

Domains Required for Transcriptional Activation Show Conservation in the Mga Family of Virulence Gene Regulators

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Mga, or the multigene regulator of the group A streptococcus (GAS) (*Streptococcus pyogenes*), is a transcriptional regulator of virulence genes important for colonization and immune evasion. All serotypes of the GAS possess one of two divergent *mga* alleles (*mga-1* or *mga-2*), and orthologues of Mga have also been identified in other pathogenic streptococci. To date, the only functional motifs established within Mga are two amino-terminal DNA-binding domains (HTH-3 and HTH-4). To uncover novel domains, a random mutagenesis screen using an M6 Mga (*mga-1*) was undertaken to find mutations leading to a defect in transcriptional activation of the Mga-regulated *emm* gene. In addition to mutations in the established DNA-binding domains, the screen also revealed mutations in a region conserved among several Mga orthologues. Alanine scanning helped resolve the boundaries of this conserved Mga domain (CMD-1) spanning from residues 10 to 15 of the protein, with the two flanking amino acid residues likely involved in protein stability. Transcriptional reporter analyses demonstrated the importance of CMD-1 for activation of *Pemm* and autoactivation of *Pmga* in the serotype M6 Mga. Mutational analyses showed that both CMD-1 and HTH-4 are also necessary for activation of the promoter target *Pmmp* in a divergent serotype M4 Mga (*mga-2*), suggesting a conserved functionality. However, in contrast to M6, the M4 Mga mutants did not show a defect in autoregulation. Mutation of similar conserved residues in the Mga-like regulator DmgB from *S. dysgalactiae* subsp. *dysgalactiae* showed that CMD-1 and HTH-4 are critical for transcriptional activation in this orthologue, implying that a common mechanism of virulence gene activation may exist for members of the Mga family of regulators.

The genus *Streptococcus* is a large heterogeneous collection of gram-positive cocci, with each species differing in its requirements for optimum growth and demonstrating a defining hemolytic reaction on blood agar plates. One unifying theme of this genus is that many of its members colonize a broad range of tissue sites in humans and/or animals (6). A clear example is the group A streptococcus (GAS) (*Streptococcus pyogenes*), which is able to cause a wide array of diseases throughout the human body ranging from self-limiting to life-threatening. Understanding how pathogenic streptococci survive and elicit disease throughout their host remains an underlying goal of research on these organisms.

Survival within different niches stems from the repertoire of virulence genes these pathogens possess and the ability to regulate their expression in response to specific environmental conditions. Differential gene expression in the GAS and other streptococci is often controlled by two-component signal transduction systems, which allow the bacteria to sense their environment and respond via activation or inactivation of the appropriate transcriptional regulators. However, not all transcriptional regulators that respond to environmental stimuli are part of an established two-component system. For example, the GAS contain several “stand-alone” response regulators, which are defined as environmentally responsive transcriptional regulators that contain no known sensory components (13).

One such stand-alone response regulator is Mga, the multigene regulator of the GAS. Although the mechanism by which

Mga can respond to environmental signals remains undefined, it has been shown to positively activate its own expression (18, 19) along with the expression of other genes within its regulon in response to increased CO₂ levels, body temperature, and exponential-phase growth (4, 15, 17, 22). In the GAS, the *mga* regulon is composed of genes important for colonization and immune evasion, such as those encoding the antiphagocytic M protein (*emm*), an immunoglobulin-binding protein (*mip*), a collagen-like protein (*scl*), and a C5a peptidase (*scpA*).

While all strains of the GAS contain an *mga* gene (3), the architecture of the *mga* regulon differs from strain to strain in both gene composition and arrangement (11, 29). Furthermore, two *mga* alleles have been described within the GAS (*mga-1* and *mga-2*) based upon the ability to hybridize to an oligonucleotide probe (12) and are associated with different gene patterns at the *mga* locus and tissue tropism of the serotype (11). The Mga proteins produced from the two alleles are most divergent within the C-terminal end of the protein (2), showing a maximal amino acid divergence of 20.7% (3). Despite the divergence, both *mga* alleles have been shown to be functionally equivalent in an *mga-1* deletion strain (2).

S. pyogenes is not the only streptococcal species that appears to contain a gene analogous to *mga*. Geyer and Schmidt identified an orthologue in *S. dysgalactiae* subsp. *equisimilis* named *mgc* (8) for the multigene regulator of the group C streptococcus (GCS). The Mgc protein is 51% identical and 64% similar to the Mga (*mga-1*) from the serotype M6 GAS strain D471 (8). Another GCS orthologue designated *dmgB*, exhibiting 45% identity and 61% similarity to Mga, has been identified in *S. dysgalactiae* subsp. *dysgalactiae* (28). The *mgc* and *dmgB* loci closely resemble that of the GAS *mga* in that a gene encoding an M protein homolog (*emm* and *demB*, respectively) lies di-

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rectly downstream of the aforementioned GCS genes. Recently, a virulence regulator showing 51% similarity and 25% identity to Mga from the GAS was found in *S. pneumoniae*, called *mgaA* for the Mga-like repressor A (9, 10). In contrast to Mga and DmgB/Mgc, MgrA appears to repress transcription of genes in the unlinked *rtxA* pathogenicity islet in the *S. pneumoniae* TIGR4 genome (10). Additional predicted regulators showing similarity to Mga can be found in the genome sequences of other streptococcal species, including *S. equi*, *S. gordonii*, and *S. mitis*.

Currently, only Mga has been extensively characterized and serves as an excellent model system for this family of Mga-like virulence regulators. A serotype M6 Mga (*mga-1*) is able to activate transcription by interacting with specific DNA sites within regulated (*Pemm*, *PscpA*, and *PsclA*) and autoregulated (*Pmga*) promoters (1, 14, 18). Two amino-terminal helix-turn-helix (HTH) domains necessary for DNA binding and transcriptional activation were found in M6 Mga: one (HTH-4) that is absolutely essential for binding to all targets and a second (HTH-3) that serves an accessory role, primarily in autoregulation from *Pmga* (16). In addition, two amino acids involved in transcriptional activation of a divergent Mga (*mga-2*) have been identified (27). Finally, two putative response regulator receiver domains have been suggested to exist in Mga based on homology to other two-component systems (20); however, no molecular evidence for these domains has been presented.

Since little is known about how Mga is able to sense environmental stimuli and activate virulence gene transcription, a genetic screen was devised to uncover functional domains within a serotype M6 Mga (*mga-1*) important for transcriptional activation. Using this approach, we have identified a novel conserved domain that is important for activation of downstream target genes not only in divergent Mga proteins from the GAS, but also for the orthologue DmgB from GCS. Our data suggest that differences exist between divergent Mga proteins and their contribution to autoregulation. Finally, we have shown that HTH-4 is necessary for transcriptional activation in two members of the Mga family of virulence regulators.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* JRS519 is a Δ *mga* kanamycin-resistant derivative of the serotype M6 strain JRS4 (17, 24). The *S. pyogenes* vectors for integration (VIT) strain RTG229 is a derivative of JRS4 (7). Both the M6 *Pemm-gusA* GAS reporter strain KSM148.174 and the M6 *Pmp-gusA* GAS reporter strain KSM149 are unmarked *mga* deletion (Δ *mga*) derivatives of the VIT strain RTG229 (27). The clinical isolate AP4 is a serotype M4 GAS strain (25). *Escherichia coli* DH5 α (*hsdR17 recA1 gyrA endA1 relA1*) was used as the host for all plasmid constructions, and protein purifications used the *E. coli* strain BL21(DE3) containing the T7 RNA polymerase (26).

GAS strains were grown in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY), while *E. coli* strains were grown in Luria-Bertani broth (LB). Growth of GAS was measured on a Klett-Summerson photoelectric colorimeter using an A filter. The following concentrations of antibiotics were used: ampicillin at 100 μ g/ml for *E. coli*; spectinomycin at 100 μ g/ml for both *E. coli* and GAS; and erythromycin at 500 μ g/ml for *E. coli* and 1 μ g/ml for GAS.

DNA manipulation. Plasmid DNA was isolated from *E. coli* using the Wizard miniprep kit (Promega) or from the GAS as described below. Genomic DNA was isolated from the GAS using the FastDNA prep and a FastPrep cell disruptor (Bio 101). DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (QIAGEN). All PCR, with the exception of that used to generate random mutations, was performed using *Pfu* Turbo DNA polymerase (Stratagene) and resulting products were purified with the QIAquick PCR pu-

riification system (QIAGEN). All site-specific mutations were generated with the QuickChange site-directed mutagenesis kit (Stratagene), using mutagenic oligonucleotides synthesized by Integrated DNA Technologies. DNA sequencing was done by the McDermott Center sequencing core facility at the University of Texas Southwestern Medical Center.

Isolation of plasmid DNA from the GAS. Cells from a 50-ml overnight culture were resuspended in P1 solution (QIAGEN) containing 20 mg/ml lysozyme, mixed, and incubated for 30 min at 37°C. Samples were lysed with the FastPrep cell disruptor (Bio 101) and allowed to settle and the supernatant was mixed with 300 μ l of a 1% sodium dodecyl sulfate–0.2 M NaOH solution. After 5 min at room temperature, 300 μ l of 2.5 M potassium acetate, pH 4.5, was added, mixed by inversion, and centrifuged (5 min, 13,000 rpm). The supernatant was applied to a miniprep column (QIAGEN) and plasmid DNA was eluted using the manufacturer's protocol.

Random PCR mutagenesis screen. Mutagenic PCR was performed across the 5' region of *mga* using the GeneMorph PCR mutagenesis kit (Stratagene) on plasmid pKSM162 (16) with primers RMut1-R2 and Mga6-150 (Table 1) and an initial amount of DNA of 0.110 μ g. The resulting PCR product was digested with *Ava*I and *Spe*I, ligated with *Ava*I- and *Spe*I-digested pKSM318 (27), transformed into *E. coli* DH5 α , and grown overnight in 10 ml LB broth containing spectinomycin. A similar procedure was performed for the 3' portion of *mga* except primers OYR-29 and RMut2-L were used on various concentrations of pKSM162 ranging from 0.0552 to 0.838 μ g. The reduction in starting concentration was to compensate for the increased size of the 3' fragment as per the manufacturer's protocol. Also, both the PCR product and the vector pKSM318 were digested with *Spe*I and *Sph*I for this fragment.

Plasmid DNA was extracted from the entire overnight culture, and 1 μ g of plasmid DNA was transformed into the reporter strain KSM148.174 (27). Following selection on THY agar containing spectinomycin and 175 μ g/ml 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc) (Gold Biotechnology), GAS transformants that were either light blue or white on plates were subjected to Western analysis to determine protein levels as described below. Plasmid DNA was extracted from the selected clones and amplified via subsequent transformation into *E. coli* DH5 α for DNA sequencing. Sequence alignments were performed with the Vector NTI software (Invitrogen) using the ClustalW algorithm, and genes containing more than two mutations were discarded from further analysis.

Whole-cell GAS protein extracts. Whole-cell GAS protein extractions were performed as previously described (16). Briefly, mid-logarithmic-phase GAS cultures were harvested by centrifugation and resuspended in saline containing 1 \times complete protease inhibitor cocktail (Roche). Cells were lysed with the FastPrep cell disruptor (Bio 101) and the soluble fraction was recovered after centrifugation. Total protein concentrations were determined using the protein assay kit (Bio-Rad).

Western blot analyses. Proteins were analyzed by Western blot as previously described (16). Blots were incubated with either a 1:2,000 dilution of anti-His tag monoclonal antibody (Novagen) or a 1:1,000 dilution anti-Mga-pep2 antiserum (16), then incubated with a 1:25,000 dilution of either anti-mouse (Chemicon) or anti-rat (Santa Cruz Biotechnologies) horseradish peroxidase-conjugated secondary antibody, respectively, and visualized using the Western Lightning chemiluminescence system (Perkin Elmer). For a loading control, blots were stripped in a solution containing 2% sodium dodecyl sulfate, 50 mM dithiothreitol (DTT), and 50 mM Tris-HCl, pH 7.0, for 30 min at 70°C and reprobed with a 1:50,000 dilution of mouse anti-Hsp60 monoclonal antibody (StressGen Biotechnologies Corp.).

GusA reporter assays. Soluble whole-cell lysates were isolated from the GAS grown to mid-logarithmic phase (65 Klett units) as described above. GusA activity was determined for each lysate (100 μ l) as described by Eichenbaum et al. (5). Total protein concentrations were determined as above. GusA units are defined as the optical density at 420 nm (OD₄₂₀)/total protein concentration of the lysate.

Construction of a *Pmga-gusA* reporter strain containing a Δ *Pmga-mga* allele. *Pmga-gusA* reporter strain KSM231.310 was generated by transforming the *mga*-deleted M6 VIT strain VIT231 with p*Pmga-gusA* (27) linearized by *Xmn*I, which created a Δ *mga* reporter strain containing a single-copy transcriptional fusion of the native M6 *mga* promoter to *gusA* (*Pmga-gusA*).

Construction of the site-specific *Pspac-mga6-his* plasmids pQ10R, pQ11A, pW12A, pW12R, pR13A, pE14A, and pL15A. Site-specific mutants of M6 Mga produced from the constitutive promoter *Pspac* and containing a carboxy-terminal 6X His tag were generated as described above using the QuickChange site-directed mutagenesis kit (Stratagene), pKSM318 (Table 2) template DNA, and the corresponding *mga6* mutagenic oligonucleotides (Table 1), resulting in the different plasmids.

TABLE 1. PCR primers and their relevant targets^a

Target and primer	Sequence	Reference
23S rRNA		
rRNA-23SL	GGAAGGTAAGCCAAAGAGAG	23
rRNA-23SR	TCCTAGTTGTCTGTGCAACC	23
<i>Pemm</i>		
Pemm-L1	GCATGGATCCCATCGCAAAGAGCTTA	16
Pemm-R1	GCGGCTCGAGTAGTGTCTATTCGTGTTATT	16
<i>Pmga</i>		
OYL-25	TACCATAAAAATACCTTTTC	16
OYR-25	GGTTGTACCATAACAGTC	16
<i>mga6</i>		
Mga6-150	<u>gcgtcaaaagcttcta</u> ATCTCCTGATACTTGTACGG	This study
Mga-Pet1_Nde	<u>ggggcat</u> ATGTATGTAAGTAAGTTGTTT	1
Mga-Pet2_Xho	<u>aactcgag</u> AGTTGTGGAGGGG	1
OYR-29	AAACCAACGCCTATTTGACGCATAC	This study
RMut1-R2	<u>ccgcctcgag</u> AAAGAAGGGTATACAAGG	This study
RMut2-L	TCGACCTGCAG <u>gcatgcaaa</u>	This study
Mutagenic <i>mga6</i>		
M6 <i>mga</i> Q10R-a	GTTGTTTACAAGTcGACAGTGGAGAGAACTAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> Q10R-b	CCGTTACGTATGAGATTAATTTTAGTTCTCTCCACTGTcGACTTGTAACAAC	This study
M6 <i>mga</i> Q11A-a	GTTGTTTACAAGTCAAGcGTGGAGAGAACTAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> Q11A-b	CCGTTACGTATGAGATTAATTTTAGTTCTCTCCACTGTGACTTGTAACAAC	This study
M6 <i>mga</i> W12A-a	GTTGTTTACAAGTCAACAGgcGAGAGAACTAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> W12A-b	CCGTTACGTATGAGATTAATTTTAGTTCTCTcGCTGTTGACTTGTAACAAC	This study
M6 <i>mga</i> W12R-a	GTTGTTTACAAGTCAACAGcGGAGAGAACTAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> W12R-b	CCGTTACGTATGAGATTAATTTTAGTTCTCTCCACTGTGACTTGTAACAAC	This study
M6 <i>mga</i> R13A-a	GTTTACAAGTCAACAGTGGgcAGAATAAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> R13A-b	CCGTTACGTATGAGATTAATTTTAGTTCTgcCCACTGTTGACTTGTAAC	This study
M6 <i>mga</i> E14A-a	GTTGTTTACAAGTCAACAGTGGAGAGcACTAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> E14A-b	CCGTTACGTATGAGATTAATTTTAGTgCTCTCCACTTGACTTGTAACAAC	This study
M6 <i>mga</i> L15A-a	GTTTACAAGTCAACAGTGGAGAGAAgcAAAATTAATCTCATAACGTAACGG	This study
M6 <i>mga</i> L15A-b	CCGTTACGTATGAGATTAATTTTgcTTCTCTCCACTGTTGACTTGTAAC	This study
Mutagenic <i>mga4</i>		
M4 <i>mga</i> Q11R-a	GCATGTAAGTAAATGTTTACTAGCCAACgATGGAGAGAATTGAAACTG	This study
M4 <i>mga</i> Q11R-b	CAGTTTCAATTCTCTCCATcGTTGGCTAGTAAACAATTTACTTACATGC	This study
M4 <i>mga</i> R13A-a	GTTTACTAGCCAACAATGGgcAGAATTGAAACTGATTTTCAATTTAACAG	This study
M4 <i>mga</i> R13A-b	CTGTTAAATATGAAATCAGTTTCAATTCTgcCCATTGTTGGCTAGTAAAC	This study
M4 <i>mga</i> HTH4-a	GCTGAAGAGCTGTTTGTcAGCgcAgCTACCCTCAAACGCC	This study
M4 <i>mga</i> HTH4-b	GGCGTTGAGGGTAGcTgcGCTGACAAACAGCTCTTCAGC	This study
Mutagenic <i>dmgB</i>		
<i>dmgB</i> Q11R-a	CTCTTTACAACAAAACgGTGGAGAGAATTGGAGCTAATTGCGC	This study
<i>dmgB</i> Q11R-b	GCGCAATTAGCTCCAATTCTCTCCACcGTTTTGTTGTAAAGAG	This study
<i>dmgB</i> R13A-a	CTCTTTACAACAAAACAGTGGgcAGAATTGGAGCTAATTGCGC	This study
<i>dmgB</i> R13A-b	GCGCAATTAGCTCCAATTCTgcCCACTGTTTTGTTGTAAAGAG	This study
<i>dmgB</i> L42A-a	GTTGAGAGATTAAACTGCTCACTCgcAACTTTACAATCATGTG	This study
<i>dmgB</i> L42A-b	CACATGATTGTAAAGTTgcGAGTGAGCAGTTTAACTCTCACAC	This study
<i>dmgB</i> HTH4-a	GAGCTGTTGTcAGCgcGgCAACACTCAAGCGTTTGATTG	This study
<i>dmgB</i> HTH4-b	CAATCAAACGCTTGAGTGTGcGcGCTGACAAACAGCTC	This study

^a Mutagenic or noncomplementary sequences are in lowercase and introduced restriction sites are underlined.

Construction of the control *Pspac-mga6-his* plasmids pKSM318.1H and pKSM318.4H. Plasmids that contained mutations to disrupt either the essential DNA binding HTH-4 motif or two nonessential residues of M6 *mga* under the constitutive *Pspac* were constructed as follows: the 464-bp HindIII-SpeI fragment containing either the HTH-4 motif or K31A/D32A from pKSM164.4c or pKSM164.1c (16) was cloned into HindIII- and SpeI-digested pKSM318 (27) to produce pKSM318.4H and pKSM318.1H, respectively.

Construction of the *Pmga4-mga4-his* mutant plasmids pKSM346, pKSM347, and pKSM348. Site-specific mutants of the class II serotype M4 Mga, Q11R and R13A, were generated as described above using the mutagenic oligonucleotides M4 *mga* Q11R-a, M4 *mga* Q11R-b, M4 *mga* R13A-a, and M4 *mga* R13A-b (Table 1) in pKSM322 (Table 2), resulting in pKSM346 and pKSM347, respectively. A mutant disrupting the HTH-4 domain, named pKSM348, was also constructed by site-specific mutagenesis of pKSM322 using the mutagenic oligonucleotides M4 *mga* HTH4-a and M4 *mga* HTH4-b (Table 1).

Construction of the *PdmgB-dmgB* mutant plasmids pKSM339, pKSM340, pKSM341, and pKSM342. Site-specific mutations at positions 11 and 13 were constructed in the Mga orthologue from *S. dysgalactiae*, DmgB, using the muta-

genic oligonucleotides *dmgB* Q11R-a, *dmgB* Q11R-b, *dmgB* R13A-a, and *dmgB* R13A-b (Table 1) in pKSM152 (Table 2), resulting in pKSM339 and pKSM340, respectively. Control plasmids containing either mutations in the HTH-4 domain, pKSM341, or an arbitrary mutation at position 42, pKSM342, were also constructed by site-specific mutagenesis of pKSM152 using the mutagenic oligonucleotides *dmgB* HTH4-a, *dmgB* HTH4-b, *dmgB* L42A-a, and *dmgB* L42A-b, respectively (Table 1).

Expression and purification of Mga-His fusion proteins from *E. coli*. Plasmids expressing carboxy-terminal 6X His fusions to M6 Mga mutants Q11R, W12R, R13A, and E14A were constructed as follows: a 1.6-kb DNA fragment of the *mga* coding sequence was amplified from plasmid DNA encoding the corresponding mutant Q11R, W12R, R13A, and E14A using primers Mga-Pet1_Nde and Mga-Pet2_Xho (Table 1). The resulting PCR fragments were purified, digested with NdeI and XhoI, and inserted into NdeI- and XhoI-digested vector pKSM170 (1) to generate the *mga-his* fusion alleles for purification of Q11R-His (pKSM344), W12R-His (pKSM354), R13A-His (pKSM345), and E14A-His (pKSM355), respectively (Table 2). Mga-His proteins were purified from *E. coli* as previously described (1).

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pQ10R	M6 <i>mga</i> mutant Q10R under constitutive <i>Pspac</i>	This study
pQ11A	M6 <i>mga</i> mutant Q11A under constitutive <i>Pspac</i>	This study
pQ11R	M6 <i>mga</i> mutant Q11R under constitutive <i>Pspac</i>	This study
pW12A	M6 <i>mga</i> mutant W12A under constitutive <i>Pspac</i>	This study
pW12R	M6 <i>mga</i> mutant W12R under constitutive <i>Pspac</i>	This study
pR13A	M6 <i>mga</i> mutant R13A under constitutive <i>Pspac</i>	This study
pE14A	M6 <i>mga</i> mutant E14A under constitutive <i>Pspac</i>	This study
pL15A	M6 <i>mga</i> mutant L15A under constitutive <i>Pspac</i>	This study
pKSM152	<i>S. dysgalactiae</i> locus containing both wild-type <i>dmgB</i> and <i>demB</i>	This study
pKSM162	Wild-type <i>mga6</i> under constitutive <i>Pspac</i>	16
pKSM170	Wild-type M6 <i>mga-his</i> in <i>E. coli</i> expression vector	1
pKSM318	Wild-type <i>mga6-his</i> under constitutive <i>Pspac</i>	27
pKSM318.1H	M6 <i>mga</i> mutant in HTH-1 under constitutive <i>Pspac</i>	This study
pKSM318.4H	M6 <i>mga</i> mutant in HTH-4 under constitutive <i>Pspac</i>	This study
pKSM322	Wild-type <i>mga4-his</i> under native <i>Pmga4</i>	27
pKSM339	<i>dmgB</i> mutant Q11R under the native promoter	This study
pKSM340	<i>dmgB</i> mutant R13A under the native promoter	This study
pKSM341	<i>dmgB</i> mutant in HTH-4 under the native promoter	This study
pKSM342	<i>dmgB</i> mutant L42A in HTH-1 under the native promoter	This study
pKSM344	M6 <i>mga-his</i> mutant Q11R in <i>E. coli</i> expression vector	This study
pKSM345	M6 <i>mga-his</i> mutant R13A in <i>E. coli</i> expression vector	This study
pKSM346	M4 <i>mga-his</i> mutant Q11R under native <i>Pmga4</i>	This study
pKSM347	M4 <i>mga-his</i> mutant R13A under native <i>Pmga4</i>	This study
pKSM348	M4 <i>mga-his</i> mutant in HTH-4 under native <i>Pmga4</i>	This study
pKSM354	M6 <i>mga-his</i> mutant W12R in <i>E. coli</i> expression vector	This study
pKSM355	M6 <i>mga-his</i> mutant E14A in <i>E. coli</i> expression vector	This study

EMSA. Promoter probes for *Pemm* and *Pmga* were generated by PCR amplification from serotype M6 strain JRS4 genome DNA using the relevant primer pairs, *Pemm*-L1 and *Pemm*-R1 and OYR-25 and OYL-25, respectively (Table 1). An electrophoretic mobility shift assay (EMSA) was performed as previously described (14). Briefly, constant amounts of probe end labeled with [γ -³²P]ATP were incubated with increasing concentrations of purified Mga-His proteins for 15 min at 16°C before being separated on a 5% polyacrylamide gel.

Northern blot analysis. Total RNA was isolated from the GAS grown to mid-logarithmic phase using the FastRNA Pro Blue kit and FastPrep cell disruptor (Bio 101). Northern blot analysis was performed using the NorthernMax system (Ambion) as previously described (23). As a loading control, blots were stripped and reprobed with a 23S rRNA probe (Table 1).

Alignment of Mga orthologues. A sequence alignment of different Mga and Mga-like protein sequences was performed using the ClustalW algorithm in the AlignX module of VectorNTI. The alignment included Mga protein sequences representing *mga-1* alleles from GAS serotypes M1 SF370 (GI-15675800) and M6 JRS4 (GI-153733) as well as *mga-2* alleles from GAS serotypes M4 AP4 (GI-1246852) and M49 531 (GI-56808536). Protein sequences for *S. pneumoniae* MgrA (GI-17368568), *S. dysgalactiae* subsp. *equisimilis* Mgc (GI-6782393), and *S. dysgalactiae* subsp. *dysgalactiae* DmgB (GI-6689248) were also utilized. Additional Mga-like proteins were obtained by BLAST interrogation of the unfinished genomes for *S. equi* (Sanger Institute), *S. mitis* (TIGR), and *S. gordonii* (TIGR). Conserved regions were defined as amino acids with $\geq 70\%$ identity among homologues and spanned more than two adjacent residues.

RESULTS

Random mutagenesis screen for Mga mutants defective in transcriptional activation. A nonbiased strategy using PCR-generated mutations was devised to identify functional residues within Mga involved in activation of Mga-dependent genes (Fig. 1). PCR amplification using a defective DNA polymerase was utilized to generate random mutations across the 1,590-base-pair *mga* gene from the serotype M6 strain JRS4 (see Materials and Methods), which was arbitrarily divided into two fragments (N and C terminal) at a unique SpeI site for ease of handling. Mutated *mga* fragments were then cloned under the constitutive *Pspac* promoter in plasmid pKSM318

(27) such that transcription levels in the screen would not reflect autoactivation from the native *Pmga* promoter. The resulting plasmids were transformed into an *mga*-deleted GAS reporter strain (KSM148.174) containing a single-copy transcriptional fusion of *gusA* to the Mga-regulated *Pemm*, allowing direct quantitation of Mga-regulated activity based on a colorimetric assay (see Materials and Methods).

Transformed GAS strains were plated onto media containing X-Gluc, which allowed visual determination of a defective Mga based on colony color (white or light blue) compared to blue seen with the wild-type control. Whole-cell lysates were prepared from clones chosen for further analysis and the steady-state level of Mga was determined using Western blot analysis. Finally, plasmid DNA from defective clones producing wild-type levels of protein was sequenced, and mutation sites were recorded (Table 3). Although identical *mga* mutations were found, possibly as a result of the amplification steps in *E. coli*, they were not included as separate entries in the overall results.

In total, 12 independent mutants were identified within the N-terminal *mga* fragment that resulted in either one or two amino acid changes per molecule and yet still produced wild-type levels of protein (Table 3). Eight mutants were identified that contained mutations within the known DNA binding domains HTH-3 and HTH-4 (16), resulting in colonies that were either light blue (HTH-3 mutations) or white (HTH-4 mutations). These activities correlated to the effects previously observed for mutations in the two Mga HTH domains (16) and served as a strong validation of the screen. Four additional mutations located outside of the known DNA binding regions were also identified that exhibited a light blue colony phenotype in the screen. Two pairs of mutants (Q11R, W12R/A38T and K33T, V30I/T139I) involved neighboring residues, sug-

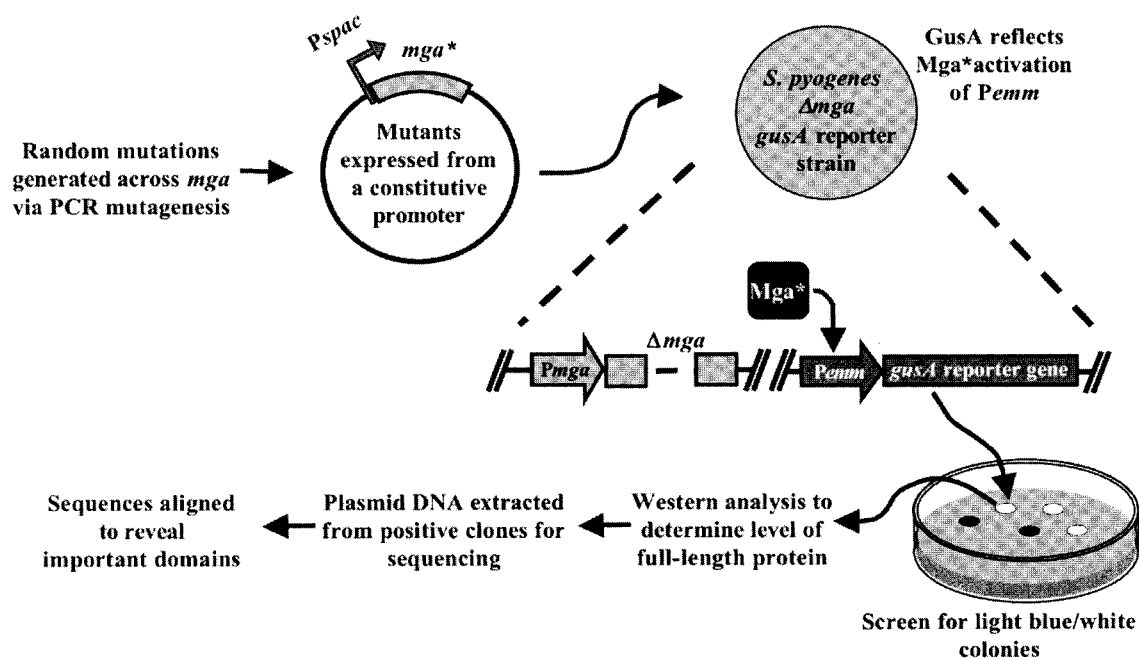


FIG. 1. Random PCR mutagenesis screen used to identify domains involved in Mga-dependent transcriptional activation. Random mutations were generated in *mga* using the GeneMorph PCR mutagenesis kit (Stratagene). Mutated M6 *mga* alleles were placed on a plasmid under the constitutive promoter *Pspac* in an *mga*-deleted strain containing a promoter fusion of the Mga-regulated *Pemm* to a promoterless *gusA* reporter gene in the chromosome of M6 GAS. Resulting strains were plated onto THY plates containing X-Gluc to reveal clones deficient in transcriptional activation (light blue or white). Whole-cell lysates from clones showing a defect in activation were extracted, and Mga protein levels were determined using Western blot analysis. Plasmid DNA from clones producing wild-type levels of protein was isolated, sequenced, and aligned to the wild-type M6 *mga* gene to identify mutations.

gesting that each might indicate potential functional domains in the N terminus of Mga.

Interestingly, no light blue or white colonies producing wild-type levels of protein were obtained from repeated screens of the C-terminal fragment of *mga*. In fact, 272 C-terminal mutants exhibiting a defect in Mga activity were analyzed via Western analysis and found to produce little to no detectable Mga (data not shown). Further analysis of a subset of these mutants found they each contained multiple amino acid changes, regardless of attempts to decrease the mutation frequency. Therefore, the C terminus of Mga appears to be quite sensitive to mutagenesis and, as a result, was not amenable to the screen used in this study.

Two mutations reside within a region conserved among Mga homologues. Conserved regions in protein families often represent important functional domains that are retained during evolution. Therefore, a protein sequence alignment was performed on Mga homologues from various streptococcal species, including Mga proteins representing divergent *mga-1* and *mga-2* alleles in the GAS, Mgc from *S. dysgalactiae* subsp. *equisimilis*, DmgB from *S. dysgalactiae* subsp. *dysgalactiae*, and MgrA from *S. pneumoniae*, as well as putative Mga-like transcriptional regulators found in the unfinished genomes of *S. equi* (Mge), *S. gordonii* (Mgg), and *S. mitis* (Mgm) via BLAST analysis (Fig. 2; see Materials and Methods).

A large region of conservation encompassed the essential HTH-4 domain of Mga, suggesting that this DNA-binding domain is likely serving a similar function in many of the orthologues (Fig. 2). Several other groups of conserved residues

were also found by the alignment outside of the HTH domains (Fig. 2). Two of the four mutants identified in our activity screen (Table 3; K33T and V30I/T139I) did not fall within one of these conserved domains and were not investigated further in this study. However, the remaining two Mga mutants (Table 3; Q11R and W12R/A38T) contained mutations within a region of amino acid conservation (noted as asterisks in Fig. 2) spanning from the glutamine at position 11 to the leucine at position 15. The glutamine at position 10 also showed a high degree of conservation, and thus the region encompassing residues 10 to 15 was named the conserved Mga domain 1 (CMD-1).

TABLE 3. Mutants found from the random PCR mutagenesis screen

Domain	Mutation	Phenotype ^a
DNA binding domain HTH-3	F55C	LB
	I62N	LB
	I71F	LB
DNA-binding domain HTH-4	S117T	W
	S117N	W
	S119L	W
	T120K	W
	R123H	W
Other	Q11R	LB
	W12R/A38T	LB
	K33T	LB
	V30I/T139I	LB

^a LB, light blue; W, white.

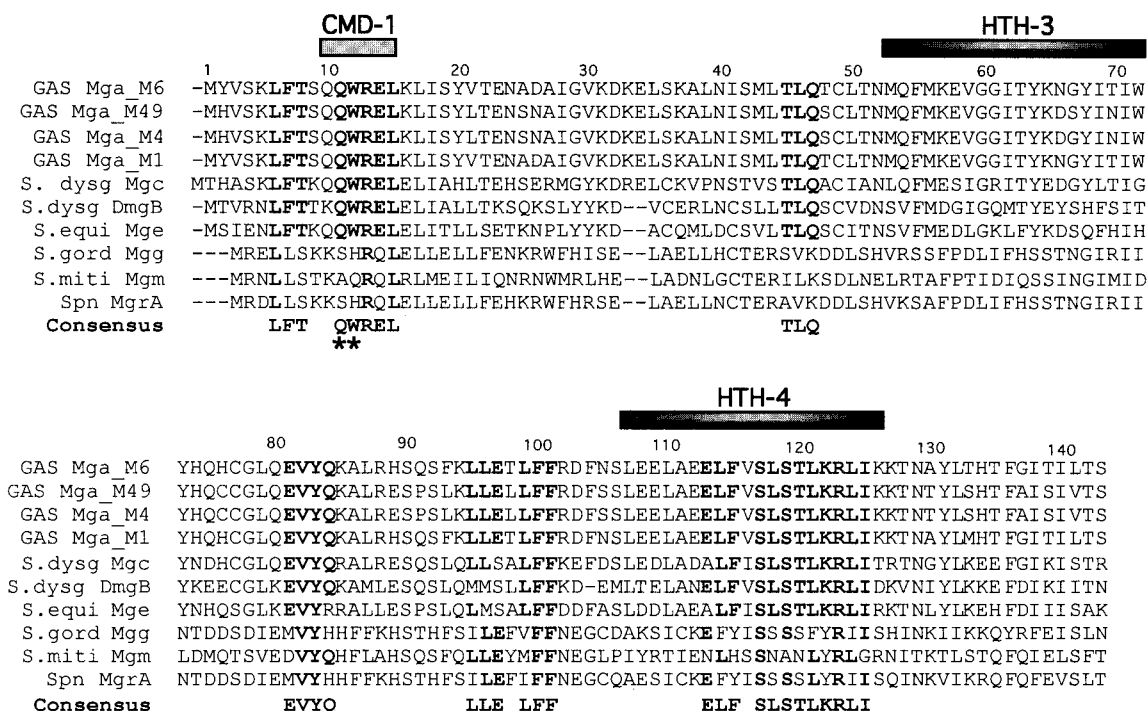


FIG. 2. Sequence alignment of Mga orthologues reveals conserved domains. A sequence alignment of the Mga orthologues from various streptococcal species, including GAS (serotypes M6, M49, M4, and M1), *S. dysgalactiae* (subsp. *equisimilis* and subsp. *dysgalactiae*), *S. equi*, *S. gordonii*, *S. mitis*, and *S. pneumoniae*, was used to derive a consensus sequence for conserved domains located within the first 143 amino acids of the proteins (see Materials and Methods). A conserved domain was defined as an area containing more than two consecutive residues exhibiting $\geq 70\%$ identity among homologues. Amino acids identical to the consensus are in bold type. Black bars depict the locations of the two known DNA-binding domains in the GAS M6 Mga, while the gray bar denotes CMD-1. The two mutations found outside of the DNA-binding domains during the random mutagenesis screen are indicated (*).

CMD-1 of an M6 Mga (*mga-1*) is involved in transcriptional activation. To establish whether the entire CMD-1 was important for Mga activity, we utilized site-specific mutagenesis to construct mutant *mga* alleles that encoded a single-amino-acid change in Mga at residues 10 to 15 (Q10R, Q11A, W12A, R13A, E14A, and L15A). In addition, the original Q11R mutant and a site-specific W12R mutant, which recapitulated the original W12R/A38T double mutant, were used. As in the initial screen, the resulting CMD-1 site-directed mutant alleles were cloned under the constitutive *Pspac* in pKSM318 to produce pQ10R, pQ11A, pQ11R, pW12A, pW12R, pR13A, pE14A, and pL15A (Table 2). A double Mga mutant, K31A/D32A (pKSM318.1H), which has been shown not to affect Mga-dependent activation, and an established HTH-4 mutation (pKSM318.4H), which leads to a DNA binding deficiency and loss of Mga-dependent activation, were constructed as controls (16). All of the plasmids were introduced into two *mga*-deleted serotype M6 GAS GusA reporter strains. The first, the Δ *mga Pemm-gusA* strain KSM148.174 (27), determined Mga-regulated activity at a downstream promoter; while the second, the Δ *mga Pmga-gusA* strain KSM231.310, assessed the ability to autoregulate.

Mga mutants Q10R, Q11A, Q11R, W12R, R13A, E14A, and L15A and the HTH-4 control mutant showed a reduction in activity at the *mga*-regulated promoter *Pemm* compared to wild-type Mga (Fig. 3A). The same mutants also showed a reduction in autoactivation at the native *Pmga* with the excep-

tion of Q11A, which appeared to have wild-type activity only in the *Pmga* strain (Fig. 3B). In contrast, mutation W12A and the negative control mutant K31A/D32A demonstrated wild-type promoter activity at both *Pemm* and *Pmga* (Fig. 3B). The steady-state levels of Mga detected in the strains expressing CMD-1 mutants in residues 11 to 14, as well as the control strains, were equivalent to the wild type compared to the loading control Hsp60 (Fig. 3C). However, flanking mutations at either end of CMD-1 (residues 10 and 15) show a reduction in protein level. Taken together, mutations in residues 11 through 14 in CMD-1 appear to affect activation without changing the overall steady-state levels of Mga, whereas amino acids 10 and 15 likely play a role in protein stabilization.

As an independent verification of the role of CMD-1 in autoregulation at *Pmga*, the Q11A, Q11R, and R13A mutants in M6 Mga were produced from the native *Pmga* in the Δ *mga Pemm-gusA* reporter strain KSM148.174 (Fig. 3D). As predicted by the *Pmga* reporter studies (Fig. 3B), those mutants showing a defect in *Pmga-gusA* activation (Q11R and R13A) did not produce wild-type levels of mutant protein from *Pmga*, whereas Q11A was normal for *Pmga* activation and showed levels of protein comparable to that of the wild-type control (Fig. 3D). Therefore, mutants in CMD-1 in M6 Mga are defective for both activation and autoregulation.

CMD-1 and HTH-4 are important for transcriptional activation in a divergent Mga. Since an *mga-2* allele has been shown to functionally complement an *mga-1* allele in vivo (2),

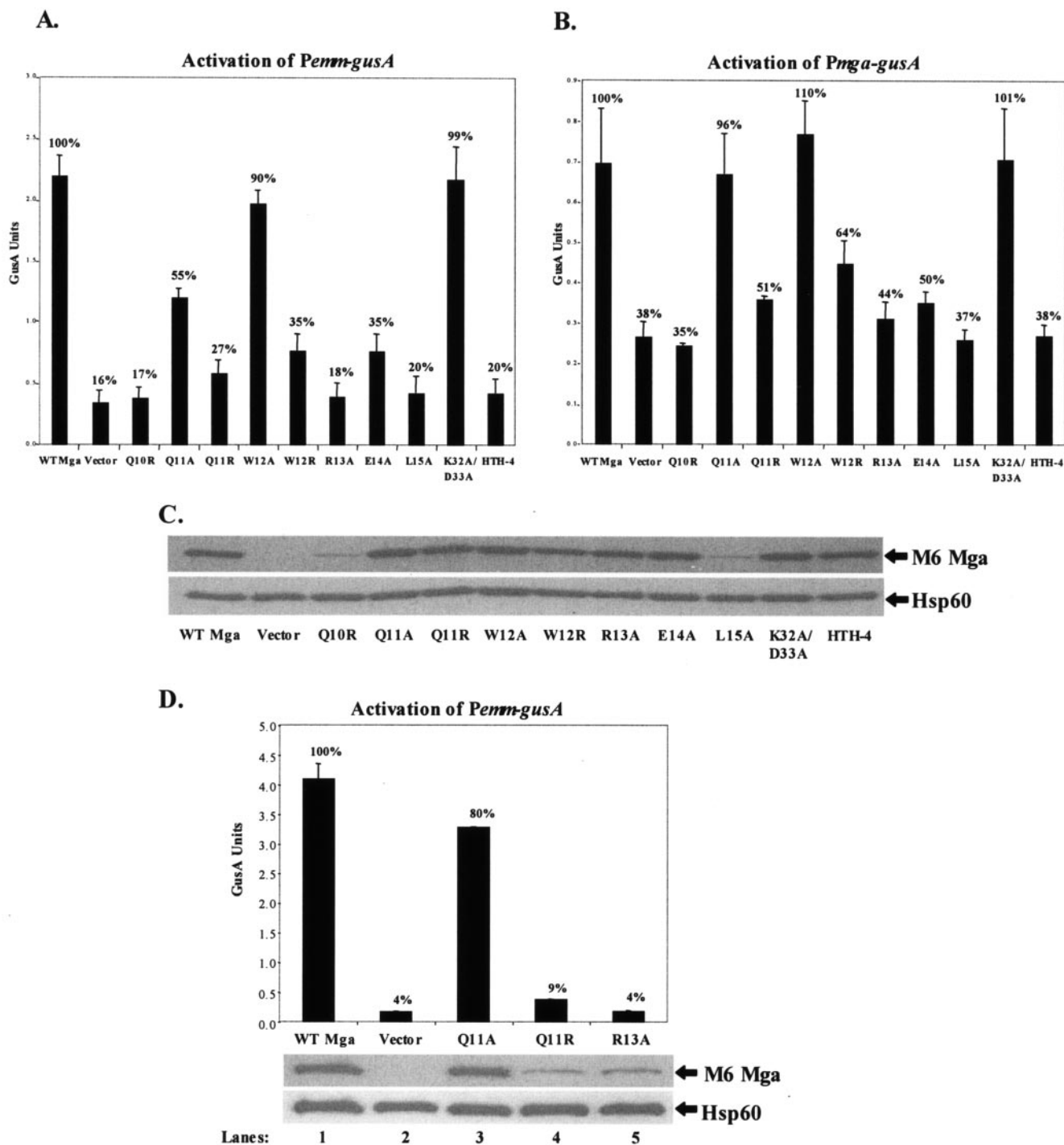


FIG. 3. In vivo transcriptional activity of mutant M6 *mga* alleles. A) GusA activity of whole-cell lysates. Production of β -glucuronidase activity was determined for lysates from an *mga*-deleted *Pemm-gusA* reporter strain KSM148.174 or B) an *mga*-deleted *Pmga-gusA* reporter strain KSM231.310 containing plasmids expressing the following M6 Mga alleles from a constitutive *Pspac* promoter: wild-type M6 Mga, vector only, the M6 Mga mutants Q10R, Q11A, Q11R, W12A, W12R, R13A, E14A, and L15A. An arbitrary mutant K32A/D33A (no defect) and a known DNA-binding mutant in HTH-4 (defective) are included as controls. GusA units represent a measure of absorbance (A_{420})/protein concentration ($\mu\text{g/ml}$) and are the average of three independent experiments. C) Western analysis was performed on whole-cell lysates from the above samples using both an anti-Mga antibody for detection of protein levels (top) and an anti-Hsp60 antibody as a control for loading (bottom). D) Activity of mutants expressed from the native promoter. Activity levels for both wild-type and the M6 Mga mutants Q11A, Q11R, and R13A were examined for each allele when expressed from the native *mga* promoter using the Δ *mga* *Pemm-gusA* reporter strain (top). Protein production was also determined via Western analysis (bottom) as described above. Percentages indicate level of GusA activity compared to that of the wild-type control.

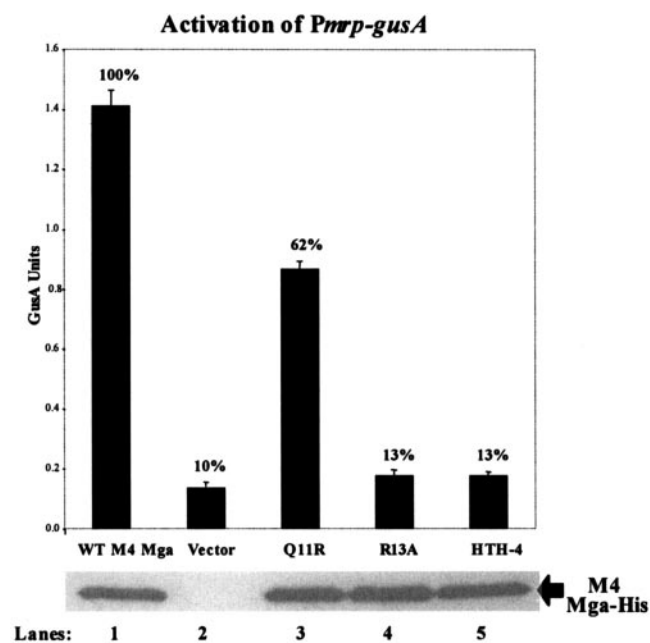


FIG. 4. In vivo transcriptional activity of mutant M4 *mga* alleles expressed from the native *Pmp* promoter: GusA activity of whole-cell lysates (top). Production of β -glucuronidase activity was determined for lysates from an *mga*-deleted *Pmp*-*gusA* reporter strain, KSM149, containing plasmids expressing the following different M4 *mga* alleles from the native *Pmga* promoter: wild-type M4 Mga (lane 1), vector only (lane 2), the M4 Mga mutants Q11R (lane 3) and R13A (lane 4), and a DNA-binding mutant in HTH-4 (lane 5). GusA units are a measure of the absorbance (A_{420})/protein concentration ($\mu\text{g/ml}$) and are the average of three independent experiments. Western analysis was performed on whole-cell lysates of each sample using an anti-His antibody for detection of Mga-His protein levels (bottom). Percentages indicate level of GusA activity compared to that of the wild-type control.

we predicted that CMD-1 would play a similar role in a divergent Mga. To investigate the effects of CMD-1 mutations in a divergent Mga, we introduced mutations into the *mga-2* allele from the serotype M4 strain AP4. Given that an antibody against the M4 Mga is not available, all of the alleles were modified to produce a carboxy-terminal 6X-His fusion to allow detection with anti-His monoclonal antibodies. Plasmids containing either the wild-type M4 *mga-his* allele or *mga-his* possessing the Q11R, R13A and HTH-4 mutations under their native *Pmga4* promoter were transformed into the *Pmp*-*gusA* GAS reporter strain KSM149. This strain has been used previously to study transcriptional activation by divergent Mga proteins (27).

GusA analysis demonstrated that the HTH-4 DNA-binding domain, which shows 100% identity within all Mga proteins from the GAS, is necessary for full transcriptional activation of *Pmp* by a divergent Mga. Furthermore, a decrease in activation at *Pmp* was also observed when the M4 Mga was mutated at either amino acid 11 or 13, implying that CMD-1 is important for Mga-specific activation in a divergent Mga as well (Fig. 4). Interestingly, all of the mutant alleles produced steady-state levels of Mga-His from *Pmga4* equivalent to wild-type protein levels (Fig. 4), suggesting that the R13A and HTH-4 mutations

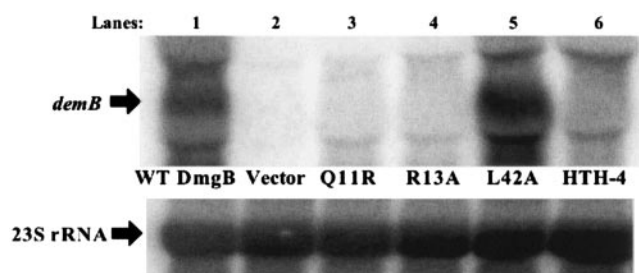


FIG. 5. Transcriptional activation of *demB* by mutant Mga orthologue DmgB from *S. dysgalactiae* subsp. *dysgalactiae*. Northern analysis of transcript levels of the DmgB-regulated gene *demB* was determined using total RNA (5 μg) isolated from the GAS *mga*-inactivated M6 strain JRS519 harboring plasmids containing *demB* and the following *dmgB* alleles under the native *dmgB* promoter: wild-type *dmgB* (lane 1), vector only (lane 2), and the *dmgB* mutants Q11R (lane 3), R13A (lane 4), an arbitrary mutant L42A (lane 5), and an HTH-4 DNA-binding domain mutant (lane 6). Blots were stripped and reprobed with 23S rRNA to serve as a loading control (directly below). The blot shown is representative of data from three independent experiments.

are not defective for autoregulation as observed for the divergent M6 Mga (Fig. 3B).

CMD-1 and HTH-4 are important for transcriptional activation in the Mga orthologue DmgB. Since the CMD-1 and HTH-4 domains are important in both *mga* alleles from the GAS, we investigated whether these domains were also necessary for transcriptional activation in an Mga orthologue from another pathogenic streptococcus. A plasmid containing both the Mga-like regulator gene *dmgB* and the linked DmgB-regulated gene *demB* from *S. dysgalactiae* subsp. *dysgalactiae* (28) under the native *PdmgB* promoter were introduced into the Δ *mga* M6 GAS strain JRS519 (17). Northern analysis was then used to monitor DmgB-mediated activation of *demB* in the Δ *mga* GAS background (Fig. 5).

Expression of wild-type *dmgB* leads to high levels of *demB* transcripts compared to either the vector alone (Fig. 5) or a deletion of *dmgB* from the plasmid (data not shown). Introduction of the HTH-4 mutation or the CMD-1 mutations Q11R and R13A into DmgB resulted in a dramatic reduction in *demB* transcript levels compared to the wild-type allele (Fig. 5). As a control, mutation of an arbitrary amino-terminal residue (L42A) of DmgB had little effect on its ability to regulate *demB* expression (Fig. 5). Overall, both CMD-1 and HTH-4 are necessary for activation of corresponding virulence genes not only in Mga, but also in other members of the Mga family of virulence regulators.

DNA-binding activity of CMD-1 Mga mutants. Binding of Mga to promoter targets is essential for transcriptional activation of Mga-regulated genes in the GAS (16). To elucidate the contribution of CMD-1 to DNA binding, electrophoretic mobility shift assays were performed to determine the ability of Mga mutants to bind to Mga-regulated DNA targets in vitro. Plasmids containing either wild-type M6 *mga-his* (pKSM170) or the mutant M6 *mga-his* alleles Q11R (pKSM344), W12R (pKSM354), R13A (pKSM345), and E14A (pKSM355) were transformed into *E. coli*, and Mga proteins purified from each lysate. Increasing amounts of the purified Mga-His proteins were incubated with a constant amount of radiolabeled pro-

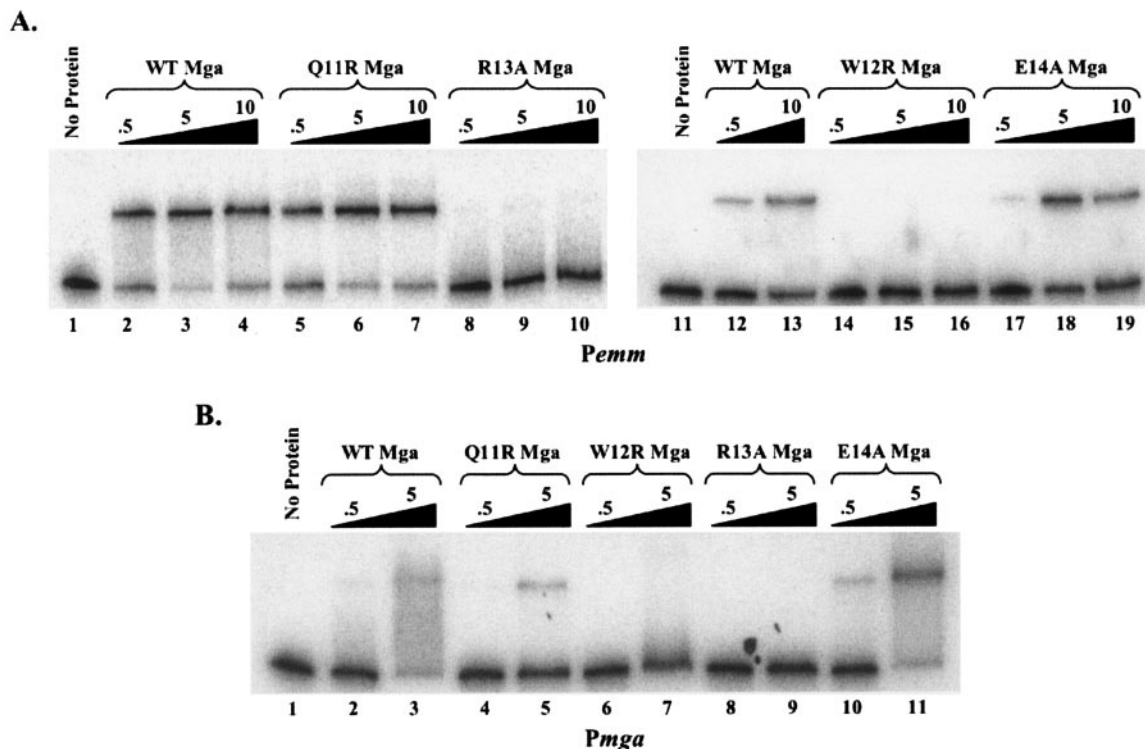


FIG. 6. Electrophoretic mobility shift assays of CMD-1 Mga mutants binding to Mga-regulated promoters. (A) Electrophoretic mobility shift assays of Mga-regulated promoter *Pemm*. C-terminal 6X-His fusion proteins (Mga-His) were purified from *E. coli* lysates. Identical amounts of the radiolabeled promoter probe *Pemm* were incubated for 15 min at 16°C with an increasing amount (0.5, 5, and 10 μ g) of either the wild-type (lanes 2 to 4 and 12 to 13), Q11R (lanes 5 to 7), R13A (lanes 8 to 10), W12R (lanes 14 to 16), E14A (lanes 17 to 19), or no M6 Mga-His (lanes 1 and 11) prior to separation on a 5% polyacrylamide gel. (B) Electrophoretic mobility shift assays of the native *Pmga* promoter were performed as described above using 0.5 and 5 μ g of the wild-type (lanes 2 and 3), Q11R (lanes 4 and 5), W12R (lanes 6 and 7), R13A (lanes 8 and 9), E14A (lanes 10 and 11), or no Mga-His protein (lane 1).

motor probe corresponding to the M6 Mga-regulated promoters *Pemm* (Fig. 6A) and *Pmga* (Fig. 6B).

EMSA reactions containing the wild-type Mga (Fig. 6A, lanes 2 to 4, 12, and 13, and Fig. 6B, lanes 2 to 3) and mutant Mga proteins Q11R (Fig. 6A, lanes 5 to 7, and Fig. 6B, lanes 4 to 5) and E14A (Fig. 6A, lanes 17 to 19, and Fig. 6B, lanes 10 to 11) showed reduced mobility of both promoter probes. However, neither W12R (Fig. 6A, lanes 14 to 16, and Fig. 6B lanes, 6 to 7) nor R13A (Fig. 6A, lanes 8 to 10, and Fig. 6B, lanes 8 to 9) were able to bind at wild-type levels to either of the two probes. Thus, two of the CMD-1 mutants (W12R and R13A) were defective for binding to DNA targets, whereas two other flanking CMD-1 mutants (Q11R and E14) exhibited normal DNA-binding activity. Therefore, a loss of DNA binding alone does not explain the defect in transcriptional activation observed with some CMD-1 mutations.

DISCUSSION

HTH-4 and CMD-1 are functional domains of Mga. Despite its established role in the pathogenesis of GAS infections, we currently know very little about how Mga functions. Previous studies using a serotype M6 Mga (*mga-1*) identified a minor (HTH-3) and a major (HTH-4) helix-turn-helix DNA-binding domain within the amino terminus of Mga (16). Importantly, mutations in HTH-4 led to a defect in M6 Mga-dependent

activation in vivo and established DNA binding as an essential function of Mga. Finding additional functional domains in Mga would provide insights into its mechanism of action.

To address this issue, we utilized a genetic screen to look for random mutations in the serotype M6 Mga (*mga-1*) that lead to a defect in its ability to activate Mga-regulated gene transcription in vivo. The screen identified both the HTH-3 and HTH-4 DNA-binding domains of M6 Mga (Fig. 2), providing further evidence of their importance and acting as a strong validation of the screen. The analysis also revealed two amino acids (Q11 and W12) involved in M6 Mga activity that were conserved among divergent Mga proteins in the GAS and orthologues found in other pathogenic streptococci. Site-directed mutagenesis within the conserved Mga domain 1 (CMD-1; amino acids 10 to 15) verified the importance of CMD-1 for transcriptional activation of M6 Mga-regulated genes (*emm*) as well as its own autoregulation (*mga*) in this background (Fig. 3). Therefore, CMD-1 represents a new Mga functional domain involved in its ability to regulate virulence gene expression in the GAS.

To date, most of the functional studies performed on Mga have been done using the serotype M6 (*mga-1*) allele (16, 18). Thus, it was important to see if functional domains identified in the M6 Mga were also required in a divergent M4 Mga (*mga-2*). Our recent studies have found that some amino acids im-

portant for M4 Mga (*mga-2*) activity did not serve the same function in a divergent M6 Mga (*mga-1*) (27). However, site-directed mutations in both the HTH-4 and CMD-1 domains of the M4 Mga (*mga-2*) resulted in a protein that was unable to activate transcription of the M4 Mga-regulated gene *mip* (Fig. 4). Therefore, both motifs appear to serve similar roles in divergent *mga* alleles and represent conserved functional domains in all Mga proteins.

Functional role for CMD-1 in Mga activity. Given that CMD-1 is important for Mga-dependent transcriptional activation, exactly how the domain contributes to Mga function is of particular interest. The mutational analysis provided some clues as to the role of specific residues. It was clear from the mutagenesis of CMD-1 that mutations in either of the two amino acids (position 10 and 15) flanking the conserved region showed diminished activation due to low steady-state protein levels (Fig. 3C), suggesting that these two residues are likely important in overall Mga stability. Most alanine substitutions within the rest of CMD-1 in M6 Mga resulted in a significant decrease in activity by as much as 83% for *Pemm* and 65% for *Pmga* without affecting the levels of protein (Fig. 3AB). Similar trends were seen for those CMD-1 mutations tested in the divergent M4 Mga, although differences in the degree of reduction were observed for the Q11R mutation (Fig. 4). One M6 Mga mutant (W12A) did not show a decrease in transcription at either promoter; however, when W12 was mutated to an arginine instead of an alanine, a loss of activity was observed. This implies that, at least for this residue in CMD-1, the ability of Mga to activate transcription is contingent upon the particular amino acid at that position, possibly reflecting a charged or polar interaction.

Because DNA binding is essential for Mga activity (16), CMD-1 M6 Mga mutants were tested for their ability to bind probes corresponding to the Mga-regulated *Pemm* and *Pmga* promoters. It was expected that all CMD-1 mutations would be either wild type or defective in their ability to bind to regulated promoter targets. Surprisingly, two of the four transcriptionally defective mutants tested (Q11R and E14A) retained the ability to bind DNA, while the other two mutants (W12R and R13A) did not (Fig. 6A and B). The inability of the W12R mutant to bind normally may be dependent upon the amino acid change at that position as discussed above. Since a W12A mutation at this position shows wild-type Mga activity (Fig. 3A and B), it is predicted to retain normal binding activity as well. The other binding mutant, R13A, occurs at an arginine residue that is 100% conserved among Mga orthologues (Fig. 2), and we can only speculate what effects different mutations at R13 would have on the ability to bind or activate Mga-regulated promoters. Therefore, at least two residues in CMD-1 are important for DNA binding in addition to the established HTH-3 and HTH-4 domains.

The ability of two mutants (Q11R and E14A) to bind DNA normally while still being defective in transcriptional activation *in vivo* clearly suggests that portions of CMD-1 contribute to Mga activity independently of DNA binding. Secondary-structure predictions (Jpred; <http://www.compbio.dundee.ac.uk/~www-jpred>) suggest that CMD-1 is part of an alpha-helix. Mutants at positions 11 and 14 not only demonstrate a similar phenotype, but also would reside on the same face of the helix. This aspect of CMD-1 potentially represents a novel function for this regulator, and

further study may provide us with new insights into the mechanism of Mga regulation.

Autoregulation and divergent Mga proteins. The M6 Mga (*mga-1*) has been shown to bind directly to its own promoter, resulting in activation of *mga* expression and amplification of the Mga response (7, 16, 18). In this study, constitutive expression of M6 Mga CMD-1 and HTH-4 mutants unable to activate *Pemm* also demonstrated a corresponding defect in autoactivating *Pmga* (Fig. 3AB). Furthermore, expression of several CMD-1 M6 Mga mutants from their native M6 *Pmga* did not produce wild-type levels of protein (Fig. 3D), further supporting a direct role of an active Mga in its own regulation.

Interestingly, the loss of Mga activity caused by the same CMD-1 mutations as well as the HTH-4 mutation in the divergent M4 Mga (*mga-2*) had no effect on their autoregulated expression from their native M4 *Pmga* (Fig. 4). In fact, we observed a similar lack of autoregulation in a recent study of naturally occurring mutants introduced into M4 Mga expressed from the M4 *Pmga* (27). Therefore, it appears that normal expression of M4 Mga from its own promoter is not dependent on an active Mga. Promoter analysis of *Pmga* from an M49 (*mga-2*) strain found significant differences at the nucleotide level compared to the same region from an M6 (*mga-1*) strain (21), including a nine-nucleotide insert into Mga binding site 2 (16). Previous studies suggested autoregulation in the M49 system based on Northern analysis (21). Given that the M4 and M49 strains share 99.5% sequence identity across *Pmga*, it will be interesting to determine whether all *mga-2* alleles share a common mechanism of *mga* regulation.

Another interesting observation involved the M6 Mga mutant Q11A produced from a constitutive promoter, which was transcriptionally defective only at *Pemm* and not *Pmga* (Fig. 3A and B), suggesting that it is possible to unlink the ability of an M6 Mga to activate downstream promoters and autoactivation. This hypothesis is supported by the differences in the number, size, and location of Mga binding sites at each promoter compared to the start of transcription (14, 16, 18).

Conserved functional domains define an Mga family of virulence regulators. Regions of amino acid conservation found between related proteins will often highlight those areas that are indispensable for function in the cell. In this study, we propose a new family of transcriptional regulators found within various pathogenic streptococcal species, including established virulence regulators from *S. dysgalactiae* and *S. pneumoniae*, based on their sequence homology to Mga proteins from the GAS. Even though individual members can vary considerably from one to another, regions of 70 to 100% identity were observed in the different regulatory proteins. Two such conserved domains, CMD-1 and HTH-4, were subsequently found in our genetic screen as being essential for transcriptional activation in all Mga alleles tested. Based on these results, one would predict that these regions might likely serve similar roles in other members of this family. Using the Mga-like transcriptional regulator DmgB from *S. dysgalactiae* subsp. *dysgalactiae* to test this hypothesis, we found that mutations in both the conserved HTH-4 and CMD-1 domains resulted in an inactive DmgB, as predicted from our results with Mga (Fig. 5).

Due to technical reasons, our screen only investigated the amino-terminal 150 residues of Mga. Thus, there may be a number of domains in the full-length molecules that will pro-

vide interesting targets for further analysis. Although functional differences are bound to exist among the proteins to correspond with the variations in regulated genes, vast amounts of knowledge applicable to the entire Mga family can be gained through exploration of the conserved regions within family members.

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