

Research article

## Mouse skin passage of a *Streptococcus pyogenes* Tn917 mutant of *sagA/pel* restores virulence, beta-hemolysis and *sagA/pel* expression without altering the position or sequence of the transposon

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### Abstract

**Background:** Streptolysin S (SLS), the oxygen-stable hemolysin of *Streptococcus pyogenes*, has recently been shown to be encoded by the *sagA/pel* gene. Mutants lacking expression of this gene were less virulent in a dermonecrotic mouse infection model. Inactivation of the *sagA/pel* gene affect the expression of a variety of virulence factors in addition to the hemolysin. Insertion of a Tn917 transposon into the promoter region of the *sagA/pel* gene of *S. pyogenes* isolate CS101 eliminated expression of SLS, as well as decreased expression of the streptococcal pyrogenic exotoxin B, streptokinase and M protein.

**Results:** In this study a mouse skin air sac model was utilized to analyze the effect of biological pressures on expression of SLS and other *sagA/pel* regulated gene products. The insertion delayed the lethal effect of *S. pyogenes* in a mouse skin infection model. Despite this, bacteria could be cultured from the kidneys 72 hours post infection. These kidney-recovered isolates were  $\beta$ -hemolytic despite the transposon being present in its original location and had equivalent virulence to the wild type isolate when re-injected into naive mice. Northern blot analysis of the kidney-recovered isolates confirmed that transcription of *sagA/pel* was restored; however the expression of all *sagA/pel* regulated genes was not restored to wild type levels.

**Conclusions:** These results show that biological pressure present in the mouse can select for variants with altered expression of key virulence factor genes in *S. pyogenes*.

### Background

*Streptococcus pyogenes* causes a variety of diseases in man ranging from mild suppurative throat and skin infections like pharyngitis and erysipelas to severe invasive conditions such as necrotizing fasciitis and streptococcal toxic shock syndrome [1]. One of the most widely recognized putative streptococcal virulence factors is the oxygen-stable hemolysin, streptolysin S (SLS). Despite the ease of measuring SLS activity the precise molecular na-

ture of the toxin is not known. This is due, in part, to the assembly requirement of a carrier molecule, e.g. double stranded RNA, and a peptide to form the functional hemolysin [2]. Recent genetic and immunochemical studies have clearly identified the *sagA/pel* gene as being responsible for the key peptide component of SLS [3–6].

The precise biological role of SLS in streptococcal infections remains controversial [7]. The original analysis of

the *sagA* gene demonstrated that inactivation of the gene encoding the polypeptide component of SLS rendered the organism less virulent in a dermonecrotic mouse model [3]. In a related series of studies, Li et al also isolated a mutant that not only lacked SLS activity but also affected other phenotypes [4]. The additional phenotypes included surface M and M-related protein as well as the secreted cysteine protease, streptococcal pyrogenic exotoxin B, SpeB [4]. The disrupted gene was termed *pel* (pleotrophic effect locus). In isolate CS101 the *pel* gene acted as a transcriptional regulator [4] while in an M6 isolate it displayed effects on secretion and membrane anchoring [8]. The transposon inserted in the *pel* gene mapped to the promoter region of the previously identified *sagA* gene (SLS-associated gene A). Since the same gene is disrupted in all of the studies we will use the designation *sagA/pel* throughout to define this regulatory region that also is directly related to the  $\beta$ -hemolytic phenotype.

In this study we have further characterized the *sagA/pel* mutant of isolate CS101 and report that it is less virulent than the wild type organism. The loss of virulence associated with the *sagA/pel* mutant can be reversed by injection of this mutant into the skin of mice and recovering a  $\beta$ -hemolytic positive variant from the kidney 72 hours later. This kidney-recovered variant restored SLS activity, and M and M-related protein expression but not SpeB or streptokinase (SK) secretion. This change in phenotype was achieved despite the continued presence of the Tn917 transposon in the promoter region of the *sagA/pel* gene.

## Results

### Selection of *sagA/pel* variants by biological pressures in the mouse

The *sagA/pel* mutant of isolate CS101 fails to express  $\beta$ -hemolysin, SpeB, SK or surface M and M-related proteins [4]. Based on prior studies from our laboratory [9,10], we predicted that this isolate would be avirulent in the mouse skin air sac model. To test this possibility, two groups of six mice were injected with  $2 \times 10^9$  cfu of either wild type or an isogenic *sagA/pel* mutant isolate and the mice observed over a 72 hour period. Surprisingly, at the conclusion of this study there was no statistically significant difference ( $p > 0.05$ ) between the mice injected with the wild type isolate and the *sagA/pel* mutant (data not shown).

This result was reminiscent of an earlier study conducted by our laboratory using the *mga* mutant of isolate 64/14 [9]. In this case, although the *mga* mutant failed to express M or M-related proteins, however, it was capable of causing a lethal infection [9]. Detailed analysis of this system indicated that the mouse was capable of selecting

an M protein over-expressing variant despite the presence of the *mga* mutation in an identical location [9].

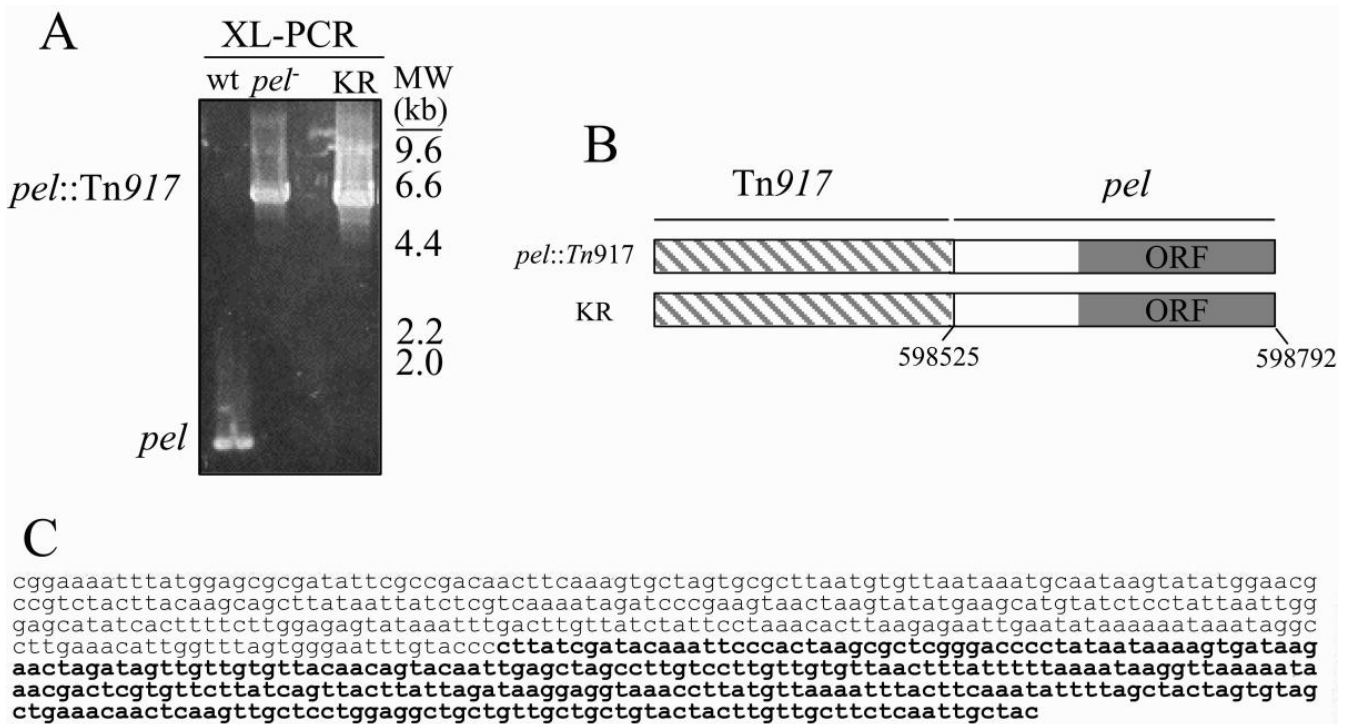
To determine if selection for a phenotypic variant was also occurring with the *sagA/pel* mutant, mice were injected in a skin air sac and recovered at varying times post infection from either the spleen, liver or kidney. Mice were euthanized at 4, 8, 12, 24, 48 and 72 hours post infection with  $10^9$  cfu. Since the study was designed to select for revertants or phenotypic variants, studies of the wild type isolate were not included. At each time point, three mice were euthanized and spleen, kidney and liver tissue samples were obtained. The samples were homogenized in sterile PBS and aliquots plated on blood agar plates containing erythromycin. The results of these studies are presented in Table 1.

**Table 1: Recovered Bacteria (CFU) from Mice infected in the skin with CS101 *sagA/pel*::Tn917\***

Time (h)	Mouse	Spleen	Kidney	Liver
4	1	0	0	0
	2	200	140	124
	3	0	0	0
8	1	0	0	0
	2	361	2	5
	3	0	3	0
12	1	0	0	0
	2	90	7	3
	3	0	0	0
24	1	15	0	1
	2	59	0	0
	3	0	0	0
48	1	0	0	0
	2	0	0	0
	3	0	0	0
72	1	0	0	0
	2	136	15	0
	3	115	2	12
	4	>>	72	76

\*The recovered isolates were erythromycin resistant ">>" = too numerous to count

At four hours, only one of the three mice showed a significant bacteremia in any sample tested. In the other two mice, three of four sites were sterile. Within eight hours only one mouse showed  $> 10$  cfu in any sample and the organisms were confined to the spleen. At 24 hours a low level of bacteria were noted in the spleen samples and by 48 hours all mice were sterile at all sites tested. Surprisingly, at 72 hours bacteria could be recovered from the spleen, kidney and liver of three of four mice. It was of interest that the majority of recovered isolates at 72 hours post-infection were  $\beta$ -hemolytic (data not shown).



**Figure 1**  
 Comparison of the *sagA/pel* region in chromosomal DNA from the non-mouse-passaged  $\beta$ -hemolysis negative *sagA/pel*::Tn917 mutant and the mouse-passaged kidney-recovered (KR)  $\beta$ -hemolysis positive variant. **A**). XL-PCR. The *sagA/pel* region from wild type CS101 (wt), *sagA/pel*<sup>-</sup> (*pel*::Tn917) and *sagA/pel*::Tn917 kidney-recovered (KR) was amplified by XL-PCR and *sagA/pel* specific primers. The position of wild type and *sagA/pel*::Tn917 amplification products are indicated to the left of the figure. Apparent molecular weights are indicated to the right of the figure. The size of the XL-PCR products was consistent with a Tn917 insertion into the *sagA/pel* region [4]. **B**). Schematic of the *sagA/pel*::Tn917 region from non mouse-passaged (*pel*::Tn917) and mouse-passaged kidney-recovered (KR) isolates. The schematic shows that the *sagA/pel* ORF (shaded box), *sagA/pel* upstream region (empty box) and the right end of the Tn917 transposon (hatched box) were identical between the two strains. **C**). Chromosomal DNA was isolated from the two strains and directly sequenced as described in Materials and Methods and the sequences were found to be identical. The bold text is 267 bases of sequence that covers the promoter and 5' end of the *sagA/pel* gene. This sequence is identical to bases 598525 through 598792 of the annotated *S. pyogenes* genome [43] The text in regular type shows 301 bases of sequence from the Tn917 transposon including the right terminal repeat and the 3' end of the transposase gene.

All of these isolates were erythromycin-resistant indicating that the Tn917 transposon was still present in these variants. No mice died prior to 72 hours in this experiment. The  $\beta$ -hemolytic positive phenotype of these recovered variants was stable for over ten passages on blood agar plates or in broth (data not shown).

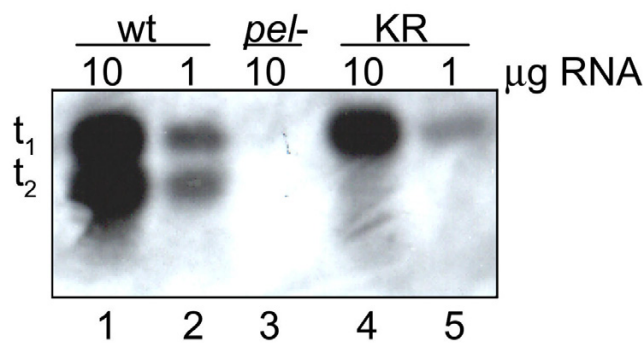
**Analysis of the chromosomal DNA of the *sagA/pel* mutant and kidney-recovered variants**

To determine whether the  $\beta$ -hemolytic positive *sagA/pel* variants recovered from the kidney of an infected mouse maintained the Tn917 transposon in the original location, we performed XL-PCR and Southern blot analysis as well as sequencing the region near the Tn917 insertion using chromosomal DNA from the parental *sagA/pel* strain and the kidney-recovered isolates as templates.

The XL-PCR profile of the parental *sagA/pel* mutant and the  $\beta$ -hemolytic kidney-recovered variants was identical (Fig. 1A). In addition, there was no difference in the DNA sequence around the transposon-*sagA/pel* junction between the original *sagA/pel* mutant and the kidney-recovered variant (Fig. 1B & 1C). Southern blotting confirmed that the location and size of the Tn917 insertion was unaltered in the kidney-recovered variant compared to the parental strain and that only a single Tn917 transposon was present (data not shown). Thus the restoration of  $\beta$ -hemolysis cannot be due to loss, rearrangement or duplication of the Tn917.

**Northern blot and primer extension analysis of *sagA/pel***

The insertion site for the Tn917 transposon is in the promoter region of the *sagA/pel* gene and no *sagA/pel* mes-



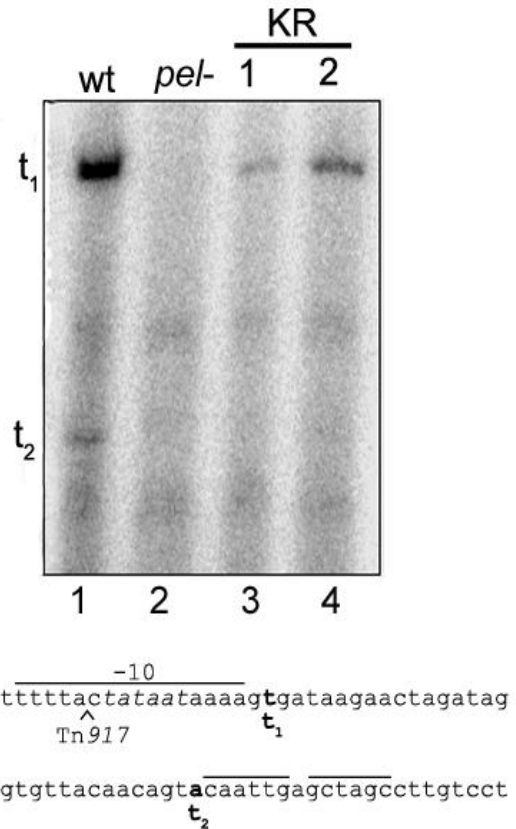
**Figure 2**  
Northern blot analysis of mRNA in CS101 (wt), CS101 *sagA/pel::Tn917* (*pel-*) and CS101 *sagA/pel::Tn917* kidney-recovered (KR). Bacteria were grown overnight at 37°C, 10% CO<sub>2</sub> in Todd-Hewitt Yeast extract broth. RNA was extracted and 10 μg or 1 μg of total RNA was loaded on a 1.0% MOPS-formaldehyde agarose gel. After blotting the RNA to a charged nylon filter, *sagA/pel* RNA was detected using a biotinylated probe. t<sub>1</sub> and t<sub>2</sub> represent the two transcripts detected in the wild type strain. Only t<sub>1</sub> was detected in the KR variant.

*sage* was detected in the original mutant [4]. RNA was isolated from the β-hemolytic positive kidney-recovered variant and the wild type isolate and analyzed for *sagA/pel* message by Northern blotting, (Fig. 2). A 500 base message was detected in both the wild type and kidney-recovered variants but not in the RNA isolated from the *sagA/pel* mutant (Fig. 2). In contrast to previous reports [3–5] a second smaller transcript was detected in the wild type strain (Fig. 2, lane 1–2). This transcript was not seen in the *sagA/pel* mutant or kidney-recovered variants grown under these conditions (Fig. 2, lane 3–5).

Primer extension analysis of the wild type and kidney-recovered variants demonstrated that the *sagA/pel* message expressed in the kidney-recovered variant had an identical transcription start site to the 500 base message present in the wild type strain (Fig. 3). The second transcript, present only in the RNA isolated from the wild type isolate (Fig. 3, lane 1), started 35 bases downstream of the longer transcript. It is not clear whether this is a second transcription start site or a processed form of the larger transcript. It is interesting to note that two 6-base palindromes are located immediately downstream of the 5'-end of the shorter transcript and a 6-base inverted repeat lies just upstream of the 5'-end of the larger transcript (Fig. 3 lower panel).

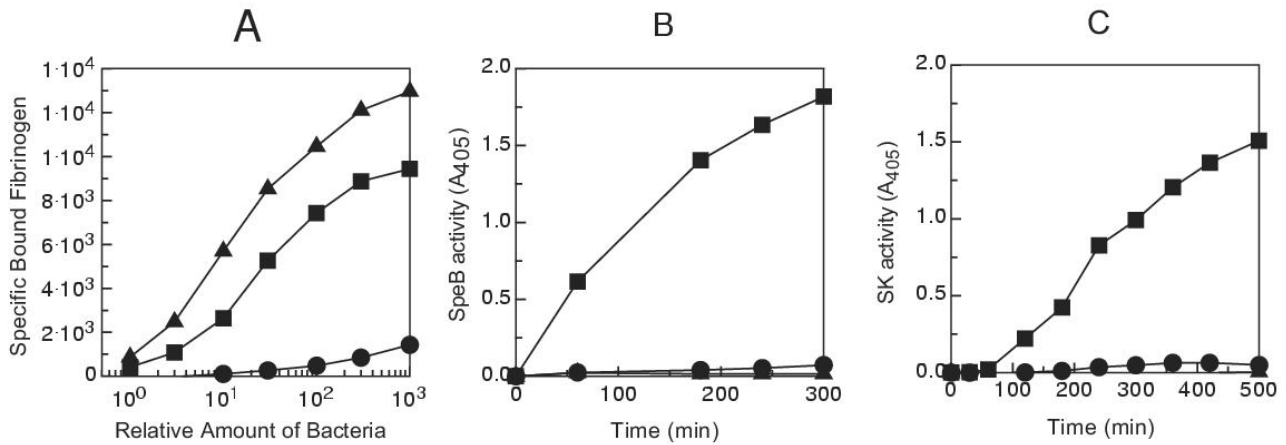
#### Analysis of other *sagA/pel* phenotypes

The presence of a *sagA/pel* transcript is consistent with the β-hemolytic phenotype of the kidney-recovered variants. Previous studies have demonstrated that the inactivation of the *sagA/pel* gene product also effects



**Figure 3**  
Primer extension analysis of *sagA/pel* from wild type, *sagA/pel* mutant and kidney-recovered strains. Bacteria were grown overnight at 37°C with 10% CO<sub>2</sub> in THY broth. RNA was extracted and *sagA/pel* was detected using a primer extension assay and *sagA/pel*-specific primers. Equal amounts of RNA were loaded in each lane. Lane 1, RNA isolated from a wild type CS101 strain. Lane 2, RNA from an isogenic *sagA/pel::Tn917* mutant. Lanes 3 and 4, RNA isolated from 2 different KR variants of the *sagA/pel* mutant. The larger *sagA/pel* transcript is indicated as t<sub>1</sub>, the smaller as t<sub>2</sub>. The DNA sequence in the lower part of the figure is from the region around the *sagA/pel* promoter. These represent bases 598514–598593 in the *S. pyogenes* genome [43]. The bases in italics are the putative -10 region of the *sagA/pel* promoter. The overlined regions are 16, 6 and 6 base inverted repeats. The bold letters are the 5'-ends of the t<sub>1</sub> and t<sub>2</sub> transcripts as determined by primer extension. The position of the Tn917 insertion is indicated by the ^ symbol. Note that the site of insertion is slightly different from what was previously reported [4]. The t<sub>2</sub> transcript was not detected in the KR variants even at longer exposures.

expression of other key streptococcal products, including surface M proteins, streptokinase (SK) and the secreted cysteine protease, SpeB [4]. Analysis of SpeB, SK and M and M-related proteins was conducted to determine if



**Figure 4**

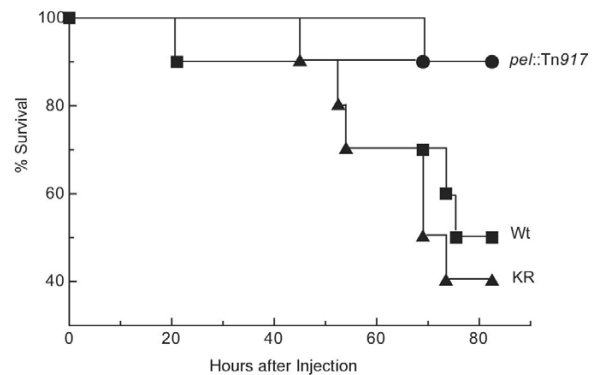
Phenotypes of CS101 wt, CS101 *sagA/pel::Tn917* and CS101 *sagA/pel::Tn917* kidney-recovered (KR) variants. Panel A. Bacterial binding of <sup>125</sup>I labeled fibrinogen CS101 wt (square), CS101 *sagA/pel::Tn917* (circle) and CS101 *sagA/pel::Tn917* kidney-recovered (triangle). Panel B. Streptococcal pyogenic exotoxin B (SpeB) activity of culture supernatants treated with DTT. CS101 wt (square), CS101 *sagA/pel::Tn917* (circle) and CS101 *sagA/pel::Tn917* kidney-recovered (triangle). Panel C. Streptokinase (SK) activity of culture supernatants grown in the presence of cysteine protease inhibitor E 64 (10 μM) to prevent destruction of SK by any secreted cysteine protease; CS101 wt (square), CS101 *sagA/pel::Tn917* (circle) and CS101 *sagA/pel::Tn917* KR (triangle).

restoration of the expression of the large *sagA/pel* transcript also reverted the other phenotypes associated with the wild type organism.

Expression of surface fibrinogen-binding M and M-related proteins was monitored by the ability of intact bacteria to bind radiolabeled fibrinogen. The kidney-recovered mutant not only recovered fibrinogen binding potential, that was lost when the *sagA/pel* gene was inactivated, but also the level of fibrinogen-binding exceeded that of the wild type isolate (Fig. 4A). Analysis of culture supernatants for the presence of SpeB (Fig. 4B) or SK (Fig. 4C) indicated that the *sagA/pel* mutant and the kidney-recovered variant displayed a similar low level of expression when compared to the wild type. There were no significant changes in fibronectin binding among any of the variants tested (see Table 2). Consequently, restoration of expression of the larger *sagA/pel* transcript (Fig. 3) was not sufficient to revert all of the *sagA/pel*-associated phenotypes to wild type levels (see Table 2).

**Restoration of virulence in the kidney-recovered *pel* mutants**

Based on the M and M-related protein phenotypic characteristics of the β-hemolytic positive kidney-recovered variant of the *sagA/pel* mutant *in vitro*, we predicted this variant would be virulent in a mouse skin infection model. To test this prediction the kidney-recovered variant,



**Figure 5**

Virulence of wild type isolate CS101 (square) and the isogenic β-hemolysis negative *sagA/pel* mutant, CS101 *sagA/pel::Tn917* (circle) and a β-hemolytic kidney recovered CS101 *pel::Tn917* KR *sagA/pel* mutant variant (triangle). Groups of 10 outbred CDI mice were injected with 1 × 10<sup>9</sup> cfu into a skin air sack. Time to death was monitored and statistical significance was determined by use of Student's t test (wt vs. *sagA/pel::Tn917* p = 0.54; *sagA/pel::Tn917* vs. KR p = 0.018).

the wild type and the original *sagA/pel* mutant were tested for virulence using the skin air sac model. The results

**Table 2: Phenotypic analysis of wild type *Streptococcus pyogenes* isolate CS101, an isogenic *sagA/pel::Tn917* mutant and a kidney-recovered variant of the *sagA/pel::Tn917* mutant.**

Phenotype	Strains		
	Wild Type	<i>sagA/pel::Tn917</i>	Kidney-recovered <i>sagA/pel::Tn917</i>
Beta hemolysis SLS	+	-	+
SK	+	-	-
Cysteine protease, SpeB	++	-	-
Fibrinogen binding %	+	±	++
Fibronectin binding %	+	±	+

++ = > 50% more than the wild type + = wild type levels ± = 15 – 30% of the wild type level - = < 10% of the wild type

present in figure 5 indicate that the kidney-recovered variant was significantly more virulent than the *sagA/pel* mutant from which it was originally selected ( $p = 0.018$ ) despite not secreting SpeB or SK in culture (see Table 2).

## Discussion

Inactivation of the *sagA/pel* locus by insertion of a Tn917 transposon into the promoter region leads to decreased expression of SLS, SpeB, SK as well as M and M-related proteins [4] and reduced virulence in a mouse model of infection using Cytodex beads [3]. In this paper we have shown that this mutation also leads to decreased or delayed virulence in a mouse air-sac model of infection. Although virulence of the *sagA/pel* mutant was decreased during the initial infection period, viable bacteria could be isolated from the spleen, kidney or liver 72 hours after infection in the skin. What was surprising was when cultured on blood agar plates at 37°C these isolates were  $\beta$ -hemolytic yet remained erythromycin resistant.

In this study we have analyzed a representative  $\beta$ -hemolytic positive kidney-recovered variant. Direct genomic sequencing of the *sagA/pel::Tn917* insertion junction in these kidney-recovered  $\beta$ -hemolysis positive variants established that the transposon was present in the genome in exactly the same location as the parental  $\beta$ -hemolytic negative *sagA/pel* mutant. Northern blot and primer extension analysis confirmed that the *sagA/pel* gene was transcribed in the  $\beta$ -hemolytic kidney-recovered variant, while *sagA/pel* message was not seen in the parental  $\beta$ -hemolysis negative *sagA/pel* mutant. Since the *sagA/pel* promoter was identical in both the parental and kidney-recovered isolates we conclude that the Tn917 was inserted into a positive regulatory site and not into an essential promoter sequence.

In previous studies only a single *sagA/pel* transcript were observed [3–5]. However, in this study we have identified two *sagA/pel* transcripts present in approximately equal concentration in the wild type parent (Figure 2). We do not know if this second shorter transcript represents a second transcription start site or is a processed form of the larger transcript. What is intriguing is that only the larger transcript is present in the  $\beta$ -hemolysis positive kidney-recovered variant. This result would be consistent with the hypothesis that *sagA/pel* has two transcriptional start sites and expression from only one site is restored after mouse selection. Other *S. pyogenes* regulators have been shown to have multiple transcription start sites that are differentially regulated. For example, Mga, a transcriptional activator of M and M-related proteins, also has two transcription start sites that are independently regulated [11] and two distinct transcription start sites are associated with expression of the streptokinase gene [12–15].

The strain CS101 *sagA/pel::Tn917* has previously been rendered  $\beta$ -hemolytic negative by a transposon insertion. To recover a  $\beta$ -hemolysis positive variant, from the mouse kidney, with the transposon in its original position was unexpected. This result indicated additional levels of regulation of the  $\beta$ -hemolysis phenotype could be selected by biological pressures in the mouse. The selected  $\beta$  hemolysis positive variant was stable and retained this phenotype even after repeated passage on laboratory media in the absence of any additional selective biological pressures.

The mouse selection process results not only in the restoration of a  $\beta$ -hemolytic positive phenotype, but also restored some, but not all, of the phenotypes known to be regulated by *sagA/pel*[4]. For example, fibrinogen-binding M and M-related protein expression was restored;

however secretion of the cysteine protease, SpeB, or SK was not. Previous studies from our laboratory have consistently demonstrated loss of the SpeB phenotype in *S. pyogenes* isolates injected in a skin air sac and recovered from the organs of lethally infected mice [10,16]. This selection was not associated with loss of  $\beta$ -hemolysis but was associated with over-expression of M and M-related proteins, which in turn are predictive of the invasive potential of the organism in a skin infection model [16]. Based on the phenotypic characteristics of the kidney-recovered variant ( $\beta$ -hemolytic positive, M and M-related protein positive and SpeB negative) we predicted that this variant would be as virulent or more virulent than the wild type organism in the mouse skin infection model. This prediction was tested experimentally and the  $\beta$ -hemolysis positive *sagA/pel* variant was found to be as virulent as the wild type isolate in the skin infection model (see figure 5).

The genetic event(s) associated with the selection of a virulent variant of the *sagA/pel* mutant without changing the site or orientation of the Tn917 transposon was reminiscent of earlier studies from our laboratory testing the virulence of *mga* mutants of isolate 64/14 [9]. In that study, injection of an *mga* mutant, that failed to express any detectable surface M or M-related protein, lead to selection of *mga* variants over-expressing M and M-related proteins that could be recovered from the spleen following a lethal skin infection. This reversion of the M and M-related protein phenotype occurred without any change in the position or orientation of the spectinomycin-resistance cassette inserted into the *mga* gene to create the original mutant [9].

Taken together, these studies suggest a complex network of positive and negative regulatory pathways controlling key virulence genes in *S. pyogenes* that can be activated or inactivated in response to certain biological pressures in the infected host. Analysis of the selected phenotypes recovered following mouse passage cannot be explained by the activities of any known regulator or combination of regulators e.g. *mga*[9,17–24], *nra*[25], *CsrRS/CovRS*[26–29], *sagA/pel*[4] *rofA*[30–32], *rgg*[33–35], *fasX* [36] or *luxS*[37,38].

It is unknown if there is a regulator or a series of regulators that are inactivated or activated after passage through the mouse; however, it is clear that key virulence factors are under a more complex pattern of regulation than previously envisaged. In related studies, the selection of stable variants of either wild type or mutant *S. pyogenes* isolates was not consistently observed when the organism was injected i.p. [39]. This may relate to either the presence of unique host factors at the skin infection site or to the kinetics of clearance of the organisms. In

studies using a tissue chamber model, Kotb and colleagues have noted changes in expression of key virulence factors as a function of time [40]. Thus, it is possible that the *in vivo* events leading to selection of stable *S. pyogenes* variants may require a dynamic interaction with the host and that only under certain experimental conditions will the stable variant population be recovered.

The unique biological pressures associated with infection in the skin and persistence in the systemic circulation seems to consistently select stable variants which over-express key surface M and M-related proteins. Organisms selected in this model are consistently negative for SpeB secretion. Selection of SpeB negative variants have also been noted following sequential human blood passage of isolates or in a mouse skin infection model [39,41]. This selective pressure can also be associated with enhanced capsular expression in SpeB negative variants [42].

Several bacteriophage and transposons were identified in the *S. pyogenes* genome [43] as well as a number of potential two-component regulatory systems whose precise function remains to be elucidated. The biological selection of phenotypic revertants of variants of *S. pyogenes* from populations with defined mutations in key regulators or promoter regions of putative virulence genes is likely to provide key insights into the pathogenesis of host-bacterial interactions.

## Conclusions

Selection of  $\beta$  hemolysis positive variants from a *sagA/pel* mutant of *S. pyogenes* isolate CS101 were identified. This change in phenotype occurred despite the presence of the Tn917 transposon in an identical position in both the  $\beta$  hemolysis negative mutant and the  $\beta$  hemolysis positive selected variant. The ability of biological pressures in the mouse to select stable variants of *S. pyogenes* expressing different patterns of virulence factors suggest the existence of more complex regulatory pathway than is currently envisaged.

## Materials and Methods

### Chemicals, Bacteria and Media

The bacteria used in this study were the opacity factor positive M49 *Streptococcus pyogenes* isolate CS101 and an isogenic  $\beta$ -hemolytic negative variant generated by transposon mutagenesis, CS101 *sagA/pel::Tn917*[4]. Todd-Hewitt broth containing 0.3% yeast extract (THY) was obtained from DIFCO (Detroit MI). Blood agar plates were obtained from BBL (Fisher, Chicago, IL).

### Mouse skin air sac procedure

A skin air sac model was used to compare the virulence of isolate CS101 and paired isogenic mutants [9]. Briefly an air- and liquid-tight connective tissue pouch was generated on the back of female, six week old, outbred CD1 mice (Charles River, Portage, MI) by slow dermal injection of 0.9 mL of air via an 0.4 mm needle on a 1.0 mL syringe. The syringe containing the air also contained 0.1 mL of an appropriately diluted suspension of *S. pyogenes*. Mice were provided with food and water ad libitum. For selection of bacterial variants, experiments were continued for 72 hours post-infection. For virulence studies death was used as the endpoint and at 144 hours post-infection the experiments terminated. For bacteremia studies surviving animals were euthanized at the times stated. Spleen, kidney and a section of the liver was removed from the animals. The tissue samples were homogenized in 1 mL of sterile 10 mM PBS, pH 7.4. An 100  $\mu$ L aliquot was cultured on blood agar plates to determine if *S. pyogenes* were present. All animal studies were conducted in accordance with protocols approved by the Medical College of Ohio's Institutional Animal Use and Care Committee.

### Southern blot analysis and XL-PCR

Analysis of chromosomal DNA for the presence of Tn917 transposon insertion was carried out as described previously [4].

XL-PCR was performed using GeneAmp XL PCR kit (PE Applied Biosystems, Foster City, CA).

### DNA Sequencing

Chromosomal DNA was isolated as described previously [44]. Genomic DNA sequencing was carried out on an Applied Biosystems 310 Genetic Analyzer (PE/Applied Biosystems) using a big dye terminator cycle sequencing ready reaction kit (PE/Applied Biosystems) according to the manufacturer's specifications. The oligonucleotide 5'-ATAAATGGACCGCATATTGA-3' (corresponding to the DNA sequence just downstream of the *SagA/Pel* open reading frame), and 5'-ATAAATGGACCGCATATTGA-3' (corresponding to the region from the right end of the Tn917 insertion) were used as primers for the sequencing reaction. The resulting DNA sequences was compared using blast 2 for pair wise comparisons. [http://www.ncbi.nlm.nih.gov/blast/Blast.cgi]

### Northern blot analysis

RNA was prepared from wild type CS101 wt, *CS101 sagA/pel::Tn917* and *CS101 sagA/pel::Tn917* kidney-recovered (KR) variants grown overnight (37°C 10% CO<sub>2</sub>) in 40 ml THY media. The bacteria were harvested 8 hours post-exponential phase by centrifugation (5 min, 4000 g, 4°C) and resuspended in 500  $\mu$ L of cell lysis buff-

er (25% glucose, 10 mM EDTA, 100 mM Tris pH 7.0). 400  $\mu$ L of a solution containing 4 mg/mL lysosyme (Sigma, St. Louis, MO.) and mutanolysin (20  $\mu$ g), was added and incubated for 20 minutes at 37°C. The bacteria were sedimented by centrifugation and resuspended in 3 mL Trizol (Gibco, Rockville, MD). RNA was isolated according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. RNA was electrophoresed in a 1% agarose gel (Molecular Biology Certified Agarose, Biorad, Hercules, CA) containing 0.66 M formaldehyde in 1 $\times$  MOPS (3-(N-morpholino)-propanesulfonic acid) buffer. Following electrophoresis, RNA was transferred to a nylon membrane (Hybond-N+, Millipore, Bedford, MA) according to the manufacturer's instructions, and hybridized with digoxigenin-dUTP-labeled probe as described previously [4]. The primers used to generate the probe were: 5'-GGAATTCACCTGCTAATTACCTGA-3' and 5'-CGCGGATCCGTTTACACATAGTTATTGATAGAATCT-3'

### Primer extension

The 5'-end of the *sagA/pel* mRNA was determined by the extension of the 5'-end <sup>32</sup>P-labeled oligonucleotides 5'-ACCTTATTTTAAAAATAAAGTTAA-3' following the method of Sambrook [45]. Oligonucleotides were labeled with [ $\gamma$ -<sup>32</sup>P] ATP (10 mCi/mL in aqueous solution) (Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Gibco BRL Life Technologies, Rockville, MD). SequiTherm EXCEL II DNA sequencing kit (Epicentre Technologies, Madison, WI) was used according to the manufacturer's instruction for the corresponding sequencing reaction using the same primer.

### Streptokinase Assay

Streptokinase activity was measured as described previously [46]. Briefly, aliquots of culture supernatants (100  $\mu$ L) were mixed with either 1  $\mu$ g of purified human plasminogen or buffer. The synthetic chromogenic substrate, S2251 (H-D-Val-Leu-Lys-paranitroanilide) obtained from Kabi Pharmacia (Franklin, OH), was added to a final concentration of 400  $\mu$ M. Plasmin generation was quantified by measuring product absorbance at 405 nm.

### Cysteine endopetidase assay

Cysteine protease activity present in culture supernatants was assayed as described [47]. Briefly, 50  $\mu$ L of culture supernatant with or without 0.1  $\mu$ M dithiothreitol, was added to the wells of a microtiter plate. Following incubation for 30 minutes at 37°C 150  $\mu$ L of the substrate buffer solution, Bz-Pro-Phe-Arg-paranitroanilide, (Sigma Chemical) was added to each well. Cleavage of the substrate was monitored by measuring the A<sub>405</sub> over time. The cysteine protease specific inhibitor, E64 (Sigma), was included in parallel assays at a concentration of



1  $\mu\text{M}$  to determine if all the enzymatic activity being measured could be attributed to the presence of a cysteine protease.

#### Binding assay for fibrinogen

The ability of bacteria to bind fibrinogen was determined by their ability to bind the specific radiolabeled ligand. Human fibrinogen was radiolabeled with  $^{125}\text{I}$  (Amersham, Chicago, IL) using Iodobeads (Pierce, Rockford, IL) as described [48]. Different numbers of bacteria were incubated with 20,000 cpm of  $^{125}\text{I}$  labeled fibrinogen for 60 min at  $37^\circ\text{C}$ . The bacteria were pelleted by centrifugation at  $5,000 \times g$  for 20 min and washed twice with 2 ml of 50 mM veronal buffer pH 7.35, containing 0.15 M NaCl and 0.1% gelatin. The radioactivity associated with the bacterial pellet was quantified in a Beckman 5500B automatic gamma counter (Beckman, Fullerton CA).

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