Role of *Streptococcus pyogenes* Two-Component Response Regulators in the Temporal Control of Mga and the Mga-Regulated Virulence Gene *emm*

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We examined the role of *Streptococcus pyogenes* two-component response regulators (SptR) in expression of Mga and the Mga-regulated gene *emm*. Both serotype M6 and serotype M1 mutants in 12 of the 13 identified *sptR* genes exhibited levels of *emm* transcripts and Mga protein comparable to those of the wild type during exponential and stationary phases of growth. Thus, temporal control of these virulence genes does not require Spt response regulators.

To successfully colonize and persist within a variety of host tissues, pathogens have developed mechanisms by which they can respond to varying environments through coordinate changes in their patterns of gene expression. Bacteria often mediate such responses by two-component signal transduction systems, a family of phosphorelay proteins known to regulate a wide variety of cellular processes (9, 10). The typical twocomponent signal transduction system is composed of a membrane-bound sensor histidine kinase that detects the specific external signal and transfers a high-energy phosphate to a cognate cytoplasmic response regulator that interacts directly with DNA to control expression of a defined set of genes.

The group A streptococcus (GAS) (*Streptococcus pyogenes*) is an important bacterial pathogen and the causative agent of numerous diseases in its human host (4). GAS has evolved mechanisms that allow it to persist in varying microenvironments by coordinately expressing virulence factors in response to its changing surroundings. Analysis of the published genomes of GAS (M1, M3, and M18) has identified an average of 13 potential S. pyogenes two-component systems (SPTs) (1, 6, 21). Furthermore, 12 of the 13 Spts found in the M1 genome can also be found in M18 and M3, indicating their potential importance for environmental regulation among all class I serotypes of GAS. However, only three of these Spts have been characterized to any degree at the molecular level. The covRS/ csrRS system represses expression of virulence genes encoding capsule and several exotoxins, as well as influencing the transcription of as much as 15% of the M6 GAS genome (2, 5, 7, 8, 13). The fasBCA system is a growth-phase-regulated SPT containing two histidine kinase components that uses a fasXeffector RNA to down-regulate genes involved in adhesion and to up-regulate those encoding aggressins during the transition from exponential- to stationary-phase growth (11). Finally, the ihk/irr Spt has been shown to allow survival of GAS following uptake by polymorphonuclear leukocytes (5, 23).

In addition to SPTs, GAS possesses "stand-alone"

global regulators that control large sets of virulence genes in response to both temporal and environmental stimuli. One such regulator is Mga, a DNA-binding protein that activates the expression of virulence genes encoding molecules important for colonization and immune evasion, such as the M protein family (emm, mrp, and enn), C5a peptidase (scpA), and collagen-like protein 1 (scl1) (12). Maximal expression of the Mga regulon occurs during exponential-phase growth and is rapidly shut down upon entering stationary phase (16). The regulon is also up-regulated by growth in elevated CO₂ and iron levels and temperature (3, 14, 19). However, the mechanism by which any of these different conditions regulates expression of mga and its regulon is not known. In this study, 12 of the 13 putative response regulator genes were inactivated in two different class I GAS strains to determine their possible role in the temporal regulation of this important virulence cascade.

KSM148, a derivative of the S. pyogenes serotype M6 strain JRS4 containing a single-copy Pemm-gusA transcriptional reporter (20), was used for insertional inactivation of the response regulator gene (sptR). Open reading frame internal fragments for 12 of the 13 putative sptR genes in the serotype M1 genome (Fig. 1), except for the essential sycF (spt3R) gene, were amplified from M1 SF370 (6) genomic DNA by highfidelity PCR using the primers listed in Table 1. Each PCR fragment was cloned into pCR-TOPO Blunt II (Invitrogen), purified following BamHI-PstI digestion, and subsequently cloned into BamHI-PstI-digested pJRS233, a temperaturesensitive integration vector (18). The 12 mutagenic plasmids (p233-spt1R, -2R, and -4R to -13R) (Fig. 1) were verified by PCR and DNA sequence analysis and integrated into the chromosome of KSM148 as previously described (18). PCR was used to verify the presence of the plasmid backbone, each integrant junction, and the absence of the wild-type sptR gene product using the appropriate primers (Table 1).

To initially characterize each M6 SptR mutant strain, growth curves were determined in Todd-Hewitt broth at 37°C and compared to those of parental KSM148 and *mga*-inactivated KSM148.586 (20). Most of the mutants exhibited rates of growth identical to that of wild-type KSM148, while KSM148.spt5R actually grew slightly faster and KSM148.spt2R (*covR*) dem-

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#	Locus	GAS M1 ORF	Homologs	Plasmid
SPT-1 (<i>fasBCA</i>)	$\xrightarrow{S1}$ $\xrightarrow{S2}$ \xrightarrow{R}	Spy0242,0244, 0245	<i>hk13, rr13</i> (Spn) <i>iH,iR2</i> (Spn)	p233-1R
SPT-2 (<i>covRS, csrRS</i>)	\xrightarrow{R} \xrightarrow{S}	Spy0336,0337	<i>etaRK</i> (Ef) <i>lisRK</i> (Lm)	p233-2R
SPT-3 (sycFG)		Spy0529,0528	yycFG (Bs) vicRS (Spn)	Essential* no plasmid
SPT-4	\xrightarrow{R} \xrightarrow{S}	Spy0874,0875	<i>phoPQ</i> (Bs) <i>trcR</i> (Mtb)	p233-4R
SPT-5	$\xrightarrow{S} \xrightarrow{R} \xrightarrow{R}$	Spy1061, 1062	yesNM(Bs)	p233-5R1
SPT-6 (<i>srtRK</i>)	\xrightarrow{R} \xrightarrow{S}	Spy1082, 1081	nisRK(Ll)	p233-6R
SPT-7	$\xrightarrow{S} \xrightarrow{R} \xrightarrow{R}$	Spy1106,1107	yufML (Bs)	p233-7R1
SPT-8	$\xrightarrow{R} \xrightarrow{S}$	Spy1236, 1237	ciaRH(Spn)	p233-8R1
SPT-9	\xrightarrow{R} $\xrightarrow{S1}$ $\xrightarrow{S2}$	Spy1553,1554, 1556	<i>yesNM (Bs)</i> <i>zmpRS; rr09 hk09</i> (Spn)	p233-9R
SPT-10	$\xrightarrow{S} \xrightarrow{R} \xrightarrow{R}$	Spy1587,1588	<i>YesNM (Bs)</i> rr07, hk07(Spn)	p233-10R
SPT-11	$\xrightarrow{S} \xrightarrow{R} \xrightarrow{R}$	Spy1621,1622	<i>rr03, hk03</i> (Spn)	p233-11R
SPT-12 (<i>salRS</i>)	$\xrightarrow{S} \xrightarrow{R} \xrightarrow{A}$	Spy1908, 1909	salRK (Ssa)	p233-12R
SPT-13 (<i>irr, ihk</i>)	$\xrightarrow{\mathbf{R}}$ $\xrightarrow{\mathbf{S}}$	Spy2026, 2027	<i>phoPQ</i> (Bs) <i>vanSR</i> (Ef) <i>arlSR</i> (Sa)	p233-13R

FIG. 1. Thirteen loci encoding known or putative *S. py*ogenes SPTs in the serotype M1 SF370 genome. The SPT loci were numbered based on their order of appearance in the M1 genome sequence, with any previously assigned names provided in parentheses. A schematic representation of the gene positions for each SPT loci (R, response regulator; S, sensor histidine kinase) is shown, along with their respective open reading frame numbers (Spy) based on the original annotation by Ferretti et al. (6). Each insertion-duplication mutation within each *sptR* gene is indicated (Δ). The homologs provided for each SPT locus represent those found in other gram-positive genomes (Bs, *Bacillus subtilis*; Spn, *Streptococcus pneumoniae*; Ef, *Enterococcus faecalis*; Lm, *Listeria monocytogenes*; Mtb, *Mycobacterium tuberculosis*; Ll, *Lactococcus lactis*; Ssa, *Streptococcus salivarus*; Sa, *Staphylococcus aureus*) that exhibit the highest similarity at the amino acid level to the respective locus. The names of the temperature-sensitive mutagenic plasmids used in the study to inactivate each *sptR* gene are listed on the right of the figure, excluding one for the essential *spt3R* locus.

onstrated an extended lag phase prior to reaching comparable growth rates (data not shown). The ability to obtain mutants for each of the 12 targeted *sptR* genes with no obvious effects on growth demonstrated that none of 12 two-component loci tested is essential for growth of GAS under normal laboratory conditions.

Since *mga* expression is optimally expressed during exponential phase and repressed during stationary phase, it was expected that the loss of an Spt necessary for temporal regulation would produce a significant decrease in expression of Mgaregulated *emm* during exponential phase and/or a dramatic increase in expression during stationary phase. Liquid GusA assays were performed, with each mutant strain isolated at exponential phase of growth and compared to both the wild-type KSM148 and *mga*-inactivated KSM148.586. All of the mutants exhibited Mga-regulated GusA activity equal to or slightly above the levels observed for wild-type KSM148 (Fig. 2A), with none showing decreased GusA activity during exponential-phase growth comparable to that of the *mga*-inactivated control, KSM148.586 (Fig. 2A). To verify that the levels

TABLE 1. Primers used in this study

spt1R (fasA) spt1R-L TACCATGTAATCAGCTTGAA This study spt1R.R AACAAACTTTGTGAGGAGT This study fasA-R1* GGATTGATTGCTCGATAAA This study spt2R (covR) covR-L TAGTGAGGAGAAAACCAATCGATG This study spt2R (covR) covR-L TAGTGAGGAGAAAATCTCATCG 5 covR-R TATGAAGTCATTGTTGAAGGT 5 covR-R1* AGGCCAATCAGTGTAAAGGCA This study spt4R spt4R-R CGGTCAAGAGGCAATTGATGTTACCAT This study spt4R-R1* GCATCTTGTGAGTTTACCAT This study spt4R-R1* spt4R-R1* GCATTTGGTGAGTTTACCAT This study spt5R-R2* GCCATTTGCGTTTGAGTTTACCAT This study spt5R-R2* GCCATTTGCGTTTGAGGTTTT This study spt6R-R2 TGACGTGACAGTAATTCTAA This study spt6R-R2 TAACCACATTATGAGACA This study spt6R-R2* TAACCACATTATGAGGACA This study spt6R-R2* TAACACCATTATGAAGCA This study spt7R spt7R-R2 TCTCAGTGACACACATTATGAG This study spt7R-R2 TCTCAGTGACACACACTTAT This study
spt1R-R AACAAACTTTGTGAAGAGT This study fasA-R1* GGGATTGATTGCTCGATAAA This study spt2R (covR) covR-L TAGTGAGAGAAAACCAATCGTGT This study spt2R (covR) covR-R TATGAAGTCATTGTGAGGT 5 covR-R1* AGGCACAAAAACCAATCGTGT This study spt4R Spt4R-L AAGGCAATCAGTGTAAAGGCA This study spt4R-R1 CGGTCAAGAGGCAATTGATA This study spt4R-R1* GGGTAATATTTCTTGTTGACT This study spt4R-R1* GCGTTCAAGAGGCAATTGATA This study spt4R-R1* GCGTTCAAGAGGCAATTGATA This study spt4R-R1* GCGATTGGTGTGAGTATTTACC This study spt4R-R2* GCCATTGCGTTTGAGTTTTAATC This study spt5R-R2 TTCGGGAATTGATGCTCATC This study spt6R-R2* TAACAACCCCATTAATCAA This study spt6R-R2* TAACAACTCCCATTAAATCCA This study spt7R-R2 TCTCAGTGACACACCATATGAAACCA This study spt7R-R2 TCTCAGTGACAACCCTTAT This study spt7R-R2 TCTCAGTGACAACCCTTAT This study spt7R-R2 CCTCAGTGACAACCCTT
fasA-R1* GGGATTGATTGCTCGATAAA This study spt2R (covR) covR-L TAGGAGAAAACCAATCGTGT This study covR-R TATGAAGTCATTGTTGAGGT 5 covR-R1* AGGCAATCAGTGTAAAGGCA This study spt4R AGGCAATCAGTGTAAAGGCA This study spt4R-L AAGGCCATTAATTTACCTTC This study spt4R-R1* GCGTCAAGAGGCAATTGATA This study spt4R-R1* GCGTCAAGAGGCAATTGATA This study spt4R-R1 CGGTCAAGAGGCAATTGACT This study spt4R-R1* GCATCTGTGTGTTACCAT This study spt5R spt5R-R1 CCTGATTAATTCTGTGACT This study spt5R-R2* GCCATTGGCGTTTGAGCTTT This study spt5R-R2* GCCATTGCGGTATGAAGCA This study spt6R-R TGACGTGACAGTAATTCTAA This study spt6R-R2* TAACATCCCATTAGAAGCA This study spt6R-R2* TAACACTCCCATTAAACC This study spt7R-R2 TCTCAGTGACAACACCTTAT This study spt7R-R2 TCTCAGTGACAACACCTAAT This study spt7R-R2 TCTCAGGAACACACCTTAA This study
fasA-L1 AGCACAAAAACCAATCGTGT This study spt2R (covR) covR-R TAGTGAAGAAAATCTCATTGT 5 covR-R TATGAAGTCATTGTTGAGGT 5 covR-R1* AAGCCATTAATTTGCAAGGCA This study spt4R spt4R-R CGGTCAAGAGGCAATTGATA This study spt4R-R CGGTCAAGAGGCAATTGATA This study spt4R-R1* GCATCTTGTGAGGTTACCAT This study spt4R-R1 CGGTCAAGGGCAATTGATA This study spt5R-R1 CCGGTTAATTTCTTGTGAGTTT This study spt5R-R1 CCGGTAAGGCAATTGAAGCC This study spt5R-R2* GCCATTTGCGTTTGAGTTTT This study spt5R-R1 CTGAAGGAACTCAATTCTAA This study spt6R-R TGACGTGACAGTAATTCTAA This study spt6R-R2* TGACGGAACTAATTCTAA This study spt6R-R2* TAACAAGCGGAACTAATTCTAA This study spt7R spt7R-R1 ATCCAGCGAACTCAATTCAAATTCCTAA This study spt7R-R2 TCTCAAGTGACAACACCCTAA This study spt8R-R1 spt8R-R1 TGAAGGTTATAGGAACACACCCAAA This study spt8R-R2* GGCTATTAGCC
spt2R (covR) covR-L TAGTGAGAGAAATCTCATCG 5 covR-R TATGAAGTCATTGTTGAGGT 5 covR-R1* AGGCAATCAGTGTAAAGGCA This study spt4R spt4R-L AAGGCCATTAATTTACCTTC This study spt4R-R CGGTCAAGAGGCAATTGATA This study spt4R-R1* GCATCTTGTGATGTTACCAT This study spt4R-L1 CGGTTAATTTCTTGTTGACT This study spt5R-R1* GCATCTTGGGATTAATCCATTGAT This study spt5R-R2* GCCATTGGGTTTGAGTTTT This study spt6R-12 ATCTGGAAGCTATGATGACAC This study spt6R-R2* GCATTTGAGAGAGAAACCTCT This study spt6R-R2* TAACAGTCCATTAAATCCAA This study spt6R-R2* TAAACATCCCATTTAAAGAA This study spt6R-R2* TAAACATCCCATTTAAAGAA This study spt6R-R2* TAAACATCCCATTAAAGAA This study spt7R-R2 TCTCAGGGAACACACCTTAT This study spt7R-L2* GTTTATGACAATTCGAACACCACTAT This study spt7R-L2* GTTTATACGAAACACCTATA This study spt8R-R1 TACAAGGGTTAAGGACACAATA This study
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spt4R-R1*GCATCTTGTGATGTTACCATThis studyspt4R-L1CGGTTAATTCTTGTTGACTThis studyspt5R-R1CCTGATTAATCCGATATTGAThis studyspt5R-R2*GCCATTTGCGTTTGAGTTTThis studyspt5R-R2*GCCATTTGCGTTGAGTTTTThis studyspt6Rspt6R-R2*TGACGTGACAGTAATGAAGACThis studyspt6R-R2*TGACGTGACAGTAATTCTAAThis studyspt6R-R2*TAAACATCCCATTTATAAACCThis studyspt7Rspt7R-L1ACAAGCGGAAGCTAATTCTAThis studyspt7Rspt7R-R2TCTCAGTGACAACACTTATGAAAGThis studyspt7R-R2TCTCAGTGACAACACCTATThis studyspt7R-R2TCTCAGTGACAACACCTATThis studyspt7R-R2GTTATGACAACATCCCATAThis studyspt7R-R2GTCATGGACAACACCTATThis studyspt8Rspt8R-L1ATCACTATCGAATCCCCATAThis studyspt8Rspt8R-L2AAACTACTGTCAAATGGGCTThis studyspt8Rspt8R-R1TGAAGGTTTAACCAAGGAGThis studyspt8Rspt8R-R2*GGCTATTAGCCTCTAAATAThis studyspt9Rspt8R-R2*GGTATTAGCCTCTAAATAThis studyspt10RSpt10R-R1*TTGAGGAAGCTAAGGGCTATThis studyspt10RSpt10R-R1*TGGGGAGTGTCATGATGAGGGThis studyspt11R-R1*AGGCATTAAACCGTATCTThis studyspt11R-R1*AGGCGTGTGGACCAAGGGGCTATThis studyspt11R-R1*AGGCATATAACCCGTATCTTThis studyspt12R-R1*TAGGAGGTGTGAGAGGGGGGGGGGGGTThis studyspt12R-R1*
spt3Rspt4R-L1CGGTTAATTTCTTGTTGACTThis studyspt5Rspt5R-L1AAGAAGGAGCTGTTTTAATCThis studyspt5R-R2*GCCATTGCGTTGAGTTTTThis studyspt5R-R2*GCCATTGCGTTGAGTCTATThis studyspt6Rspt6R-LATTCTGAAGCTAATGAAGACThis studyspt6R-R2*GCATTGGGTGACAGTAATTCTAThis studyspt6R-R2*TAAACATCCAATTAAACCThis studyspt7Rspt7R-L1ACAAGCGGAAGCTAATTCTTThis studyspt7R-R2TCTCAGTGACAACACCCTATThis studyspt7R-R2TCTCAGTGACAACACCCTATThis studyspt7R-R2GTTATGACAATTAGGGTCThis studyspt8Rspt7R-L2*GTTATGCAAATCGCAATTAGGGTCThis studyspt8Rspt8R-R1TGAAGGTTTATACGAAGCAGThis studyspt8Rspt8R-R1TGAAGGTTATACCAACTGACAGAThis studyspt9Rspt7R-R2GCCATTAGCAACTGTCAAATAThis studyspt8Rspt8R-R1TGAAGGTTTATACGAAGCAGThis studyspt8R-R1TGAAGGTTATAGCCTCTAAATAThis studyspt9Rspt7R-R2GTCATTAGCCAGTCAACTGACGAThis studyspt9R-R1TTAATCAAGAGCTAAAGGGTAAThis studyspt9R-R2GTGTATTGAGCAGTCAAAGGGCTAAThis studyspt10Rspt10R-R1*TTGGTAGCAGTCAAGGGCTATThis studyspt10R-R1*TTGAGGGAGTCAAGGAGCThis studyspt11R-R1*AGCGATATAACCGTATCTTThis studyspt11R-R1*AGGCGATATAACCGTATCTTThis studyspt11R-R1*AGCGATATAACCGTATCTTThis study <td< td=""></td<>
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spt8R-L2 AAACTACTGTCAACTGACGA This study spt8R-R2* GGCTATTAGCCTCTTAAATA This study spt9R spt9R-L TTTTAATCAAGACGCTAAGG This study spt9R GTTGATTTAGCCAGTTCAA This study spt9R-R GTTGATTTAGCCAGTCAA This study spt9R-R1* ATATGGAAGCTAAGGGCTAT This study spt9R-L1 TACGAGAACTCGTCTGGTAA This study spt10R spt10R-L AGTGGATGTCATGATTTCAG This study spt10R-R CGCTTTTGTAGATCATAGGT This study spt10R-R1* TTGGTAGCCAGTCATGATGTCT This study spt10R-R1* TCAGGGTGTTGAGATGAGAGG This study spt11R-R CGCCATGGGACTCAAGAGT This study spt11R-R GTCAGGGTGTTGATCATGCG This study spt11R-R GTCAGGGTGTTGATCATGGG This study spt11R-R1* AGGCATATAACCCGTATCTT This study spt12R spt12R-R1* TTCCGGTCAGTTGATGATGG This study spt12R Spt12R-R1 TTCCGGTCAGTTAAGATG This study spt12R-R1 TTCCGGTCAGTTAAAGATT This study spt12R-R1 TTCCGGTCAGTAAACACTAGATG This study spt12R-R1 TTCCGGTCAGTTAAAGATT This study spt12R-R1 TTCCGGTCAGTTAAAGATT<
spt9R Spt8R-R2* GGCTATTAGCCTCTTAAATA This study spt9R spt9R-L TTTTAATCAAGACGCTAAGG This study spt9R-R GTTGATTTAGCCAGTCAA This study spt9R-R1 ATATGGAAGCTAAGGGCTAT This study spt9R-R1 ATATGGAAGCTAAGGGCTAT This study spt9R-R1 TACGAGAACTCGTCTGGTAA This study spt10R spt10R-L1 AGTGGATGTCATGATTTCAG This study spt10R-R1* TTGGTAGCCAGTCATGATT This study spt10R-R1* TTGGTAGCCAGTCATGAGATC This study spt10R-R1* TCAAGGCTTTAGATGAGAGCG This study spt11R-R1* TCCGCATGGGACTCAAGAGT This study spt11R-L1 TCCGCATGGGACTCAAGAGT This study spt11R-R1* AGCAATATAACCCGTATCTT This study spt12R spt12R-R1* TTCCGGTCAGTTGATGATGG This study spt12R spt12R-R1 TCCGGTCAGTATAACCCGTATCTT This study spt12R TTCCTGGTCAGTAACACACTGTT This study spt12R-R1* TTCCTGGTCAGTTAAAGATT This study spt12R-R1 TTCCTGGTCAGTATAACCCGTT This study spt12R TTCCTGGTCAGCACACAACTATGATG This study spt12R-R1 TTCCTGGTCAGTTAAAGATT This study spt
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spt9R-R GTTGATTTTAGCCAGTTCAA This study spt9R-R1* ATATGGAAGCTAAGGGCTAT This study spt9R-L1 TACGAGAACTCGTCTGGTAA This study spt10R spt10R-L AGTGGATGCATGGATTCAG This study spt10R-R1* CGCTTTGTAGATCATAGGT This study spt10R-R1* TTGGTAGCCAGTCATGGTT This study spt10R-R1* TTGGTAGCCAGTCATGGGT This study spt10R-L1 TCAAGGCTTAGATCATAGGT This study spt11R-R1* TTGGTAGCCAGTCAAGAGT This study spt11R-R1* GTCAGGGGTGTGACAGAGGT This study spt11R-L1 TCCGCATGGGACTCAAGAGT This study spt11R-L1 TTGAAGAGTGATGATGATGG This study spt12R-R1* AGCAATATAACCCGTATCTT This study spt12R-R1* TTCCTGGTCAGTTAAAGATG This study spt12R-R1* TACCAGGACACAACTATCCAACTGTT This study spt12R-R1* TACCAGGACACAACAACTATCAACATT This study spt12R-R1* TACCAGGACACAAGTATAACCGTT This study spt12R-R1* TACCAGGACACAAGTAACAAT This study spt12R-R1* TACCAGGACACAAGTAAAAAAAAAAAAAAAAAAAAAAAA
spt9R-R1* ATATGGAAGCTAAGGGCTAT This study spt9R-L1 TACGAGAACTCGTCTGGTAA This study spt10R spt10R-L AGTGGATGTCATGATTTCAG This study spt10R-R1* CGCTTTTGTAGATCATAGGT This study spt10R-R1* TTGGTAGCCAGTCATGATGTC This study spt10R-L1 TCAAGGCTTAGATCATAGGT This study spt10R-L1 TCAAGGCTTAGATCATGAGACG This study spt11R Spt11R-R GTCAGGGACTCAAGAGT This study spt11R-R1* AGCCATATAACCGTATCTT This study spt11R-R1* AGCCATATAACCCGTATCTT This study spt12R-R1* AGGCATATAACCCGTATCTT This study spt12R-R1* TTCCAGGACACAAGTATCCAACTGTT This study spt12R-R1* TACCAGGACACAACTAGCATT This study spt12R-R1* TACCAGGACACAAGTATCCAACTGTT This study spt12R-R1* TACCAGGACACAAGTAACATT This study spt12R-R1* TACAAGAACACTAGCACTAACAAT This study spt13R (irr) irr-L GGTGACGTTTGCCAAATAA 5
spt10R spt9R-L1 TACGAGAACTCGTCTGGTAA This study spt10R-L AGTGGATGTCATGATTTCAG This study spt10R-R CGCTTTTGTAGATCATAGGT This study spt10R-R1* TTGGTAGCCAGTCATAGGT This study spt10R-L1 TCAAGGCTTTAGATGAGACG This study spt10R-L1 TCCGCATGGGACTCAAGAGT This study spt11R spt11R-L TCCGCATGGGACTCAAGAGT This study spt11R-R GTCAGGGTGTTGATCATGCG This study spt11R-R1* AGGCATATAACCCGTATCTT This study spt12R Spt12R-L1 TTGAAGATGATGATGATGG This study spt12R TACCAGCACAACTACACTGTT This study spt12R-L1 TCCTGGTCAGTTAAAGATT This study spt12R-R1* TAGCAACGACACAAGTAAAA This study spt12R-R1* TAGCAACGACACAAGTAAAA This study spt12R-R1* TAGCAACGACACAAGTAAAA This study spt12R-R1* TAGCAACGACACAAGTAAAA This study spt13R (irr) irr-L GGTGACGTTTGCTAAGAGCT This study
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spt10R-R1* TTGGTAGCCAGTCACTGTCT This study spt10R-L1 TCAAGGCTTAGATGAGACG This study spt11R-L TCCGCATGGGACTCAAGAGT This study spt11R-L TCCGCATGGGACTCAAGAGT This study spt11R-R1* GTCAGGGTGTTGATCATGCG This study spt11R-L1 TTGAAGAGTGATCATGCG This study spt12R-L1 TGAAGAGTGATTGATGATGGT This study spt12R-L1 TGAAAGAGTATCCCAACTGTT This study spt12R-R1* AGGCAATATAACCCGTATCTT This study spt12R-L1 TTGAAGAGAGTATCCCAACTGTT This study spt12R-R1* TACCAAGGACACAAGTATCCAACTAT This study spt12R-R1* TAGCAACGACACAAGTAAAA This study spt12R-R1* TAGCAACGACACAAGTAAAAAAAAAAAAAAAAAAAAAAA
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spt13R (irr) irr-L GGTGACGTTTTGCTAAATAA 5
irr-R AAAGCGAATAACTATGATCC 5
irr-R1* TGTCTTTGGACTATTACCAG This study
irr-L1 CTTCTTTGTCTTTGACTTTG This study
M13 1201 AACAGCTATGACCATGATTACG Clontech
1211 GTAAAACGACGGCCAGT Clontech
pWV01 ori pLZ12ori-L TTATATCCTGACTCAATTCC This study
pLZ12ori-R CTCAAACCATAATCTAAAGG This study
emm OM6-35 AACAGCAAATTAGCTGCTC 15
OM6-16 GTTTCCTTCATTGGTGCT 15
23S rRNA rRNA_23S-L GGAAGGTAAGCCAAAGAGAG 20
rRNA_23S-R TCCTAGTTTCTGTGCAACC 20

^{*a*} Boldface indicates wild-type *sptR* gene pair. An asterisk indicates integrant junction primer.

of GusA activity observed reflected Mga-regulated gene expression, Northern blot analysis using an *emm6* probe was carried out, and the results were normalized using a probe for 23S RNA as a control for loading (20). As observed in the

GusA assays, each of the 12 SPTR mutant strains showed levels of Mga-regulated *emm6* transcripts similar to those of wild-type KSM148 (Fig. 2B). In addition, the Mga protein was found in each mutant strain lysate at levels comparable to that for the wild type, as determined by Western blot analysis (Fig. 2B) (15). These data clearly demonstrate that none of the 12 *sptR* genes examined are necessary for exponential-phase expression of Mga or *emm* in the serotype M6 GAS strain KSM148.

To investigate whether the results obtained with the M6 strain were serotype specific, we inactivated the comparable *sptR* genes in the sequenced serotype M1 strain SF370 (6, 22)as described for KSM148. Growth curves for the M1 mutants were almost identical to those observed for the M6 KSM148 mutants (data not shown), demonstrating that these response regulators are not required for in vitro growth in rich medium with either serotype. Furthermore, inactivation of spt2R (covR/ csrR) in SF370 and KSM148 resulted in a colony phenotype on agar plates that was highly mucoid compared to that of the parental strain, as expected (data not shown) (13). Since a Pemm-gusA reporter fusion is not available in the SF370 background, Northern blot analysis using an emm1 probe was performed, and the results were normalized using a probe for 23S RNA as a control for loading. Each of the 12 SPTR mutant strains showed levels of Mga-regulated emm1 transcripts comparable to those for wild-type SF370 and significantly higher than the levels seen for mga-inactivated KSM165-L (Fig. 2C). Therefore, there is no major requirement for SPTRs in exponential-phase expression of Mga or the Mga-regulated emm gene in either of the two class I strains of GAS.

As GAS cells enter into stationary-phase growth, a dramatic decrease in the levels of mga and emm is observed that is mediated through the mga promoter (16, 17). To investigate whether SPTRs may be involved in negatively influencing expression of *emm* late in the growth cycle, Northern blot analysis using an emm6 probe was performed. Total RNA from the 12 M6 KSM148 sptR mutants, wild-type KSM148, and mgainactivated KSM148.586 was isolated at 6 h after reaching stationary phase (90 to 100 Klett units), and results were normalized using a probe for 23S RNA as a control for loading. Comparison of emm transcript levels from previously isolated logarithmic-phase cells (1 µg of RNA) to levels in stationaryphase cells (5 µg of RNA) clearly shows the expected dramatic reduction in Mga-regulated gene expression following entry of GAS into the later phase of growth (Fig. 3A). Importantly, no detectable increase in emm transcripts during stationary-phase growth was observed for any of the sptR mutant strains compared to levels for wild-type KSM148 (Fig. 3A). Levels of Mga protein detected in the same stationary-phase lysates were similar to wild-type levels as assessed by Western blotting using an Mga-specific antibody (Fig. 3B). Interestingly, comparable

FIG. 2. Analysis of exponential-phase Mga-regulated gene expression in serotype M6 KSM148 *sptR* mutants. (A) GusA activity of whole-cell lysates from wild-type KSM148 (148), *mga*-inactivated KSM148.586 (586), and the 12 *sptR* mutants (1R, 2R, and 4R to 13R). Whole-cell lysates from exponential-phase cells were examined in a liquid GusA assay for the production of β -glucuronidase. (B) Northern analysis (1 μ g of total RNA) of serotype M6 KSM148 *sptR* mutants for *emm6* transcript levels (top). Northern blots were stripped and reprobed for 23S RNA as a control for loading. Mga protein levels (bottom) in each lysate were determined by Western blotting (7 μ g of total protein), with an antibody to a peptide of Mga used as a probe. (C) Northern analysis (1 μ g of total RNA) of serotype M1 SF370 *sptR* mutants for *emm1* transcript levels (top). Northern blots were stripped and reprobed for 23S RNA as a control for loading (bottom).





В.



C.





Β.



FIG. 3. Analysis of stationary-phase Mga-regulated gene expression in serotype M6 KSM148 *sptR* mutants. (A) Northern analysis of wild-type KSM148 (148), *mga*-inactivated KSM148.586 (586), and the 12 *sptR* mutants (1R, 2R, and 4R to 13R). Total RNA (1 μ g of total RNA for exponential-phase KSM148; 5 μ g of total RNA for all stationary-phase samples) was probed for *emm6* transcript levels. Northern blots were stripped and reprobed for 23S RNA as a control for loading. (B) Mga protein levels in each stationary-phase lysate were determined by Western blotting (7 μ g of total protein), with an antibody to a peptide of Mga used as a probe.

levels of protein were observed in both exponential- and stationary-phase cells, indicating that Mga is stably maintained in the cell during times when transcription of Mga-regulated genes is absent (Fig. 3A). Taken together, these data strongly suggest that repression of Mga-regulated *emm* transcription upon entry into stationary phase does not require the involvement of the SPTR response regulators studied.

Since spt3RS (sycFG) (Fig. 1) appears to be essential for growth in GAS, its role in Mga regulon expression was not investigated in these studies and we cannot rule out that this two-component locus may be involved in regulating expression of mga and emm in response to growth-phase signals. Additionally, the apparent lack of input from sptR components in the growth-phase regulation of mga expression does not rule out the possibility that Mga itself may directly interact with one of the sensor kinase components (sptS), resulting in a modification of Mga activity. Although half of our sptR mutations are likely to confer a polar effect on its downstream sptS gene (Fig. 1), we did not directly ask whether the cognate sptS genes in the remaining loci were required for Mga-regulated gene control. Therefore, the direct interaction of Mga with a sensor kinase may still represent a valid model for temporal regulation of the pathway. Finally, the potential role of SPTs in the response of the Mga pathway to other stimuli, such as CO_2 levels and temperature, remains to be investigated.

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