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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 two-component 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 *fasX* effector 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 CO_2 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 P*emm-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 high-fidelity 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 temperature-sensitive 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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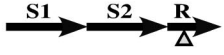
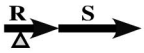
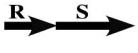
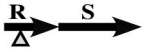
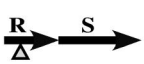
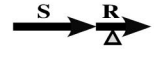
#	Locus	GAS M1 ORF	Homologs	Plasmid
SPT-1 (*fasBCA*)		Spy0242, 0244, 0245	*hk13, rr13* (Spn) *iH,iR2* (Spn)	p233-1R
SPT-2 (*covRS, csrRS*)		Spy0336, 0337	*etaRK* (Ef) *lisRK* (Lm)	p233-2R
SPT-3 (*sycFG*)		Spy0529, 0528	yycFG (Bs) vicRS (Spn)	Essential* no plasmid
SPT-4		Spy0874, 0875	*phoPQ* (Bs) *trcR* (Mtb)	p233-4R
SPT-5		Spy1061, 1062	*yesNM* (Bs)	p233-5R1
SPT-6 (*srtRK*)		Spy1082, 1081	*nisRK* (Ll)	p233-6R
SPT-7		Spy1106, 1107	*yufML* (Bs)	p233-7R1
SPT-8		Spy1236, 1237	*ciaRH* (Spn)	p233-8R1
SPT-9		Spy1553, 1554, 1556	*yesNM (Bs)* *zmpRS; rr09 hk09* (Spn)	p233-9R
SPT-10		Spy1587, 1588	*YesNM (Bs)* *rr07, hk07* (Spn)	p233-10R
SPT-11		Spy1621, 1622	*rr03, hk03* (Spn)	p233-11R
SPT-12 (*salRS*)		Spy1908, 1909	salRK (Ssa)	p233-12R
SPT-13 (*irr, ihk*)		Spy2026, 2027	*phoPQ* (Bs) *vanSR* (Ef) *arlSR* (Sa)	p233-13R

FIG. 1. Thirteen loci encoding known or putative *S. pyogenes* 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 (△). 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 Mga-regulated *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

Target	Primer[a]	Sequence (5′–3′)	Reference
spt1R (*fasA*)	spt1R-L	TACCATGTAATCAGCTTGAA	This study
	spt1R-R	AACAAACTTTGTGAGGAGT	This study
	fasA-R1*	GGGATTGATTGCTCGATAAA	This study
	fasA-L1	AGCACAAAAACCAATCGTGT	This study
spt2R (*covR*)	covR-L	TAGTGAGAGAAATCTCATCG	5
	covR-R	TATGAAGTCATTGTTGAGGT	5
	covR-R1*	AGGCAATCAGTGTAAAGGCA	This study
	covR-L1	AATCCTTTTGCTAGCTTGCA	This study
spt4R	spt4R-L	AAGGCCATTAATTTACCTTC	This study
	spt4R-R	CGGTCAAGAGGCAATTGATA	This study
	spt4R-R1*	GCATCTTGTGATGTTACCAT	This study
	spt4R-L1	CGGTTAATTTCTTGTTGACT	This study
spt5R	spt5R-L1	AAGAAGGAGCTGTTTTAATC	This study
	spt5R-R1	CCTGATTAATCCGATATTGA	This study
	spt5R-R2*	GCCATTTGCGTTTGAGTTTT	This study
	spt5R-L2	TTCGGGAATTGATGCTCATC	This study
spt6R	spt6R-L	ATTCTGAAGCTTATGAAGAC	This study
	spt6R-R	TGACGTGACAGTAATTCTAA	This study
	spt6R-L2	AGAGATCTTGAGTGATAGCA	This study
	spt6R-R2*	TAAACATCCCATTTATAACC	This study
spt7R	spt7R-L1	ACAAGCGGAAGCTAATTCTT	This study
	spt7R-R1	ATCCAGCGACTCTATGAAAG	This study
	spt7R-R2	TCTCAGTGACAACACCTTAT	This study
	spt7R-L2*	GTTTATGACAATTTAGGGTC	This study
spt8R	spt8R-L1	ATCACTATCGAATCCCCATA	This study
	spt8R-R1	TGAAGGTTTATACGAAGCAG	This study
	spt8R-L2	AAACTACTGTCAACTGACGA	This study
	spt8R-R2*	GGCTATTAGCCTCTTAAATA	This study
spt9R	spt9R-L	TTTTAATCAAGACGCTAAGG	This study
	spt9R-R	GTTGATTTTAGCCAGTTCAA	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*	TTGGTAGCCAGTCACTGTCT	This study
	spt10R-L1	TCAAGGCTTTAGATGAGACG	This study
spt11R	spt11R-L	TCCGCATGGGACTCAAGAGT	This study
	spt11R-R	GTCAGGGTGTTGATCATGCG	This study
	spt11R-R1*	AGGCATATAACCCGTATCTT	This study
	spt11R-L1	TTGAAGATGATTGATGATGG	This study
spt12R	spt12R-L	GCAAAGAGTATCCAACTGTT	This study
	spt12R-R	TTCCTGGTCAGTTAAAGATT	This study
	spt12R-R1*	TAGCAACGACACAAGTAAAA	This study
	spt12R-L1	TTCAAGAAACACTAGCAGCT	This study
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

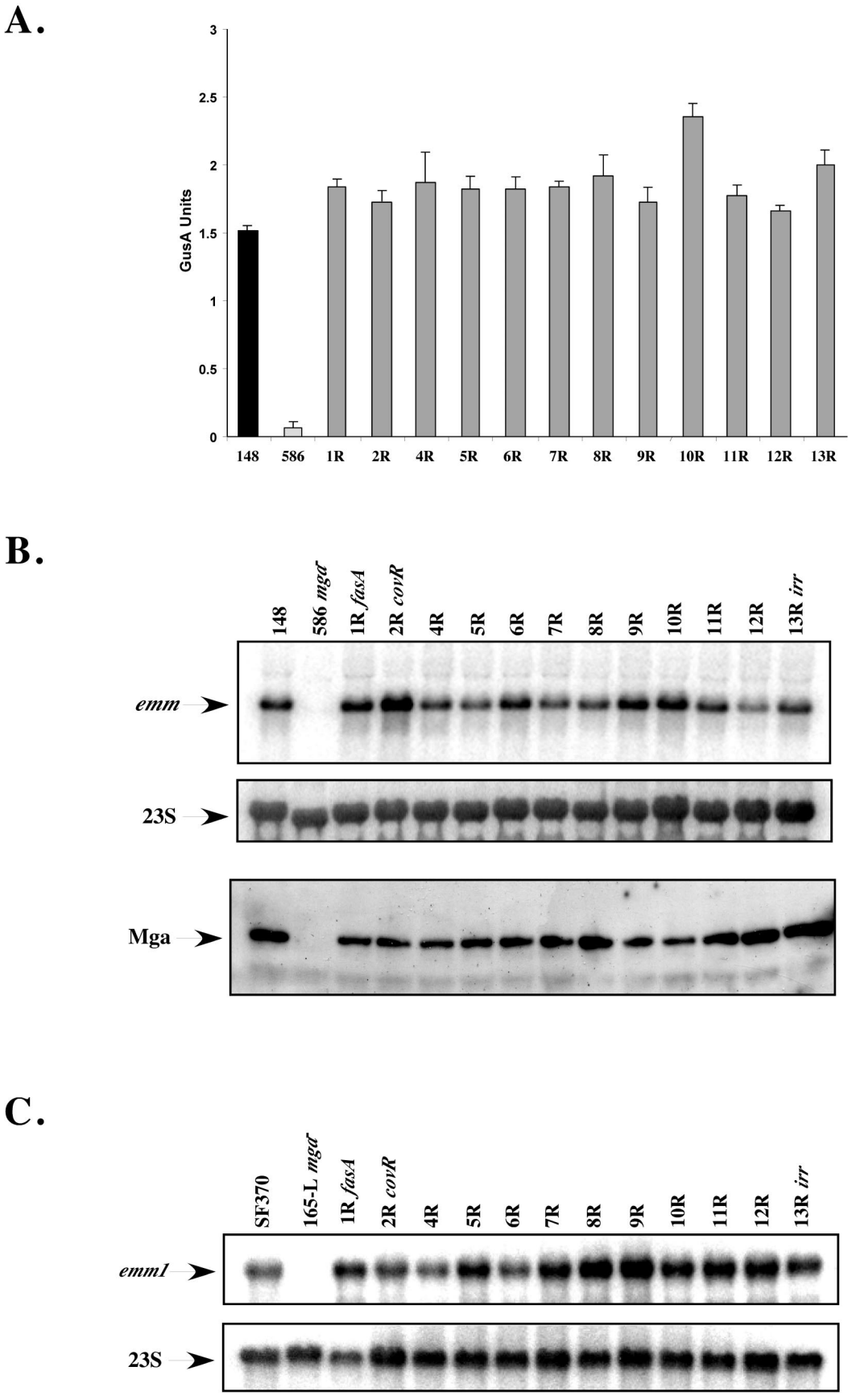
[a] Boldface indicates wild-type *sptR* gene pair. An asterisk indicates integrant junction primer.

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 P*emm-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 *mga*-inactivated 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 stationary-phase 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 μg 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 μg of total protein), with an antibody to a peptide of Mga used as a probe. (C) Northern analysis (1 μg 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).


A.
GusA Units
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1R fasA
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4R
5R
6R
7R
8R
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10R
11R
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13R irr
emm
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165-L mga
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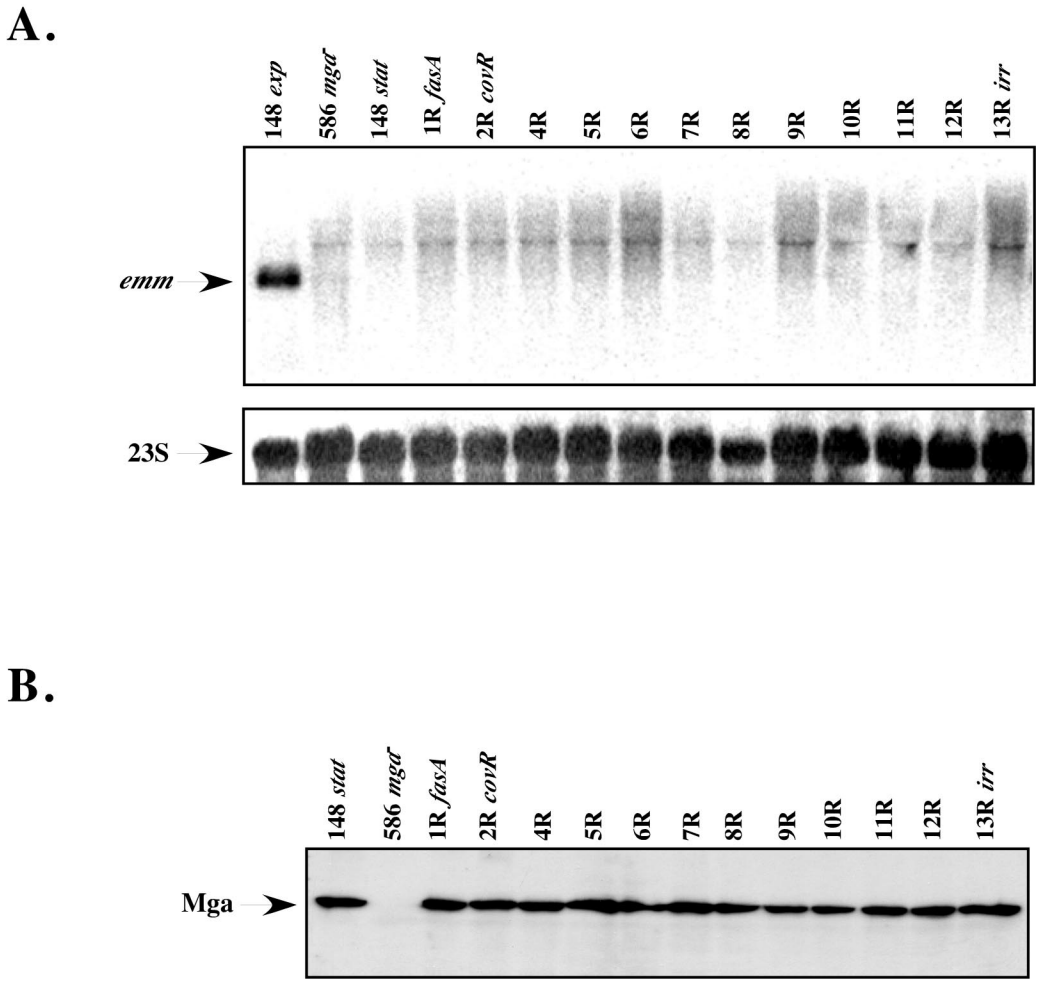

FIG. 3. Analysis of stationary-phase Mga-regulated gene expression in serotype M6 KSM148 *sptR* mutants. (A) Northern analysis of wild-type KSM148 (148), *mga*-inactivated KSM148.586 (586), and the 12 *sptR* mutants (1R, 2R, and 4R to 13R). Total RNA (1 μg of total RNA for exponential-phase KSM148; 5 μg 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 μg 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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