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Journal Title: Applied and Environmental Microbiology
Volume: Volume 77, Number 13
Publisher: American Society for Microbiology | 2011-07, Pages 4422-4428
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/AEM.00554-11
Permanent URL: http://pid.emory.edu/ark:/25593/f24j6

Final published version: http://aem.asm.org/content/77/13/4422

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Accessed December 14, 2018 2:42 PM EST
The Histone-Like Protein Hlp Is Essential for Growth of *Streptococcus pyogenes*: Comparison of Genetic Approaches To Study Essential Genes

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Received 10 March 2011/Accepted 21 April 2011

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].
P₄₄ 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) [P₄₄(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 P₄₄ 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 P₄₄ constructs: mjcA, encoding RNase J1, and mjB, encoding RNase J2, which we previously showed are essential in GAS by using a chromosomally inserted P₄₄(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 (27) 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 P₄₄ and tested conditional activation of hlp from both P₄₄(O)₂ and P₄₄(O)₃. We also tested inactivation of hlp by expressing its antisense RNA from P₄₄(O) in a plasmid. In addition, we compared expression of hlp from these three constructs with its production under the control of a synthetic riboswitch that we engineered into the chromosome to replace the native ribosome binding site. We concluded that the use of P₄₄ 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 P₄₄-gusA fusion plasmids.** (i) pEU9705. A fragment containing the inducible promoter (P₄₄) and the tet repressor (tetR) was amplified from plasmid pTetE (42), and the gusA (β-glucuronidase) reporter gene was amplified from plasmid pJKS462 (16). These two fragments were fused by overlapping PCR and cloned into pJRS4508 (2), a derivative of the low-copy-number stable GAS plasmid pREG969 (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 P₄₄(O)₃ promoter with the Tet repressor region amplified from pEU8S18 (5) were fused by overlapping PCR, and the fusion was inserted into pREG969 (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 Rnas J1 and Rnas J2 mutants.** To generate strains JRS7315 (P_{tet}(O)2; mRJ/A in MGA315) and JRS7315 [P_{tet}(O)2; mRJ/B in MGA315], plasmids pEU8512 and pEU8510 (5) were linearized and electroporated separately into GAS strain MGA315. The double-crossover transformants in GAS were selected in the presence of AHT (25 ng/ml) and chloramphenicol for the mRJ J1 or kanamycin for the mRJ J2 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 pCar.** 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_{hlp}(O)1 cassette was amplified from pEU8512, and the P_{hlp}(O)2 cassette was amplified from pEU8517 (5). The two hlp fragments were fused with each other, cassette fragment by overlapping PCR, cloned into pCR-XL-TOPO (Invitrogen), and transformed into strain MGA2221 to generate the inducible hlp mutant strains JRS2514 and JRS2518 (see Fig. 4A) as described for the Rnas J1 mutant.

To generate pJRST555, 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 cloned 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_{hlp}(O)2 and the antisense hlp sequence was then moved from this plasmid to the high-copy-number plasmid pLZ12 (19) (see Fig. 4B).

To construct plasmid pRS6334, which contains the P_{hlp}(O)1 promoter with no gene expressed from it, pJRST555 was digested to remove the antisense hlp fragment and then religated (see Fig. 4B).

To generate pJRST559, a plasmid with terminators surrounding the region expressing the hlp antisense sequence under the control of the P_{hlp}(O)1 promoter, the fragment containing P_{hlp}(O)1 and the antisense hlp sequence was recloned from plasmid pJRST555 into plasmid pLZ12 (26) to produce pEU8335. The fragment containing P_{hlp}(O)2 and the antisense hlp sequence surrounding 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 pJRST559 (see Fig. 4C).

**Insertion of a riboswitch to control expression of Hlp.** To generate JRS2663 (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 MGA2221, chromosomal DNA, a fragment carrying the chloramphenicol resistance gene from pLZ12, the hlp promoter region from MGA2221, riboswitch E (41), and a region beginning with the start codon of Hlp and including the sequence downstream of hlp. These five DNA fragments were fused in the order described above, and the resulting fragment was cloned into pCR-XL-TOPO (Invitrogen), and transformed into strain MGA2221 to generate the inducible hlp mutant strains JRS2514 and JRS2518 (see Fig. 4A) as described for the Rnas J1 mutant.

**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 (O2), and in the other plasmid, pEU9711, by three operators (O3). 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} (>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)2 construct, pEU9711, Gus activity was undetectable in the absence of inducer. However, induction from this construct was not as high as from the P_{tet}(O)2 construct.
We recently demonstrated that Ptet(O)3 can be used successfully to control chromosomal expression of the essential genes, since it was inducible to a higher level than Ptet(O)2. We inserted the cassette containing Ptet(O)2 in front of the ATG translation start for the rnjA or rnjB gene in the chromosome of MGAS315 as described in Materials and Methods. The generated strains, JRS7314 [Ptet(O)2 rnjA] and JRS7315 [Ptet(O)2 rnjB], contained an antibiotic resistance gene for selection (chloramphenicol for rnjA and kanamycin for rnjB), following tetR and separated from it by transcriptional terminators (5). This Ptet cassette also contained a strong ribosome binding site (AGGAGG) to drive expression of rnjA or rnjB. 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 Ptet(O)2 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 Ptet at the cost of reducing the amount of expression upon induction.

To quantify the amounts of rnjA and rnjB transcripts from Ptet(O)3 in each of these strains, we used quantitative real-time RT-PCR (Q-RT-PCR). We used strains JRS7316 and JRS7317/pJRS9508 [with Ptet(O)3 rnjA and Ptet(O)3 rnjB, 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 rnjA and rnjB 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 rnjB 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 rnjB operon showed that all three transcripts (two of which included downstream genes) (5) appeared to be full length. Thus, Ptet(O)3 seems a better choice for inactivation and regulation of the RNase J genes, which code for essential enzymes in GAS.

Use of Ptet 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 Ptet 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 Ptet(O)2 was integrated in front of the translation start ATG for hlp in the

![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. ○, 0 ng/ml; ■, 500 ng/ml; ▲, 750 ng/ml; ●, 1,000 ng/ml.](image)
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 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-

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**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, $tetO$.

**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, $P_{tet}(O)_3$ $hlp$. 
Ptet(O)2, pJRS8334 contains Ptet(O)2 but no (41) to regulate translation of this gene. In this riboswitch was attempted. We used a newly described synthetic riboswitch protein below that in the wild type, an alternative approach to generate a significant decrease in the amount of Hlp...
sufficient 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 cas-sette 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.

ACKNOWLEDGMENTS

We are grateful to V. Pancholi for his generous gift of anti-Hlp antiserum. We are also grateful to C. Reynoso and J. Gallivan for the riboswitch.

This work was supported in part by NIH grant AI20723.

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