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Serine/Threonine Protein Kinase Stk Is Required for Virulence, Stress Response, and Penicillin Tolerance in *Streptococcus pyogenes*▽

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Genes encoding one or more Ser/Thr protein kinases have been identified recently in many bacteria, including one (*stk*) in the human pathogen *Streptococcus pyogenes* (group A streptococcus [GAS]). We report that in GAS, *stk* is required to produce disease in a murine myositis model of infection. Using microarray and quantitative reverse transcription-PCR (qRT-PCR) studies, we found that Stk activates genes for virulence factors, osmoregulation, metabolism of α-glucans, and fatty acid biosynthesis, as well as genes affecting cell wall synthesis. Confirming these transcription studies, we determined that the *stk* deletion mutant is more sensitive to osmotic stress and to penicillin than the wild type. We discuss several possible Stk phosphorylation targets that might explain Stk regulation of expression of specific operons and the possible role of Stk in resuscitation from quiescence.

Streptococcus pyogenes (the group A streptococcus [GAS]) is a Gram-positive bacterium that causes a variety of diseases in humans. GAS can cause pharyngitis, cellulitis, invasive diseases like myositis and fasciitis, and immune-mediated poststreptococcal sequelae, such as rheumatic heart disease and glomerulonephritis (52). In addition, GAS can reside in some individuals in the carrier state, in which the infected person is completely free of disease symptoms (62). GAS infections may originate by colonization of the respiratory mucosa or the skin but apparently can also be activated from the carrier state to cause serious invasive disease (70). Although all GAS strains are sensitive to penicillin, there are reports of as much as 37% treatment failure (29, 53), and it is estimated that 500,000 people die each year from GAS infections worldwide (5). Therefore, both a vaccine and better therapeutic agents are needed to improve protection from GAS diseases. The development of improved treatment regimens requires a greater understanding of the pathophysiology of GAS.

As for most bacterial pathogens, GAS virulence is multifactorial, and there are many virulence factors that contribute to different disease syndromes. The success of this important human pathogen in the different ecological niches it can occupy depends on its ability to respond to the environment by differential regulation of its many virulence factors. An understanding of this regulatory network is therefore critical to combating GAS disease.

Considerable study has been focused on the regulation of gene expression at the level of initiation of transcription of known and presumed virulence factors of GAS. In addition to multiple stand-alone transcriptional regulators, 13 two-component sensor-transducer regulatory systems (TCSs) have been identified in GAS genomes (31). A TCS typically comprises a membrane-associated sensor histidine kinase that autophosphorylates a conserved histidine residue in response to an environmental signal. This phosphate is then transferred to a conserved aspartate residue on its cognate response regulator, which alters the conformation of this protein and enhances its ability to bind to its specific recognition sequence(s) in the region of the promoter that it regulates (30). For most histidine kinases, this process is also reversible, since the histidine kinase can act as a phosphatase to remove the phosphate from the aspartate residue of its cognate response regulator. One of the TCSs studied in GAS is CovRS (control of virulence) (17), which is critical for growth under many stress conditions (general stress response) (8, 9, 18) and appears to respond to membrane stress. The CovRS system appears to be pivotal in determining disease outcome (24, 28, 69). CovR represses many virulence factors, and CovS reverses this repression by inactivating CovR, presumably by dephosphorylation (9).

Recently, there has been an additional focus on posttranslational control of the activity of bacterial proteins, including some that regulate transcription of other genes. For TCSs, posttranslational modification is critical for activity, since phosphorylation of histidine kinases on a conserved histidine residue is essential for transmitting the signal to the response regulator, and phosphorylation of response regulators on a key aspartate dramatically affects their DNA binding ability. In eukaryotic systems, phosphorylation of proteins on other residues, including serine, threonine, and tyrosine, is important for activity (40). Recently, genes homologous to those encoding eukaryotic-type serine/threonine kinases (Stks) have been recognized in bacterial genome sequences (11). Such Stks all contain kinase domains, which are required for phosphorylation of target proteins (74). In addition, some Stks, including those of streptococci (GAS, *Streptococcus agalactiae* [group B streptococcus, GBS], and *Streptococcus pneumoniae*), contain penicillin-binding and Ser/Thr kinase-associated (PASTA) domains which bind to peptidoglycan and β-lactam antibiotics

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(81). Although bacteria may have multiple Ser/Thr kinases, streptococci have only one copy of Stk and of its cognate phosphatase Stp (26, 55, 60). Stk in GAS is a membrane-associated protein, with its N terminus, which contains the kinase domain, facing inside the cell, and its C terminus, which contains three PASTA domains, facing outside (26).

In streptococci, carried just upstream of *stk* and overlapping with it, is a gene encoding a serine threonine phosphatase, *stp* (26, 46, 54). The product of this gene constitutes a signaling pair with Stk and is responsible for inactivation of Stk by dephosphorylation. In streptococci, *stp* cannot be deleted if *stk* is present, suggesting that Stk is detrimental to growth when it is in a permanent phosphorylated state (26, 48). This implies that regulation of the activity of Stk by Stp in response to appropriate cues is required to allow normal bacterial growth.

Bacterial Stks are typically involved in regulation of growth, metabolism, and pathogenesis. This regulation is accomplished by phosphorylation of protein substrates at conserved Ser and/or Thr residues (30). When the Stk substrate is an enzyme, phosphorylation may affect its activity (46, 78), and when the substrate is a transcriptional regulator, phosphorylation may affect its binding to DNA (35, 75), which then alters transcription of the promoters in its regulon. The transcriptional regulator of the sensor-transducer two-component system, CovR, is a phosphorylation substrate for Stk in GBS (55), and its homolog RitR is phosphorylated by Stk in *S. pneumoniae* (75). In GBS, CovR phosphorylation by Stk on a Thr65 residue interferes with phosphorylation by the cognate sensor kinase, CovS, on an Asp53 residue and reduces binding of CovR to DNA (35). CovR has not yet been identified as an Stk substrate in GAS.

In GAS, the only target of Stk phosphorylation identified so far is Hlp, which is phosphorylated on a Thr residue (49). Hlp, a small, abundant protein, is required for growth in several bacteria, including GAS (4), *Streptococcus intermedius* (36), and *Bacillus subtilis* (43). Hlp was identified as a target for phosphorylation by Stk in whole-cell GAS lysates as the protein that becomes phosphorylated upon incubation with ^{32}P-labeled Stk (26). Because Hlp is abundant, it is possible that in GAS, Stk has additional phosphorylation target proteins that were unrecognized because they are much less plentiful than Hlp. Hlp binds to many regions of DNA and thus controls expression of many genes (14), so Stk could be expected to regulate expression of these genes indirectly.

To understand the role of Stk in GAS and the mechanism of Stk control of gene expression, we constructed and studied a mutant deleted for *stk*. By comparing the transcriptome of a Δ*stk* strain with its wild-type (wt) parent, we identified genes whose expression is regulated by Stk. We also determined how this regulation affects biological functions of GAS, including pathogenesis in a mouse model of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless indicated otherwise, M1 GAS strain MGAS2221 (69) and its derivatives were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY). For the osmotic stress experiments, NaCl was added to THY at 0.5 M. For the starch degradation test, THY was supplemented with 0.3% soluble starch prior to autoclaving. *Escherichia coli* Top10 cells (Invitrogen) were used for the cloning of plasmids. Antibiotics were used for selection at the following concentrations: spectinomycin, 100 μg/ml for GAS and 50 μg/ml for *E. coli*; kanamycin, 200 μg/ml for GAS and 50 μg/ml for *E. coli*; chloramphenicol, 5 μg/ml for GAS and 20 μg/ml for *E. coli*.

Construction of an *stk* deletion mutant. The promoterless spectinomycin resistance gene *aad9*, amplified from pUC-Spec (25), was cloned with the regions flanking *stk* and used to replace *stk* in GAS strain MGAS2221. Sequencing of this region of the generated strain, JRS2516, confirmed that the replacement had occurred.

Replacement to restore *stk*. The chloramphenicol resistance gene *cat* with its own promoter, from pLZ12 (12), was cloned between the *stk* gene and a region downstream of the *stk* gene, and this construct was used to replace the *aad9* gene in the Δ*stk* strain JRS2516. This resulted in insertion of *stk* followed by *cat* in the chromosome. The correct sequence of this region in the resulting strain, JRS7322, was confirmed by sequence analysis.

Construction of a Δ*covR* mutant. The plasmid pJRS7562, a derivative of pJRS948 (17), was used to generate a *covR* deletion mutation in strain MGAS2221. The resulting strain, JRS2517, contains the kanamycin resistance gene *aphA3* in place of *covR*. The correct sequence of this region in JRS2517 was confirmed by sequence analysis.

Microarray. Microarray slides for *Streptococcus pyogenes* were from the Pathogen Functional Genomic Resource Center at the J. Craig Venter Institute. RNA was purified from triplicate mid-exponential-phase cultures of strains MGAS2221 and JRS2516 using CsCl (42), and contaminating DNA was removed with the Turbo DNA-free kit (Ambion) according to the manufacturer's instructions. Labeled cDNA probes were generated from the RNA using the FairPlayIII microarray labeling kit (Agilent Technologies) and Cy3 and Cy5 monoreactive dyes (GE Healthcare Biosciences) as recommended by the manufacturers. The labeled probes were hybridized to microarray slides, and the slides were scanned and analyzed as described previously (1). RNA from three independent cultures for each strain was analyzed using two microarray slides each.

Quantitative RT-PCR (qRT-PCR). RNA was prepared as described for microarrays, and transcripts were quantified using the iScript one-step reverse transcription (RT)-PCR kit with SYBR green (Bio-Rad) and LightCycler (Roche) as described previously (58).

RT-PCR. RNA prepared for microarrays was used for transcript analysis of the *fab* region by RT-PCR with the iScrip Select cDNA synthesis kit (Bio-Rad) followed by PCR with primers described in Results.

Mouse model of myositis. For each bacterial strain, 10 7- to 8-week-old female CD1 mice (Charles River Lab) were injected intramuscularly in the right hind limb with 10^8 CFU/mouse in 100 μl saline suspension. Mice were observed twice a day for 5 days after the infection. In addition to mice that died between observations, moribund mice with signs of terminal disease, which were euthanized, were included in the nonsurviving group.

Assays for starch degradation activity. To quantify starch degradation activity, GAS strains were grown in starch-containing broth, cultures were collected at time points indicated in Results, and cells were removed by centrifugation. Aliquots of 12.5 μl and 50 μl of each of the supernatants were mixed with 1 ml starch-staining solution (prepared by mixing 3 ml Gram's iodine solution and 50 ml 3.8 mM HCl), and optical density at 660 nm (OD_{660}) was measured. The decrease in starch concentrations in the supernatants compared to those of the original starch-containing medium was calculated using a calibration curve produced with known amounts of starch.

Penicillin sensitivity. Overnight cultures grown in THY were diluted 1:20 into prewarmed THY in stoppered side-arm flasks, which were used to allow determination of optical density without admitting extra oxygen. Cell growth was monitored using a Klett-Summerson photoelectric colorimeter with the red filter. Once the cultures reached early log phase, the culture was split in half and penicillin G was added to a concentration of 5 μg/ml to one half of the culture while the other half served as a control. To quantitate CFU/milliliter, the cells were precipitated by centrifugation to remove penicillin and plated on THY.

Microarray data accession number. Data are deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) with the accession no. GSE28881.

RESULTS

Stk is required for GAS to produce disease in a myositis model of infection in mice. To study the role of the serine/threonine kinase Stk in GAS, we generated an *stk* deletion mutant in strain MGAS2221. The serine/threonine phosphatase (Stp) that dephosphorylates Stk is encoded upstream of

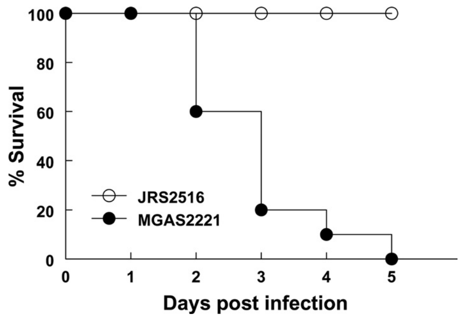

FIG. 1. Survival of mice following intramuscular inoculation. CD1 mice were injected intramuscularly with 10^8 CFU MGAS2221 (wild-type) (closed circles) or with 10^8 CFU JRS2516 (Δ*stk* mutant) (open circles) resuspended in saline. Ten mice were infected with each GAS strain, and percent survival was plotted for each day for five consecutive days after infection.

stk, and these genes overlap by 1 nucleotide (26). We designed the *stk* mutation in strain JRS2516 to leave *stp* and the gene downstream of *stk* intact by constructing an in-frame deletion within the *stk* gene. The growth rate in THY of JRS2516 was indistinguishable from that of its wild-type parent, strain MGAS2221, but the mutant never attained the same final concentration as its parent (OD_{660} of 0.7 versus 1.1). In addition, we found that there are about 6- to 7-fold more CFU in an overnight culture of MGAS2221 than in its Δ*stk* derivative, JRS2516, indicating that the Δ*stk* mutant is significantly less viable in an overnight stationary-phase culture. Morphologically, the strains differed significantly, as originally observed by Pancholi's group (26, 49). Microscopic examination shows that the cells of the Δ*stk* strain appear larger and more elongated.

To determine whether Stk affects virulence, we used the murine myositis model of infection (32, 66, 73) in which bacteria are injected directly into the muscle. The rapid dissemination of the bacteria within this tissue mimics necrotizing fasciitis, a serious disease caused by GAS in humans (47). We found that by day 5 following inoculation with strain MGAS2221, all the mice died, while none of the mice inoculated with its Δ*stk* derivative, JRS2516, showed any visible signs of illness (Fig. 1). Therefore, we conclude that Stk is required for GAS to produce disease in this model.

Stk activates expression of genes in GAS. Since Stk had a dramatic effect on virulence, we wished to identify the known and putative virulence genes whose expression is regulated by Stk. For this analysis, we compared the transcriptomes of the M1 strain MGAS2221 with that of its Δ*stk* derivative, strain JRS2516. Cells were collected at mid-exponential phase, RNA was harvested, and transcripts were analyzed by microarray. For some of the regulated genes, independent RNA preparations made from freshly grown cultures were used to confirm the microarray results by qRT-PCR (Table 1). From this analysis, Stk appears to be an activator of gene expression in GAS, since all transcripts affected showed a decrease in the absence of *stk*.

Since Stk phosphorylates CovR in GBS (35) and its homolog in *S. pneumoniae* (75), and this phosphorylation reduces binding of CovR to DNA, it seemed possible that GAS Stk activates genes by reducing the repression caused by CovR bind-

TABLE 1. Genes regulated by Stk and CovR in GAS as determined by microarray and qRT-PCR for cells from mid-exponential phase of growth in standard laboratory medium

Gene name	M5005_Spy gene no.	Putative function	Fold change in transcription[a]		
			Δ*stk* mutant/wt		Δ*covR* mutant/wt
			Microarray	qRT-PCR	qRT-PCR
Activated by Stk and CovR					
cdhA	17	Cell wall hydrolase	0.26	0.2	0.58
opuAA	157	Glycine betaine/proline transport	0.29	0.34	0.33
opuABC	158	Glycine betaine/proline transport	0.23	ND	ND
Activated by Stk, repressed by CovR					
grab	1106	Protein G-related alpha 2 M-binding protein	0.43	0.36	2.28
Activated by Stk, no effect of CovR					
mur1.2	664	Glucosaminidase	0.49	0.44	0.73
malD	1063	α-Glucan metabolism/uptake	0.60	ND	ND
malC	1064		0.61	0.43	0.93
amyA	1065		0.45	ND	ND
malX	1067		0.52	0.5	0.84
accA	1485	Fatty acid biosynthesis	0.61	ND	ND
accC	1486		0.60	ND	ND
fabZ	1487		0.58	ND	ND
accB	1488		0.53	ND	ND
fabF	1489		0.48	ND	ND
fabK	1492		0.59	0.55	1.15
acpP	1493		0.62	ND	ND
fabT	1495		0.65	0.57	1.14

[a] ND, not determined; wt, MGAS2221; Δ*stk* mutant, JRS2516; Δ*covR* mutant, JRS2517.

ing. Therefore, for some Stk-regulated genes, we assayed the amount of transcript in a Δ*covR* strain as well (Table 1). We found that some, but not all, of the Stk-regulated genes are also regulated by CovR (Table 1) (17, 66). We confirmed the finding of Pancholi's group that Stk activates *cdhA* (49) and found that transcription of this gene is also activated by CovR. CdhA is a member of the CHAP family (cysteine, histidine-dependent aminohydrolases/peptidases) of enzymes that cleave within peptide cross-bridges of cell wall peptidoglycan (3, 57), and its deletion appears to have a major effect on the morphology of GAS (49).

Expression of several known and putative virulence-associated genes is activated by Stk, as expected from the large effect Stk has in a murine model of GAS virulence (Fig. 1). One virulence-associated gene that is activated by Stk but is repressed by CovR is *grab*, which encodes a G-related α_2-macroglobulin-binding protein (Grab). This protein, which is covalently attached to the bacterial surface (2, 56), acts as a protease inhibitor in human blood, and this inhibition is thought to protect bacterial surface antigens, including several important virulence factors, from proteolytic degradation during infection (56). In agreement with this, Grab is required for full virulence of GAS in two models of infection: intraperitoneal inoculation and subcutaneous injection (56, 71). Grab has not yet been examined following intramuscular inoculation, so its contribution to the virulence effect of Stk in this model is not yet clear.

Another group of Stk-activated genes includes those whose transcription is not affected by CovR (Table 1). These include *mur1.2*, which encodes a secreted autolysin belonging to the family of glucosaminidases that can hydrolyze peptidoglycans by cleaving the bond between *N*-acetyl-muramic acid and the peptide cross-link (33, 79). The homologs of this enzyme in *S. pneumoniae*, *lytA* and *lytB*, affect separation of dividing cells (10, 59). Thus, if Mur1.2 has a similar function in GAS, its depletion in the GAS Δ*stk* strain might contribute to the unusual appearance of the cells of this mutant (26, 49). In addition, this peptidoglycan hydrolase may affect virulence in GAS, as do its homologs in *S. pneumoniae* (21, 23).

Stk is required for osmoregulation in GAS. Another genetic locus we found to be activated by Stk and by CovR is the *opu* locus. We found 3-fold less transcript of both genes in this locus, *opuAA* and *opuABC*, in the Δ*stk* strain than in its wild-type parent (Table 1). These genes code for the two-protein Opu osmosensing glycine betaine transport system, which mediates the uptake of the osmolytes glycine betaine and proline in response to increased extracellular osmolarity (37). By this means, bacterial cells are protected from osmotic shrinkage in the presence of high salt concentrations (80). Our transcription results suggest that the amount of the protective Opu proteins should be reduced in the Δ*stk* strain, so we predicted that the Δ*stk* mutant might show osmotic sensitivity. To test this, we wished to compare growth in high salt concentrations of the mutant with that of its wild-type parent and with that of a strain in which *stk* expression was restored. To avoid overexpression of Stk by complementation with a plasmid, we reintroduced *stk* into the deletion strain JRS2516 by replacing the deleted *stk* allele with the wild-type *stk* allele to produce strain JRS7322. The morphological appearance of JRS7322 was similar to that of its wild-type parent, and like its parent, it grew to an OD_{660} of 1.1.

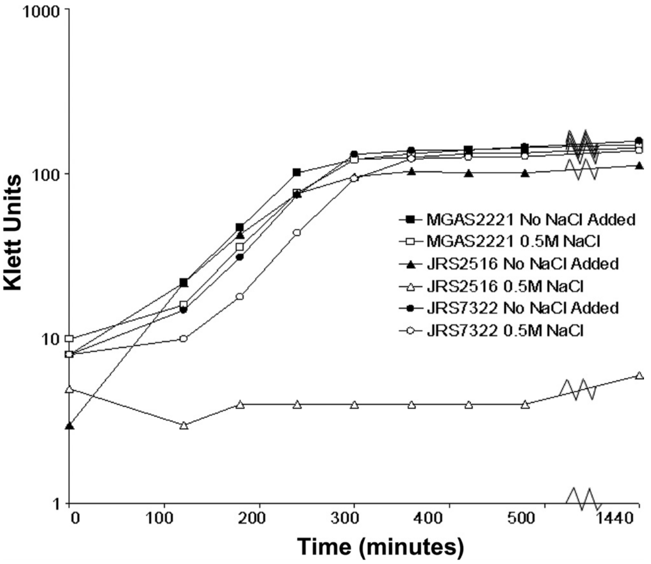

FIG. 2. Stk is required for growth in high salt concentrations. Strains MGAS2221, JRS2516 (Δ*stk* mutant), and JRS7322 (Δ*stk*::*stk* mutant) were grown overnight without extra salt in THY and then diluted into THY with and without 0.5 M NaCl.

We grew the three strains overnight in THY, diluted them into THY with and without 0.5 M NaCl, and followed growth (Fig. 2). In confirmation of our prediction based on transcription assays, we found that growth of the Δ*stk* strain JRS2516 was inhibited in high-salt conditions, while that of its wild-type parent, MGAS2221, and of the derivative of JRS2516 with *stk* expression restored, JRS7322, was not (Fig. 2). We conclude that Stk appears to be required for regulation of the response to osmotic stress in GAS.

Stk regulates the entire locus for fatty acid biosynthesis. Analysis by microarray indicated that transcription of genes encoding the enzymes constituting the fatty acid biosynthesis pathway was about 2-fold lower in the Δ*stk* mutant than in its wild-type parent (Table 1). The genes encoding these proteins are grouped within a single region in the GAS genome (68), in the same order as in the *S. pneumoniae* genome (38) (Fig. 3). In *S. pneumoniae*, there are at least three promoters in the *fab* region, located in front of *fabM*, *fabT*, and *fabK* (38). In this organism, the *fabT* transcript includes *fabH* and *acpP* but does not include either *fabM* or *fabK*, as shown by Northern blotting (38). To determine whether the transcriptional arrangement of the *fab* genes in GAS differs from that in *S. pneumoniae*, we performed RT-PCR analysis of the *fab* region with GAS RNA (Fig. 3C). We found that in GAS, *fabM* is present on the same transcript as *fabT*, and *fabT* is present on the same transcript as *fabK*, indicating a difference in transcription of the *fab* region from *S. pneumoniae*. Potential mechanisms for activation of *fab* genes by Stk in GAS and possible difference from that in *S. pneumoniae* will be considered in the Discussion.

Stk activates genes for metabolism and transport of α-glucans. Our transcription studies also demonstrated that Stk activates genes from M5005_Spy_1063 to M5005_Spy_1067 (Table 1), which are all in the same orientation. These genes

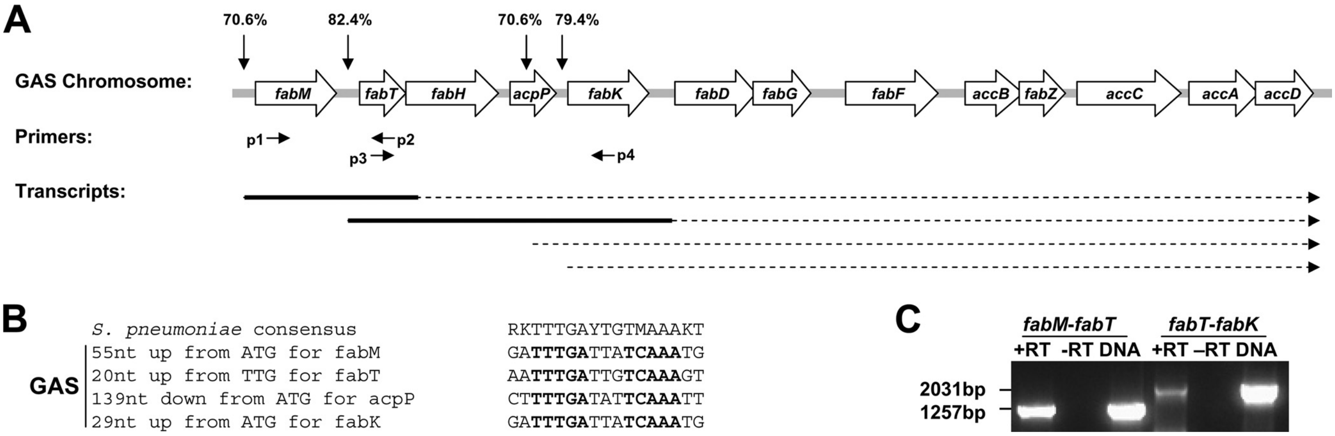
A
70.6%
82.4%
70.6%
79.4%
GAS Chromosome:
fabM
fabT
fabH
acpP
fabK
fabD
fabG
fabF
accB
fabZ
accC
accA
accD
Primers:
p1
p2
p3
p4
Transcripts:
B
S. pneumoniae consensus RKTTTGAYTGTMAAAKT
GAS
55nt up from ATG for fabM GATTTGATTATCAAATG
20nt up from TTG for fabT AATTTGATTGTCAAAGT
139nt down from ATG for acpP CTTTTGATATTCAAATT
29nt up from ATG for fabK GATTTGATTATCAAATG
C
fabM-fabT
fabT-fabK
+RT -RT DNA +RT –RT DNA
2031bp
1257bp

FIG. 3. Fatty acid biosynthesis (*fab*) region in GAS. (A) Predicted transcriptional organization for the *fab* region in GAS. Vertical arrows point to the predicted binding sites for the transcriptional repressor FabT in MGAS5005 with % homology to the consensus *S. pneumoniae* binding site shown above each arrow. Small horizontal arrows indicate location of primers used for detection of *fab* transcripts in GAS (p1 to p4). Dashed lines depict predicted transcripts for the *fab* region, and bold lines delineate the transcripts detected by RT-PCR (see panel C). (B) Sequence and location of the predicted FabT binding sites in MGAS5005. The palindromic sequence repeat that is conserved in GAS is delineated in bold letters. (C) RT-PCR analysis of transcripts for the *fab* region in GAS. The reaction was performed with (+RT) or without (−RT) reverse transcriptase using MGAS2221 RNA and primers p1 and p2 to identify a transcript that includes both *fabM* and *fabT* (*fabM-fabT*) or primers p3 and p4 to identify a transcript including *fabT*, *fabH*, *acpP* and *fabK* (*fabT-fabK*). DNA of MGAS2221 was used as a positive control for the PCR.

are part of a larger locus encoding proteins involved in glucan transport/metabolism (Table 1) (64). One of these genes encodes AmyA, an enzyme that degrades long-chain α-glucan starch (64). If the amount of AmyA is proportional to the amount of its transcript, there should be less of this enzyme in the Δ*stk* strain than in its parent. We found no significant difference in starch hydrolysis between the strains: at 30 min into stationary phase, the wild-type strain hydrolyzed 12% ± 2% of the starch originally present in the growth medium, and the Δ*stk* strain hydrolyzed 10% ± 6% of the starch. At 15 h into stationary phase, the wild-type hydrolyzed 24% ± 5%, and the Δ*stk* mutant hydrolyzed 18% ± 3% of the starch. Since deleting *stk* significantly reduced the transcript for these enzymes but did not alter the ability of GAS to degrade starch, it is possible that there are additional enzymes that compensate for the decrease in AmyA in GAS.

Stk regulates sensitivity to penicillin in GAS. We found that Stk activates the peptidoglycan hydrolase gene *mur1.2* and also confirmed the finding that Stk activates *cdhA* (49). CdhA is predicted to cleave within the peptide cross-link in the cell wall peptidoglycan, and Mur1.2 is expected to cleave the bond between *N*-acetyl-muramic acid and the peptide cross-link (79). Therefore, both of these enzymes are expected to affect cell wall expansion during growth. We therefore hypothesized that the absence of *stk* might change the sensitivity of GAS to antibiotics that target cell wall synthesis. To test this, we analyzed sensitivity to penicillin. Exponential-phase cultures of MGAS2221, its Δ*stk* derivative, and the strain in which the wild-type gene replaced the *stk* deletion were grown in THY and split, and penicillin was added to half of each culture. Growth in a closed flask was followed, and viable cells were assayed by diluting each culture and plating for colonies in the absence of penicillin. We found that the mutant had about 10-fold fewer CFU than its wild-type parent at both 2 and 4 h after the addition of penicillin (Fig. 4). The addition of 50 μg/ml penicillin gave the same results as 5 μg/ml penicillin (data not shown).

In strain JRS7322, in which the wild-type *stk* gene has been reintroduced by homologous recombination, the level of pen-

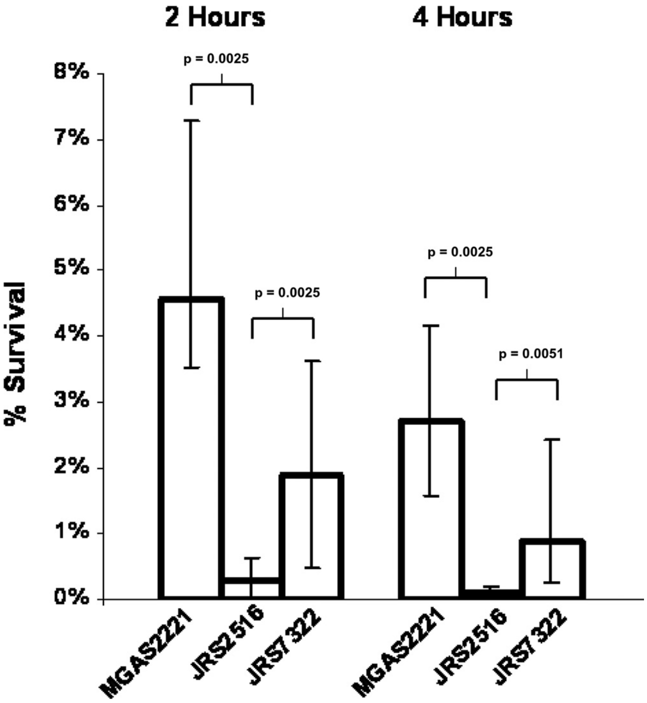

FIG. 4. Stk is required for full penicillin tolerance in GAS. Percent survival, measured by colony counts, is presented for MGAS2221 (wild type), JRS2516 (its Δ*stk* derivative), and JRS7322 (Δ*stk*::*stk* mutant). Vertical bars indicate the range of values in at least 5 separate experiments. Nonparametric statistics (Wilcoxon test) were used to calculate *P* values.

icillin tolerance is about 5-fold higher than that of the mutant. The incomplete restoration of penicillin tolerance in the replacement strain could be due to the presence of the chloramphenicol resistance gene downstream of *stk*. Our results suggest that Stk is required for the normal level of penicillin tolerance of GAS.

DISCUSSION

Traditionally, sequence-specific transcriptional regulators, such as CovR, have been the focus of investigation for GAS virulence gene regulation. More recently, in other Gram-positive pathogens, the signaling input that occurs posttranslationally by modification of transcriptional regulators is beginning to be appreciated. Ser/Thr kinases have recently been demonstrated to provide critical signaling that alters expression of genes in bacteria, including genes encoding virulence factors. In this work, we found that in the human pathogen GAS, the Ser/Thr kinase Stk regulates expression of genes involved in virulence, glucan metabolism, fatty acid biosynthesis, osmoregulation, cell division/separation, and penicillin tolerance. Stk regulates gene expression by phosphorylation of proteins controlling transcription of specific operons in a process that may be direct or indirect.

The only protein currently shown to be phosphorylated by Stk in GAS is Hlp (26). Since Hlp controls expression of many genes by binding to their promoter and/or coding regions, it is possible that Stk regulates expression of some genes indirectly by phosphorylating Hlp. This phosphorylation could be expected to change the affinity with which Hlp binds to DNA and therefore to alter its ability to influence gene expression. An additional potential target for Stk phosphorylation in GAS is CovR, a global transcriptional regulator in GAS (7) and GBS (34), which is phosphorylated by Stk in GBS (55). However, since our transcriptome analysis indicates that Stk regulates genes that are not in the Cov regulon, as well as some genes that are regulated by CovR, phosphorylation of CovR, if it occurs in GAS, cannot be the only mechanism for Stk control of gene expression in this organism. In addition, phosphorylation by Stk of enzymes involved in metabolic pathways controlled by feedback loops may alter transcription of genes in these pathways (44, 78).

Regulation by Stk is important for virulence. We found that in the human pathogen GAS, Stk is required for virulence, as it is in other pathogenic bacteria (16, 19, 54). Deletion of *stk* had a dramatic effect on virulence in the murine myositis model we used. Several of the genes we found to have reduced expression in the Δ*stk* mutant (Table 1) have been implicated in virulence in GAS. For example, Grab, which we found is activated by Stk, protects GAS surface proteins, including those required for virulence, from degradation (56) and might, in part, mediate the effect of Stk on GAS virulence.

Regulation of metabolism of α-glucans. Another locus activated by Stk which appears to be involved in pathogenesis encodes the enzymes responsible for metabolism and transport of α-glucans. Deletion of *amyA*, a gene within this locus, decreases GAS virulence in the murine mucosal infection model, in which mice are infected intranasally (64). The *amyA* mutant also shows reduced transepithelial migration (64), and higher *amyA* transcript levels are found in GAS strains isolated from invasive infections than in GAS strains isolated from pharyngeal disease (64). This suggests that Stk-mediated activation of the α-glucan locus might facilitate invasiveness of GAS. Although it is not known whether the genes in this locus constitute a single operon, our transcriptome analysis indicates that most are activated by Stk. Further work will be needed to determine the mechanism by which Stk interacts with the other regulators of the α-glucan locus, which include Rgg (RopB) (13) and CodY (39), as well as the response regulator, SptR (65, 67), to coordinate α-glucan metabolism in response to the environment and contribute to virulence.

Regulation of fatty acid biosynthesis. Stk in GAS appears to activate transcription of the enzymes in the pathway responsible both for biosynthesis of fatty acids and for the ratio of saturated to unsaturated fatty acids. Although the enzymes of this pathway in GAS are encoded in a single locus very similar to that of *S. pneumoniae* (38), our work suggests the presence of a long transcript, including *fabM*, with the other genes of the pathway in GAS (Fig. 3). The simplest hypothesis for the mechanism of Stk activation of the *fab* locus in GAS is that Stk phosphorylates FabT, inactivating this transcriptional repressor. Another possibility is that Stk phosphorylates several enzymes in the pathway and thus reduces their activity, as in mycobacteria (44, 78), and the presence of a feedback inhibition loop results in increased transcription resulting from Stk inactivation of the Fab enzymes.

Mutations in one of the genes in this cluster, *fabH*, are associated with more invasive strains of GAS (63), so this biosynthetic pathway is expected to have a significant impact on GAS virulence. In addition, membrane fluidity, controlled by this pathway, is important for response to stresses like high osmolarity. In GAS, which lacks secondary sigma factors like those that regulate response to stress in other bacteria, stress response is mediated by the two-component system CovRS (9). Since the signal for CovS activation appears to involve membrane stress (9), Stk might be involved indirectly in signaling to the Cov regulon by controlling membrane fluidity and therefore might be a key player for coordination of the response to stress.

In the *S. pneumoniae fab* locus, there are at least three promoters, one each in front of *fabM*, *fabT*, and *fabK*, and the transcripts synthesized from these promoters do not overlap (38). In this organism, repression by FabT results in a greater proportion of unsaturated fatty acids, because it represses *fabK*, whose product diverts fatty acid biosynthesis to saturated fatty acid formation, but not *fabM*, whose product drives the pathway to produce unsaturated fatty acids and thus to increase membrane fluidity. In GAS, we expect FabT to repress both *fabK* and *fabM* because, in addition to the FabT binding sequences in front of *fabT* and *fabK*, we find one with 70% identity 55 bases upstream of the start of *fabM*. It will be interesting to learn whether Stk and FabT differentially affect expression of these two enzymes in GAS and thus regulate the stress response of this pathogen.

Osmoregulation. We found that Stk is required for GAS to grow under osmotic stress, as is also true for *S. pneumoniae* (60). This adaptation to osmotic stress in GAS is aided by Stk-mediated regulation of the Opu transporter system. The osmotic conditions on skin, at which many GAS infections are initiated, are expected to differ significantly from osmotic conditions of internal tissues encountered by GAS during invasion. Therefore, the ability to respond to the changes in microenvironment during infection through the Opu system is expected to be vital for survival and growth of GAS.

There are at least two regulators of the Opu system that might be targets for Stk phosphorylation. One, the response regulator VicR, downregulates the *opu* locus (37). Phosphorylation by Stk may reduce the affinity of VicR for the promoter of this locus, leading to activation of *opu* gene expression. However, it should be kept in mind that both VicR and Stk activate *cdhA* (37, 49), indicating that different mechanisms for activation by Stk are involved at the promoters for *cdhA* and *opu*. We also found that CovR activates *opu* genes (Table 1), but activation of genes by CovR in GAS has so far been found to be indirect, involving interference with binding of a repressor (22). This suggests that if Stk acts through CovR to activate *opu*, this process is likely to be indirect.

Regulation of cell division and separation. Two genes activated by Stk in GAS, *cdhA* and *mur1.2*, are likely to affect cell division and separation of dividing cells and thus to contribute to the unusual morphology of the *stk* mutant strain. Similar morphology has been seen previously for GAS mutants lacking either *stk* (26) or *cdhA* (49) and also for *S. pneumoniae* mutants lacking the *cdhA* homolog *pcsB* (20) or the *mur1.2* homologs *lytA* (59) and *lytB*. In GAS, deletion of *stk* or *cdhA* results in aggregation instead of formation of the long chains characteristic of this organism, and the *stk* mutant cells also have incomplete division septa (26, 49). In the GAS Δ*stk* mutant, when *cdhA* was provided on a plasmid, the chains were restored to their normal length, but cell septation was still abnormal (49). Since we found that both *mur1.2* and *cdhA* are activated by Stk, we suggest that full complementation of the cell division defect of the Δ*stk* GAS strain requires expression of Mur1.2, in addition to expression of CdhA.

Penicillin tolerance and the role of Stk in signaling and virulence. All GAS strains are sensitive to penicillin. However, during growth of GAS in the laboratory, a phenomenon known as "penicillin tolerance" (i.e., ability to form colonies after exposure to penicillin) has been observed (53, 76, 77). We found that about 1 to 5% of wild-type GAS in the exponential phase of growth were tolerant (able to form colonies) after 4 h of treatment with penicillin (Fig. 4). Furthermore, the fraction of tolerant cells did not depend on the dose of penicillin, suggesting that these cells constitute a physiologically resistant subpopulation of the bacteria in the culture. The kinetics of loss of viability during penicillin treatment is biphasic, consistent with the presence of a subpopulation of resistant bacteria. These tolerant cells are not mutants that are resistant to penicillin, and no such GAS mutants have ever been isolated. Since penicillin, which targets cell wall synthesis, rapidly kills growing cells, it is likely that the tolerant bacteria are quiescent (not growing) or are growing much more slowly than the majority of the population. When penicillin is removed from the culture, the quiescent cells that survive must have recovered the ability to replicate (emerged from the dormant state). We found that the Δ*stk* mutant showed a substantial decrease in penicillin tolerance compared to its wild-type parent. This may result from the inability of the Δ*stk* mutant to recover from the quiescent or slow-growing state that allows the bacteria to survive in the presence of penicillin. Our findings suggest that the ability to recover, i.e., penicillin tolerance, apparently depends on Stk.

Autophosphorylation of Stk is activated by an environmental signal sensed by its carboxy-terminal PASTA domains, which are C-terminal to a membrane-spanning domain and are presumed to be extracellular. PASTA domains are characteristic of proteins involved in the transpeptidation reaction in cell wall synthesis, and they interact with peptidoglycan (PG) fragments (27, 61). During normal bacterial growth, PG fragments are released to allow insertion of new PG material as the cell expands and divides. In Gram-positive bacteria, large quantities of PG fragments are released, because these bacteria have no system to recycle them (15, 41). Homologs of Stk in other organisms are activated by PG fragments, which bind to the extracellular PASTA domain of this protein (50, 51, 61, 81).

Many bacteria, including GAS, enter a quiescent state during which they do not actively replicate. Emergence from quiescence, in several bacteria, is stimulated through a serine threonine kinase that responds to the presence of a resuscitation factor (6, 45, 61, 72). In *B. subtilis* it has been shown that the presence of PG fragments results in activation of Stk, which leads to germination of the dormant spore (61). We suggest that, similarly, GAS Stk might be activated by PG fragments released by the fraction of the population that does not survive penicillin treatment. From our work, it appears that Stk is involved in this resuscitation process as it is in other bacteria.

In the human host, GAS also appears to be able to enter a quiescent state. Quiescence is probably responsible for the carrier state in people, in which the GAS bacteria persist without causing disease. Quiescence is also the probable explanation of the high rate of penicillin treatment failure in clinical practice, since GAS recovered from patients treated with penicillin remain sensitive to this antibiotic when tested in the laboratory. During GAS infection, activation of Stk may result from binding of PG fragments released both by GAS itself and by other Gram-positive bacteria in the same environment. This could correspond to a kind of quorum sensing in which resuscitation of quiescent bacteria responds to the presence of growing bacteria in the environment. We anticipate that the microbiome at the site of infection by GAS will be found to be critical in triggering Stk signaling, and we look forward to further analyses of the mechanisms by which Stk exerts its important effects on virulence.

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