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## The Histone-Like Protein Hlp Is Essential for Growth of *Streptococcus pyogenes*: Comparison of Genetic Approaches To Study Essential Genes<sup>∇†</sup>

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**Selection of possible targets for vaccine and drug development requires an understanding of the physiology of bacterial pathogens, for which the ability to manipulate expression of essential genes is critical. For *Streptococcus pyogenes* (the group A streptococcus [GAS]), an important human pathogen, the lack of genetic tools for such studies has seriously hampered research. To address this problem, we characterized variants of the inducible  $P_{tet}$  cassette, in both sense and antisense contexts, as tools to regulate transcription from GAS genes. We found that although the three-operator  $P_{tet}$  construct [ $P_{tet}(O)_3$ ] had low uninduced expression, its induction level was low, while the two-operator construct [ $P_{tet}(O)_2$ ] was inducible to a high level but showed significant constitutive expression. Use of  $P_{tet}(O)_3$  in the chromosome allowed us to demonstrate previously that RNases J1 and J2 are required for growth of GAS. Here we report that the uninduced level from the chromosomally inserted  $P_{tet}(O)_2$  construct was too high for us to observe differential growth. For the highly expressed histone-like protein (Hlp) of GAS, neither chromosomal insertion of  $P_{tet}(O)_2$  or  $P_{tet}(O)_3$  nor their use on a high-copy-number plasmid to produce antisense RNA specific to *hlp* resulted in adequate differential expression. However, by replacing the ribosome binding site of *hlp* with an engineered riboswitch to control translation of Hlp, we demonstrated for the first time that this protein is essential for GAS growth.**

*Streptococcus pyogenes* (the group A streptococcus [GAS]) is a Gram-positive, exclusively human pathogen. GAS produces a wide range of diseases, including superficial infections of the skin (impetigo) and throat (pharyngitis) and severe invasive infections (necrotizing fasciitis and toxic shock syndrome) (7, 31, 40). In some individuals, superficial GAS infections can also lead to complications that involve the immune system (glomerulonephritis and acute rheumatic fever) (7). Although GAS currently remains sensitive to penicillin, the rate of penicillin treatment failure is as high as 37% (23, 33), and allergy to this antibiotic precludes its use in many people (32). Therefore, GAS remains a major public health problem, with an estimated toll of over 500,000 deaths each year worldwide (6) and an annual estimated cost of over \$1 billion. To reduce this disease burden, prevention of infection is desirable. Development of appropriate therapeutic interventions and of vaccines to prevent infection requires an understanding of the physiology of the bacterial pathogen. Although genetic analyses of GAS have progressed extensively in the last few years and many tools for study of GAS are now available, until recently there have been no suitable techniques for studying genes essential for growth of GAS, although such genes are obviously appropriate therapeutic targets.

For studies of the functions of essential genes, a promoter

that can be regulated under laboratory conditions would be helpful. In GAS, the regulated promoter that has been employed previously is the nisin-inducible lactococcal  $P_{nisA}$  promoter (11), which requires the additional introduction into the strain to be studied of *nisR* and *nisK*, the two-component system that controls expression from  $P_{nisA}$  in response to exogenously added nisin (24). Although induction of this promoter in GAS by nisin is about 10-fold, the background level of expression is high. Furthermore, nisin is bactericidal to GAS, and the appropriate concentration for its use as an inducer varies with the strain studied (11). Thus, this system has not been employed extensively for work with GAS.

In many organisms, including several Gram-positive bacteria, modifications of the tetracycline-inducible promoter  $P_{tet}$ , which is based on the promoter of *Tn10* from *Escherichia coli*, can be employed for conditional expression of genes (14). In this system, the *tet* repressor, TetR, controls expression (18). In the absence of an inducer, TetR binds to the *tet* operator *tetO*, a 19-nucleotide (nt) palindromic sequence that overlaps the promoter for the regulated gene, to prevent expression of this gene. When an inducer is present, it binds to TetR, releasing it from the operator and allowing expression of the regulated gene.

The tetracycline-inducible system was adapted from the Gram-negative bacterium *E. coli* for the Gram-positive bacterium *Bacillus subtilis* by (i) inserting poly(A) blocks upstream of *tetO*, (ii) altering the promoter sequence to increase its strength for *B. subtilis*, and (iii) replacing the *tetR* ribosome-binding site (RBS) with a strong consensus RBS (AGGAGG) (14). The *tet* system adapted for Gram-positive bacteria comprises *tetR*, with its RBS, and two *tet* operators, the first of which overlaps the promoter for *tetR* and the second of which overlaps the promoter being studied [ $P_{tet}(O)_2$  in Fig. 1]. This

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† We dedicate this paper to the memory of Wolfgang Hillen, who graciously encouraged us in our work with the  $P_{tet}$  system and supplied us with useful hints and tips as we proceeded.

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$P_{tet}$  system has been used successfully to control gene expression in many Gram-positive bacteria, including *B. subtilis* (14), *Staphylococcus aureus* (3), *Streptococcus mutans* (42), and *Streptococcus intermedius* (27), and has even been used to control Gram-positive bacterial gene expression during animal infections (3, 13, 21).

The system with two *tet* operators can be induced >50-fold by addition of the inducer in *B. subtilis*, but it shows expression even in the absence of the inducer (14). To eliminate expression in the absence of inducer, a third *tetO* was inserted following the first two *tet* operators (14) [ $P_{tet}(O)_3$  in Fig. 1]. Although this version of the *tet* cassette has no detectable expression in the absence of inducer, the highest level of induction achieved with it is at least 20 times lower than that for the induced double-*tetO* system (14). We therefore tried both the two- and three-operator  $P_{tet}$  cassettes for use in GAS.

An alternative to regulation at the level of transcription by an inducible promoter is use of a riboswitch, which takes advantage of the universal bacterial conservation of translational machinery. A riboswitch consists of a *cis*-acting mRNA domain that alters its conformation to allow ribosome binding in response to the intracellular concentration of a small molecule. Recently, Topp et al. (41) constructed a synthetic riboswitch responsive to the small molecule theophylline and showed that this riboswitch can be regulated in many different bacteria, both Gram negative and Gram positive. When this riboswitch was used in GAS in conjunction with a very strong promoter on a plasmid, a reporter showed very little expression in the absence of theophylline, while in the presence of this molecule, an increase in the reporter gene product of at least 50-fold was seen (41). Because this riboswitch appears to be active in GAS, at least on a plasmid, we also tested its use in this work.

We selected the following three candidate genes in GAS for inactivation by use of variants of  $P_{tet}$  constructs: *mjA*, encoding RNase J1, and *mjB*, encoding RNase J2, which we previously showed are essential in GAS by using a chromosomally inserted  $P_{tet}(O)_3$  construct (5); and *hlp*, which encodes the histone-like DNA-binding protein Hlp. Unlike the RNases, histone-like proteins are among the most abundant in bacteria, with about 60,000 molecules per cell in *E. coli* (1) and in *B. subtilis* (12, 36). Hlp serves as a DNA chaperone, folding and compacting DNA by binding to it with little nucleotide sequence specificity (10). This process affects transcription of genes and thus is important in regulating gene expression (9). Although nonessential in the Gram-negative bacterium *E. coli*, the Hlp homolog is essential for growth of many other bacteria, including *B. subtilis* (29), *Deinococcus radiodurans* (30), and *Streptococcus intermedius* (27). GAS strains carry a homolog of Hlp that was identified as the primary phosphorylation target of the serine threonine kinase (Stk) of this organism (22). Because Stk affects the GAS transcriptome (our unpublished work), we wished to delete *hlp* to examine its role in regulation by Stk. We were unable to delete *hlp*, so we surmised that it might be essential for growth of GAS as well.

In this work, we compared expression in GAS from different variants of  $P_{tet}$  and tested conditional activation of *hlp* from both  $P_{tet}(O)_2$  and  $P_{tet}(O)_3$ . We also tested inactivation of *hlp* by expressing its antisense RNA from  $P_{tet}(O)_2$  on a plasmid. In addition, we compared expression of *hlp* from these three constructs with its production under the control of a synthetic

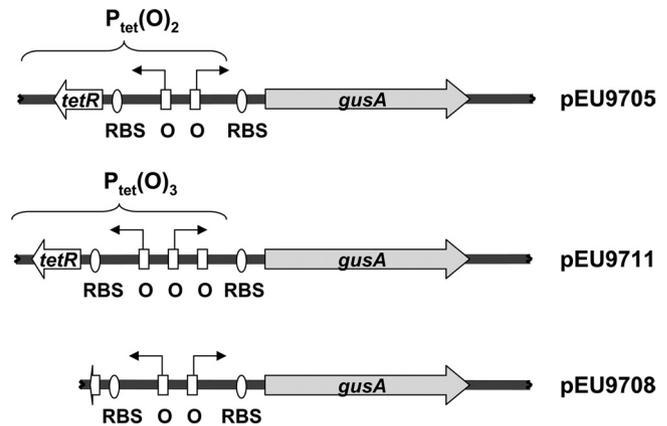


FIG. 1. Diagrams of  $P_{tet}$ -*gusA* fusions. The plasmid pEU9705 contains the  $P_{tet}(O)_2$  cassette, which includes the gene *tetR* (encoding the repressor) with its RBS followed by two *tet* operators (each has the sequence ACTCTATCATTGATAGAGT). The first *tet* operator overlaps the promoter for *tetR*, and the second overlaps the promoter for *gusA*. The distance between these two operators is 66 nt. The *gusA* gene has the consensus RBS (AGGAGG). pEU9711 is similar to pEU9705 but has an additional *tetO* inserted 14 nt after the first two operators [ $P_{tet}(O)_3$  cassette]. pEU9708 is the same as pEU9705 but has the internal region of *tetR* deleted. Promoters are designated by bent arrows. O, *tetO*. Note that the diagrams are not drawn to scale.

riboswitch that we engineered into the chromosome to replace the native ribosome binding site. We concluded that the use of  $P_{tet}$  may be appropriate in specific situations, but limitations of its dynamic range preclude its universal use. On the other hand, the theophylline-regulated riboswitch can be used in this important human pathogen to study genes that encode proteins required in large quantities as well as those that are essential for growth.

## MATERIALS AND METHODS

**Bacterial growth conditions.** GAS strains MGAS315 (serotype M3) (4), JRS4 (serotype M6) (37), MGAS2221 (serotype M1) (38), and their derivatives were grown in Todd-Hewitt medium with 0.2% yeast extract (THY) at 37°C. To determine optical density without admitting extra oxygen, the cultures were grown in stoppered side-arm flasks and cell growth was monitored using a Klett-Summerson photoelectric colorimeter with a red filter. *E. coli* Top10 cells (Invitrogen), used for cloning, were grown in LB medium. For *E. coli* cultures, concentrations of antibiotics were as follows: spectinomycin, 50 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 20 µg/ml. For GAS cultures, antibiotics were used at the following concentrations: spectinomycin, 100 µg/ml; kanamycin, 200 µg/ml; and chloramphenicol, 5 µg/ml.

To quantify Hlp RNA and protein, overnight cultures grown in THY, with or without 500 ng/ml anhydrotetracycline (AHT), were diluted 1/20 in prewarmed THY broth containing the same concentration of AHT, and growth was monitored in a colorimeter. When cultures reached mid-exponential phase, two aliquots were used, one to harvest RNA for quantitation of message and antisense RNA and the other for protein determination.

**Construction of  $P_{tet}$ -*gusA* fusion plasmids.** (i) **pEU9705.** A fragment containing the inducible promoter ( $P_{tet}$ ) and the *tet* repressor (*tetR*) was amplified from plasmid pTetE (42), and the *gusA* ( $\beta$ -glucuronidase) reporter gene was amplified from pJRS462 (16). These two fragments were fused by overlapping PCR and cloned into pJRS9508 (2), a derivative of the low-copy-number stable GAS plasmid pREG696 (15), to create pEU9705 (Fig. 1).

(ii) **pEU9708.** The *tet* repressor was deleted from pEU9705 by use of a QuikChange site-directed mutagenesis kit (Agilent Technologies) to amplify the entire pEU9705 plasmid minus 660 bp within *tetR*, creating pEU9708 (Fig. 1).

(iii) **pEU9711.** The *gusA* reporter amplified from pEU9703 and the  $P_{tet}(O)_3$  promoter with the Tet repressor region amplified from pEU8518 (5) were fused by overlapping PCR, and the fusion was inserted into pREG696 (15) to create

pEU9711 (Fig. 1). The plasmids generated, pEU9705, pEU9708, and pEU9711, were electroporated into JRS4, and transformants were selected on THY plates containing spectinomycin.

**Construction of inducible RNase J1 and RNase J2 mutants.** To generate strains JRS7314 [ $P_{tet}(O)_2$  *mjA* in MGAS315] and JRS7315 [ $P_{tet}(O)_2$  *mjB* in MGAS315], plasmids pEU8512 and pEU8510 (5) were linearized and electroporated separately into GAS strain MGAS315. The double-crossover transformants in GAS were selected in the presence of AHT (25 ng/ml) and chloramphenicol for the *mjA* mutant or kanamycin for the *mjB* mutant. The final result was the chromosomal integration of the  $P_{tet}(O)_2$  cassette in front of the ATG translation start codon of the gene for RNase J1 or RNase J2.

**Construction of inducible Hlp mutants using  $P_{tet}$ .** Two fragments, one containing the region upstream of *hlp* and another containing a region that included *hlp* and the sequence downstream of *hlp*, were amplified from JRS4 genomic DNA in separate PCRs. The  $P_{tet}(O)_2$  cassette was amplified from pEU8512, and the  $P_{tet}(O)_3$  cassette was amplified from pEU8517 (5). The two *hlp* fragments were fused with either  $P_{tet}$  cassette fragment by overlapping PCR, cloned into pCR-XL-TOPO (Invitrogen), and transferred into strain MGAS2221 to generate the inducible *hlp* mutant strains JRS2514 and JRS2518 (see Fig. 4A) as described for the RNase J1 mutant.

To generate pJRS7555, which expresses *hlp* antisense RNA under the control of the  $P_{tet}(O)_2$  promoter, the fragment containing *hlp* (including 17 nucleotides upstream of the *hlp* translation start codon and 282 nucleotides beyond the *hlp* stop codon) was amplified by PCR, using JRS4 genomic DNA as a template. The generated fragment was cloned in place of *gusA* in plasmid pEU9705 (Fig. 1), in the antisense orientation. The fragment containing  $P_{tet}(O)_2$  and the antisense *hlp* sequence was then moved from this plasmid to the high-copy-number plasmid pLZ12Spec (19) (see Fig. 4B).

To construct plasmid pJRS8334, which contains the  $P_{tet}(O)_2$  promoter with no gene expressed from it, pJRS7555 was digested to remove the antisense *hlp* fragment and then religated (see Fig. 4B).

To generate pJRS7559, a plasmid with terminators surrounding the region expressing the *hlp* antisense sequence under the control of the  $P_{tet}(O)_2$  promoter, the fragment containing  $P_{tet}(O)_2$  and the antisense *hlp* sequence was recloned from plasmid pJRS7555 into plasmid pTL61t (26) to produce pEU8335. The fragment containing  $P_{tet}(O)_2$  and the antisense *hlp* sequence surrounded by terminators was amplified from pEU8335 and cloned into pLZ12 (pLZ12 is pNZ12 [8] with a multiple cloning site) (obtained from B. Chassy) to generate pJRS7559 (see Fig. 4C).

**Insertion of a riboswitch to control expression of Hlp.** To generate JRS2653 (in which translation of Hlp is controlled by a riboswitch), five DNA fragments were isolated using PCR: a fragment upstream of the *hlp* promoter region from MGAS2221 chromosomal DNA, a fragment carrying the chloramphenicol resistance gene from pLZ12, the *hlp* promoter region from MGAS2221, riboswitch E (41), and a region beginning with the start codon of Hlp and including the *hlp* gene and sequences downstream of *hlp* from MGAS2221. These five DNA fragments were fused in the order described above, and the resulting fragment was cloned into pCR-XL-TOPO (Invitrogen), the plasmid was linearized, and the DNA was electroporated into MGAS2221. Double-crossover transformants in GAS were selected in the presence of 2 mM theophylline and chloramphenicol. The correct insertion of the riboswitch was confirmed by sequencing.

**$\beta$ -Glucuronidase assay.** Overnight cultures grown in THY broth with spectinomycin were washed at room temperature and diluted 1/20 in prewarmed THY containing different concentrations of doxycycline or anhydrotetracycline as described in Results and Discussion. Aliquots of log-phase cultures were pelleted and resuspended in ice-cold Z buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{Na}_2\text{H}_2\text{PO}_4$ , 10 mM KCl, and 1 mM  $\text{MgSO}_4$ ). The cells were lysed by vortexing with glass beads (lysing matrix B; MP Biochemicals) for 10 min at the highest setting at 4°C. Debris was removed by centrifugation, and the supernatant was transferred to a clean tube at 4°C. Kinetic GusA assays were performed as described previously (11). GusA activity was normalized to the amount of total protein determined by the Pierce bicinchoninic acid (BCA) protein microassay.

**RNA preparation and analysis.** RNA was prepared by the CsCl method as described previously (5). To quantify the amounts of *mjA*, *mjB*, and *proS* transcripts, we used real-time reverse transcription-PCR (RT-PCR) with a One Step iScript SYBR green kit (Bio-Rad). Controls and normalization of data were performed as described previously (35).

To quantify the amounts of *hlp* sense and antisense transcripts separately, a two-step real-time RT-PCR method was used. First, we used the avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and either the reverse primer to generate cDNA from the *hlp* sense transcript or the forward primer to generate cDNA from the *hlp* antisense RNA. Following cDNA synthesis, the reverse transcriptase was inactivated by incubation at 95°C for 10 min. In the

second step, cDNA was quantified by real-time PCR. The method for Northern blots to detect *mjB* transcripts was described previously (5).

**Western blot analysis of Hlp.** Mid-exponential-phase cells were resuspended in lysis buffer A (50 mM  $\text{NH}_4\text{OAc}$ , pH 6.2, 10 mM  $\text{CaCl}_2$ , and 1 mM dithiothreitol [DTT]) to which recombinant lysis from bacteriophage B30 of *Streptococcus agalactiae* (34) was added to a concentration of 200 U/ml. The cells were incubated at 30°C for 30 min with shaking at 700 rpm (Eppendorf Thermoblock) and boiled for 10 min in SDS-PAGE sample buffer. Proteins were resolved in a Bis-Tris 4 to 12% acrylamide gel (Invitrogen) with morpholineethanesulfonic acid (MES) running buffer (Invitrogen), transferred to nitrocellulose membranes (Bio-Rad), and incubated with anti-*S. pyogenes* Hlp antibody (obtained from V. Pancholi [22]). Bands were detected using alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma).

**Effect of theophylline on growth.** Overnight cultures of strains MGAS2221 and JRS2653, grown in THY broth plus 2 mM theophylline and THY broth plus 2 mM theophylline and chloramphenicol, respectively, were washed once to remove the theophylline and counted in a Petroff-Hausser chamber. Cells were resuspended to the same cell number, and 10-fold serial dilutions were spotted on THY agar with and without 2 mM theophylline. Following incubation at 37°C for 24 h, plates were photographed.

## RESULTS AND DISCUSSION

**$P_{tet}$  with two or three operators can be induced to different levels in GAS.** For quantitative comparison of the expression of the tetracycline-inducible promoter  $P_{tet}$ , we constructed transcriptional fusions to the reporter gene *gusA* on a low-copy-number plasmid that is maintained stably in GAS. To compare the effects of two versus three operators, we constructed two different plasmids, each of which contained the gene for the repressor of  $P_{tet}$  (*tetR*) with its own promoter and its native RBS. In one plasmid, pEU9705, this was followed by two *tet* operators ( $O_2$ ), and in the other plasmid, pEU9711, by three operators ( $O_3$ ). To determine the induction level in the complete absence of repression, we constructed plasmid pEU9708, which is identical to pEU9705 but lacks *tetR* (Fig. 1). These plasmids were electroporated separately into strain JRS4 to study the induction of  $P_{tet}$  in GAS.

We then identified a concentration of inducer sufficient to activate  $P_{tet}$  without inhibiting GAS growth. We investigated the effects of two different inducers, doxycycline and AHT, both of which have been used for  $P_{tet}$  activation in other bacteria (13, 25, 27, 42), on growth of the GAS strain JRS4/pEU9705 in liquid medium. We found that at the lowest concentration sufficient to produce detectable activation of  $P_{tet}$  ( $\geq 2$  ng/ml), doxycycline inhibited cell growth (data not shown). However, AHT at concentrations as high as 500 ng/ml had no effect on the growth of GAS in liquid culture (Fig. 2). Therefore, unless otherwise noted, we used this concentration of AHT for induction.

We found that  $P_{tet}(O)_2$  was induced about 5-fold in JRS4/pEU9705 (Table 1), suggesting that this promoter would be useful for further studies. However, in JRS4 with the plasmid lacking the *tet* repressor (pEU9708), GusA activity was >3-fold higher than that in the induced strain carrying plasmid pEU9705 (Table 1). This indicates that the highest concentration of AHT that could be used without inhibiting cell growth, 500 ng/ml, was inadequate for complete induction of  $P_{tet}(O)_2$ . Furthermore, there was significant Gus activity expressed from  $P_{tet}(O)_2$  in the absence of inducer, which might limit the value of this construct in genetic analyses. In the  $P_{tet}(O)_3$  construct, pEU9711, Gus activity was undetectable in the absence of inducer. However, induction from this construct was not as

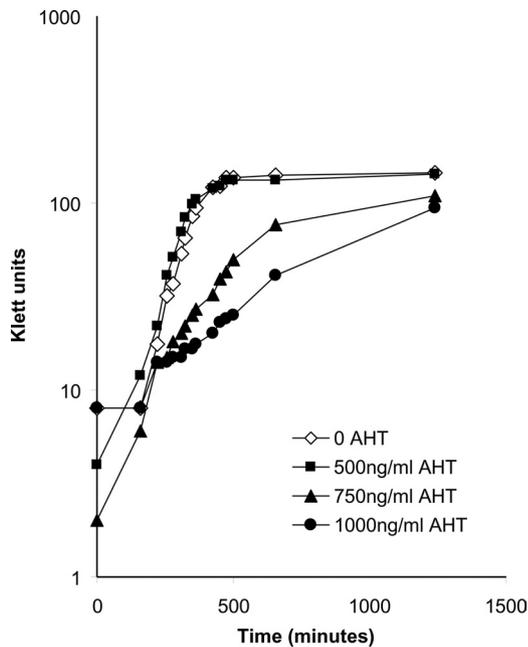


FIG. 2. Effect of AHT on growth of GAS. Strain JRS4/pEU9705 was grown at 37°C in THY broth containing the indicated concentrations of AHT.  $\diamond$ , 0 ng/ml;  $\blacksquare$ , 500 ng/ml;  $\blacktriangle$ , 750 ng/ml;  $\bullet$ , 1,000 ng/ml.

great (Table 1). In summary, both promoter constructs [P<sub>tet</sub>(O)<sub>2</sub> and P<sub>tet</sub>(O)<sub>3</sub>] demonstrated regulated activity in GAS, and although both had disadvantages as well as advantages, both can be used to control expression of genes in this bacterium.

**Use of P<sub>tet</sub> in the chromosome of GAS for expression of essential genes.** We recently demonstrated that P<sub>tet</sub>(O)<sub>3</sub> can be used successfully to control chromosomal expression of the genes encoding RNase J1 (*mjA*) and RNase J2 (*mjB*) in GAS strain MGAS315, the strain that we used for mRNA decay studies (5). Here we determined whether the two-operator construct P<sub>tet</sub>(O)<sub>2</sub> could be used in the chromosome of GAS for conditional expression of essential genes, since it was inducible to a higher level than P<sub>tet</sub>(O)<sub>3</sub>. We inserted the cassette containing P<sub>tet</sub>(O)<sub>2</sub> in front of the ATG translation start for the *mjA* or *mjB* gene in the chromosome of MGAS315 as described in Materials and Methods. The generated strains, JRS7314 [P<sub>tet</sub>(O)<sub>2</sub> *mjA*] and JRS7315 [P<sub>tet</sub>(O)<sub>2</sub> *mjB*], contained an antibiotic resistance gene for selection (chloramphenicol for *mjA* and kanamycin for *mjB*), following *tetR* and separated from it by transcriptional terminators (5). This P<sub>tet</sub> cassette also contained a strong ribosome binding site (AG-

TABLE 1.  $\beta$ -Glucuronidase activities of strains in this study

Strain description	Plasmid	$\beta$ -Glucuronidase activity (GU/mg protein)	
		0 ng/ml AHT	500 ng/ml AHT
P <sub>tet</sub> (O) <sub>2</sub> <i>gusA</i>	pEU9705	303 $\pm$ 27	1,446 $\pm$ 3
P <sub>tet</sub> (O) <sub>3</sub> <i>gusA</i>	pEU9711	<5	113 $\pm$ 47
$\Delta$ <i>tetR</i> P <sub>tet</sub> (O) <sub>2</sub> <i>gusA</i>	pEU9708	4,310 $\pm$ 268	ND <sup>a</sup>

<sup>a</sup> ND, not determined.

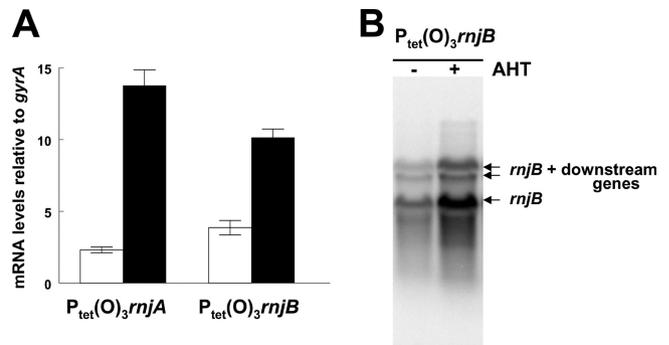


FIG. 3. Amounts of transcripts for RNase J1 and RNase J2 in the conditional mutants and the wild-type strain. (A) Real-time RT-PCR analysis of transcript amounts of *mjA* in strain JRS7316 [P<sub>tet</sub>(O)<sub>3</sub> *mjA*] and of *mjB* in strain JRS7317/pJRS9508 [P<sub>tet</sub>(O)<sub>3</sub> *mjB*]. The conditional mutants were grown with 0.1 ng/ml of AHT (white bars) or with 25 ng/ml of AHT (black bars). Averages  $\pm$  standard deviations relative to the amount of *gyrA* are presented. Each bar represents four technical replicates from two independent cultures. (B) Northern blot analysis with 5  $\mu$ g of total RNA for the strain JRS7317/pJRS9508 [P<sub>tet</sub>(O)<sub>3</sub> *mjB*] grown with 0.1 ng/ml of AHT (-) or with 25 ng/ml AHT (+). The probe was specific for *mjB*.

GAGG) to drive expression of *mjA* or *mjB*. As reported previously (5), neither of the strains with three operators grew without inducer, but both were viable in the presence of AHT. However, we now report that for both J1 and J2, expression from P<sub>tet</sub>(O)<sub>2</sub> was high enough in the absence of induction to allow growth of GAS (data not shown). Thus, in this context, it was preferable to reduce constitutive expression from P<sub>tet</sub> at the cost of reducing the amount of expression upon induction.

To quantify the amounts of *mjA* and *mjB* transcripts from P<sub>tet</sub>(O)<sub>3</sub> in each of these strains, we used quantitative real-time RT-PCR (Q-RT-PCR). We used strains JRS7316 and JRS7317/pJRS9508 [with P<sub>tet</sub>(O)<sub>3</sub> *mjA* and P<sub>tet</sub>(O)<sub>3</sub> *mjB*, respectively] grown with 25 ng/ml AHT (“induced”), which was more than sufficient to restore growth of the mutants to wild-type rates, and with 0.1 ng/ml AHT (“uninduced”), which allowed some growth of the mutants (5). We found that the amounts of *mjA* and *mjB* transcripts were 3- to 6-fold greater following induction than in the uninduced strain (Fig. 3A). This is much less than the induction we observed for the *gusA* reporter on a plasmid (Table 1). Quantitation of *mjB* transcripts in a Northern blot corroborated the results from Q-RT-PCR, i.e., induction produced a 5-fold increase in the amount of transcripts (Fig. 3B). In addition, Northern blot analysis of the *mjB* operon showed that all three transcripts (two of which included downstream genes) (5) appeared to be full length. Thus, P<sub>tet</sub>(O)<sub>3</sub> seems a better choice for inactivation and regulation of the RNase J genes, which code for essential enzymes in GAS.

**Use of P<sub>tet</sub> to regulate expression of hlp.** Because *hlp* is essential for growth in other bacteria, including *S. intermedius* (27), and because we were unable to delete it, we anticipated that it might also be required in GAS. To determine whether this was the case, we inserted the P<sub>tet</sub> cassettes into the chromosome in front of *hlp*, following the same strategy as that used for the RNase J genes (Fig. 4). The cassette containing the chloramphenicol resistance gene, *tetR*, and P<sub>tet</sub>(O)<sub>2</sub> was integrated in front of the translation start ATG for *hlp* in the

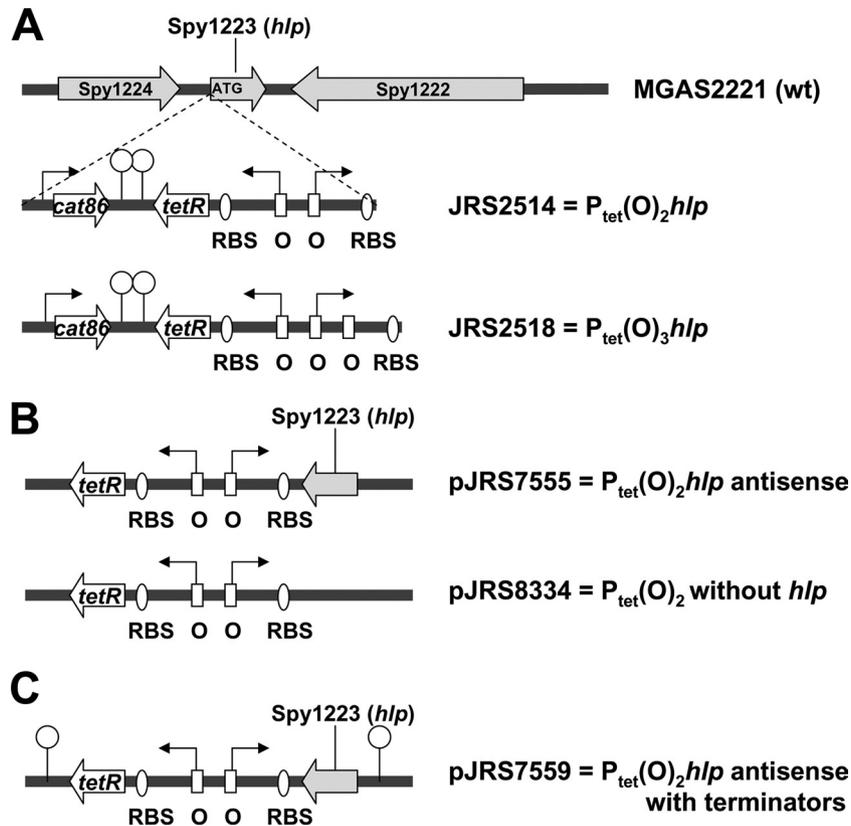


FIG. 4. Diagrams of Hlp conditional mutants. (A) Chromosomal mutants. The tetracycline-inducible mutants for Hlp contained the  $P_{tet}$  cassettes inserted directly in front of the ATG translation start codon for *hlp*. Spy numbers are from strain MGAS5005, whose sequence is identical in this region to that of MGAS2221 (39). (B) Plasmid expressing inducible *hlp* antisense RNA and control plasmid without *hlp*. (C) Plasmid expressing inducible *hlp* antisense RNA with transcriptional terminators flanking the cloned  $P_{tet}(O)_2 hlp$  fragment. These diagrams are not drawn to scale. Bent arrow, promoter; lollipop, transcription terminator; O, *tetO*.

chromosome of the GAS strain MGAS2221, a serotype M1 strain, to generate JRS2514, and the same strategy with  $P_{tet}(O)_3$  was used to generate JRS2518 (Fig. 4A). To evaluate the effect of induction of each *tet* cassette on expression of Hlp, we used Western blotting (Fig. 5). This showed that induction with AHT increased the amounts of Hlp in the  $P_{tet}$  strains, while AHT had no effect on the amount of Hlp in the wild-type parental strain. Although the amount of Hlp in the absence of induction in the  $P_{tet}(O)_3$  strain was significantly lower than that

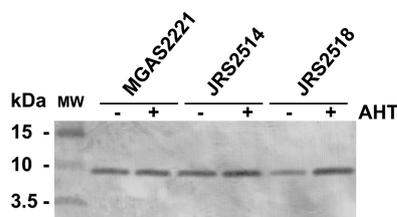


FIG. 5. Western blot showing the expression of Hlp. Total cell lysates from log-phase cultures grown without AHT (-) and in the presence of 500 ng/ml AHT (+) were resolved in a Bis-Tris 4 to 12% acrylamide gel (Invitrogen). Bands for Hlp were detected using rabbit anti-Hlp antiserum. MW, Novex Sharp standard; molecular sizes are shown to the left of the figure. MGAS2221, wild type; JRS2514,  $P_{tet}(O)_2 hlp$ ; JRS2518,  $P_{tet}(O)_3 hlp$ .

in the  $P_{tet}(O)_2$  strain, as expected, it was still significant and allowed normal growth (data not shown).

**Use of  $P_{tet}$  to produce *hlp* antisense RNA.** Since the constitutive level of Hlp even in the  $P_{tet}(O)_3$  strain seemed likely to be too high to be useful, we attempted an antisense approach to reduce Hlp expression further. We constructed pJRS7555 by removing the native promoter and inserting  $P_{tet}(O)_2$  oriented to produce antisense RNA for *hlp* (Fig. 4B). Although the addition of AHT to MGAS2221/pJRS7555 or strain JRS4/pLZ12spec prevented or reduced growth on plates (Fig. 6A and B), strain MGAS2221, containing the control plasmid pJRS8334 (Fig. 4B) in which the *hlp* gene was deleted, also showed sensitivity to addition of AHT (Fig. 6C). Therefore, no conclusion about the essentiality of Hlp in GAS can be drawn from this experiment.

The effect of pJRS8334 on growth of GAS suggested that a transcript resulting from readthrough from the induced  $P_{tet}$  into the plasmid was detrimental to GAS growth. To test this idea, we inserted multiple terminators upstream of *tetR* and downstream of *hlp* in the  $P_{tet}(O)_2 hlp$  antisense construct to give plasmid pJRS7559 (Fig. 4C) as a source of *hlp* antisense RNA. We evaluated the effect of AHT induction on production of both sense and antisense *hlp* RNAs and normalized these to the amount of *proS* message. Quantitation of the antisense *hlp* RNA in MGAS2221/pJRS7559 showed an in-

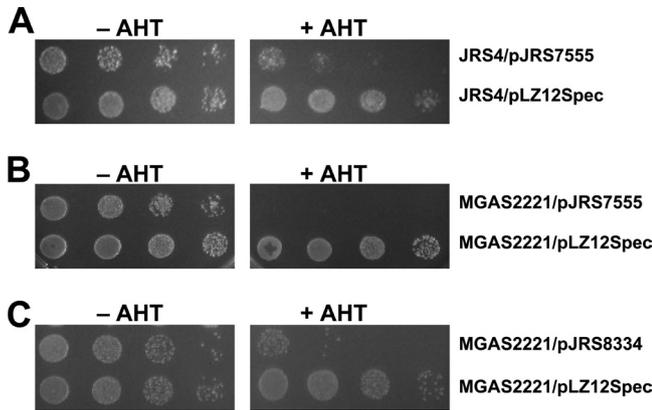


FIG. 6. Effect of Hlp on growth on THY plates. Tenfold serial dilutions of overnight cultures were spotted on plates with and without AHT (500 ng/ml). The plates were incubated at 37°C with 5% CO<sub>2</sub> for 18 h (A) or for 23 h (B and C) and then photographed. Plasmid pJRS7555 expresses antisense RNA for *hlp* from the promoter P<sub>tet</sub>(O)<sub>2</sub>, pJRS8334 contains P<sub>tet</sub>(O)<sub>2</sub> but no *hlp*, and pLZ12Spec is the empty vector.

crease of 9-fold following AHT induction, while the effect of AHT in MGAS2221 with the empty vector (pLZ12) was <2-fold, suggesting that this approach was effective. In agreement with this, the sense *hlp* transcript in MGAS2221/pJRS7559 decreased 7-fold with induction, while AHT had little effect on the sense *hlp* transcript in MGAS2221/pLZ12. However, although induction of the *hlp* antisense transcript was successful, it had very little effect on the amount of Hlp protein present (Fig. 7, compare lanes 1 and 2) and no effect on cell growth (data not shown). The strain producing the least Hlp protein was uninduced JRS2518, which had P<sub>tet</sub>(O)<sub>3</sub> inserted into the chromosome in front of *hlp* (Fig. 7, lane 3).

**Use of a riboswitch shows that Hlp is essential for GAS growth.** Because we were unable to use P<sub>tet</sub> in any configuration to generate a significant decrease in the amount of Hlp protein below that in the wild type, an alternative approach was attempted. We used a newly described synthetic riboswitch (41) to regulate translation of this gene. In this riboswitch

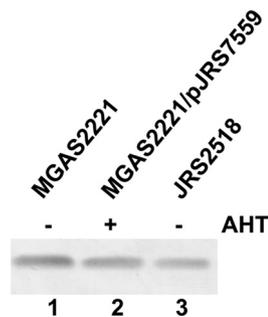


FIG. 7. Western blot showing the expression of Hlp in the *hlp* chromosomal mutant and the strain expressing antisense RNA for *hlp*. Strains MGAS2221 (wild type) and JRS2518 [P<sub>tet</sub>(O)<sub>3</sub> *hlp*] were grown without AHT (-), and strain MGAS2221/pJRS7559 [P<sub>tet</sub>(O)<sub>2</sub> *hlp* antisense] was grown in the presence of 500 ng/ml AHT (+). Total cell lysates prepared from the three strains were resolved in a Bis-Tris 4 to 12% acrylamide gel (Invitrogen). Hlp was detected using rabbit anti-Hlp antiserum.



FIG. 8. Hlp is required for growth of GAS. Overnight cultures of strain JRS2653, expressing Hlp under the control of the riboswitch, and its parent strain, MGAS2221, were pelleted, and the pellets were washed with saline to remove the theophylline. The two cultures were adjusted to the same cell number, and serial 10-fold dilutions (columns) were spotted in duplicate (rows) on THY plates with 2 mM theophylline and without theophylline. Plates were incubated at 37°C for 24 h.

system, the RBS is occluded by spontaneous folding of the mRNA. The RBS is freed to bind ribosomes and translate the message when a small-molecule ligand, theophylline, is added to the medium. To avoid the use of plasmids, we inserted the riboswitch directly upstream of the *hlp* open reading frame in the chromosome of strain MGAS2221 to generate strain JRS2653, which was isolated and maintained in the presence of theophylline. To determine whether theophylline was required for growth of JRS2653, in which Hlp translation was under the control of the riboswitch, overnight cultures of JRS2653 and the wild-type strain MGAS2221 grown in the presence of theophylline were washed to remove the theophylline and diluted to the same cell number. Dilutions of both strains were spotted onto plates with and without theophylline and incubated for 24 h at 37°C. As shown in Fig. 8, theophylline was required for growth of JRS2653, demonstrating that in GAS, *hlp* is an essential gene. As might be expected for a gene encoding an essential protein, extended periods of incubation of JRS2653 in the absence of theophylline led to outgrowth of mutants (data not shown).

**Conclusions.** To evaluate the role of any specific gene in the pathophysiology of GAS, tools that allow the experimenter to regulate gene expression are required. We evaluated four approaches to achieve this goal: we expressed genes under the control of two variants of the inducible P<sub>tet</sub> promoter, we used antisense RNA to reduce expression, and we used a synthetic riboswitch to control translation in response to a small-molecule ligand. We found that the P<sub>tet</sub> cassette with three operators was effective for study of RNase J1 (*mjA*) and RNase J2 (*mjB*), which are expressed at low levels, while the same cassette with two operators had an uninduced background level that was too high for studies of proteins required in very small amounts. However, the two-operator *tet* cassette can be induced to a higher level than the three-operator cassette and thus might be useful in the antisense configuration. We found that although the amount of the sense transcript for *hlp* in a cell was significantly reduced when antisense transcript for *hlp* was expressed under the control of P<sub>tet</sub>(O)<sub>2</sub>, there was still too much Hlp protein to determine whether Hlp is required for GAS growth. The last method we evaluated was use of a newly developed riboswitch in which the small-molecule theophylline controls translation of a protein by regulating the access of ribosomes to the ribosome binding site. The addition of theophylline led to at least a 50-fold increase in the amount of protein translated from the riboswitch (41), and this was suf-

ficient for expression of Hlp in the amount required for GAS growth. Of all the gene regulation methods tested, only the riboswitch, which has very low background expression in the absence of the ligand (41), reduced the amount of the abundant Hlp protein sufficiently to demonstrate that Hlp is essential for growth of GAS. We anticipate that in future studies several of these methods may be combined to allow investigation of several genes simultaneously. In addition, the *tet* cassette has already been used in murine infection models (3, 13, 17, 20, 21, 28), and future work will determine whether the riboswitch can also be used in animal models of infection.

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