

Role of *mga* in Growth Phase Regulation of Virulence Genes of the Group A Streptococcus

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To determine whether growth phase affects the expression of *mga* and other virulence-associated genes in the group A streptococcus (GAS), total RNA was isolated from the serotype M6 GAS strain JRS4 at different phases of growth and transcript levels were quantitated by hybridization with radiolabeled DNA probes. Expression of *mga* (which encodes a multiple gene regulator) and the Mga-regulated genes *emm* (which encodes M protein) and *scpA* (which encodes a complement C5a peptidase) was found to be maximal in exponential phase and shut off as the bacteria entered stationary phase, while the housekeeping genes *recA* and *rpsL* showed constant transcript levels over the same period of growth. Expression of *mga* from a foreign phage promoter in a *mga*-deleted GAS strain (JRS519) altered the wild-type growth phase-dependent transcription profile seen for *emm* and *scpA*, as well as for *mga*. Therefore, the temporal control of *mga* expression requires its upstream promoter region, and the subsequent growth phase regulation of *emm* and *scpA* is Mga dependent. A number of putative virulence genes in JRS4 were shown not to require Mga for their expression, although several exhibited growth phase-dependent regulation that was similar to *mga*, i.e., *slo* (which encodes streptolysin O) and *plr* (encoding the plasmin receptor/glyceraldehyde-3-phosphate dehydrogenase). Still others showed a markedly different pattern of expression (the genes for the superantigen toxins MF and SpeC). These results suggest the existence of complex levels of global regulation sensitive to growth phase that directly control the expression of virulence genes and *mga* in GAS.

The group A streptococcus (GAS) *Streptococcus pyogenes* is an important human pathogen that causes a variety of infections at several different locations within the host. A primary site of infection for GAS involves colonization of the upper respiratory tract, where this organism is the most frequent bacterial cause of acute pharyngitis in children. Localized purulent GAS infections of the skin, or pyoderma, can begin either from colonization of unbroken skin or by secondary infection of wounds or burns. Severe invasive GAS infections of skin and soft tissues, such as myositis (muscle), cellulitis (dermis), and necrotizing fasciitis (subcutaneous tissue and fascia), can sometimes become life threatening (37, 59). Streptococcal toxic shock syndrome, a soft-tissue infection characterized by shock, bacteremia, multiorgan failure, and a 30% mortality rate, has also been reported with increasing frequency in recent years (24, 37, 59, 60). In some cases, GAS infections can lead to serious nonsuppurative sequelae such as rheumatic fever, acute glomerulonephritis, and rheumatic heart disease.

During the initiation of different types of human disease and their subsequent course of infection, GAS strains utilize a large number of secreted and surface-exposed virulence factors. The organism is enveloped in a hyaluronic acid capsule that may help retard phagocytosis by polymorphonuclear leukocytes (PMNs) and macrophages (4). The fibrillar, cell wall-anchored M protein confers resistance to uptake by phagocytic cells and is the major virulence antigen of GAS (28). A C5a peptidase molecule, located on the cell surface, is able to cleave and inactivate the C5a chemotactic factor of complement to prevent recruitment of PMNs and macrophages to the site of infection (64, 65). Many GAS strains produce M-like proteins on their surface that are similar in structure to M protein and

are able to bind the Fc portion of immunoglobulins A and/or G (14, 58). In addition to cell surface products, GAS also secretes numerous virulence-associated exoproducts. The streptococcal pyrogenic exotoxins (Spe) are responsible for the rash seen in scarlet fever and, along with the mitogenic factor (MF) and streptococcal superantigen, can also function as bacterial superantigens that may be responsible for the shock and organ failure characteristic of streptococcal toxic shock syndrome (5, 23, 52). Two distinct cytolysins are elaborated, streptolysins O and S, as well as streptokinase, which catalyzes the conversion of plasminogen to plasmin (4). The plasmin receptor (Plr) or surface glyceraldehyde-3-phosphate dehydrogenase (SDH) is a multifunctional protein that is the major protein on the surface of GAS grown in lab medium but is released extracellularly under iron-restricted growth conditions (17, 31, 41).

The ability of GAS strains to grow and persist in such varied locations in the human host suggests that expression of their virulence factors is controlled by a global regulatory circuit sensitive to the organism's changing environment. A major component of this global circuit is likely to be the multiple gene regulator of GAS, Mga. Mga activates expression of several GAS virulence genes in an environmentally controlled regulon that includes the genes encoding M protein (*emm*) and C5a peptidase (*scpA*) (8, 10, 12, 48). The Mga regulon can also include the genes encoding M-like proteins (e.g., *fcrA*, *enn*, and *sph*), the opacity factor gene (*sof*), and a secreted inhibitor of the complement cascade (*sic*) when these are present in a GAS strain (26, 35, 47). Transcription of *emm*, *scpA*, and *mga* is stimulated by growth in elevated carbon dioxide levels (8, 38, 48). In addition, *emm* expression is also sensitive to changes in osmolarity, temperature, and iron limitation (34).

Pathogenic bacteria have developed different types of global regulatory circuits to activate and/or repress expression of their virulence genes in response to changing environments during infection. The simplest scenario, exemplified by *Bordetella per-*

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tussis, involves direct activation of virulence genes by a single two-component signal transduction system (BvgA-BvgS) that senses changes in growth conditions (15, 57). More complex control networks are seen in other organisms. For example, some genes in *Salmonella typhimurium*, including those encoding virulence factors, respond directly to the two-component system PhoP-PhoQ, which senses environmental changes, and others respond indirectly, via PhoP-PhoQ regulation of another two-component system, PmrA-PmrB (21, 56). In the gram-positive human pathogen *Staphylococcus aureus*, expression of many virulence genes is coordinately controlled in a growth phase-dependent manner by a complex of three regulatory loci known as *agr*, *sar*, and *xpr* (13, 27, 36, 42, 49, 55). Genes encoding secreted virulence proteins are activated at entry into stationary phase, while genes encoding cell surface virulence proteins are expressed mainly during exponential growth and are negatively regulated as they enter stationary phase (6, 29, 63).

At this time, little is known about the different environmental growth conditions that may affect expression of the Mga regulon genes. Previous studies of GAS using a transcriptional fusion of a chloramphenicol acetyltransferase (*cat86*) gene to the *emm* promoter indicated that *emm* exhibits growth phase-

dependent expression and reaches maximal levels at late-exponential-phase growth (8). Since *emm* encodes a cell surface virulence factor, the similarity to the growth phase-dependent regulation of cell surface proteins by *sar-agr-xpr* in *S. aureus* is intriguing. To further investigate this phenomenon, we have begun to look directly at transcript levels of the Mga regulon genes and other virulence-associated genes of GAS at different phases of growth to identify potential temporal regulation.

MATERIALS AND METHODS

Bacterial strains and media. GAS strain JRS4 is a streptomycin-resistant derivative of serotype M6 strain D471 (54). *Escherichia coli* DH5 α (22) was used as the host for plasmid constructions. *E. coli* was grown in Luria broth (53), and *S. pyogenes* was grown in Todd-Hewitt medium supplemented with 0.2% yeast extract (THYB). Antibiotics were used at the following concentrations: ampicillin, 100 μ g/ml for *E. coli*; erythromycin, 750 μ g/ml for *E. coli* and 1 μ g/ml for *S. pyogenes*; kanamycin, 30 μ g/ml for *E. coli* and 500 μ g/ml for *S. pyogenes*; and spectinomycin, 100 μ g/ml for *E. coli* and *S. pyogenes*.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by using the Wizard Maxiprep and Miniprep system (Promega). Genomic DNA was isolated from GAS by the method of Chassy (11). DNA fragments were isolated from agarose gels by electroelution (32). The Klenow fragment of DNA polymerase I or mung bean nuclease (New England Biolabs) was used to blunt restriction nuclease-digested DNA ends. Colony hybridization was performed with the DIG system (Boehringer Mannheim) by using PCR-labeled DNA probes. DNA se-

TABLE 1. PCR primers used in this study

Target	Primer name	Sequence (5'→3') ^a	Source or reference
<i>mga</i>	OYR-2	ACCTAATTAAGTTAGTGAC	This laboratory
	OYR-4	GTACCATCAACATTGCG	This laboratory
	OYL-13	GACGGCAGAGTATCCCTTGT	This laboratory
	Mgadel-L1	cgggaattcagatctGGCATCAGCGTTTTCTGTTACGTATGAGAT	This study
	Mgadel-R1	cgggaattccccgggCAAAATGATTCCAGAAAGCGATTATTGA	This study
	Mga-X1	cgcgctgcacAAAGAAGGGTATACAAGGTA	This study
	Mga-X2	gcggaagcttAGCCAAAAGGTAAAGACCAG	This study
<i>emm6</i>	OM6+28	TAGTGTCTATTTCGTG	This laboratory
	OM6-30	GAGTGTAATAGGGGCAGGA	This laboratory
	OM6-19	TGTTCTGTAACCACGGGAA	This laboratory
<i>scpA</i>	SCPA-1	CTAAAACACTACTGATACGCCAGC	This laboratory
	SCPA-10	ACCGTCTTTTCGACTGATAAAG	This laboratory
<i>recA</i>	SRA-L	GCGTTCAGGAAGTCTAGCTC	17
	SRA-R	CTGATGCTACTGCCATAGCAG	17
<i>slo</i>	SLO-L1	TGCCACTAGAATCTGCAGAAAA	This study
	SLO-R1	CCCAAAGGATTTTCATATTGAGC	This study
<i>ska</i>	SKA-L2	CGGGATCCTGGGATGTTTGCACTGCTG	17
	SKA-REV	GGTGTCCCTGTATAACGCA	47
<i>plr</i>	PLR-L	CGTCTTGCATTCCGCCG	17
	PLR-R	ATCGTGAAGAGCTTTAGCC	17
<i>hasA</i>	HASA-L	GGTGTAATTGTGCCTATTTTTTA	This study
	HASA-R	GGCAAAAAGTTTAATAAGGTCTA	This study
<i>speB</i>	SPEB-1	GATAACCATACGATTCAGCT	This laboratory
	SPEB-2	TCTGTGTCTGATGGATAGC	This laboratory
<i>speC</i>	SPEC-L1	CATAATTACAGTCACTGATT	This laboratory
	SPEC-R1	ATCGAAATGACTAAAGTTCTTCAT	This laboratory
MF gene	SPEMF-L2	cgggatccTGGGATGTTTGCCTGCTG	This laboratory
	SPEMF-R1	AACGATCCATCATATGTTTC	This laboratory
P _{φSKHG}	PSK2G-L	gcctaggCAACTTGAAGGAACTGCA	This study
	PSK2G-R	cggacgtCGTCTTTTCTACTGCCTT	This study

^a Uppercase letters indicate homologous sequences; lowercase letters indicate nonhomologous sequences and restriction enzyme sites.

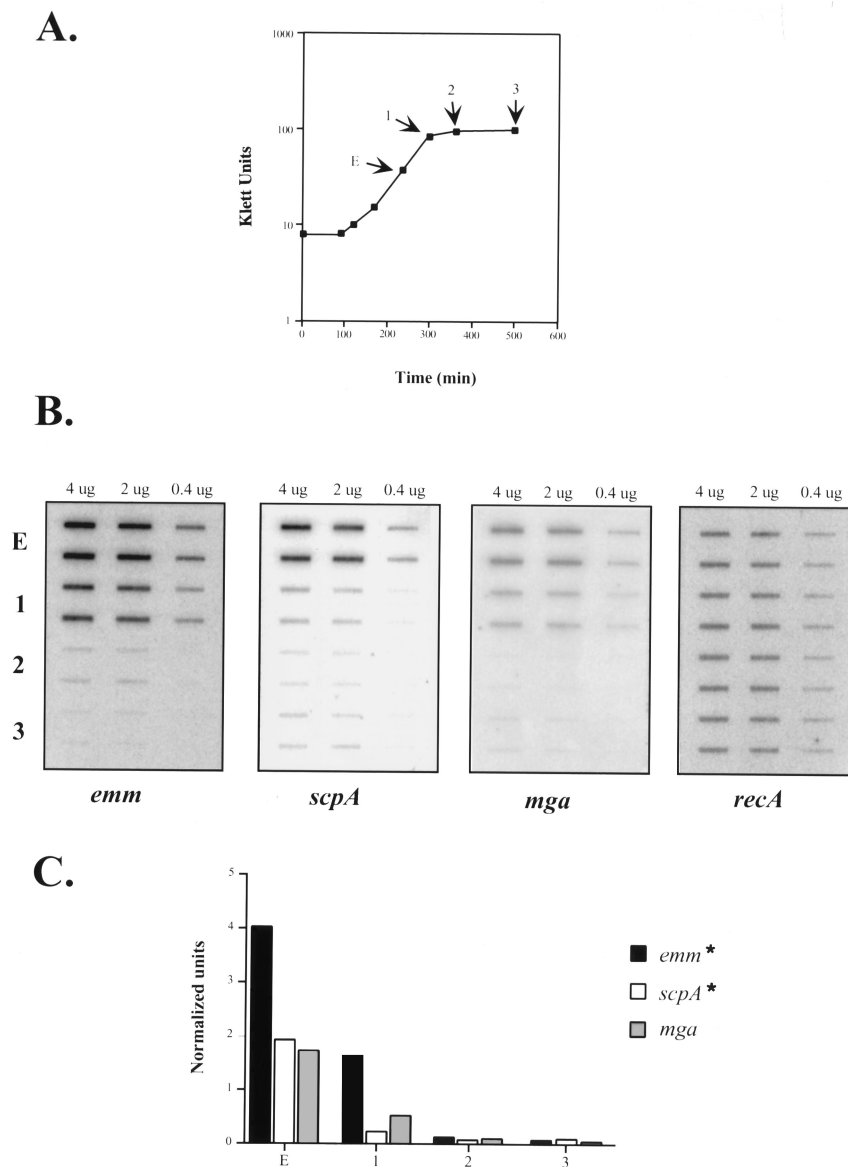


FIG. 1. Expression of Mga regulon genes in GAS strain JRS4 at different points during cell growth. (A) Growth curve of JRS4 for isolation of total RNA. Cells were grown in THYB in tightly capped flasks without agitation at 37°C, and growth was determined by measuring turbidity (Klett units) with a Klett-Summerson colorimeter as described in Materials and Methods. Arrows indicate phases of growth at which samples were removed for total RNA isolation. E, mid-exponential; 1, late exponential; 2, stationary; 3, 2 h into stationary phase. (B) Hybridization of specific DNA probes to RNA isolated from JRS4 at different times during growth. Total RNA was isolated at the time points indicated by arrows in panel A, and 4, 2, and 0.4 μ g of RNA were loaded per slot in duplicate for each time point. The membranes were reacted with specific DNA probes internal to the coding regions of *emm*, *mga*, *scpA*, and *recA* as shown. (C) Quantitation of transcript levels from hybridization analyses. Data are represented as normalized units defined as the band intensity (pixel volume) for any experimental probe at a particular RNA concentration divided by that of a *recA* internal control probe at the same RNA concentration. The stars denote that actual values are 10-fold higher than values shown. Data shown are representative of several independent experiments.

quencing was done by the method of Sanger (50) by using Sequenase (U.S. Biochemicals).

RNA slot blot analyses. *S. pyogenes* strains were grown in THYB at 37°C without agitation as previously described (8). Growth was assayed by measuring absorbance with a Klett-Summerson photoelectric colorimeter with the A filter. When not indicated otherwise, RNA was isolated in late exponential phase. Total RNA was extracted from samples as described by McDonald et al. (33), except that glycine was omitted from the medium. RNA was pelleted by sedimentation through 5.7 M CsCl (19), transferred to a Zeta probe membrane (Bio-Rad), and hybridized with a labeled DNA probe as previously described (62). Labeled membranes were autoradiographed by using phosphor exposure screens and scanned at 176- μ m resolution with a PhosphorImager 445SI (Molecular Dynamics), and the resulting data were analyzed with ImageQuANT software (version 4.1). Normalized units were defined as the band intensity (pixel

volume) for any experimental probe at a particular RNA concentration divided by that of a *recA* control probe at the same RNA concentration.

DNA probes internal to the coding region for each gene were generated by PCR amplification from GAS strain JRS4 genomic DNA using Ultra polymerase (Bethesda Research Laboratories, Inc.) and the primer pairs shown in Table 1. Resulting fragments were radiolabeled with [α - 32 P]dATP by random priming (32).

Construction of the *mga*-deleted GAS strain JRS519. The *mga-9* allele containing a 1.4-kb deletion of the *mga* gene from GAS strain JRS4 was constructed as follows. The plasmid pJRS2050 (1) contains a 4.2-kb *Bam*HI-*Hind*III insert from pJRS1006.5 (9) which includes the 3' end of *isp*, all of *mga* and its upstream region, and the 5' end of the *emm6* gene. This 4.2-kb fragment was cloned into pBluescript II KS- (Stratagene) to create the plasmid pJRS515. From this plasmid, inverse PCR with two diverging PCR primers, Mgadel-L1 and

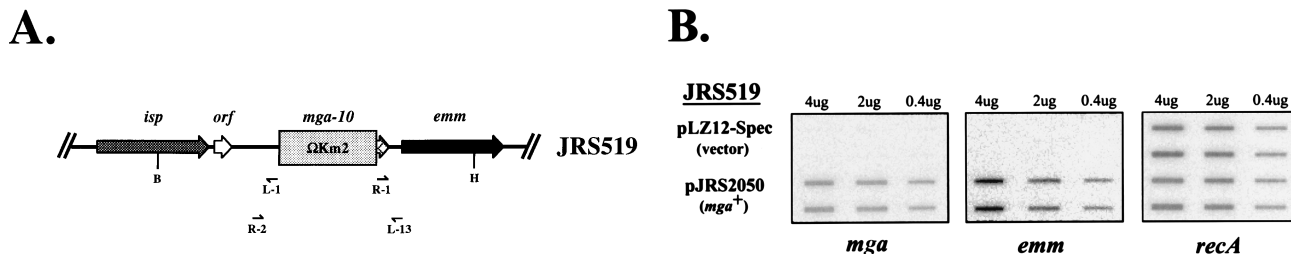


FIG. 2. Diagram of *mga*-deleted GAS strain JRS519 and complementation with wild-type *mga* in *trans* from plasmid. (A) Diagram showing region surrounding *mga-10* mutant allele in the chromosome of JRS519. The *mga-10* allele represents a 1.4-kb deletion of the *mga* coding region and insertion of the Ω Km2 interposon as represented by the shaded box. Gene replacement of wild-type *mga* by *mga-10* was performed as described in Materials and Methods. Adjacent genes are designated by large arrows, and their names are shown above the arrows. Small arrows denote primer pairs (Table 1) as follows: R-1, Mga-del-R1; L-1, Mga-del-L1; R2, OYR-2; and L-13, OYL-13. B, *Bam*HI restriction site; H, *Hind*III restriction site. (B) Hybridization of specific DNA probes to RNA isolated from JRS519 containing either pLZ12-Spec (vector) or pJRS2050 (wild-type *mga*) plasmids. Total RNA was isolated from late-exponential-phase cultures, and 4, 2, and 0.4 μ g of RNA were loaded per slot in duplicate for each sample. The membranes were reacted with specific DNA probes internal to the coding regions of *mga*, *emm*, and *recA* as shown.

Mga-del-R1 (Table 1), was used to amplify a 6.2-kb product (Mga-del-PCR) lacking the *mga* coding region located between the 5' ends of the two primers (1.4 kb). New *Eco*RI, *Sma*I, and *Bgl*II restriction sites were also introduced on the primers at the site of the deletion. The Mga-del-PCR product was blunted with the Klenow fragment of DNA polymerase I and religated to create the *mga-9* allele in pJRS516. Sequence analysis showed that pJRS516 contained the predicted *mga-9* deletion; however, only the *Bgl*II restriction site was present, which suggests loss of the other sites during Klenow treatment and ligation.

To introduce the *mga-9* allele into the temperature-sensitive shuttle vector pJRS233 (44), the 3.1-kb *Bam*HI-*Hind*III fragment of pJRS516 containing the *mga-9* allele was cloned into *Bam*HI-*Hind*III-digested pJRS233, creating the plasmid pJRS517. To construct the *mga-10* allele in which the interposon Ω Km2 (43) is inserted at the site of the deletion in *mga-9*, the 2.3-kb *Sma*I fragment of pUC4 Ω Km2 was cloned into the blunted *Bgl*II site of pJRS517. The temperature sensitivity conferred by the pWV01 origin (40) of pJRS519 was used to substitute the *mga-10* allele for wild-type sequences in the GAS JRS4 chromosome to produce the mutant GAS strain JRS519 (see Fig. 2A) as described previously (44). Introduction of *mga-10* into JRS519 was verified by PCR analysis across *mga* with the primers OYR2 and OM6+28 (Table 1).

Construction of pJRS542 (P ϕ 2-*mga*). The *mga* gene was placed under the control of the heterologous lactococcal phage promoter P ϕ SKIIG (46) as follows. pJRS524 and pJRS525 are modified versions of pLZ12-Spec (25) that contain both the *lacZ* α gene, to give blue or white screening capability on plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -galactopyranoside), and the multiple cloning site (MCS: *Spe*I, *Kpn*I, *Sac*I, *Avr*II, *Xba*I, *Age*I, *Aat*II, *Nco*I, *Hind*III, *Bam*HI, *Eco*RV, *Pst*I, *Eco*RI, *Xho*I, *Bsi*WI, *Bss*HII, *Nsi*I, *Bgl*II) of pLITMUS29 (New England Biolabs). The 0.5-kb *Aff*III-*Hpa*I MCS fragment of pLITMUS29 and *Pst*I-digested pLZ12-Spec were blunted with mung bean nuclease and ligated to produce clones with each of the inserted *lacZ* α gene-MCS fragments in two different orientations with respect to the spectinomycin resistance gene *aad9* (pJRS524 [-] and pJRS525 [+]).

The P ϕ SKIIG promoter was amplified from pNZ278 (46) with the PSK2G-L and PSK2G-R primers (Table 1). This removed the 182-bp open reading frame at the 3' end and introduced *Avr*II and *Aat*II restriction sites at the 5' and 3' ends, respectively. The resulting promoter fragment, termed P ϕ 2, was cloned into *Avr*II-*Aat*II-digested pJRS525 to create the vector pJRS540. A promoterless *mga* gene was amplified by PCR from JRS4 genomic DNA with primers Mga-X1 and Mga-X2 (Table 1) to generate a 1.8-kb *Sal*I-*Hind*III fragment (MgaXA). MgaXA was blunted with the Klenow fragment of DNA polymerase I and cloned into the *Eco*RV site of pJRS540 to create the plasmid pJRS542 (P ϕ 2-*mga*). Clones were identified by colony hybridization, and orientation was determined by PCR analysis.

RESULTS

The *emm*, *scpA*, and *mga* genes are growth phase regulated.

To begin a study of the regulation of expression of some of the virulence genes in GAS, the growth stage at which Mga regulon genes are maximally expressed was determined. The cells were grown in a sealed flask in THYB at 37°C, and aliquots were removed at mid-exponential phase, the end of exponential phase, early stationary phase, and late stationary phase (Fig. 1A). The RNA was transferred to nylon membranes, and the amount of *emm*-specific transcript of each sample was determined by hybridization with a radiolabeled DNA probe corresponding to an internal region of *emm*. As an internal

control for the amount of RNA used, the membrane was stripped and reprobbed for *recA* (Fig. 1B) or *rpsL* (data not shown). Transcript levels for these genes remained constant over the entire growth period studied, and the amount of *recA* message was of the same order of magnitude as that of *emm*. Therefore, the data are expressed relative to the amount of *recA* message in each case.

As the cells entered stationary phase, the amount of *emm* transcript dropped about 2.5-fold (compare E and 1 in Fig. 1B and C). The amount of *emm*-specific message had dropped about 40-fold by early stationary phase and remained low in late stationary phase (Fig. 1B and C). Therefore, *emm* transcription is maximal in exponential phase and is turned off at later times during growth.

Both *emm* and *mga* are part of the Mga regulon that includes other putative virulence factors, including the C5a peptidase encoded by *scpA*. To determine whether other genes that are positively regulated by *mga* also demonstrate this growth phase regulation of transcript levels, the membrane was stripped again and hybridized with a probe specific for *scpA*. Transcripts of *scpA* also showed a dramatic decrease as the bacteria entered stationary phase (Fig. 1B and C). A 9.5-fold drop in the amount of *scpA* transcript was seen between mid- and late exponential phase, and the amount of *scpA* message had declined by about 47-fold at early stationary phase and remained low in late stationary phase. The similar decreases in message at later phases of growth for both Mga regulon genes suggest that such decreases may result from regulation of the *mga* gene itself.

A *mga*-specific probe hybridized to the same membrane showed a decrease in transcript production similar to that of the other *mga*-regulated genes, *emm* and *scpA* (Fig. 1B and C). A 3.4-fold reduction in the amount of *mga* transcripts occurred between mid-exponential and late exponential phase. This dropped 28-fold by stationary phase and remained low thereafter. The simplest explanation for the similar growth phase-dependent pattern of transcript production for *mga* and Mga regulon genes is that a growth phase regulator acts directly on *mga* and Mga regulates the expression of the other genes.

Construction of JRS519, a GAS strain with *mga* deleted. To learn whether the growth phase regulation of *mga* transcription depends on the presence of the *mga* promoter and adjacent upstream DNA (Pmga region), we replaced this region with DNA of a heterologous promoter. To allow for detection of *mga* expression from a plasmid, we first needed to construct a strain deleted for the *mga* gene (JRS519). The GAS strain JRS519 was constructed from strain JRS4 as described in Ma-

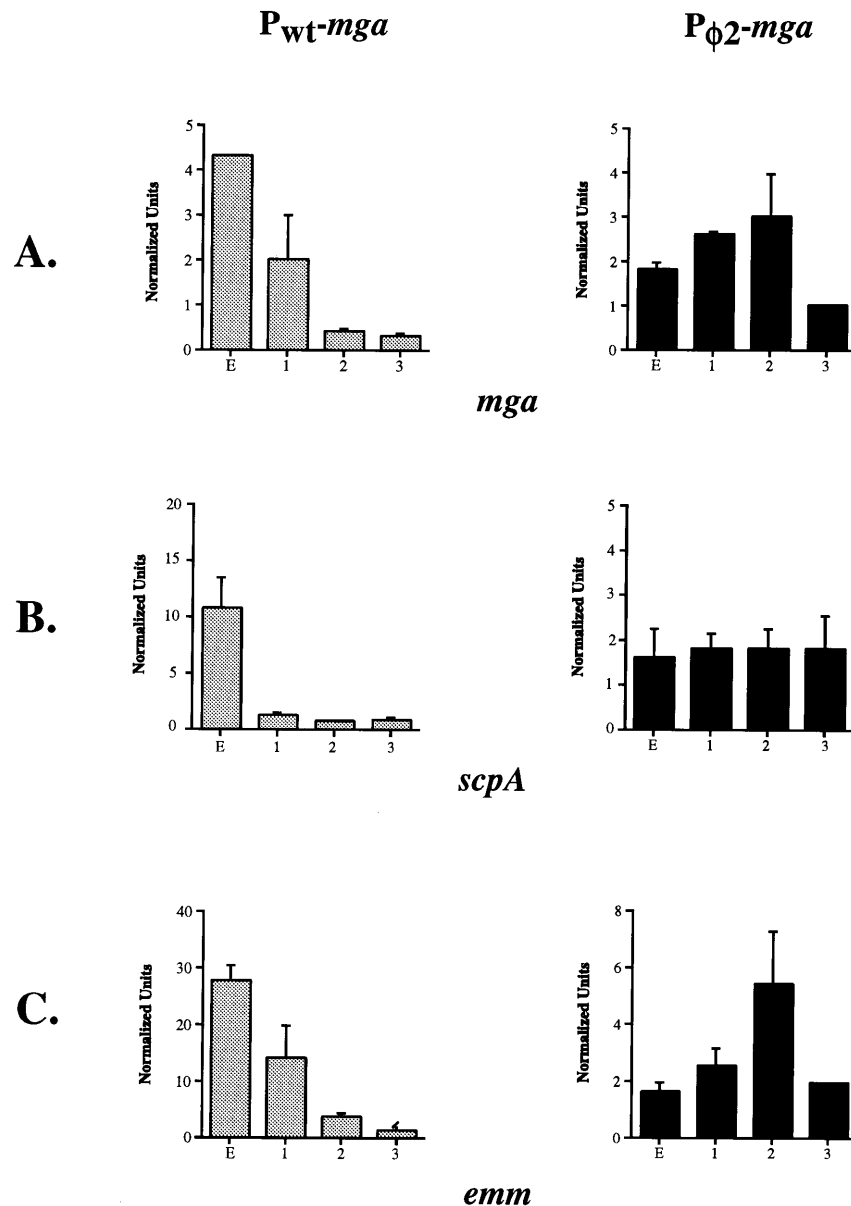


FIG. 3. Effect of *mga* expressed from a heterologous $P_{\phi 2}$ promoter on the transcript levels of *mga* and *emm* during cell growth. Total RNA was isolated from JRS519 containing either pJRS2050 (P_{wt-mga}) or pJRS542 ($P_{\phi 2-mga}$) at different times during the growth of the cell as described in Materials and Methods. DNA probes representing fragments internal to the *mga* and *emm* coding sequences were hybridized to RNA isolated from these samples. Data are represented as normalized units defined as the band intensity (pixel volume) for any experimental probe at a particular RNA concentration divided by that of a *recA* internal control probe at the same RNA concentration. Normalized unit values, shown on the vertical axis, are not the same for different graphs. Graphs represent at least two independent experiments and standard error bars are shown.

terials and Methods. Briefly, 85% of the *mga* coding region was deleted and replaced with the Ω Km2 interposon, resulting in the *mga-10* allele (see Materials and Methods). In a derivative of strain JRS4, named JRS519, PCR across the *mga* region showed a product of the size expected for the replacement of *mga* by *mga-10* (Fig. 2A and data not shown).

Strain JRS519 was tested to verify that the *mga-10* allele could be complemented for *emm* expression by a plasmid containing *mga* under the control of its native promoter. The plasmid pJRS2050, containing all of *mga* and the regulatory region upstream of *mga* in the shuttle vector pLZ12-Spec, was used. Both pJRS2050 and the vector pLZ12-Spec were electroporated into JRS519, and their effects on chromosomal

emm expression were investigated by Western immunoblot analysis. Surface proteins were isolated from both strains, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody to M protein. M protein was found in the surface extract of JRS519(pJRS2050) but not that of JRS519(pLZ12-Spec) (data not shown). In addition, total RNA was also isolated from these two strains at late exponential phase and assayed by hybridization for *mga*, *emm*, and *recA* transcripts. No detectable *mga* or *emm* transcripts were observed in JRS519 containing the vector alone, while *recA* RNA was easily detected (Fig. 2B). However, introduction of pJRS2050 into JRS519 resulted in expression of both *mga* and

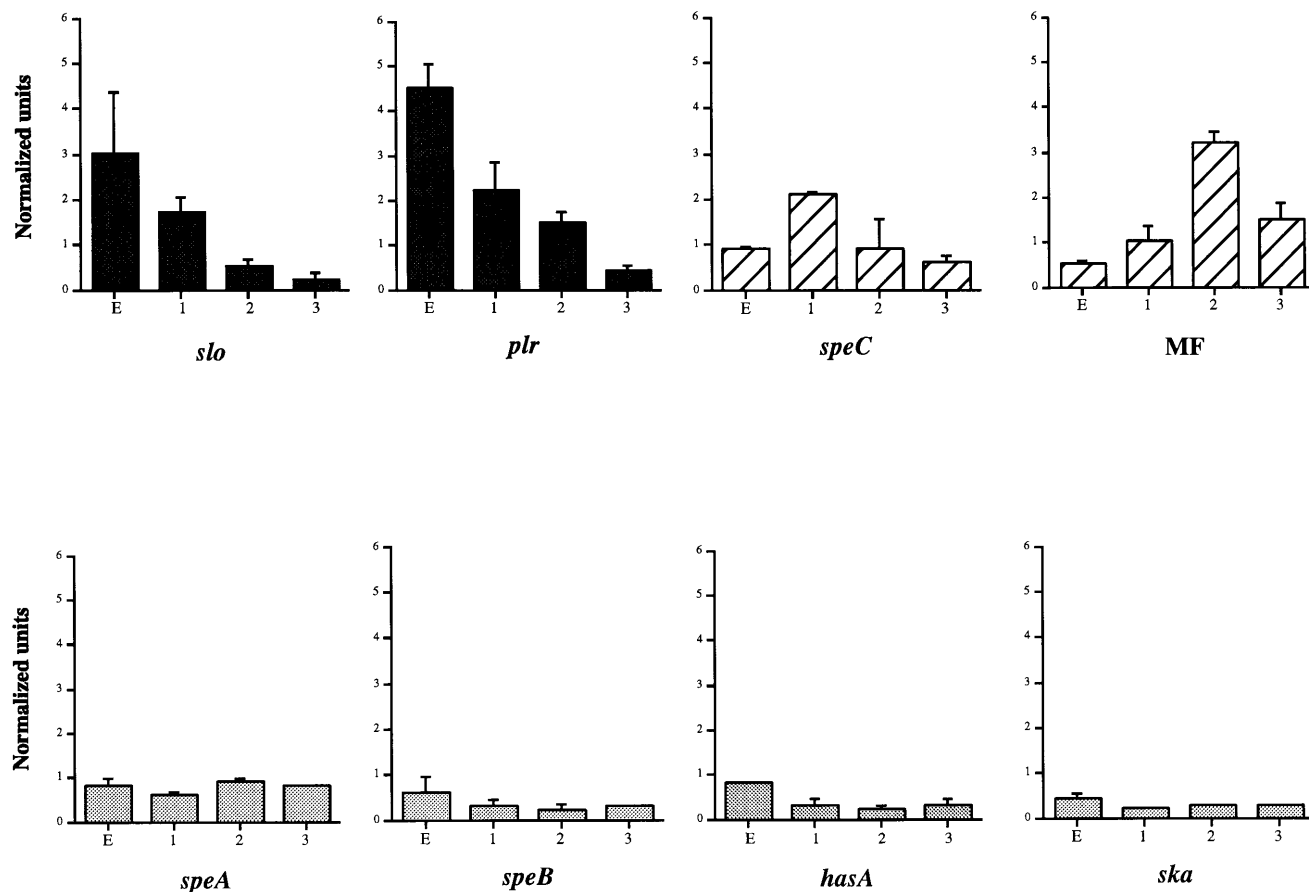


FIG. 4. Effect of growth phase on the transcript levels of different GAS virulence genes. Total RNA was isolated from JRS4 at different times during the growth of the cell as described in Materials and Methods. Slot blot analyses were performed on these samples using probes specific to *hasA*, *slo*, *plr*, *speC*, *speB*, *speA*, *ska*, and the MF gene. Data are represented as normalized units defined as the band intensity (pixel volume) for any experimental probe at a particular RNA concentration divided by that of a *recA* internal control probe at the same RNA concentration.

emm (Fig. 2B). These data demonstrate that the chromosomal *mga-10* allele of JRS519 can be complemented by wild-type *mga* on a plasmid.

The growth phase regulation of *mga* requires the Pmga region. If the Pmga region is the target for the growth phase regulation observed for *mga* and the genes it regulates, placing *mga* under the control of a heterologous promoter should alter the observed regulation. To test this, a promoterless *mga* gene retaining its ribosome binding site was cloned downstream of the P ϕ 2 promoter derived from the *Lactococcus lactis* phage SKIIG (see Materials and Methods). The resulting P ϕ 2-*mga* plasmid, pJRS542, was then introduced into JRS519 (*mga-10*). Total RNA was isolated from both JRS519(pJRS2050), which carries *mga* under its native promoter (Pwt-*mga*), and JRS519(pJRS542) (P ϕ 2-*mga*) at the phases of growth shown in Fig. 1A and hybridized to a probe specific for *mga*. The pattern of expression for *mga*-specific message from JRS519 harboring the Pwt-*mga* plasmid pJRS2050 (Fig. 3A, Pwt-*mga*) was very similar to that of the parental strain JRS4 (Fig. 1C): both showed a significant decrease in transcript levels at entry into stationary phase. However, in JRS519 containing the P ϕ 2-*mga* plasmid pJRS542, a very different pattern of growth phase-dependent *mga* expression was seen (Fig. 3A, P ϕ 2-*mga*). The amount of *mga* transcript in this strain increased as the cells entered stationary phase, and later in stationary phase the transcript levels dropped only twofold below the levels in ex-

ponential phase. This demonstrates that the growth phase regulation of *mga* is dependent upon its native promoter and/or the region upstream of *mga*.

Growth phase regulation of the Mga regulon genes requires the Pmga region. To test whether growth phase regulation of *emm* and *scpA* depends on the temporal regulation of *mga*, we examined the levels of *emm* and *scpA* transcripts in JRS519 (pJRS542) in which *mga* is expressed from the P ϕ 2 promoter. When *mga* was expressed from its native promoter in JRS519(pJRS2050), regulation of *emm* and *scpA* was similar to that in the parental strain JRS4 as expected (compare Fig. 3B and C, Pwt-*mga*, with Fig. 1C). However, in JRS519(pJRS542), expression of *mga* from P ϕ 2 dramatically altered the growth phase pattern of *emm* and *scpA* transcripts from that observed when *mga* was expressed from its native promoter (compare Fig. 3A to C, P ϕ 2-*mga*). The *scpA* transcript levels remained constant during both the exponential and stationary phases (Fig. 3B, P ϕ 2-*mga*). For *emm*-specific transcripts, the growth-related expression pattern more closely resembled that for *mga* transcripts, in which the amount was maximal upon entry into stationary phase and low in exponential phase, than that for *scpA* (compare Fig. 3A to C, P ϕ 2-*mga*). However, all three showed similar growth-related patterns of expression when experimental error was taken into account (Fig. 3, P ϕ 2-*mga*). It therefore appears that the growth phase regulation of *mga* and

the other Mga regulon genes results from the growth phase dependence of *mga* transcription.

Other putative GAS virulence genes are not *mga* regulated. Many potential virulence genes have been identified for the GAS, most of which encode proteins that are secreted or found on the streptococcal surface. In addition to the genes for the C5a peptidase and the M protein(s), Mga may also regulate other virulence genes found in GAS in a growth phase-dependent manner. Whether Mga regulates other putative virulence genes had not previously been tested except in a GAS strain of serotype M49, where Mga is not required for expression of the genes encoding streptokinase (*ska*) and streptolysin O (*slo*) (47). To test whether these and other potential virulence genes require *mga* for expression in the serotype M6 GAS strain JRS4, radiolabeled probes specific for *ska* and *slo*, as well as for the genes encoding *plr*, the pyrogenic exotoxins A (*speA*), B (*speB*), and C (*speC*); MF; and the first gene in the operon for synthesis of the hyaluronate capsule (*hasA*) were generated by PCR with the primer pairs shown in Table 1. To determine if these genes are *mga* regulated, RNA was isolated from wild-type JRS4 and the *mga-10* strain JRS519 at late exponential phase (Fig. 1A) and transcript levels for each gene were compared. All of the genes tested were transcribed as well in the *mga*-deleted strain JRS519 as in JRS4, indicating that they are not regulated by *mga* under the conditions tested (data not shown).

Growth phase regulation of other putative virulence genes. To determine whether the other putative virulence genes examined above demonstrate growth phase regulation independent of *mga*, the same probes were used for hybridization analyses of JRS4 RNA samples from each phase of growth. Like the Mga regulon genes, *slo* and *plr* showed maximal expression in exponential phase (compare Fig. 1C to Fig. 4), although the decline in transcript levels was more gradual for these than for genes of the Mga regulon.

The chromosomally encoded MF gene and the prophage-encoded *speC* showed a pattern of growth phase-dependent expression different from *slo*, *plr*, and the Mga regulon genes. Maximal steady-state RNA levels of *speC* were found at late exponential phase, while the MF gene exhibited maximum transcript levels as cells entered stationary phase (Fig. 4). Because the remaining genes tested, *speA*, *speB*, *hasA*, and *ska*, are expressed weakly in GAS strain JRS4 under the growth conditions used, it is difficult to determine whether they are differentially expressed in different phases of growth. However, in a strain expressing higher levels of *hasA*, Crater et al. (16) reported a dramatic decrease in *hasA*-specific message as cells enter stationary phase, similar to the pattern we found with *mga*-regulated genes.

DISCUSSION

Expression of *mga* is shut off in stationary phase. The multiple gene regulator of GAS, called Mga, controls the expression of many important streptococcal virulence factors in response to changing environmental conditions. To help further our understanding of the way that GAS interacts with its host during the course of an infection, we have begun to identify the different signals that affect expression of *mga* and of virulence genes. We report here that in the serotype M6 GAS strain JRS4, transcription of *mga* and of the *emm* and *scpA* genes it regulates is active during exponential phase but is then turned off as the bacteria enter into stationary phase. This result differs from those of previous studies which used transcriptional promoter fusions of Pemm to a *cat86* reporter gene. In JRS145, the GAS derivative of strain JRS4 which contains a

Pemm-*cat86* transcriptional fusion, Cat-specific enzyme activity showed that expression from Pemm peaks at the transition to stationary phase and exhibits no significant decrease after 18 h of growth (8). Similarly, in studies using *cat86* transcriptional fusions to Pemm and PscpA from a serotype M12 GAS strain, both promoter fusions showed significant expression after overnight growth (48). However, by direct assays of *emm*-specific RNA, we found that these transcripts are barely detectable by early stationary phase. It seems likely that this discrepancy results from the stability of the Cat86 protein. Thus, although the transcript for the protein is no longer detectable in stationary phase, the enzyme is present long after expression of its gene has been turned off. Because specific enzyme activity does not correlate directly with the amount of RNA made from the promoter responsible for its transcription, conclusions from experiments in which *cat86* reporter fusions were used to estimate gene expression (8, 34, 48) must be reexamined.

In other bacterial pathogens, many virulence-associated genes are activated when the cells enter stationary phase and nutrients are limiting. Stationary-phase activation is often due to the production of a new sigma factor needed for transcription of a class of genes. However, few bacterial genes whose expression is shut off in stationary phase have been described. Furthermore, this type of regulation is not typical of genes in GAS strain JRS4 either, since we found that the expression of two housekeeping genes, *recA* and *rpsL*, remained constant from mid-exponential phase until at least 2 h into stationary phase. The few examples of genes in other bacteria that are expressed only in exponential phase include those encoding small DNA-binding proteins, such as the nucleoid-associated proteins Fis in *E. coli* and the histone H1 homolog BPH1 in *B. pertussis* (51, 67), genes encoding cell-surface virulence factors in *S. aureus* (6, 29, 63), and the regulator *abrB* in *Bacillus subtilis* (39, 61). The mechanism involved in stationary-phase shutoff has not been completely elucidated in any case, although in *S. aureus* it involves the complex control network including the regulatory factors Sar, Agr, and Xpr (13, 27, 55).

It seems likely that the dramatic decrease in *mga* transcription in stationary phase is not due to a general slowdown of cell transcription or overall RNA stability, because other genes (e.g., *recA* and *rpsL*) do not behave similarly and because *mga* shutoff depends on the presence of the *mga* promoter and/or upstream region. We therefore believe that an active mechanism is needed to turn off *mga* transcription in stationary phase. The simplest mechanism for such regulation would be controlled by a repressor synthesized predominantly in stationary phase that acts at or upstream of the *mga* gene promoter. Synthesis of such a repressor might be dependent on production of a stationary-phase sigma factor, like σ^S of *E. coli* and other gram-negative bacteria (30) or σ^B in *B. subtilis* and *S. aureus* (3, 7, 66). Alternatively, production of this hypothetical repressor might be regulated by a quorum-sensing system which responds not to growth stage per se but to cell density. Such a system is used for the regulation of development of competence for genetic transformation in the gram-positive pathogen *Streptococcus pneumoniae* (20, 45) and in the postexponential-phase expression of virulence genes regulated by *agr* in *S. aureus* (2). For *mga*, this model would involve accumulation in the medium of a diffusible inducer that reaches a concentration high enough to activate expression of the hypothetical repressor of *mga* transcription when the cells reach the high density characteristic of late exponential phase.

Growth phase control of the Mga regulon genes is Mga dependent. To test whether growth phase control of *emm* and *scpA*, the two Mga regulon genes we studied, is a consequence

of the amount of Mga or whether these genes are independently responsive to growth phase, we expressed Mga from a foreign promoter, that of the phage SKIIG. By comparison with expression of Mga from its native promoter, the growth phase-dependent expression of *emm*, *scpA*, and *mga* was altered. With Mga under the foreign promoter, expression of these three genes appears to show the same dependence on growth phase, within experimental error. Therefore, it seems that the stationary-phase shutoff of the Mga regulon genes is a result of that of Mga itself, and these genes are not independently responsive to the phase of growth. The regulator *abrB* of *B. subtilis* may play a role similar to that of Mga. There is a set of genes requiring AbrB for expression which is not transcribed in stationary phase because *abrB* transcription is shut off post-exponentially (39, 61).

Thus, Mga falls within a regulatory hierarchy in which control of its transcription by a regulatory circuit sensitive to growth phase results in the subsequent growth phase-dependent regulation of all Mga regulon genes. Since there are additional Mga-regulated genes whose products have not yet been identified (18), the full impact of this Mga-dependent regulatory cascade on GAS virulence is still not known.

Stationary-phase repression of other GAS virulence genes. In this work, we have also determined that many presumed virulence genes of GAS are not regulated by Mga. When *mga* was deleted, expression of *slo*, *ska*, *speA*, *speB*, *speC*, *plr*, and the MF gene was unaffected.

However, several of these genes exhibit growth phase-dependent regulation even though they do not require Mga for their expression. Transcription of the genes *slo*, encoding streptolysin O, and *plr*, encoding the plasmin receptor/glyceraldehyde-3-phosphate dehydrogenase, is shut off during stationary phase in a manner similar to the Mga regulon genes. Although in JRS4 expression of the *hasA* gene, which is required for hyaluronate capsule synthesis, was too weak to analyze, in a highly encapsulated GAS strain its expression has also been demonstrated to be shut off at entry into stationary phase (16). The stationary-phase repression of these different genes suggests the possibility of a level of global regulation higher than that of Mga. We propose that there may be a repressor expressed exclusively in stationary phase that controls expression of *slo*, *plr*, and *hasA*. This repressor may be the same as that proposed above for regulation of *mga*, or it may be an additional repressor. Such a system might be similar to that of *Salmonella typhimurium* in which the two-component system PhoP-PhoQ, which responds to changes in environmental conditions, controls a second two-component regulatory system, PmrA-PmrB, and also directly controls expression of a set of virulence genes. Like Mga, PmrA-PmrB directly regulates an additional group of virulence genes (21, 56). This type of control may allow the bacteria greater flexibility in response to different environmental signals.

Growth phase control of other GAS virulence genes. Expression of other presumptive GAS virulence genes, including those for the superantigens SpeC (*speC*) and MF, is also growth phase sensitive. However, transcription of these genes is maximal at different stages of growth from those of *mga*, *slo*, *plr*, and *hasA*. These different types of Mga-independent regulation suggest that other regulatory circuits are involved in control of these genes. Therefore it appears that virulence genes of GAS may be regulated by complex control networks involving interactions between two or more separate regulatory units that process environmental signals.

What selective advantage does GAS gain by expressing certain virulence genes only in exponential phase? Genes whose expression is shut off in stationary phase include *slo*, *plr*, *hasA*,

and the Mga-regulated genes *scpA* and *emm*. One possible reason for their lack of expression in stationary phase may be to save energy for the cell under starvation conditions. These genes encode very different types of products which have very different functions. For example, Plr (also called SDH) encodes an enzyme essential for intermediary metabolism in the cytoplasm that is also a potential virulence factor on the streptococcal cell surface. It may be that the amount of the enzyme needed is greatly reduced in the stationary phase when metabolism is less active. Furthermore, the products of several of these genes, which are located on the external surface of the cell, may be very stable, so further transcription may not be needed. The GAS may have an adequate amount of M protein on its surface by the time it reaches stationary phase and more may not be needed until the cells divide. We have found that the promoter for *emm* is very strong in comparison to the other GAS promoters we have investigated (see above and compare Fig. 1C and Fig. 4), so repressing transcription of this gene may save significant energy.

It may also be possible that a window of time exists during exponential phase within which these proteins must be produced in order to interact properly with other cell components. For example, the proteins that are located on the GAS surface, including the M protein, C5a peptidase, and Plr, might need to be produced early in the growth cycle to allow correct interaction with the machinery responsible for anchoring them to the cell wall.

A comparison of proteins in the cell surface fraction of GAS strain JRS4 and the *mga*-deleted strain JRS519 showed that some proteins are present only in the presence of Mga and others are found only in its absence (18). This suggests that Mga induces several genes, including some whose products are unknown, and represses the expression of others, like *abrB* in *B. subtilis* (61). It is possible that the true target of the growth phase-dependent regulation of *mga* may be one or more of these unknown gene products. It might be an advantage to the GAS to produce a Mga-repressed protein in stationary phase, and this could be accomplished by turning off expression of Mga at this stage of the growth cycle. A better understanding of the regulation of Mga and the way it controls expression of other genes will help us comprehend the adaptation of the GAS to its pathogenic lifestyle.

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