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**

Thomas H Eberhard, Darren D Sledjeski and Michael DP Boyle*

Address: Department of Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio, 43614, USA
E-mail: Thomas H Eberhard - teberhard@mco.edu; Darren D Sledjeski - DSledjeski@mco.edu; Michael DP Boyle* - Mboyle@mco.edu
*Corresponding author

Published: 17 December 2001
BMC Microbiol 2001, 1:33
Received: 20 August 2001
Accepted: 17 December 2001

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 β-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 nature 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 (pleiotrophic 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 β-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 β-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 β-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. Based on prior studies from our laboratory hemolysin, SpeB, SK or surface M and M-related proteins [4]. Based on prior studies from our laboratory, we predicted that this isolate would be avirulent. Results

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       | 0     | 0      | 0      | 0     |
| 3       | 0     | 0      | 0      | 0     |
| 1       | 0     | 0      | 0      | 0     |
| 8       | 2     | 361    | 2      | 5     |
| 3       | 0     | 0      | 3      | 0     |
| 1       | 0     | 0      | 0      | 0     |
| 12      | 2     | 90     | 7      | 3     |
| 3       | 0     | 0      | 0      | 0     |
| 1       | 1     | 15     | 0      | 1     |
| 24      | 2     | 59     | 0      | 0     |
| 3       | 0     | 0      | 0      | 0     |
| 1       | 0     | 0      | 0      | 0     |
| 48      | 2     | 0      | 0      | 0     |
| 3       | 0     | 0      | 0      | 0     |
| 1       | 0     | 0      | 0      | 0     |
| 72      | 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 β-hemolytic (data not shown).
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 β-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 β-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 β-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 β-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-
sage was detected in the original mutant [4]. RNA was isolated from the \( \beta \)-hemolytic positive kidney-recovered variant and the wild type isolate and analyzed for \( \text{sagA/pel} \) 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 \( \text{sagA/pel} \) 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 \( \text{sagA/pel} \) 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 \( \text{sagA/pel} \) 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 \( \text{sagA/pel} \) phenotypes**

The presence of a \( \text{sagA/pel} \) transcript is consistent with the \( \beta \)-hemolytic phenotype of the kidney-recovered variants. Previous studies have demonstrated that the inactivation of the \( \text{sagA/pel} \) gene product also effects 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
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, the wild type and the original sagA/pel mutant were tested for virulence using the skin air sac model. The results
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 airsac 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 β-hemolytic yet remained erythromycin resistant.

In this study we have analyzed a representative β-hemolytic positive kidney-recovered variant. Direct genomic sequencing of the sagA/pel::Tn917 insertion junction in these kidney-recovered β-hemolysin positive variants established that the transposon was present in the genome in exactly the same location as the parental β-hemolytic negative sagA/pel mutant. Northern blot and primer extension analysis confirmed that the sagA/pel gene was transcribed in the β-hemolytic kidney-recovered variant, while sagA/pel message was not seen in the parental β-hemolysin 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 β-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 β-hemolytic negative by a transposon insertion. To recover a β-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 β-hemolysis phenotype could be selected by biological pressures in the mouse. The selected β 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 β-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 β-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 (β-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 β-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 β 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 β hemolysis negative mutant and the β 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 β-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-infec-tion the experiments terminated. For bacteremia studies surviving animals were euthanized at the times stated. Spleen, kidney and a section of the liver were removed from the animals. The tissue samples were homogenized in 1 mL of sterile 10 mM PBS, pH 7.4. An 100 µL 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’-ATAAATGGACCCGATTTGA-3’ (corresponding to the DNA sequence just downstream of the SagA/Pel open reading frame), and 5’-ATAAATGGACCCGATTTGA-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 were compared using blast 2 for pair wise comparisons.  

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% CO₂) 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 µL of cell lysis buff-
er (25% glucose, 10 mM EDTA, 100 mM Tris pH 7.0). 400 µL of a solution containing 4 mg/mL lysozyme (Sigma, St. Louis, MO,) and mutanolysin (20 µg), was added and incubated for 20 minutes at 37°C. The bacteria were sedimneted 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 electro-phoresed in a 1% agarose gel (Molecular Biology Certified Agarose, Biorad, Hercules, CA) containing 0.66 M formaldehyde in 1x 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 digoxigen-dUTP-labeled probe as described previously [4]. The primers used to generate the probe were: 5’-GGATTTACCT-GTAAATTACCTGA-3’ and 5’-CGCGGATCCGTTTACA-CATGTTATTGATAGAT-3’

Primer extension
The 5’-end of the sagA/pel mRNA was determined by the extension of the 5’-end 32P-labeled oligonucleotides 5’-ACCTATTTTTAATBAAAGTAA-3’ following the method of Sambrook [45]. Oligonucleotides were labeled with [γ-32P] 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 (Epichem Defence 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 µL) were mixed with either 1 µg 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 µM. Plasmin generation was quantified by measuring product absorbance at 405 nm.

Cysteine endopeptidase assay
Cysteine peptidase activity present in culture supernatants was assayed as described [47]. Briefly, 50 µL of culture supernatant with or without 0.1 µM dithiothreitol, was added to the wells of a microtiter plate. Following incubation for 30 minutes at 37°C 150 µL 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 A405 over time. The cysteine peptidase specific inhibitor, E64 (Sigma), was included in parallel assays at a concentration of...
1 μM 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 125I (Amer sham, Chicago, IL) using iodobeads (Pierce, Rockford, IL) as described [48]. Different numbers of bacteria were incubated with 20,000 cpm of 125I labeled fibrinogen for 60 min at 37°C. The bacteria were pelleted by centrifugation at 5,000 × 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).

**Acknowledgements**
The authors would like to thank Carol Hepner for typing the manuscript and Terence Romer and Amanda Meeker for expert technical assistance.

**References**
1. Cunningham MW: Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 2000, 13:470-511
2. Ginsburg I: Streptolysin. In: Microbial Toxins Edited by T Monteil, DE, Low, JC De Azavedo, New York: Academic Press; 1976:617-717
3. Betshel SD, Borgia SM, Barg NL, Low DE, De Azavedo JC: Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. Infect Immun 1998, 66:1671-1679
4. Li Z, Sledjeski DD, Kreikemeyer B, Podbielski A, Boyle MD: Identification of pel, a Streptococcus pyogenes locus that affects both surface and secreted proteins. J Bacteriol 1999, 181:609-612
5. Nizet V, Beall B, Dress J, Datta V, Kilburn L, Low DE, De Azavedo JC: Genetic locus for streptolysin S production by group A streptococcus. Infect Immun 2000, 68:4245-4254
6. Carr A, Sledgeski DD, Podbielski A, Boyle MD, Kreikemeyer B: Similarities between Complement-mediated and Streptolysin S-mediated Hemolysis. J Biol Chem 2001, 276:14790-14796
7. Ginsburg I: Is streptolysin S of group A streptococci a virulence factor? APMS 1999, 107:1051-1059
8. Stawas I, Germon P, McDade K, Scott JR: Generation and Surface Localization of Intact M Protein in Streptococcus pyogenes Are Dependent on sagA. Infect Immun 2001, 69:7029-7038
9. Boyle MD, Raeder R, Flosdorff A, Podbielski A: Role of emm and mpr genes in the virulence of group A streptococcal isolate 64/14 in a mouse model of skin infection. J Infect Dis 1998, 177:991-997
10. Schmidt KH, Podbielski A, Raeder R, Boyle MD: Inactivation of single genes within the virulence regulon of an M2 group A streptococcal isolate result in differences in virulence for chicken embryos and for mice. Microb Pathog 1997, 23:347-355
11. Bormann NE, Cleary PP: Transcriptional analysis of mga, a regulatory gene in Streptococcus pyogenes: identification of monocistronic and bicistronic transcripts that phase vary. Gene 1997, 200:123-134
12. Gase K, Elinger T, Malke H: Complex transcriptional control of the streptokinase gene of Streptococcus equisimilis H46A. Mol Gen Genet 1995, 247:749-758
13. Malke H, Ferretti JJ, Podbielski A, Suvorov A, Trieu-Cuot P: Summary of the round table discussion on genome structure and regulation of gene expression in streptococci and enterococci. Adv Exp Med Biol 1997, 418:1051-1056
14. Malke H, Steiner K, Gase K, Mechoul D, Elinger T: The streptok inase gene: allelic variation, genomic environment and expression control. Dev Biol Stand 1995, 85:183-193
15. Malke H, Steiner K, Gase K, Frank C: Expression and regulation of the streptokinase gene. Methods 2000, 21:111-124
16. Raeder R, Boyle MD: Properties of IgG-binding proteins expressed by Streptococcus pyogenes isolates are predictive of invasive potential. J Infect Dis 1996, 173:888-895
17. Simpson WJ, LaPenta D, Chen C, Cleary PP: Coregulation of type 12 M protein and streptococcal CSa peptidase genes in group A streptococci: evidence for a virulence regulon controlled by the virR locus. J Bacteriol 1990, 172:692-700
18. Simpson WJ, Cleary PP: Expression of M type 12 protein by a group A streptococcus exhibits phase-like variation: evidence for coregulation of colony opacity determinants and M protein. Infect Immun 1987, 55:2448-2455
19. Okada N, Geist RT, Caparon MG: Positive transcriptional control of mga regulates expression in the group A streptococcus. Mol Microbiol 1993, 7:893-903
20. Perez-Casal J, Caparon MG, Scott JR: Mry, a trans-acting positive regulator of the M protein gene of Streptococcus pyogenes with similarity to the receptor proteins of two-component regulatory systems. J Bacteriol 1993, 175:178-187
21. McIver KS, Scott JR: Role of mga in group A streptococcus. J Bacteriol 1997, 179:517-5187
22. Podbielski A, Kaufhold A, Lutzeck R: Das Vir-Regulon von Streptococcus pyogenes: Koordinierte Expression wichtiger Virulenzfaktoren. Immun Infekt 1992, 20:161-168
23. Podbielski A, Flosdorff A, Weber-Heynemann J: The group A streptococcal virR49 gene controls expression of four structural vir regulon genes. Infect Immun 1995, 63:9-20
24. Podbielski A, Flosdorff A, Weber-Heynemann J: Molecular characterization of the type M group 49 A streptococcal (GAS) virR gene. Dev Biol Stand 1995, 85:153-157
25. Podbielski A, Woischnik M, Leonard BA, Schmidt KH: Characterization of nra, a global negative regulator gene in group A streptococci. Mol Microbiol 1999, 31:1051-1064
26. Levin JC, Wessels MR: Identification of csrR/csrS, a genetic locus that regulates hyaluronic acid capsule synthesis in group A streptococci. Mol Microbiol 1998, 30:209-219
27. Federle PJ, McIver KS, Scott JR: A response regulator that represses transcription of several virulence operons in the group A streptococcus. J Bacteriol 1999, 181:3649-3657
28. Heath A, DiRita VJ, Barg NL, Engleberg NC: A two-component regulatory system, CsrR-CsrS, represses expression of three Streptococcus pyogenes virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun 1999, 67:5298-5305
29. Bernish B, van de Rijn I: Characterization of a two-component system in Streptococcus pyogenes which is involved in regulation of hyaluronic acid production. J Biol Chem 1999, 274:4786-4793
30. VanHeyningen T, Fogg G, Yates D, Hanski E, Caparon M: Adherence and fibronectin binding are environmentally regulated in the group A streptococcus. Mol Microbiol 1993, 9:1213-1222
31. Fogg GC, Gibson CM, Caparon MG: The identification of rofA, a positive-acting regulatory component of prfE expression: use of an mrg-based shuttle mutagenesis strategy in Streptococcus pyogenes. Mol Microbiol 1994, 11:671-684
32. Granok AB, Parsonage D, Ross RP, Caparon MG: The RofA binding site in Streptococcus pyogenes is utilized in multiple transcriptional pathways. J Bacteriol 2000, 182:1529-1540
33. Chauasse MS, Aijdic D, Ferretti JJ: The rgg gene of Streptococcus pyogenes NZ131 positively influences extracellular SPE B production. Infect Immun 1999, 67:1715-1722
34. Chauasse MS, Watson RO, Smoot JC, Musser JM: Identification of Rgg-regulated exoproteins of Streptococcus pyogenes. Infect Immun 2001, 69:822-831
35. Lyon WR, Gibson CM, Caparon MG: A role for trigger factor and an rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of Streptococcus pyogenes. Embio J 1998, 17:6263-6275
36. Kreikemeyer B, Boyle MD, Buttaro BA, Heinemann M, Podbielski A: Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas) with homologies to two-component-type regulators requires a small RNA molecule. Mol Microbiol 2001, 39:392-406
37. Lyon WR, Madden JC, Levin JC, Stein JL, Caparon MG: Mutation of luxS affects growth and virulence factor expression in Streptococcus pyogenes. Mol Microbiol 2001, 42:145-157
38. Steiner K, Malke H: **relA-Independent Amino Acid Starvation Response Network of Streptococcus pyogenes.** J Bacteriol 2001, 183:7354-7364

39. Raeder R, Boyle MD: **Association of type II immunoglobulin G-binding protein expression and survival of group A streptococci in human blood.** Infect Immun 1993, 61:3696-3702

40. Kazmi SU, Kansal R, Aziz RK, Hooshdaran M, Norrby-Teglund A, Low DE, Halim AB, Korb M: **Reciprocal, temporal expression of speA and speB by invasive M1T1 group a streptococcal isolates in vivo.** Infect Immun 2001, 69:4988-4995

41. Raeder R, Boyle MD: **Association between expression of immunoglobulin G-binding proteins by group A streptococci and virulence in a mouse skin infection model.** Infect Immun 1993, 61:1378-1384

42. Raeder R, Harokopakis E, Hollingshead S, Boyle MD: **Absence of SpeB production in virulent large capsular forms of group A streptococcal strain 64.** Infect Immun 2000, 68:744-751

43. Ferretti JJ, McShan WM, Ajdic D, Savic DJ, Savic G, Lyon K, Primeaux C, Sezace S, Suvorov AN, Kenton S, et al: **Complete genome sequence of an M1 strain of Streptococcus pyogenes.** Proc Natl Acad Sci U S A 2001, 98:4658-4663

44. Caparon MG, Scott JR: **Genetic manipulation of pathogenic streptococci.** Methods Enzymol 1991, 204:556-586

45. Sambrook J, Fitsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, second edn. Cold Spring Harbor: Cold Spring Harbor Press 1989

46. Christner R, Li Z, Raeder R, Podbielski A, Boyle MD: **Identification of key gene products required for acquisition of plasmin-like enzymatic activity by group A streptococci.** J Infect Dis 1997, 175:1115-1120

47. North MJ: **Cysteine endopeptidases of parasitic protozoa.** Methods Enzymol 1994, 244:523-539

48. Markwell MA: **A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum.** Anal Biochem 1982, 125:427-432

Publish with BioMed Central and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with BMC and your research papers will be:

• available free of charge to the entire biomedical community
• peer reviewed and published immediately upon acceptance
• cited in PubMed and archived on PubMed Central
• yours - you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/manuscript/editorial@biomedcentral.com

Page 10 of 10