

Transcriptional Regulation of a Bacteriophage Encoded Extracellular DNase (Spd-3) by Rgg in *Streptococcus pyogenes*

Srivishnupriya Anbalagan, Michael S. Chaussee*

Division of Basic Biomedical Sciences, The Sanford School of Medicine of the University of South Dakota, Vermillion, South Dakota, United States of America

Abstract

The *Streptococcus pyogenes* transcriptional regulator Rgg controls the expression of virulence-associated genes encoded both within the core genome and within horizontally transmissible DNA such as temperate bacteriophage. Previously, we showed that Rgg binds to the non-coding DNA upstream of the bacteriophage gene encoding an extracellular DNase Spd-3. In the current study, we further characterized Rgg-mediated regulation of *spd-3* expression. Two *spd-3* transcripts were identified by northern blotting. The 5' ends were 27 and 594 nucleotides upstream of the start codon as determined with primer extension analysis and 5' RACE (rapid amplification of c-DNA ends), respectively. Results obtained with gel shift assays showed that purified Rgg bound specifically to non-coding DNA containing the promoters of both transcripts. Transcriptional fusion analyses confirmed the presence of Rgg-repressible promoters within these DNA regions. In addition, repression was associated with direct DNA binding by Rgg as determined with chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR). The results show that the chromosomally encoded transcriptional regulator, Rgg, directly represses both bacteriophage promoters controlling the expression of Spd-3. The results provide new information regarding the regulation of prophage encoded virulence factors of *S. pyogenes* and highlight the complex evolutionary history of *S. pyogenes* and temperate bacteriophage.

Citation: Anbalagan S, Chaussee MS (2013) Transcriptional Regulation of a Bacteriophage Encoded Extracellular DNase (Spd-3) by Rgg in *Streptococcus pyogenes*. PLoS ONE 8(4): e61312. doi:10.1371/journal.pone.0061312

Editor: Indranil Biswas, University of Kansas Medical Center, United States of America

Received: January 2, 2013; **Accepted:** March 7, 2013; **Published:** April 17, 2013

Copyright: © 2013 Anbalagan, Chaussee. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding was provided by the Sanford School of Medicine of the University of South Dakota and by National Institutes of Health grant 2 P20 RR016479 from the INBRE Program of the National Center for Research Resources. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Michael S. Chaussee serves as a PLOS ONE Editorial Board member. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: Michael.Chaussee@usd.edu

Introduction

Streptococcus pyogenes causes several human diseases ranging in severity from self-limiting pharyngitis to life-threatening necrotizing fasciitis and streptococcal toxic shock syndrome [1]. The virulence of the pathogen varies temporally over the course of decades due to changes in both the pathogen and human immunity [2,3]. The determination of the genome sequences of several isolates revealed a theoretically limitless *pan*-genome, or mobilome, comprised of mobile genetic elements (MGE) including bacteriophages, integrative conjugative elements, transposons, and insertion sequences [3–5]. MGEs significantly alter the composition of the chromosome. For example, between two and eight complete or partial bacteriophage genomes are present in the genomes of *S. pyogenes* and bacteriophage DNA can account for up to 12% of the chromosome [6,7]. Often, the bacteriophage encode virulence factors including superantigens [8] and extracellular nucleases [9,10], which profoundly influence interactions between the pathogen and its human host. Thus, chromosomal heterogeneity, including variation in the number and types of prophage within a chromosome, is responsible for much of the genetic diversity observed among clinical isolates and contributes to the clinical and temporal variation in the outcome of human colonization with *S. pyogenes* [7,11–13].

S. pyogenes can produce up to four extracellular DNases [14,15]. One (MF-1/DNaseB) is chromosomally encoded and is adjacent to *rgg* (also known as *ropB* [16]), which encodes a global transcriptional regulator [17]. The remainder are encoded by prophage. Therefore, the number of extracellular DNases potentially produced by an isolate varies depending on the prophage content of the chromosome.

Extracellular DNases have long been thought to be important in liquefying pus to promote bacterial dissemination. In addition, prophage-encoded extracellular DNase degrades bacterial DNA following induction of the lytic phase, which can further reduce viscosity at the site of colonization and promote transmission of the progeny virions to new bacterial hosts [9]. More recently, the prophage encoded extracellular nuclease Sda1 was found to degrade neutrophil extracellular traps (NETs) [18]. NETs are a component of innate immunity and are composed of a scaffold of neutrophil-derived chromatin and antimicrobial peptides, which entraps and kills microbes, including *S. pyogenes* [19]. Degradation of bacterial DNA also decreases macrophage mediated killing of *S. pyogenes* due to decreased stimulation of toll-like receptor 9, which recognizes unmethylated CpG-rich DNA [20]. Thus, extracellular DNase's promote pathogen dissemination and survival, although some appear to be relatively more important than others [18,21]. Importantly, the exoproteins contribute to virulence in both mouse

models of invasive infection [20] and in a cynomolgus macaque model of pharyngitis [21].

The serotype M49 strain NZ131 possesses three prophages [22], including one consisting of only 16 kb that presumably has decayed. The remaining two prophages, NZ131.2 and NZ131.3, are 37,895 and 47,501 bp, respectively. NZ131.2 encodes a superantigen known as streptococcal pyrogenic exotoxin H (SpeH; [23]) and NZ131.3 encodes an extracellular nuclease known as Spd-3. Thus, strain NZ131 has two extracellular nucleases, the chromosomally encoded SdaB (MF-1) and the prophage encoded Spd-3.

Inactivation of the gene encoding the transcriptional regulator Rgg increased expression of both SdaB (Spy49_1692c; Mf-1) and Spd-3 (Spy49_1455) in the post-exponential phase of growth [17]. Subsequently, we found that Rgg binds to non-coding prophage DNA upstream of *spd-3* [24]. The purpose of the current study was to characterize further the role of Rgg in the regulation of the prophage encoded DNase Spd-3.

Results

Identification of *spd-3* Transcripts

As an initial step to characterize the regulation of *spd-3* expression, northern blotting was done using RNA isolated during the post-exponential phase of growth from both the wild-type *S. pyogenes* strain NZ131 and an *rgg* mutant. Two distinct transcripts were detected and both were more abundant in the mutant strain compared to the wild-type strain (Fig. 1), which was consistent with our previous finding that Rgg represses *spd-3* expression [17]. The more abundant transcript was approximately 925 bp in length and accounted for 65% of the transcript signal, as determined by densitometry.

Mapping the *spd-3* Transcriptional Start Sites

Primer extension analysis was used to determine the 5' termini of the two transcripts. Extension with primer spd3PEc_96 (Table 1) showed a transcript that originated 27 bp upstream of the predicted *spd-3* open reading frame (ORF) (Fig. 2). This origin, coupled with a putative transcriptional terminator 91 bp down-

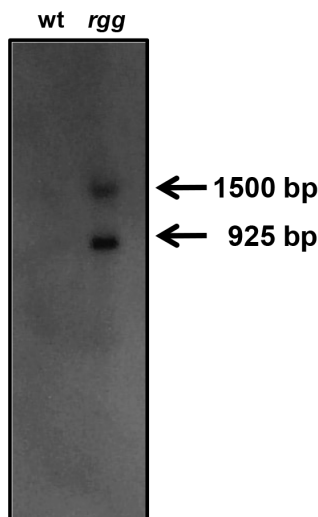


Figure 1. Detection of *spd-3* transcripts. Northern blotting using an *spd-3* specific probe and RNA isolated from the wild type (wt) and the *rgg* mutant (*rgg*) strains showed two transcripts in the sample obtained from the mutant strain. The approximate sizes were determined by using an RNA ladder (not shown). doi:10.1371/journal.pone.0061312.g001

stream of the *spd-3* ORF, predicted a 919 bp transcript, which corresponded to the more abundant approximately 925 bp transcript detected by northern blotting (Fig. 1). A variety of primers were used in attempts to identify the start site of the longer transcript by using primer extension; however, we were unable to do so, possibly due to secondary structure formed within the large untranslated region. As an alternative approach, 5' RACE was used and the results showed that the 5' terminus was 594 bp upstream of the *spd-3* start codon (Fig 3). The results predicted a 1,487 bp transcript, which also correlated with the size of the larger transcript identified with northern blotting (Fig. 1). In addition, the start of transcription coincided with the non-coding DNA region previously shown to be bound by Rgg *in vivo* during the exponential phase of growth [24]. Analyses of the DNA proximal to the transcriptional start sites revealed the presence of putative -10 and -35 RNA polymerase binding sites (Fig. 3). The two transcriptional start sites were designated P₁ and P₂ (Fig. 3).

Rgg Binds Specifically to DNA Containing the P1 and P2 Promoters

ChIP coupled with DNA genechips (ChIP-chip) and gel-shift assays previously showed that Rgg binds to P₁ [25]; however, we did not previously detect binding to P₂. Therefore, we re-analyzed Rgg binding to both sites by using the more sensitive ChIP-qPCR procedure. As expected, DNA containing P₁ was enriched by approximately 9-fold in strain SA5, which encodes an Rgg-Myc fusion protein used to facilitate immunoprecipitation, compared to the control strain (Fig. 4). In addition, the P₂ region was enriched nearly 75-fold (Fig. 4). The results show that Rgg binds to both the P₁ and P₂ DNA (Fig. 4). Binding near P₂ was further evaluated with gel-shift assays and the results confirmed that Rgg specifically binds to DNA containing P₂ (Fig. 5).

Rgg Directly Regulates *spd-3* Expression

To characterize the two *spd-3* promoters further, prophage DNA regions bound by Rgg (P₁ and P₂) were cloned adjacent to a promoterless firefly luciferase (*luc*) gene present in the shuttle plasmid pKSM720 [26]. The recombinant plasmids were used to transform both the wild-type and the *rgg* mutant strains to determine if expression from P₁ and P₂ was regulated by Rgg. The use of the plasmid based reporter system also allowed us to measure promoter activity apart from the prophage, thus avoiding confounding factors potentially associated with prophage induction, such as variation in gene copy number. Promoter activity was detected with P₂ DNA and the presence of Rgg in the wild-type strain decreased transcription, consistent with other data indicating that Rgg represses *spd-3* transcription (Table 2). Similarly, a fragment containing both *spd-3* promoters (P₁ and P₂), showed significantly more activity compared to the fragment containing only P₂ (Table 2). Again, more promoter activity was detected in the *rgg* mutant strain, consistent with Rgg-dependent repression of *spd-3* expression by direct binding to the promoter regions (Table 2).

Rgg Acts in *cis* to regulate *spd-3* Promoters

Finally, we used the transcriptional reporter system and ChIP-qPCR to determine if Rgg bound in *cis* to regulate expression of the *luc* gene. One PCR primer was specific to *luc* and a second was upstream of P₁. The results showed that Rgg bound directly to the plasmid DNA to repress expression of *luc* (Fig. 6).

Table 1. Oligonucleotides.

Primer	Sequence ^a (5'–3')	Reference or source
spd3fwd	GCCAGACCCTTGCTGCTAATCCA	24
spd3rev	GGTGCCTGTAAAATACGAATAAATAAGT	24
spd3fwd1	GGCGTAGCATTAAATAAACGGAA	This study
spd3rev1	GGCAAGGAGGGTAAAAATGCTAAC	This study
pspd3_IRfwd	<u>GCGGATCCGTCGGACTAGTCTATGACAAA</u>	24
pspd3_IRrev	<u>GCCTCGAGATCCATGTCCTCCTTTATTATTAC</u>	24
spd3_237fwd	TTTTACCTCCTACTTATTATTTCG	This study
spd3_300_BamHlfwd	<u>GCGGATCCCGGAATTAATAAAATATTTTGTCC</u>	24
spd3_378_Xholrev	<u>GCCTCGAGAAGTAGCGGGAGAAGTCTAATGG</u>	This study
groEL2fwd	GCTACTCGACGTAACTTGTG	24
groEL2rev	GGAGCCTTCGTACCCAGCAT	24
spd3_cfwd	<u>GCGGATCCATGCTAAATCAAATCGTCGTA</u>	This study
spd3_crev	<u>GCCTCGAGTTCGGTTCTAAATTACTATCTTC</u>	This study
spd3_PEc_96	TCTGGCTGCCGTAACAGTACTTGTG	This study
spd3prx36	GAAGTAGCGGGAGAAGTCTAATGG	This study
AAP-G	GGCCACGCTCGACTAGTACGGGGGGGGGG	This study
LucRev1	GCCAAGCTGGAATTCGAGCTCCCAT	This study
Nested prx	GGACAAAATATTTTAAATTAATTCCG	This study
AUAP	GGCCACGCTCGACTAGTAC	This study

^aUnderlined nucleotides are restriction sites incorporated into primer.
doi:10.1371/journal.pone.0061312.t001

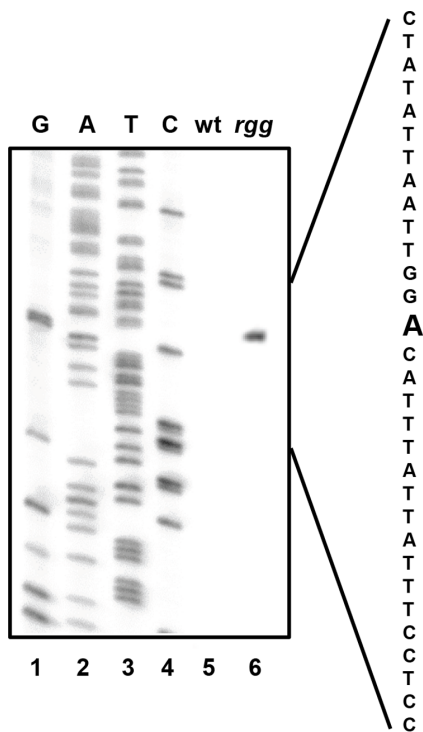


Figure 2. Primer extension analysis of *spd-3* transcripts. RNA isolated from the wt (lane 5) and *rgg* mutant strain (lane 6) was used to identify the 5' end of the *spd-3* transcript. The DNA sequence (G A T C) generated from the non-coding DNA upstream of *spd-3* (lanes 1–4) is shown. The antisense strand DNA sequence proximal to the 5' end is shown to the right and bold type indicates the start of the transcript.
doi:10.1371/journal.pone.0061312.g002

Discussion

Temperate bacteriophage can ferry virulence-associated genes encoding a variety of toxigenic and enzymatic exoproteins among bacteria. In *S. pyogenes*, prophages encode superantigens, which are responsible for toxic shock syndrome; extracellular DNases, which assist in the pathogen's escape from the innate immune response; and surface exposed proteins, which have not been characterized. Despite the importance of the gene products to human diseases caused by *S. pyogenes*, comparatively little is known about the regulation of their expression. Here, we examined the role of Rgg in the repression of the bacteriophage encoded extracellular DNase Spd-3. Two *spd-3* transcripts were identified and the 5' termini determined. The abundance of both transcripts was elevated in an *rgg* mutant strain and Rgg bound to DNA containing the promoters of both transcripts, indicating that Rgg binding represses expression. To analyze regulation outside the context of the prophage, which can excise from the chromosome and thereby increase *spd-3* copy number, a plasmid-based reporter system was used to measure *spd-3* promoter activity. The results confirmed that Rgg repressed expression from both promoters. Finally we showed with ChIP-qPCR that Rgg bound to DNA *in cis* to effect transcription of the reporter gene. The results show that the chromosomally encoded regulator Rgg represses expression of the prophage-encoded virulence gene *spd-3* expression by direct binding to prophage promoters.

Rgg regulates dozens of genes in strain NZ131 in a growth-phase associated manner, including many known to contribute to virulence [16,27,28]. It does so, in part, by changing its DNA binding specificity in response to glycolytic flux via direct protein-protein interaction with LacD.1 [29]. In addition, Rgg binds to hydrophobic peptides, which modulates regulatory activity [30,31]. The genes controlled by Rgg are functionally diverse and include both cell-associated proteins and soluble exoproteins

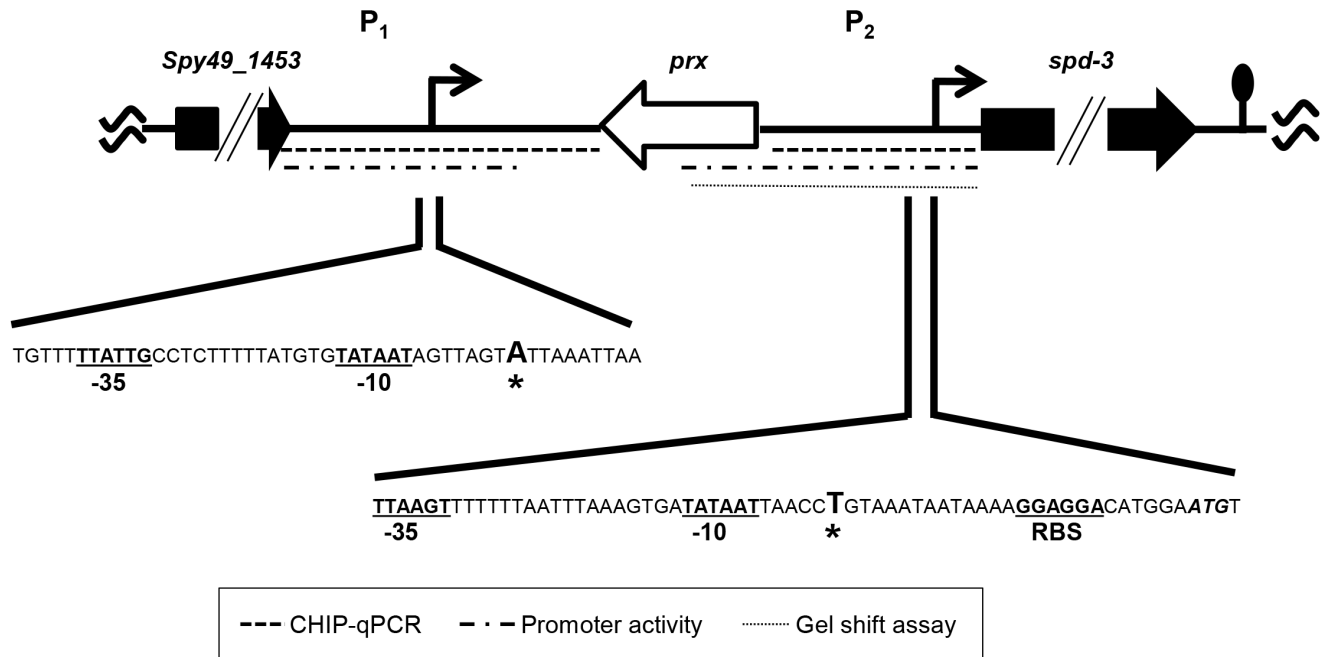


Figure 3. Schematic location of the two *spd-3* promoters. Transcriptional start sites are indicated by asterisks and bold face. The predicted -10 and -35 RNA polymerase binding sites of each promoter are indicated with bold type and underlined. A putative ribosome binding sites (RBS) associated with P₂ is similarly indicated. The Spd-3 start codon is shown in italics and bold type. Dotted lines indicate the target DNAs used with ChIP-qPCR, gel-shift assays, and transcriptional fusion assays. The lollipop symbol indicates the location of a putative transcriptional terminator. doi:10.1371/journal.pone.0061312.g003

associated with virulence [17,25]. For example, Rgg represses expression of the cell wall-associated antiphagocytic M protein in the exponential phase of growth and activates expression of a secreted cysteine protease SpeB in the post-exponential phase [16,27,32]. The results of the current study, together with results from previous studies, show that Rgg coordinates the expression of both prophage and chromosomally-encoded virulence genes in response to environmental cues. Consistent with these findings, Rgg contributes to the outcome of *S. pyogenes* infection as assessed with animal models and as observed in human epidemics of invasive disease [33] [34].

Excision of prophage from the chromosome is associated with increased gene copy number and often with simultaneous expression of prophage-encoded virulence genes. In some instances the repressor of the lytic phase also directly represses expression the prophage-encoded virulence genes. For example, expression of the prophage-encoded Shiga toxin (StxAB) in *Escherichia coli* is primarily controlled by a prophage repressor that also controls lysogeny [35,36]; thus *stxAB* expression is dependent on prophage induction. In contrast, expression of the prophage-encoded cholera toxin (Ctx) is controlled by several chromosomally encoded regulators and is not dependent on induction of the

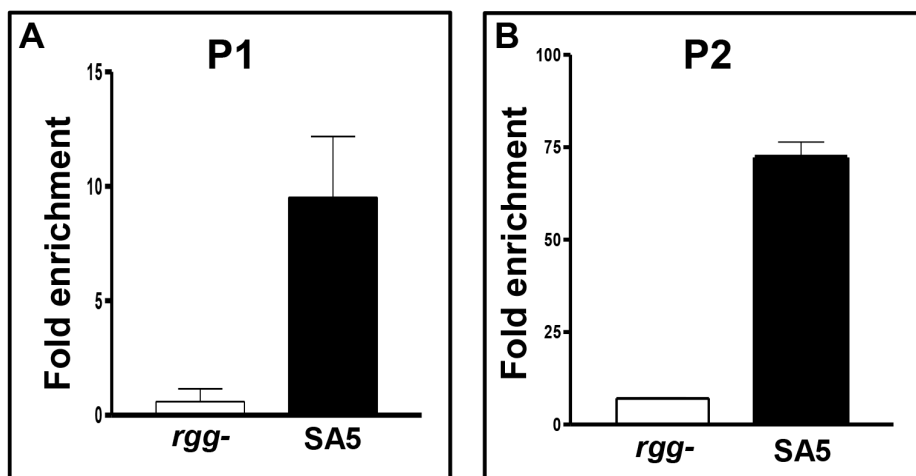


Figure 4. Rgg binds *in vivo* to *spd-3* promoter regions P1 and P2. ChIP and qPCR was used to measure the amount of P1 (Panel A) and P2 (Panel B) containing DNA bound by Rgg. Experiments were conducted at least three times, and the means and standard deviations are shown. doi:10.1371/journal.pone.0061312.g004

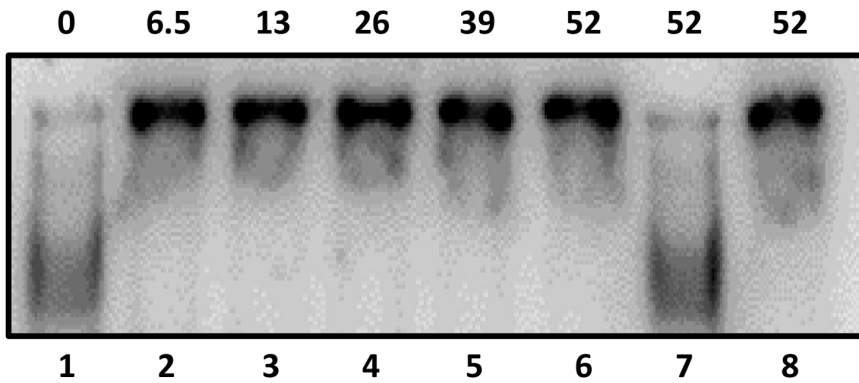


Figure 5. Rgg binds specifically to P2. Rgg binding to the non-coding DNA upstream of *spd-3* containing P2 was assessed by incubating with 0, 6.5, 26, 39 and 52 pmoles (Lanes 1–6) of purified Rgg with radiolabeled target DNA. Lane 7) Specific un-labeled competing DNA was added to the reaction. Lane 8) Labeled non-specific DNA (*groEL*) was added to the reaction.
doi:10.1371/journal.pone.0061312.g005

encoding prophage [37]. Thus two paradigms have emerged from the study of the regulation of prophage encoded virulence factors.

In *S. pyogenes*, expression of prophage encoded virulence genes, including those encoding superantigens, has been associated with the induction of prophage [4,8,9]; however, induction does not appear to be necessary for expression [4]. Moreover, expression of the phage-encoded extracellular DNase Sdn (SpyM3_1409) decreases following mitomycin C induction of prophage, despite an increase in *sdn* copy number [4]. In this study, we showed that Rgg directly represses the promoters controlling *spd-3* expression, even in the absence of prophage induction, as determined by using a plasmid based reporter system. Although our investigation focused on the regulation of *spd-3* expression, the results are consistent with a model in which the expression of prophage encoded virulence factors in *S. pyogenes* is not dependent on induction the lytic cycle and is controlled, at least in part, by chromosomally encoded regulatory loci. Given the relevance of prophage encoded superantigens and secreted DNases to human disease, additional investigation into the regulation of other prophage-encoded virulence factors is warranted.

Phenotypic variation is a hallmark of many pathogens, including *S. pyogenes*, and can result in heterogeneous clinical outcomes of infection. We previously showed that Rgg binds to non-coding DNA upstream of a prophage integrase/excisionase (Spy49_0746c) to repress expression [17,24]. Increased expression of the integrase/excisionase in an *rgg* mutant strain was associated with a decrease in the frequency of prophage excision from the chromosome [17]. The current study extends the idea that Rgg regulates specific prophage encoded genes, which alters the

phenotype of the pathogen. Given the tremendous variation in the number and composition of bacteriophage among different isolates of *S. pyogenes* [7], we speculate that the direct regulation of MGEs by Rgg contributes, directly or indirectly, to the variation in the Rgg regulon observed among various isolates of *S. pyogenes* [38] and potentially the clinical outcome of human infection.

Materials and Methods

Bacterial Strains and Culture Conditions

S. pyogenes strain NZ131 (serotype M49) was isolated from a patient with acute post-streptococcal glomerulonephritis (Table 3) [22]. NZ131 and its genetic derivatives including *rgg*- (32) and SA5 and have been previously described (24). *S. pyogenes* strains were grown with Todd-Hewitt broth (Becton Dickinson, Spark, MD) containing 0.2% (wt/vol) yeast extract at 37°C in a 5% CO₂ atmosphere without agitation. *E. coli* DH5α was grown with Luria-Bertani medium at 37°C with agitation. When necessary, antibiotics was added to the growth media at the following concentrations: carbenicillin at 100 µg/ml for *E. coli*; spectinomycin at 100 µg/ml for both *E. coli* and *S. pyogenes*; erythromycin at 2.5 µg/ml for *S. pyogenes*; kanamycin at 50 µg/ml for *E. coli* and 500 µg/ml for *S. pyogenes*.

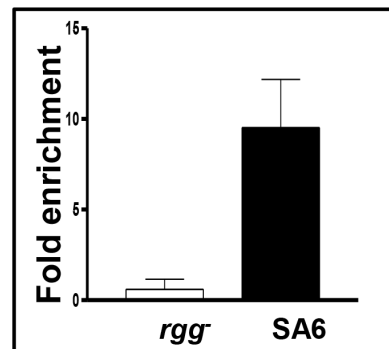


Figure 6. Rgg binds to episomal P2 containing DNA. The amount of the non-coding DNA upstream of *spd-3* cloned into a transcriptional reporter fusion plasmid was measured by quantitative PCR, as a negative control, *groEL* in ChIP samples obtained from the *rgg* mutant and strain SA6. Experiments were conducted at least three times, and the means and standard deviations are shown.
doi:10.1371/journal.pone.0061312.g006

Table 2. Both *spd-3* promoters (P1 and P2) are repressed by Rgg.

Promoter	[Luciferase] units	
	wild-type	<i>rgg</i> mutant
P2 ^a	145 (69)	35,857 (20,462)
P1 & P2 ^a	1,486 (855)	335,979 (61,184)

^aA 457 bp fragment containing the P2 promoter or a 761 bp fragment containing both the P1 and P2 promoters was cloned adjacent to the luciferase reporter gene. The mean (standard deviation) from independent experiments is shown.

doi:10.1371/journal.pone.0061312.t002

Table 3. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	<i>hsdR17 recA1 gyrA endA1 relA1</i>	Invitrogen
<i>S. pyogenes</i>		
NZ131	M49 serotype	D.R. Martin, New Zealand
<i>rgg</i> -	NZ131 <i>rgg</i> mutant, Em ^R	32
SA5	NZ131 <i>rgg</i> ⁻ complemented with pSA3, Em ^R , Kan ^R	24
SA6	SA5 transformed with pSA12	This study
wt:: <i>luc</i>	NZ131 transformed with pKSM720, Spec ^R	24
wt::Pspd3- <i>luc</i>	NZ131 transformed with pSA6, Spec ^R	24
wt::Pspd3-379 <i>luc</i>	NZ131 transformed with pSA27, Spec ^R	This study
wt::Pspd3-457 <i>luc</i>	NZ131 transformed with pSA28, Spec ^R	This study
<i>rgg</i> :: <i>luc</i>	NZ131 <i>rgg</i> mutant transformed with pKSM720, Spec ^R	24
<i>rgg</i> ::Pspd3- <i>luc</i>	NZ131 <i>rgg</i> mutant transformed with pSA12, Spec ^R	24
<i>rgg</i> ::Pspd3-379 <i>luc</i>	NZ131 transformed with pSA27, Spec ^R	This study
<i>rgg</i> ::Pspd3-457 <i>luc</i>	NZ131 transformed with pSA28, Spec ^R	This study
Plasmids		
pGEM-T-Easy		
pKSM720	GAS replicating plasmid with firefly luciferase and RBS, Spec ^R	26
pSA12	Non-coding region upstream of <i>spd-3</i> was cloned into pKSM720, Spec ^R	24
pSA27	Non-coding region between -761 and -382 upstream of <i>spd-3</i> was cloned into pKSM720, Spec ^R	This study
pSA28	Non-coding region between -1 and -457 upstream of <i>spd-3</i> was cloned into pKSM720, Spec ^R	This study
pSA29	Non-coding region between -225 and -472 upstream of <i>spd-3</i> was cloned into pGEM-T-easy vector, Amp ^R	This study

doi:10.1371/journal.pone.0061312.t003

DNA Manipulation

To isolate plasmid DNA from *E. coli*, either the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) or Maxi/Midi prep purification systems (Qiagen) was used. DNA fragments were PCR amplified with GoTaq DNA polymerase (Promega, Madison, WI) and the amplified DNA was separated by using agarose gel electrophoresis and purified using the SpinPrep Gel DNA kit (EMD Millipore, Darmstadt, Germany). DNA sequencing to confirm various constructs was done at Iowa State University (Ames, IA).

Promoter Activity Assays

A shuttle plasmid (pKSM720) containing *luc* [26], which encodes firefly luciferase, was used to construct transcriptional fusions. Two DNA regions upstream of *spd-3* (-761 to -382 and -1 to -237) were amplified using pspd3_IRfwd and spd3_378_XhoIrev; spd3_237fwd and pspd3_IRrev primer combinations, respectively (Table 1). The 379 bp and 237 bp DNA fragments were gel purified, digested with *Bam*HI and *Xho*I, and cloned 5' to *luc* between *Bg*II and *Xho*I of pKSM720 (Table 3) to create pSA27 and pSA28, respectively (Table 3). NZ131 was transformed with pKSM720, pSA27, and pSA28 by electroporation to create wt::*luc*, wt::Pspd3-379*luc*, and wt::Pspd3-457*luc*, respectively (Table 3). Similarly, the NZ131 *rgg* mutant was transformed with pKSM720, pSA27, and pSA28 to create *rgg*::*luc*, *rgg*::Pspd3-379*luc*, and *rgg*::Pspd3-457*luc*, respectively (Table 3). Construction of the recombinant plasmids was confirmed by PCR.

The *S. pyogenes* strains containing the transcriptional fusion plasmids were grown with THY broth to the exponential phase of growth ($A_{600} \sim 0.35$) and luciferase activity was measured according to manufacturer's instructions (Promega).

Electrophoretic Mobility Shift Assays (EMSA)

An Rgg-maltose binding protein fusion protein (Rgg-MBP) was expressed in *E. coli* and purified as previously described [24]. Non-coding DNA upstream of *spd-3* (corresponding to nucleotides -1 to -457 bp relative to the *spd-3* start codon) was amplified by using primers spd3fwd1 and spd3rev1 primers and NZ131 genomic DNA as a template (Table 1). The fragment was isolated by using agarose gel electrophoresis, purified, and cloned into pGEM-T-easy (Promega, Madison, WI) to create pSA29. As a non-specific control of DNA binding, a similarly sized fragment was similarly prepared by using groEL2fwd and groEL2rev primers, which are specific to the *groEL* ORF (Table 1). The fragments were excised from pGEM-T-easy, gel purified, dephosphorylated, and end labeled with [γ ³²P] ATP using polynucleotide kinase. Different amounts of Rgg-MBP were incubated in 25 μ l of binding buffer (25 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 0.5 μ g/ml of calf thymus DNA) at room temperature for 20 min. Competition experiments were conducted by including unlabeled DNA prior to protein addition. The reaction mixtures were separated with a 6% nondenaturing polyacrylamide gel. The gels were dried, exposed to an Amersham Biosciences storage

phosphor screen and imaged with a Typhoon 9400 instrument (GE Healthcare, Piscataway, N.J.).

RNA Isolation and Northern Blotting

Overnight cultures of NZ131 and the *rgg* mutant were inoculated into 40-ml THY broth to an A_{600} of 0.08. The cultures were grown to the post-exponential phase of growth ($A_{600} \sim 0.6$). Total RNA was isolated as described previously [17]. The concentration and quality of RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) using an RNA 6000 Nano LabChip kit (Agilent). Fifteen micrograms of total RNA from each strain was separated with a 1.5% agarose-0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA; pH 7.0). RNA was blotted onto Hybond N⁺ membranes (GE Healthcare Biosciences, Pittsburgh, PA.) with the Turbo-blotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.), according to the manufacturer's instructions. RNA was fixed to the membrane by baking at 80°C for 30 min. The *spd-3* coding DNA was amplified using *spd3_c fwd* and *spd3_c rev* primers (Table 1), separated by agarose gel electrophoresis, the fragment excised from the gel, and purified as described above. Using the purified *spd-3* coding DNA as a template, [α -³²P]dCTP radiolabelled probes were synthesized by the random-primed method (Ready-To-Go Labeling Kit; Pharmacia). Membranes were hybridized under aqueous conditions at 65°C with the radiolabelled probes. The blots were washed and exposed to an Amersham Biosciences storage phosphor screen and imaged with a Typhoon instrument.

Primer Extension Analysis

The 5' end of the *spd-3* transcript was determined with 15 μ g of RNA isolated from NZ131 and the *rgg* mutant using an AMV reverse transcriptase primer extension kit (Promega) according to manufacturer's instructions. The *spd3PEc_96* primer (Table 1) was end-labeled with [γ -³²P] ATP using polynucleotide kinase and the extension products were separated with a 6% polyacrylamide-urea sequencing gel. The 5' end was mapped by comparison to a sequencing reaction generated with a SequiTherm EXCELTM II DNA Sequencing Kit (Epicentre Biotechnologies, Madison, Wisconsin) with the end-labeled *spd3PEc_96* and a DNA template that contained the entire non-coding DNA upstream of *spd-3*.

5' Rapid Amplification of c-DNA Ends (5'-RACE)

The 5' end of the larger *spd-3* transcript was determined using the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen, Carlsbad, California) according to

manufacturer's instructions. First strand cDNA was synthesized using 15 μ g of total RNA, the gene specific primer *spd3prx36*, and Superscript III Reverse Transcriptase (Invitrogen). After the first strand cDNA synthesis, the mRNA template was removed with the RNase cocktail (Ambion, Austin, Texas). Single stranded cDNA was purified using a DNA Clean and Concentrator-5 kit (Zymo Research Irvine, California). A homopolymeric tail was added to the 3'-end of the cDNA using recombinant TdT (Invitrogen) and dCTP. Five μ L of dC-tailed single stranded cDNA was amplified using abridged anchor primer (AAP-G) and nested *prx* primers (Table 1). The amplified product was diluted 100-fold and 5 μ L was used in a second-round of PCR amplification with abridged universal amplification primer (AUAP) and nested *prx* primers. The PCR products were separated by electrophoresis on a 1% agarose gel, purified, and sequenced at the Iowa State Sequencing facility.

ChIP-qPCR

A ChIP assay was performed using the experimental protocol previously described [24]. Briefly, cultures of *S. pyogenes* strains *rgg*⁻ [32] and SA5 (encoding an Rgg-myc fusion protein [24]) were grown to an A_{600} of approximately 0.6, which corresponds to the post-exponential phase of growth. The cultures were treated with 1% formaldehyde (w/v). DNA bound to Rgg-Myc was immunoprecipitated with a monoclonal antibody to Myc (Invitrogen, Carlsbad, CA). Control samples were similarly prepared from an NZ131 *rgg* mutant strain. Immunoprecipitated DNA was purified and specific regions quantitated with PCR using ABsolute SYBR Green ROX Mix (ABgene House, Surrey, United Kingdom). Primer pairs *pspd3_IR fwd*, *spd3_378_XhoI rev* and *Spd3_237 fwd*, *pspd3_IR rev* were used to quantitate DNA containing P1 and P2, respectively (Table 2). Primers *pspd3_IR fwd* and *LucRev1* were used to measure DNA cloned upstream of the *luc* gene (Table 2). For the control region, primers *groEL2 fwd* and *groEL2 rev* were used (Table 2). Enrichment was normalized to the amount of non-specific *groEL* DNA in precipitated samples, as previously described [24].

Acknowledgments

We thank Dr. Adhar Manna for technical assistance.

Author Contributions

Conceived and designed the experiments: MSC SA. Performed the experiments: SA. Analyzed the data: MSC SA. Contributed reagents/materials/analysis tools: SA MSC. Wrote the paper: SA MSC.

References

- Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13: 470–511.
- Stevens DL (1992) Invasive group A streptococcus infections. Clin Infect Dis 14: 2–11.
- Musser JM, Shelburne SA, 3rd (2009) A decade of molecular pathogenomic analysis of group A Streptococcus. J Clin Invest 119: 2455–2463.
- Banks DJ, Lei B, Musser JM (2003) Prophage induction and expression of prophage-encoded virulence factors in group A Streptococcus serotype M3 strain MGAS315. Infect Immun 71: 7079–7086.
- Medini D, Donati C, Tettelin H, Massignani V, Rappuoli R (2005) The microbial pan-genome. Curr Opin Genet Dev 15: 589–594.
- Ferretti JJ, Ajdic D, McShan WM (2004) Comparative genomics of streptococcal species. Indian J Med Res 119 Suppl: 1–6.
- Banks DJ, Beres SB, Musser JM (2002) The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence. Trends Microbiol 10: 515–521.
- Zabriskie JB (1964) The Role of Temperate Bacteriophage in the Production of Erythrogenic Toxin by Group A Streptococci. J Exp Med 119: 761–780.
- Broudy TB, Pancholi V, Fischetti VA (2002) The in vitro interaction of Streptococcus pyogenes with human pharyngeal cells induces a phage-encoded extracellular DNase. Infect Immun 70: 2805–2811.
- Ferretti JJ, McShan WM, Ajdic D, Savić DJ, Savić G, et al. (2001) Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc Natl Acad Sci U S A 98: 4658–4663.
- Aziz RK, Kotb M (2008) Rise and persistence of global MIT1 clone of Streptococcus pyogenes. Emerg Infect Dis 14: 1511–1517.
- Beres SB, Carroll RK, Shea PR, Sitkiewicz I, Martinez-Gutierrez JC, et al. (2010) Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. Proc Natl Acad Sci U S A 107: 4371–4376.
- Beres SB, Sylva GL, Barbian KD, Lei B, Hoff JS, et al. (2002) Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc Natl Acad Sci U S A 99: 10078–10083.
- Wannamaker LW (1958) The differentiation of three distinct desoxyribonucleases of group A Streptococci. J Exp Med 107: 797–812.

15. Winter JE, Bernheimer AW (1964) The Deoxyribonucleases of *Streptococcus Pyogenes*. *J Biol Chem* 239: 215–221.
16. Lyon WR, Gibson CM, Caparon MG (1998) A role for trigger factor and an rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*. *Embo J* 17: 6263–6275.
17. Dmitriev AV, McDowell EJ, Kappeler KV, Chaussee MA, Rieck LD, et al. (2006) The Rgg regulator of *Streptococcus pyogenes* influences utilization of nonglucose carbohydrates, prophage induction, and expression of the NAD-glycohydrolase virulence operon. *J Bacteriol* 188: 7230–7241.
18. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, et al. (2006) DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* 16: 396–400.
19. Amulic B, Hayes G (2011) Neutrophil extracellular traps. *Curr Biol* 21: R297–298.
20. Uchiyama S, Andreoni F, Schuepbach RA, Nizet V, Zinkernagel AS (2012) DNase SdaI allows invasive MIT1 Group A *Streptococcus* to prevent TLR9-dependent recognition. *PLoS Pathog* 8: e1002736.
21. Sumbly P, Barbican KD, Gardner DJ, Whitney AR, Welty DM, et al. (2005) Extracellular deoxyribonuclease made by group A *Streptococcus* assists pathogenesis by enhancing evasion of the innate immune response. *Proc Natl Acad Sci U S A* 102: 1679–1684.
22. McShan WM, Ferretti JJ, Karasawa T, Suvorov AN, Lin S, et al. (2008) Genome sequence of a nephritogenic and highly transformable M49 strain of *Streptococcus pyogenes*. *J Bacteriol* 190: 7773–7785.
23. Profit T, Moffatt SL, Berkahn CJ, Fraser JD (1999) Identification and characterization of novel superantigens from *Streptococcus pyogenes*. *J Exp Med* 189: 89–102.
24. Anbalagan S, McShan WM, Dunman PM, Chaussee MS (2011) Identification of Rgg binding sites in the *Streptococcus pyogenes* chromosome. *J Bacteriol* 193: 4933–4942.
25. Anbalagan S, Dmitriev A, McShan WM, Dunman PM, Chaussee MS (2012) Growth phase-dependent modulation of Rgg binding specificity in *Streptococcus pyogenes*. *J Bacteriol* 194: 3961–3971.
26. Kinkel TL, McIver KS (2008) CcpA-mediated repression of streptolysin S expression and virulence in the group A *Streptococcus*. *Infect Immun* 76: 3451–3463.
27. Chaussee MS, Somerville GA, Reitzer L, Musser JM (2003) Rgg Coordinates Virulence Factor Synthesis and Metabolism in *Streptococcus pyogenes*. *J Bacteriol* 185: 6016–6024.
28. Chaussee MS, Watson RO, Smoot JC, Musser JM (2001) Identification of Rgg-regulated exoproteins of *Streptococcus pyogenes*. *Infect Immun* 69: 822–831.
29. Loughman JA, Caparon MG (2006) A novel adaptation of aldolase regulates virulence in *Streptococcus pyogenes*. *Embo J* 25: 5414–5422.
30. Shelburne SA, 3rd, Olsen RJ, Makthal N, Brown NG, Sahasrabhojane P, et al. (2011) An amino-terminal signal peptide of Vfr protein negatively influences RopB-dependent SpeB expression and attenuates virulence in *Streptococcus pyogenes*. *Mol Microbiol* 82: 1481–1495.
31. Federle M (2012) Pathogenic streptococci speak, but what are they saying? *Virulence* 3: 92–94.
32. Chaussee MS, Ajdic D, Ferretti JJ (1999) The rgg gene of *Streptococcus pyogenes* NZ131 positively influences extracellular SPE B production. *Infect Immun* 67: 1715–1722.
33. Olsen RJ, Laucirica DR, Watkins ME, Feske ML, Garcia-Bustillos JR, et al. (2012) Polymorphisms in regulator of protease B (RopB) alter disease phenotype and strain virulence of serotype M3 group A *Streptococcus*. *J Infect Dis* 205: 1719–1729.
34. Carroll RK, Shelburne SA, 3rd, Olsen RJ, Suber B, Sahasrabhojane P, et al. (2011) Naturally occurring single amino acid replacements in a regulatory protein alter streptococcal gene expression and virulence in mice. *J Clin Invest* 121: 1956–1968.
35. Wagner PL, Neely MN, Zhang X, Acheson DW, Waldor MK, et al. (2001) Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. *J Bacteriol* 183: 2081–2085.
36. Neely MN, Friedman DI (1998) Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Mol Microbiol* 28: 1255–1267.
37. Waldor MK, Friedman DI (2005) Phage regulatory circuits and virulence gene expression. *Curr Opin Microbiol* 8: 459–465.
38. Chaussee MS, Liu J, Stevens DL, Ferretti JJ (1996) Genetic and phenotypic diversity among isolates of *Streptococcus pyogenes* from invasive infections. *J Infect Dis* 173: 901–908.