nutrient-rich medium were indistinguishable (Fig. S2).

MalR-inactivation results in reduced colonization of the mouse oropharynx and decreased persistence in human saliva but does not affect invasive GAS disease

To examine the role of MalR in GAS host-pathogen interaction in the oropharynx, we compared the ability of strain MGAS2221 and its MalR-inactivated derivatives to colonize the mouse oropharynx. Following intranasal inoculation, the wild-type strain MGAS2221 was recovered from the oropharynx of a significantly higher proportion of mice over the time course of the study compared to either strains 2221 $\Delta malR$ -1 or 2221 $\Delta malR$ -2 ($P < 0.05$ for difference between strain MGAS2221 and either strain 2221 $\Delta malR$ -1 or 2221 $\Delta malR$ -2, Fig. 1A). Similarly, significantly more colony forming units (CFUs) were recovered over the course of the study from the mice infected with strain MGAS2221 compared to mice infected with either of the MalR-inactivated strains ($P < 0.05$ for difference between strain MGAS2221 and either strain 2221 $\Delta malR$ -1 or 2221 $\Delta malR$ -2, Fig. 1B).

Next, we tested the ability of the wild-type and MalR inactivated strains to grow in human saliva, an *ex vivo* model of human oropharyngeal conditions (Shelburne *et al.*, 2005a). Strain MGAS2221 and its MalR-inactivated derivative strains proliferated in human saliva to a similar degree (Fig. 1C). However, the MalR-inactivated mutant strains were unable to persist at high levels compared to wild-type strain MGAS2221 ($P < 0.05$ for difference between strain MGAS2221 and either strain 2221 $\Delta malR$ -1 or 2221 $\Delta malR$ -2, Fig. 1C). To determine if MalR affects the virulence of strain MGAS2221 at multiple infection sites, we next challenged mice intraperitoneally with the wild-type and MalR-inactivated strains. There was no statistically significant difference in survival times for mice challenged with the three strains ($P = 0.84$ for difference among the three strains, Fig. 1D). Taken together, these data indicate that MalR contributes to GAS pathogenesis in a site-specific fashion.

The MalR transcriptome includes genes putatively involved in polysaccharide catabolism and genes of unknown function

The regulon of MalR or a related orthologue is unknown. Therefore, to increase understanding of mechanisms by which MalR influences GAS host-pathogen interaction, we

determined the MalR transcriptome using a custom-made Affymetrix GeneChip® that contains 100% of the open reading frames of strain MGAS2221. Given that the two MalR inactivated mutant strains had identical phenotypes under multiple in vitro and in vivo conditions (Fig. 1 and Fig. S2), we used strain 2221 $\Delta malR$ -1 as the sole MalR inactivated strain for the remainder of the studies. Strains MGAS2221 and 2221 $\Delta malR$ -1 were grown in triplicate to mid-exponential phase (see Fig. S2A for RNA isolation point). Out of the 1,851 GAS genes present on the array, there were only 25 genes whose transcript levels were significantly different between the two strains, with 21 of the genes having higher transcript levels in the MalR-inactivated strain (Table 2). The majority of genes affected by MalR-inactivation encode proteins known to be or putatively involved in polysaccharide transport and metabolism, whereas most of the remaining genes encode hypothetical or phage proteins (Table 2). We conclude that the MalR transcriptome is relatively limited in GAS with most MalR-influenced genes being involved in carbon source acquisition and catabolism.

Identification of a putative MalR consensus DNA binding sequence

In low G+C Gram-positive organisms, MalR is thought to function mainly as a repressor protein (Shelburne et al., 2007b, Andersson & Radstrom, 2002, Nieto *et al.*, 2001). Consistent with this idea, the microarray data revealed six operons for which the transcript levels of all genes in the operon were significantly higher in the MalR-inactivated strain indicating direct or indirect MalR repression of these genes (spy0428-30, spy1057-8, spy1060-2, spy1679-80, spy1681, and spy1691-3). The putative promoter regions of each of the six operons contained the sequence $N_1N_2G_3C_4A_5A_6R_7C_8G_9N_{10}T_{11}T_{12}G_{13}C_{14}Y_{15}R_{16}$ (where N stands for any base, R for A or G, and Y for C or T) suggesting a conserved MalR binding site (Fig. 2A). A search of multiple GAS genomes did not identify any additional locations with the aforementioned sequence. Bioinformatic analysis found that all GAS genomes published to date contain the identical putative MalR binding sequences in the six identified operons (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Moreover, the putative GAS MalR consensus DNA binding sequence is in good agreement with the MalR DNA binding sequence previously identified for two *Streptococcus pneumoniae* maltose utilization operons (Nieto et al., 2001).

Identification of nucleotide residues critical to MalR DNA binding

Our proposed consensus MalR binding sequence is pseudopalindromic in nature similar to the CcpA consensus binding sequence from *Bacillus* species $W_1T_2G_3N_4N_5A_6R_7C_8G_9N_{10}W_{11}W_{12}W_{13}C_{14}A_{15}W_{16}$ (W = A or T) (Weickert & Chambliss, 1990, Miwa *et al.*, 2000). However, at the 4th/13th positions of the DNA binding sequence, each of the six promoters from the MalR-influenced operons (Fig. 2A) contain a C/G whereas the CcpA consensus sequence tolerates significant heterogeneity in base composition at this site (N/W). We have previously demonstrated that recombinant MalR binds to DNA containing the putative consensus sequence from the *malT* promoter (Shelburne et al., 2008b). To test the hypothesis that the C/G at the 4th/13th base locations are critical for MalR DNA binding, we compared recombinant MalR binding to the *malT* site and to an altered *malT* site in which the 4th/13th base pairs had been changed from C/G to A/T. Consistent with our hypothesis, altering the 4th/13th base pairs decreased the DNA binding affinity of recombinant MalR (K_D of 17.5 nM for wild-type *malT* sequence vs. 74.2 nM for altered *malT* sequence, Fig. 2B). In contrast, changing the *malT* sequence at the 4th/13th base positions did not markedly alter the DNA-binding affinity of recombinant CcpA (K_D of 23.9 nM for wild-type *MalT* sequence and 29.8 nM for the altered *MalT* sequence) consistent with the flexible nature of CcpA for base pair composition at the 4th/13th position (Fig. 2C).

To determine whether the lack of a C/G at 4th/13th position impedes MalR-mediated gene regulation, we next examined the promoter of a gene influenced by CcpA but not MalR. CcpA, but not MalR, is known to affect expression of the arginine deiminase operon through the binding sequence A₁T₂G₃T₄A₅A₆G₇C₈G₉A₁₀T₁₁T₁₂A₁₃C₁₄T₁₅C₁₆ upstream of the *arcA* gene (Shelburne *et al.*, 2010). The *arcA* CcpA binding site differs from a MalR consensus site in the presence of a T/A rather than C/G at the 4th/13th position. Therefore, we tested the hypothesis that changing the 4th/13th base positions of the *arcA* DNA binding site would increase the binding affinity of recombinant MalR. As predicted, substituting C/G for T/A at the 4th/13th base positions increased MalR affinity for the *arcA* site (K_D decreased from 260 nM to 61 nM, Fig. 2D). However, no change in CcpA DNA binding affinity was observed between the wild-type and altered *arcA* binding sequences (Fig. 2E). Taken together, these data indicate that MalR tolerates less variability compared with CcpA at the 4th/13th position of the DNA binding sequence, which likely contributes to the comparatively narrow MalR transcriptome.

***pulA* transcript levels are elevated in human saliva and in humans with pharyngitis**

Having established a site-specific contribution of MalR to GAS pathogenesis and having determined the MalR transcriptome, we next sought to better understand the mechanism by which specific MalR-regulated genes influences GAS host-pathogen interaction. The MalR regulon contains two putative cell-surface proteins, MalE, which encodes a maltodextrin-binding lipoprotein (Shelburne *et al.*, 2007a, Shelburne *et al.*, 2006), and PulA, which encodes a cell-wall anchored carbohydrate binding and degrading enzyme (van Bueren *et al.*, 2007, Hytonen *et al.*, 2006, Hytonen *et al.*, 2003). Inasmuch as we had previously investigated the role of MalE in GAS virulence (Shelburne *et al.*, 2008b, Shelburne *et al.*, 2006) and that the role of PulA has yet to be determined, we next tested the hypothesis that PulA contributes to GAS pathogenesis.

To begin to investigate the contribution of PulA to host-pathogen interaction we determined *pulA* transcript level in serotype M1 strains of defined clinical sources (Table 1). During cultivation in THY broth, *pulA* transcript was essentially undetectable except in pharyngitis strains during the stationary phase of growth (Fig. 3A). Consistent with *pulA* being positively influenced by CovS, at all growth phases *pulA* transcript levels were very low in the CovS-inactivated derivative of strain MGAS2221 (strain 2221 $\Delta covS::7bp$, Fig. 3A). When the pharyngitis strain MGAS2221 was grown in human saliva or a maltotriose-medium (i.e. a chemically defined medium in which maltotriose is the sole carbon source), *pulA* transcript level was significantly elevated compared to growth in THY broth or a glucose-medium (Fig. 3B). As expected, the increased *pulA* transcript level observed during growth in human saliva compared to growth in THY broth was translated into increased PulA cell-surface expression as determined by flow cytometry (Fig. 3C, Fig. 3D). Moreover, PulA cell-surface expression was easily detectable during growth in THY broth in the MalR-inactivated strain consistent with the idea that MalR represses PulA expression under nutrient-rich conditions (Fig. 3E). Finally, *pulA* transcript was readily detected in RNA extracted from oropharyngeal swabs collected from patients with confirmed GAS pharyngitis, suggesting in vivo derepression of *pulA* expression (Fig. 3F). These data indicate that the nutrient-limited conditions encountered in the human oropharynx result in increased PulA expression compared to a nutrient-rich environment such as a standard laboratory medium.

Inability to detect a role for PulA in GAS α -glucan catabolism

PulA contains an amino-terminal domain previously shown to be involved in carbohydrate binding (van Bueren *et al.*, 2007) and a carboxy-terminal domain putatively involved in carbohydrate degradation. Thus, one possible role for PulA is to allow GAS to use

carbohydrates, specifically α -glucans such as glycogen, pullulan, or maltodextrins (i.e. maltose, maltotriose, etc.), as an energy source. We used non-polar insertional mutagenesis to independently create two PulA-inactivated strains from the parental strain MGAS2221 (growth curves for the PulA-inactivated strains in nutrient rich medium are shown in Fig. S2). We found no growth of strain MGAS2221 or its PulA(-) derivative strains in a chemically defined medium in which glycogen or pullulan were the sole carbon source (Fig. S2 and data not shown). Similarly, we observed no significant difference in growth between MGAS2221 and the PulA-inactivated strains when maltose, maltotriose, or maltotetraose were the sole carbon source (Fig. S2 and data not shown). Thus, under the conditions studied we could not discern a role for PulA in GAS α -glucan catabolism.

PulA contributes to GAS epithelial cell adhesion following growth in human saliva

Some microbial carbohydrate binding proteins facilitate adherence to eukaryotic cells by binding to cell-surface carbohydrates (Bouckaert *et al.*, 2005, Uchiyama *et al.*, 2009). Therefore, we next sought to test the hypothesis that PulA participates in GAS epithelial cell adherence (Hytonen *et al.*, 2003, Hytonen *et al.*, 2006). We speculated that low PulA expression on the GAS cell surface during growth in THY broth may have resulted in the modest role observed for PulA in previous eukaryotic cell adherence assays (Hytonen *et al.*, 2006). Indeed, there was no significant difference in the adherence of strain MGAS2221 and its two isogenic Δ pulA derivatives to D562 pharyngeal epithelial cells when GAS was grown under standard laboratory conditions (i.e. in THY, Fig. 4A–C). However, when grown *ex vivo* in human saliva, adherence of strain MGAS2221 to D562 cells was significantly increased compared to strains 2221 Δ pulA-1 and 2221 Δ pulA-2 as measured by standard light microscopy (Fig. 4A, 4D–E). Similarly, after being grown in human saliva, strain MGAS2221 adhered significantly better than the Δ pulA strains to normal human tracheobronchial epithelial (NHTBE) cells (Fig. 4F–J). The increased adherence of GAS to D562 or NHTBE cells following growth in saliva did not result from the presence of saliva, as washing GAS grown in THY or a maltodextrin-medium (done to increase pulA expression) with human saliva decreased subsequent adherence compared to GAS not washed with saliva (data not shown). To determine whether our method of preparing or counting cells via light microscopy might have accounted for the observed differences, we next used fluorescence microscopy to study the adherence of the three strains transformed with a plasmid expressing green fluorescent protein (Gfp). Similar to our findings with light microscopy, strain MGAS2221(pSB100) grown in human saliva had increased adherence to D562 and NHTBE cells compared to strains 2221 Δ pulA-1(pSB100) or 2221 Δ pulA-2(pSB100) (Fig. 4K–P). There was no significant difference in the adherence of the three strains cultivated in THY to either D562 or NHTBE cells (data not shown).

α -glucans and antibody to the PulA N-terminal α -glucan binding domain interfere with GAS eukaryotic cell adherence

To further investigate the mechanism by which PulA mediates GAS eukaryotic cell adhesion, we overexpressed and purified the PulA N-terminal carbohydrate binding domain designated as SpyDx in accordance with a previous publication (Fig. 5A) (van Bueren *et al.*, 2007). We confirmed the functional activity of SpyDx by demonstrating its ability to bind the α -glucans glycogen and maltotetraose whereas no specific binding was observed for glucose (Fig. 5B–D). Following growth in human saliva, the addition of purified SpyDx significantly inhibited the ability of strain MGAS2221 to bind D562 eukaryotic cells (Fig. 5F) whereas the presence of recombinant HPr (Fig. 5E), a GAS cytosolic protein (i.e. a negative control), had no significant effect. Similarly, polyclonal rabbit antibody raised against SpyDx significantly reduced GAS eukaryotic cell adhesion (Fig. 5F) whereas the presence of anti-HPr polyclonal antibody did not significantly affect GAS adherence (Fig. 5F). Finally, the presence of the α -glucans glycogen and maltotetraose significantly reduced

GAS eukaryotic cell adherence (Fig. 5G) whereas glucose did not. These data strongly suggest that specific α -glucan binding by the N-terminal portion of PulA contributes to GAS eukaryotic cell adherence following growth in human saliva.

PulA affects GAS oropharyngeal colonization but not invasive GAS disease

Given that PulA contributes to the epithelial cell adherence of strain MGAS2221, we next determined whether genetic inactivation of PulA affected the ability of strain MGAS2221 to colonize the mouse oropharynx. Following nasopharyngeal inoculation, strain MGAS2221 was recovered from more mice and at a higher density compared to strain 2221 $\Delta pulA$ at each time point tested (Fig. 6A, 6B). Similar to what we observed for MalR, however, PulA inactivation did not significantly affect the virulence of strain MGAS221 following intraperitoneal inoculation (Fig. 6C). Consistent with these findings, in strain MGAS2221 the *pulA* transcript level was significantly higher among GAS recovered from the mouse oropharynx compared to GAS isolated from the bloodstream of mice infected intraperitoneally (Fig. 6D). These data indicate that the contribution of PulA to GAS virulence and the expression of *pulA* occurs in a niche-specific fashion during GAS infection.

Discussion

LacI/GalR-type transcriptional regulators have long served as prototypes for studying basic physiologic processes in prokaryotes (Jacob & Monod, 1961). Recently, however, this large family of regulatory proteins has also gathered significant attention for their contribution to the virulence of numerous bacterial pathogens (Iyer *et al.*, 2005, Li *et al.*, 2010, Chapuy-Regaud *et al.*, 2003, Shelburne *et al.*, 2008a, Allen *et al.*, 2000). In Gram-positive organisms, CcpA has been the best studied LacI/GalR member from a virulence standpoint, but Gram-positive pathogens generally contain multiple LacI/GalR family regulators whose pathogenic role has been only minimally investigated (Shelburne *et al.*, 2008b). Herein we show that the LacI/GalR family member MalR plays a role in GAS virulence that is site-specific thereby extending knowledge of role of LacI/GalR family members in bacterial pathogenesis.

One of our key discoveries was that the GAS MalR transcriptome was quite limited, consisting of only 25 genes. This stands in marked contrast to the hundreds of genes influenced by CcpA-inactivation in GAS and related organisms despite the fact that the CcpA and MalR DNA binding motifs are quite similar (Fig. S1) (Shelburne *et al.*, 2010, Shelburne *et al.*, 2008a, Seidl *et al.*, 2009, Kinkel & McIver, 2008, Abranches *et al.*, 2008). The relatively few genes influenced by MalR appears to be explained by the highly conserved nature of the MalR consensus binding site (Fig. 2). For GAS, such binding sites only occur in the promoter regions of a select number of operons, most of which involved in maltodextrin transport (Fig. 2). In contrast, we found that GAS CcpA has the flexibility to bind with high affinity to a less stringent consensus sequence providing a potential functional basis for the observation of the large CcpA transcriptome. Our data elucidate a mechanism for hierarchical control of carbon source utilization in which the global regulator CcpA can bind to a diverse array of promoters. In contrast, other LacI/GalR family members, such as MalR or the sucrose repressor ScrR, may target the same DNA sequences as CcpA but are capable of only binding to promoters that contain highly homologous sequences. Such a system would limit off-target binding of the numerous LacI/GalR family members while still permitting CcpA to be a master regulator.

Another key discovery of the work presented herein was the finding that the MalR-regulated carbohydrate-binding protein PulA contributes to GAS epithelial cell adhesion and to GAS oropharyngeal colonization but not invasive GAS disease. The cell-surface location of PulA

means that it may contribute to GAS host-pathogen interaction (Reid *et al.*, 2002), and it has been suggested that PulA could be part of a streptococcal vaccine strategy (Santi *et al.*, 2008). A previous genome-wide approach to identify new GAS vaccine candidates did not study PulA because it was not found on the GAS cell-surface following growth in standard laboratory medium (Rodriguez-Ortega *et al.*, 2006). We found that PulA cell-surface expression is minimal in a standard laboratory medium but increases markedly in distinct laboratory conditions and during oropharyngeal but not bacteremic infection (Figs. 3 and 6). The *in vivo* expression of PulA has been previously demonstrated by the finding that GAS infected human patients have anti-PulA antibodies (Reid *et al.*, 2001, Fritzer *et al.*, 2010). However, active immunization with recombinant PulA did not protect mice against intraperitoneal challenge with an invasive serotype M1 strain (Reid *et al.*, 2002) or following intravenous challenge with GAS strains of three distinct M protein serotypes (Fritzer *et al.*, 2010). Our data showing that PulA is expressed at low levels in mouse blood and that PulA inactivation did not affect GAS disease following intraperitoneal challenge provide potential explanations for the previous immunization studies. Moreover, our finding that PulA contributes to GAS epithelial cell adhesion only following growth in a medium that induces PulA expression may provide a rationale for why PulA-inactivation only slightly affected GAS adherence in a previous investigation (Hytonen *et al.*, 2006). Our data suggest that the pro-adherence effect of PulA is mediated via its amino-terminus carbohydrate binding domain. The specific carbohydrate epitopes on human epithelial cells targeted by amino-terminus of PulA are not currently known but probably are part of cell-surface glycoproteins.

Analysis of the multi-domain structure of PulA suggests that its carboxy-terminus is involved in α -glucan degradation and catabolism. However, under the conditions studied GAS did not proliferate in a defined medium in which α -glucans were the sole carbon source. A previous study in group B *Streptococcus* (GBS) found that GBS was able to grow in a medium in which pullulan was the sole carbon source and that inactivation of *pulA* abrogated such growth (Santi *et al.*, 2008). However, in that study, the observed growth phenotype consisted of only a two-fold increase in colony forming unit (CFU) density, as opposed to the 50–100 fold increase typically seen in streptococcal growth studies (Santi *et al.*, 2008). Thus whether PulA-mediated carbon source catabolism is physiologically relevant in streptococci remains unclear. The carboxy-terminus of PulA is highly conserved among various GAS strains (BLAST analysis of publicly available GAS PulA sequences), however, indicating selective pressure to maintain the putative α -glucan degrading domain of PulA which suggests a currently unknown role for this portion of the protein.

In summary, we have discovered that the LacI/GalR regulatory protein MalR contributes to GAS virulence in a site-specific fashion and that at least part of this contribution is likely to be mediated by the carbohydrate binding activity of PulA. These findings shed new light into the molecular mechanisms of selective carbon source utilization in pathogenic bacteria and how such processes influence bacterial pathogenesis.

Experimental procedures

Bacterial strains and culture media

Bacterial strains and plasmids used in this study are listed in Table 1. The genome of the pharyngeal isolate, serotype M1 strain MGAS2221 has been sequenced (Sumby *et al.*, 2006). The additional serotype M1 strains and CovS-inactivated derivative of strain MGAS2221 (strain 2221 $\Delta covS::7bp$) studied herein have been previously described (Sumby *et al.*, 2006). Isogenic MalR- and PulA-inactivated strains were created by non-polar insertional mutagenesis from strain MGAS2221 using the PCR splicing overlap extension method described by Kuwayama *et al.* with the spectinomycin resistance cassette

being amplified from plasmid pSL60 (Kuwayama *et al.*, 2002, Lukomski *et al.*, 2000). Primers used to create the isogenic mutant strains are listed in Table S1.

Growth of GAS strains in various media

GAS was grown on Trypticase soy agar containing 5% sheep blood (BSA; Becton Dickinson) or in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY; Difco). Spectinomycin (Sigma) and chloramphenicol (Sigma) was added when appropriate to THY agar or broth at a concentration of 150 µg/mL and 4 µg/mL, respectively. Ultra-pure carbohydrates (Sigma) were added at a concentration of 0.5% (wt/vol) to a carbohydrate-free preparation of a commercially available chemically-defined medium (CDM; JR Biosciences) (Vise *et al.*, 2003). For the purposes of this manuscript, we have used the terms glucose-medium, maltotriose-medium, pullulan-medium etc., to refer to the carbohydrate-free CDM supplemented with the specified carbohydrate. We have previously demonstrated that GAS cannot grow in the CDM without the addition of exogenous carbohydrates (Shelburne *et al.*, 2007a). All comparative growth experiments in THY or CDM were done as previously described in triplicate on three separate occasions for a total of nine replicates (Shelburne *et al.*, 2006). GAS was grown in human saliva as previously described (Shelburne *et al.*, 2005a). Healthy volunteers who gave their informed consent prior to participation in the study donated saliva according to a protocol for human subjects approved by the Baylor College of Medicine Institutional Review Board.

Overexpression and purification of GAS proteins

GAS MalR, CcpA, and HPr were overexpressed and purified via C-terminus His-tags as described (Shelburne *et al.*, 2008b, Shelburne *et al.*, 2008a, Shelburne *et al.*, 2010). HPr phosphorylated at Ser46 (HPr-Ser46-P) was created as described using recombinant GAS HPr kinase/phosphorylase (Shelburne *et al.*, 2010). To work with recombinant SpyDx protein that lacked a tag, we cloned the N-terminal portion of the *pulA* gene from MGAS2221 into plasmid pTXB1 (New England BioLabs) which resulted in a fusion protein with an intein tag and a chitin binding domain. Recombinant SpyDx was obtained following the manufacturer's instructions with release of the intein tag using DTT (Fig. 5A).

Fluorescence polarization

The DNA binding activities of MalR and CcpA were measured by fluorescence polarization on a Beacon 2000 fluorescence polarization instrument (Pavera, Madison, WI) as described (Shelburne *et al.*, 2008b). For CcpA, 50 µM phosphorylated HPr (HPr-Ser46-P), which is key to high affinity DNA binding, was included in the binding buffer (Shelburne *et al.*, 2008b). Equilibrium binding constants were determined in triplicate as described with all experiments carried out at 25 °C (Newberry and Brennan, 2004).

Mouse challenge studies

All mouse experiments were performed according to protocols approved by the Methodist Hospital Research Institute Institutional Animal Care and Use Committee and were conducted with adult (18- to 20-g) female outbred CD-1 Swiss mice (Harlan Sprague-Dawley Inc.). For the oropharyngeal challenge studies, both nares of each mouse were inoculated with 50 µL of the GAS suspension (total inoculation 100 µL, 1×10^7 CFU) with 35 mice in each group (Shelburne *et al.*, 2006). The throat of each mouse was swabbed before inoculation and then at specific time periods thereafter, spread onto BSA, incubated overnight, and tested for GAS as described (Shelburne *et al.*, 2006). For the invasive challenge studies, 1×10^7 CFUs of each GAS strain were inoculated intraperitoneally and mice were monitored for near mortality (Shelburne *et al.*, 2008a).

RNA isolation and transcript level analysis

For transcript level analysis under diverse growth conditions, various GAS strains were grown as indicated. RNA was isolated and purified using an RNeasy kit (Qiagen) (Shelburne *et al.*, 2005b). To determine *pulA* transcript levels *in vivo*, GAS RNA was isolated from throat swabs obtained from six patients with GAS pharyngitis and analyzed as previously described (Virtaneva *et al.*, 2003). The throat swabs were obtained from patients who gave their informed consent prior to participation in the study according to a protocol for human subjects approved by the Baylor College of Medicine Institutional Review Board. Similarly, GAS RNA was isolated from the mouse oropharynx using swabs obtained 48 hrs after challenge. GAS RNA was isolated from mouse blood 48 hrs after inoculation using the QIAamp RNA Blood MiniKit (Qiagen). The quality and the concentration of RNA were assessed with an Agilent 2100 Bioanalyzer and by analysis of the A₂₆₀/A₂₈₀ ratio. cDNA was reverse transcribed from RNA using Superscript III (Invitrogen) following the manufacturer's instructions. TaqMan quantitative real-time PCR (QRT-PCR) was performed with an ABI Thermocycler 7700 (Applied Biosystems) using the ΔC_T method of analysis with the *proS* gene used as the internal control (Chaussee *et al.*, 2001). Primers and probes used are listed in Table S1. Unless indicated, transcript level analyses were performed in duplicate biologic replicates on two separate occasions and analyzed in duplicate (8 total data points).

Expression microarray analysis

A custom-made Affymetrix GeneChip[®] that contains probes for 100% of the genes encoded by strain MGAS2221 was used for expression microarray (transcriptome) studies using three biologic replicates of indicated strains grown to mid-exponential growth phase (Fig. S2A). (44). To compare gene transcript levels between the wild-type and mutant strains, a two-sample *t*-test (unequal variance) was applied followed by a false discovery rate correction ($Q < 0.05$) to account for multiple testing using Partek Genomics Suite version 6.4. Transcript levels were considered significantly different when the corrected *P* value was < 0.05 and the mean difference was at least 2-fold.

Creation of anti-SpyDx and anti-HPr antibodies

Purified recombinant SpyDx and HPr were provided to Covance Laboratories for generation of polyclonal antibody using immunization in rabbits (Reid *et al.*, 2002). Purified antibody was obtained from rabbit hyperimmune serum using purified protein (either SpyDx or HPr) as an immunosorbent (Reid *et al.*, 2002).

Assessment of Pula on cell-surface

Surface localization of Pula was assessed with an LSR-II flow cytometer (BD Biosciences) using affinity-purified SpyDx-specific polyclonal antibody (Covance Laboratories) as described (Shelburne *et al.*, 2006). Antibody against HPr, a GAS cytosolic protein, was used as a control for nonspecific antibody binding.

Eukaryotic cell adherence assays

The ability of GAS to adhere to D562 pharyngeal cells was determined by growing various GAS strains to mid-logarithmic phase in either THY or saliva. GAS cells were pelleted, washed in PBS, resuspended in antibiotic free minimal medium and added to the D562 cells at 1:100 multiplicity of infection (MOI). After 2 hrs, non-adherent bacteria were removed via triplicate washing with PBS, and the cells were fixed by adding 4% formaldehyde (VWR) for 1 hr. Formaldehyde was removed, a Gram-stain was performed using standard protocol (Becton-Dickinson), and GAS cells were counted in five high power fields (1000 $\times$) by two independent observers blinded as to the strains being analyzed. The same protocol

was employed to analyze GAS adhesion to normal human tracheobronchial epithelial cells (NHBTE, Lonza). A similar protocol was used to compare adherence of strains MGAS2221(pSB100), 2221 Δ pulA-1(pSB100), and 2221 Δ pulA-2(pSB100) expressing Gfp via epifluorescence except that the Gram-stain step was omitted. The effect of purified proteins on GAS adhesion was determined as above with the additional step of adding 20 μ g of the indicated recombinant protein to the D562 cells for 15 mins prior to adding GAS (Jensch *et al.*, 2010). Similarly, when indicated polyclonal antibody from rabbits immunized with purified, recombinant GAS proteins was added to GAS cell suspensions at a final concentration of 10 μ g/mL for 15 mins prior to adding GAS to the D562 cells. The effect of various carbohydrates on GAS adhesion was performed by pre-incubating GAS in the indicated carbohydrate (25 mM for maltotetraose or glucose and 5 mg/mL for glycogen) as described (Ryan *et al.*, 2001). All adherence studies were done in triplicate on four separate occasions with microscopy done on a Zeiss AxioStarPlus microscope.

Isothermal titration calorimetry

Analysis of SpyDx binding to various carbohydrates purchased from Sigma was performed as described (Shelburne et al., 2007a) using a VP-ITC (GE Healthcare) in 20 mM Tris (pH 8.0) and 0.5 M NaCl at 25 °C. For glycogen, 2 mM SpyDx was titrated into type IV glycogen (bovine liver, 0.84 mg/ml) in the same buffer as SpyDx (van Bueren et al., 2007). For maltotetraose and glucose, 13.5 mM of the indicated carbohydrate dissolved in the same buffer was titrated into 400 μ M SpyDx (van Bueren et al., 2007). Each experiment was performed on at least three occasions with representative data being presented in Fig. 5.

Statistical analyses

Growth, RNA transcript levels, and eukaryotic cell adhesion assays were compared using Student's two-sided *t*-test. For the murine mucosal challenge model, a repeated measures analysis was used to test for differences in the percent of animals colonized and in the number of CFU recovered per animal. Statistical significance was assigned a two-sided *P* value of 0.05 using Bonferroni's adjustment for multiple comparisons when appropriate. Statistical calculations were performed using GraphPad version 5.01.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

MalR affects GAS pathogenesis in a site-specific fashion. (A, B) Indicated GAS strains were inoculated into the nares of 35 CD-1 outbred mice per strain. (A) Percent of mice colonized in the oropharynx with GAS by day. (B) Average number of GAS CFUs isolated from the oropharynx of mice by day with data graphed being mean $\pm$ standard error the mean. (C) Indicated GAS strains were grown in human saliva as described (Shelburne et al., 2005a). Strains were grown in duplicate on two separate occasions with data graphed being mean $\pm$ standard deviation. For (A–C) *P* values were derived from a repeated measures analysis followed by Bonferroni's correction for multiple comparisons. (D) Indicated GAS strains were inoculated intraperitoneally into 20 CD-1 outbred mice per strain. Data graphed are Kaplan-Meier survival analysis with *P* value derived from a log-rank test.

Fig. 2.

Identification of MalR consensus binding sequence and functional MalR binding studies. (A) Schematic of putative MalR binding sites in the promoter regions of the indicated operons that were negatively influenced by MalR in the transcriptome analysis. Numbers below genes refer to designation in the MGAS5005 genome (Sumby et al., 2005). (B, C) Recombinant GAS MalR (B) or CcpA (C) was titrated into a binding buffer containing 1 nM fluorescein-labeled *maltT* promoter DNA (circle) or 1 nM fluorescein labeled *maltT* promoter DNA with the 4th/13th C/G changed to A/T (called *maltT* C to A, triangle, dashed line). Note that the changed nucleotides are bold in the *maltT* portion of (A). Millipolarization units are plotted against the protein concentration (nM). (D, E) Recombinant GAS MalR (D) or CcpA (E) was titrated into a binding buffer containing 1 nM fluorescein-labeled *arcA* promoter DNA (circle, dashed line) or 1 nM fluorescein labeled *arcA* promoter DNA with the 4th/13th T/A changed to C/G (called *arcA* T to C, triangle, solid line). The changed nucleotides are bold in the *arcA* promoter region schematic shown above the binding data. For (B-E), each experiment was done on three occasions with representative data from one experiment shown. Lines indicate fit of binding data derived from non-linear regression.

Fig. 3.

Analysis of *pulA* transcript levels in laboratory conditions and *in vivo*. (A) *pulA* transcript level in serotype M1 GAS strains of defined patient and CovR/S phenotype (Table 1). (B) *pulA* transcript level in strain MGAS2221 in indicated growth medium. For (A) and (B) white bars indicate early exponential growth, grey bars mid-exponential growth, striped bars late exponential growth, and black bars stationary phase. For (A) and (B) data graphed are mean $\pm$ standard deviation of 8 data points. (C–E) Flow cytometric analysis of PulA cell-surface expression. Light grey represents anti-PulA antibody whereas dark grey indicates a control antibody. (C) Strain MGAS2221 grown in THY. (D) Strain MGAS2221 grown in human saliva. (E) Strain 2221 *malR*-1 grown in THY. (F) *pulA* transcript levels for 6 patients with GAS pharyngitis were determined by TaqMan real-time PCR. The M serotype of the infecting GAS strain is shown in the circle. Error bars represent standard deviation with each experiment done in quadruplicate on two separate occasions.

Fig. 4.

PulA contributes to GAS eukaryotic cell adherence. (A) Adherence of wild-type (MGAS2221 – white bars) and $\Delta pulA$ derivative strains (2221 $\Delta pulA$ -1, grey bars; 2221 $\Delta pulA$ -2, striped bars) to D562 pharyngeal epithelial cells following cultivation in THY or human saliva as indicated. Representative pictures of adherence of strains 2221 (B) and 2221 $\Delta pulA$ -1 (C) to D562 cells following cultivation in THY. Representative pictures of adherence of strains 2221 (D) and 2221 $\Delta pulA$ -1 (E) to D562 cells following cultivation in human saliva. (F) Adherence of wild-type (MGAS2221 – white bars) and $\Delta pulA$ derivative strains (2221 $\Delta pulA$ -1, grey bars; 2221 $\Delta pulA$ -2, striped bars) to normal human tracheobronchial epithelial (NHTBE) cells following cultivation in THY or human saliva as indicated. Representative pictures of adherence of strains 2221 (G) and 2221 $\Delta pulA$ -1 (H) to NHTBE cells following cultivation in THY. Representative pictures of adherence of strains 2221 (I) and 2221 $\Delta pulA$ -2 (J) to NHTBE cells following cultivation in human saliva. For (A) and (F) data graphed are mean numbers of adherent bacteria with error bars indicating standard error of the mean with each experiment done in triplicate on four separate occasions. (K–P) Representative pictures of GAS adherence using strains expressing Gfp and fluorescence microscopy. (K–M) Adherence of strain MGAS2221(pSB100) to NHTBE cells following cultivation in human saliva. (K) Fluorescence alone; (L) Differential phase microscopy; (M) Merge of (K) and (L). (N–P) Adherence of strain 2221 $\Delta pulA$ -1(pSB100) to NHTBE cells following cultivation in human saliva. (N) Fluorescence alone; (O) Differential phase microscopy; (P) Merge of (N) and (O).

Fig. 5.

SpyDx contributes to GAS epithelial cell adhesion through binding of α -glucans. (A) The N-terminus, carbohydrate binding portion of PulA (SpyDx) was overexpressed and purified to apparent homogeneity (shown is purified SpyDx run on 4–15% gradient SDS-PAGE). (B–D) Isothermal titration calorimetry data of recombinant SpyDx for (B) glycogen, (C) maltotetraose, and (D) glucose. Note binding shape curve for (B) and (C) indicates heat-release upon binding whereas (D) shows no evidence of specific binding. (E) Recombinant HPr was purified to apparent homogeneity and analyzed as in (A). (F, G) Binding of strain MGAS2221 to D562 human epithelial cells following growth in human saliva. Indicated recombinant proteins (F), antibodies (F), and carbohydrates (G) were added as described in Material and Methods. For (F and G) data graphed are mean numbers of adherent bacteria with error bars indicating standard error of the mean with each experiment done in triplicate on four separate occasions.

Fig. 6.

PulA contributes to GAS oropharyngeal colonization but not invasive disease. (A, B) Indicated GAS strains were inoculated into the nares of 35 CD-1 outbred mice per strain. (A) Percent of mice colonized in the oropharynx with GAS by day. (B) Average number of GAS CFUs isolated from the oropharynx of mice by day with data graphed being mean $\pm$ standard error the mean. For (A, B) *P* values were derived from a repeated measures analysis. (C) Indicated GAS strains were inoculated intraperitoneally into 20 CD-1 outbred mice per strain. Data graphed are Kaplan-Meier analysis with *P* value derived from log-rank test. (D) GAS RNA was isolated from mouse oropharynx on day 2 following intranasal inoculation and from mouse blood on day 2 following intraperitoneal inoculation. Data graphed are mean transcript level $\pm$ standard deviation for four animals analyzed in duplicate.

Table 1

Strains and plasmids.

Strain or plasmid	Genotype	Reference
Strains		
MGAS2221	Pharyngeal isolate, serotype M1, wild-type CovS	(Sumbly et al., 2006)
2221 Δ malR-1	MGAS2221 Δ malR::aad9, Spc ⁺	This study
2221 Δ malR-2	MGAS2221 Δ malR::aad9, Spc ⁺	This study
MGAS2221	Pharyngeal isolate, serotype M1	This study
MGAS2221(pSB100)	MGAS2221 <i>gfp</i> , Cm ⁺	This study
2221 Δ pulA-1	MGAS2221 Δ pulA::aad9, Spc ⁺	This study
2221 Δ pulA-2(pSB100)	MGAS2221 Δ pulA::aad9 <i>gfp</i> , Spc ⁺ , Cm ⁺	This study
2221 Δ pulA-2	MGAS2221 Δ pulA::aad9, Spc ⁺	This study
2221 Δ pulA-2(pSB100)	MGAS2221 Δ pulA::aad9 <i>gfp</i> , Spc ⁺ , Cm ⁺	This study
2221 Δ covS::7bp	MGAS2221 with 7 bp insertion in <i>covS</i>	(Sumbly et al., 2006)
MGAS294	Invasive isolate, serotype M1, V286F in CovS	(Sumbly et al., 2006)
MGAS2217	Invasive isolate, serotype M1, 1 bp insert in <i>covS</i>	(Sumbly et al., 2006)
MGAS5005	Invasive isolate, serotype M1, 1 bp deletion in <i>covS</i>	(Sumbly et al., 2005)
MGAS5322	Pharyngeal isolate, serotype M1, wild-type CovS	(Sumbly et al., 2006)
MGAS5392	Pharyngeal isolated, serotype M1, wild-type CovS	(Sumbly et al., 2006)
MGAS6184	Invasive isolate, serotype M1, A397V in CovS	(Sumbly et al., 2006)
MGAS9506	Pharyngeal isolate, serotype M1, wild-type CovS	(Sumbly et al., 2006)
Plasmids		
pSL60-1	Vector containing <i>aad9</i> gene, Spc ⁺	(Lukonski et al., 2000)
pSB100	Gfp expressing plasmid, Cm ⁺	This study

Table 2

Microarray analysis of gene transcript level in strains MGAS2221 vs. 2221 $\Delta malR$ -1.

ORF in strain MGAS5005	Putative function of encoded protein (name)	Fold change ^a	Putative MalR binding site ^b
spy0015	hypothetical protein	4.2	No
spy0150	3-keto-L-gluconate phosphotransferase	0.42	No
spy0177	bioY protein	0.46	No
spy0428	daunorubicin resistance ATP-binding protein	0.16	Yes
spy0429	daunorubicin resistance transmembrane protein	0.19	Yes
spy0430	ABC transporter permease protein	0.13	Yes
spy1021	phage protein	0.46	No
spy1029	phage protein	0.42	No
spy1049	phage protein	0.43	No
spy1055	maltodextrin phosphorylase (MalP)	0.15	Yes
spy1056	4-alpha-glucanotransferase (MalQ)	0.09	Yes
spy1058	maltodextrin binding protein (MalE)	0.40	Yes
spy1059	maltodextrin permease protein (MalF)	0.38	Yes
spy1060	maltodextrin permease protein (MalG)	0.39	Yes
spy1456	phage protein	0.32	No
spy1574	universal stress response protein	5.1	No
spy1575	MFS family transporter (NorA)	7.1	No
spy1632	6-phospho-beta-galactosidase (LacG)	4.7	No
spy1662	putative transport protein	0.34	No
spy1678	Thioredoxin	0.48	No
spy1679-80	pullulanase (PulA)	0.10	Yes
spy1681	1,6-alpha-glucosidase	0.07	Yes
spy1691	exonuclease family protein	0.43	Yes
spy1692-3	maltose phosphotransferase (MalT)	0.17	Yes
spy1829	phage protein	0.20	No

^a Ratio indicates gene level in strain MGAS2221 relative to strain 2221 $\Delta malR$ -1

^b Indicates whether a putative MalR binding site (NNGCAAYCGNTTGCYR) is present in the promoter region of the indicated gene