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Identification of the role of the sal locus in streptococcus pyogenes virulence during host-pathogen interactions

Phanramphoei Namprachan-Frantz
Wayne State University

IDENTIFICATION OF THE ROLE OF THE SAL LOCUS IN *STREPTOCOCCUS PYOGENES* VIRULENCE DURING HOST-PATHOGEN INTERACTIONS

by

PHANRAMPHOEI NAMPRACHAN-FRANTZ

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GENERAL INTRODUCTION

Group A Streptococcus (GAS), also known as *Streptococcus pyogenes*, is a Gram-positive bacterium that is strictly a human pathogen. While it can reside within the host carrier silently without causing symptomatic disease, it can also cause a wide array of diseases ranging from mild external infections, pharyngitis (‘strep throat’) and impetigo, to invasive infections such as necrotizing fasciitis (‘flesh-eating disease’) and streptococcal toxic shock syndrome. Moreover, it is a common cause of post-infection sequelae; acute rheumatic fever and acute glomerulonephritis, which is a leading cause of mortality due to invasive group A streptococcal infections. Globally, there are 600 million pharyngitis cases and 1.78 million new cases of invasive GAS infection each year, causing at least 500,000 deaths. In the United States alone, the medical burden caused by GAS infection is approximately 2 billion dollars annually [1]. Comparison of the epidemiology studies of invasive GAS infections occurring in the United States from1995-1999 to the more recent study between 2000-2004 revealed that the deaths from invasive GAS infection increased by 19% (from an average 1200 deaths to 1435 deaths, respectively) [1-3]. This suggests that the infections are becoming more severe, which may be due to the bacteria becoming more virulent. The mortality in these studies was most often from cases of toxic shock syndrome, necrotizing fasciitis, and some complications from pneumonia, bacteremia and meningitis. *S. pyogenes* is classified by the M protein, an outer

membrane protein, referred to as the M serotype [4]. Even though certain serotypes are more frequently associated with an invasive infection, most of the serotypes can cause similar severity of disease. In spite of many research efforts, an effective vaccine for GAS has not yet been fully developed. With increasing evidence of more virulent strains and the appearance of antibiotic resistant streptococcus [5, 6], the GAS vaccine is an urgent need. Therefore, a more complete understanding of the molecular pathogenesis of GAS is required in order to ultimately develop a successful vaccine.

S. pyogenes is an extracellular pathogen that is also capable of invading host cells during infection [7-9]. The invasive diseases require a combination of host cell invasion and circumvention of the host immune response. *S. pyogenes* possesses several virulence factors that not only facilitate host cell invasion, but also suppress and surpass host immune mechanisms. The major surface proteins, M protein and fibronectin binding protein, along with a cell wall component, lipoteichoic acid, assist in bacterial adherence and internalization into host epithelial cells [10-13]. Several streptococcal proteases, Hyaluronidase, Streptokinase and the SpeB cysteine protease, are reported to cause tissue destruction and allow the spreading of bacteria throughout the host cells [14-16].

Multiple GAS virulence gene products also function to overcome host immune mechanisms. M protein also has the property of an antiphagocytic molecule against polymorphonuclear leukocytes (PMNs) and macrophages [10, 17, 18]. C5a peptidase cleaves complement factors and inhibits complement

activation [19]. The bacterial exotoxins, streptolysin S (SLS) and streptolysin O (SLO), have a cytotoxic effect on both epithelial cells and neutrophils [20-23]. In addition to cell lysis and impairment of PMN-mediated killing of GAS, SLS also inhibits neutrophil recruitment to the site of infection [24]. In addition to SLS, a recently identified virulence factor, streptococcal protease (SpyCEP), inactivates CXC chemokines and inhibits the recruitment of neutrophils [25-27]. Another group of virulence factors are the streptococcal superantigens (SAgs). The superantigens function to non-specifically stimulate the activation of T-cells, causing a massive inflammatory response, which is a hallmark of streptococcal toxic shock syndrome [28].

In order to survive in various sites of infection, the bacteria must be able to adapt to different physiological conditions inside the human host, especially when in the hazardous environments created by the host immune response. The production of virulence factors is metabolically costly for bacteria. Therefore, coordination of virulence factor production to specific environmental conditions is necessary. Many bacterial pathogens are equipped with two-component signaling systems to orchestrate virulence gene expression in response to host conditions.

Two-component systems (TCSs) are a common mechanism used by prokaryotic cells to trigger an adaptive response to environmental changes in order to thrive and persist in a particular niche [29]. The TCSs are generally composed of a membrane sensor kinase that senses an environmental stimulus

and a cytoplasmic response regulator that governs the changes in gene expression at the transcriptional level. In Gram-positive bacteria, the sensor histidine kinase contains an extracellular domain, which is responsible for sensing the signal, and a conserved histidine residue, which is auto-phosphorylated during activation by the environmental signal. The cognate response regulator contains a conserved aspartic acid, which receives the phosphoryl group from the sensor kinase. The phosphotransfer process induces conformational changes in the regulator protein, affecting the DNA binding domain (H-T-H domain), resulting in a change in gene expression, and ultimately affecting in cell physiology [30].

There are 13 conserved two-component systems (TCSs) identified from the complete genome sequencing of M1, M3, M5, M6, M18 and M28 serotypes of *S. pyogenes* [31-37]. So far, only three Streptococcal TCSs have been studied extensively for their roles in the pathogenicity of the bacteria during host-pathogen interactions. The FasBCA system mainly regulates expression of genes involved in adhesion and invasion of host cells, such as the fibronectin binding protein, streptokinase and SLS [38, 39]. The CovRS system (also known as CrsRS) negatively regulates genes encoding capsule (*hasABC*) and several exotoxins (*skaA*, *sagA*, and *speB*) [40, 41] along with 15% of the entire transcriptome [42]. Deletion of the *covRS* genes conferred a hypervirulent phenotype during infection of mice [42]. Moreover, CovRS also controls the genes that contribute to *S. pyogenes* resistance to the human antimicrobial

peptide, LL-37, as well as to bacterial survival under iron starvation [43]. The Ihk/Irr system upregulates several secreted proteins that are involved in resistance to PMN-mediated killing [44, 45]. Any mutations in these described TCSs would cause a change in virulence due to the loss of regulation required for resistance to the host immune response. Therefore, to fully understand the pathogenesis of *S. pyogenes,* it is important to not only study virulence factors but also understand how these virulence factors are regulated during host infection.

The research presented in this dissertation analyzes the role of the *S. pyogenes sal* locus, a putative lantibiotic locus, in virulence. We have data to support the hypothesis that the function of this locus has evolved from its originally identified function in the commensal *S. salivarius* from providing a competitive advantage while residing in a polymicrobial environment to supporting virulence of *S. pyogenes* in a pathological niche.

Lantibiotics are bacteriocin-like inhibitory peptides that are ribosomally synthesized and produced exclusively by Gram-positive bacteria. Their unique structure contains lanthionine or methyl-lanthionine residues formed by the cross-linking of cysteine to either dehydrolamine or dehydrobutyrine [46, 47]. The mode of action of the peptide is as a bactericidal pore-forming toxin against closely related Gram-positive bacteria, providing an ecological advantage to the producing bacterium, especially those inhabiting competitive environments such as mucosal surfaces [48, 49]. There are common genetic components found in

most lantibiotic loci, which include a gene to encode LanA, the pre-lantibiotic peptide, which is modified and transported by LanM, modifying protein, and LanT, transport protein, respectively. To prevent the bactericidal effect of its own lantibiotic, this locus also encodes an immunity protein LanI or a specialized ABC transporter, LanEFG, providing an immunity function [47, 50-53]. The production of the lantibiotic is tightly regulated through the activation of a two-component signaling system, LanKR. The cognate lantibiotic also functions as a cell-density signaling molecule for the TCS similar to a quorum sensing system [52, 54].

Most Gram-positive bacteria that harbor lantibiotic loci are commensal bacteria. However, it has been reported that some pathogenic bacteria also have a lantibiotic locus that functions in virulence. The cytolysin locus in *Enterococcus faecalis* is also described as a lantibiotic locus. The activity of the cytolysin is targeted against both bacteria and eukaryotic cells. The cytolysin has a toxic effect against several cell types including phagocytic cells like neutrophils and macrophages [55]. This scenario provides evidence that bacteria can evolve to use the product of a lantibiotic locus for their advantage against the toxic environment inside the host.

The *sal* locus is a lantibiotic locus encoding proteins that produce and regulate a type 1 lantibiotic, salivaricin A [56]. This lantibiotic was first identified in *S. salivarius*, a commensal bacterium, which is predominantly found in oral and pharyngeal sites. Salivaricin A has an inhibitory function against several Streptococci, such as *S. mutans* and *S. pyogenes,* which can inhabit the same

host sites [57, 58]. Interestingly, *S. pyogenes* also harbors the *sal* locus and has ~85% homology to the *sal* locus of *S. salivarius.* However, *S. pyogenes* does not produce an active salivaricin A and is not immune to salivaricin A produced by *S. salivarius* [59]. Despite the lack of lantibiotic functions, the *sal* locus is highly conserved with >92% homology in all *S. pyogenes* serotypes sequenced. The retention and conservation of this locus, in the absence of lantibiotic production, suggests that the change in the function of the *sal* locus in *S. pyogenes* is toward bacterial fitness in a different ecological niche.

Zebrafish has been used as an animal model for several bacterial infection models because it has both innate and adaptive immune response [60]. It is also an execellent model for necrotizing fasciitis due to the similar clinical pathology observed in human. A previous transposon mutagenesis screen [61] identified a mutant with a transposon inserted into the *salK* gene of the *S. pyogenes sal* locus. The *salK* gene encodes a putative histidine kinase of a TCS and when mutated in *S. pyogenes* is highly attenuated in the zebrafish infection model. This suggests a role for the *sal* locus in pathogenesis of *S. pyogenes*. In these present studies, we demonstrated how the *sal* locus contributes to resistance of *S. pyogenes* to PMN-mediated killing during host-pathogen interactions as well as the transcriptional analysis of *sal* locus in *S. pyogenes*.

CHAPTER 1

THE ROLE OF THE SALKR TWO COMPONENT SYSTEM IN INTRACELLULAR SURVIVAL IN NEUTROPHILS

ABSTRACT

Streptococcus pyogenes can cause severe invasive diseases, in part, due to its ability to evade the host immune response. Immune evasion is facilitated by the adaptability of *S. pyogenes* to survive phagocytosis and killing by neutrophils. A previous mutagenesis screen identified the *salK* gene, which is required for virulence in a zebrafish infection disease model. SalK of *S. pyogenes* has the putative function of a histidine kinase of the salivaricin lantibiotic locus, and is located upstream of a response regulator, SalR, constituting a putative two-component signal transduction system. The complete deletion of the *salKR* genes in *S. pyogenes* conferred a mutant strain that was highly attenuated in a zebrafish infection due to inability to survive killing by neutrophils. This resulted in an inability to survive in the blood stream and disseminate to the spleen. The attenuation of the *ΔsalKR* mutant in the neutrophil infection assays was a consequence of the sensitivity of the mutant to cell lysis by lysozyme. A difference in cell wall modification processes between the wild type and the *ΔsalKR* strains was suggested by an overall net charge difference on the bacterial cell surface, which could result in lysozyme sensitivity. This difference

was SalKR dependent, suggesting that the SalR protein may function in regulation of the genes involved in physical changes on the cell surface. Furthermore, SalKR regulation may also be playing an important role in adaptation of *S. pyogenes* to the host while residing intracellularly in macrophages. After passage through macrophages, survival of the wild-type strain increased approximately ten-fold in a human neutrophil assay compared to survival without passage, whereas the survival of the Δ*salKR* strain was unchanged. These results suggest that SalKR may sense the host environment and respond by upregulating survival mechanisms.

INTRODUCTION

S. pyogenes is exclusively a human pathogen that produces a number of virulence factors that allow it to survive and persist in host cells. The first line of defense of the host innate immune system that responds to most bacterial infections includes macrophages and polymorphonuclear neutrophils (PMNs). Several of *S. pyogenes* virulence factors inhibit and interfere with the microbial killing mechanisms used by these innate immune cells.

Resident macrophages residing in tissues are the first host immune cells that interact with *S. pyogenes* after the bacteria transition from mucosal sites to a tissue infection [62]. Resident macrophages have been shown to be able to phagocytose and kill *S. pyogenes* in a mouse model of infection [63]. The destruction of the bacteria occurs within the phagolysosome, which contains several antimicrobial agents including reactive oxygen species, nitric oxides, antimicrobial peptides and enzymes. The activated macrophages, after exposure to *S. pyogenes*, also play a role in recruiting inflammatory cells to the site of infection by secreting cytokines and chemokines. Unregulated expression of these inflammatory mediators can result in septic shock, which worsens the outcome of the infection. During interaction with *S. pyogenes*, an unusual activation program of macrophages was observed. In addition to differential gene expression of cytokine production, the typical Nitric oxide synthase (iNOS) production was not induced. [64]. Therefore, only reactive oxygen species and

other antimicrobial peptides and enzymes in macrophages are responsible for controlling *S. pyogenes* infection.

While several studies have shown that murine macrophages can efficiently kill *S. pyogenes in vivo*, there is evidence of intracellular survival of the bacteria in human monocyte-derived macrophages and during human soft tissue infection [65, 66]. Another role of macrophages may also be to serve as a reservoir for *S. pyogenes* during antibiotic treatment as a safe environment for replication and multiplication [65, 67]. Moreover, the ability to survive intracellularly in a macrophage may also allow the bacteria to disseminate from the primary site of infection, which has been observed in patients with severe invasive streptococcal infection from which the bacteria can be isolated from the blood stream [68]. The data presented in this study also suggests that *S. pyogenes* residing in macrophages upregulate gene products that provide resistance to subsequent neutrophil killing.

The role of neutrophils during *S. pyogenes* infection has been extensively studied. Human neutrophils have both intracellular and extracellular killing mechanisms for bacterial pathogens. After phagocytosis of the bacteria in the presence of complement factors, neutrophil phagosomes fuse with granules that contain several bactericidal agents including both oxygen-dependent and oxygen-independent molecules. The production of reactive oxygen species (ROS) is triggered by NADPH oxidase after bacterial ingestion. The difference between the ROS produced by macrophages and neutrophils is nitric oxide

production, which is only present in macrophages [69, 70]. Degranulation occurs during the fusion of the phagolysosome with azurophilic and secondary granules containing non-oxidative peptides and enzymes that have potent antimicrobial activity, such as defensins, cathepsins, lysozyme, elastase and cathelicidins. In addition to intracellular killing, neutrophils can kill bacteria extracellularly by releasing Neutrophil Extracellular Traps (NETs). The fiber-like structure of NETs is composed of antimicrobial granules and chromatin, which trap and kill the bacteria [71, 72].

S. pyogenes is also able to survive intracellularly in neutrophils, which allows the bacteria to survive in the bloodstream as well as disseminate to different sites of infection [73, 74]. Several virulence factors and the ability to adapt to a changing environment allow *S. pyogenes* to survive neutrophil killing mechanisms. DNase, secreted by *S. pyogenes*, degrades the secreted chromatin and allows bacteria to escape killing in NETs [75]. A regulator, PerR, of group A streptococcus is shown to assist in the survival of the bacteria under oxidative stress conditions [76]. *S. pyogenes* also becomes resistant to neutrophil cationic antimicrobial peptides and enzymes by modifying its cell wall. Addition of a D-alanyl group onto the highly negatively charged lipoteichoic acid molecule on the cell wall reduces overall net charge on the bacterial cell surface as well as decreasing the attraction of cationic bactericidal peptides [77, 78]. Another streptococcal cell wall modification, O-acetylation of the C6 position on N-acetyl-muramic acid of peptidoglycan, functions to block the host lysozyme cleavage

site, thereby conferring bacterial resistance [79]. In addition to the ability to survive neutrophil killing, intracellular bacteria also exhibited higher capsule production, creating a more virulent *S. pyogenes* [80]. Increased virulence from human leukocytes was observed when *S. pyogenes* is exposed to subinhibitory concentration of a human antimicrobial peptide, LL-37 [81]. These data suggest that an overall bacterial adaptation is required for the resistance to host immune responses.

In this study, we demonstrate that the SalKR two-component system also has a function in pathogenesis of *S. pyogenes*, facilitating bacterial survival in human polymorphonuclear leukocytes and allowing bacterial dissemination. Moreover, we also observed a role for SalKR in a bacterial adaptation mechanism that was induced while residing in macrophages. This adaptation subsequently increased *S. pyogenes* resistance to human neutrophil killing suggesting a possible role for SalKR in global gene regulation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All plasmids were maintained in either *Escherichia coli* DH5α cells or *E. coli* TOP10 cells (Invitrogen), which were cultured aerobically in Luria-Bertani (LB) medium (BBL) supplemented with either 25 μg/ml kanamycin, or 20μg/ml chloramphenicol at 37°C as required. The *Streptococcus pyogenes* M14 serotype, HSC5 [82, 83] strain, used in this study was cultured anaerobically in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (BBL) (THY medium) or THY medium supplemented with 2% proteose peptone (TP) (BBL), and incubated in 15-ml conical tubes at 37°C under static conditions. Plasmids in *S. pyogenes* were maintained in media supplemented with 500 μg/ml kanamycin and 3 μg/ml chloramphenicol. *S. pyogenes* cultured on solid medium (THY supplemented with 1.4% agar) was incubated in an anaerobic gas chamber with GasPak (BBL) cartridges at 37°C. Mid-log phase cultures were grown by diluting overnight cultures of *S. pyogenes* 1:50 into fresh THY medium. When necessary, *S. pyogenes* mutants were selected on THY plates that contained chloramphenicol (Cam) (3 μg/ml), kanamycin (Kan) (500 μg/ml), or erythromycin (Erm) (1 μg/ml) as appropriate.

Manipulation of DNA. Chromosomal DNA was purified from *S. pyogenes* as described previously [84]. Plasmid DNA was isolated using Pure Yield Plasmid

Mini Prep (Promega) and transformed into *E. coli* and *S. pyogenes* by electroporation as described previously [84]. Restriction endonucleases, ligases, and polymerases were used according to the manufacturers recommendations. When required, DNA fragments were purified using a gel extraction kit (Fermentas).

Disruption of *salKR*. An inframe deletion of the *salKR* region was constructed by joining the 5' end of the *salK* gene along with ~500 bp of sequence uptstream with the 3' end of the *salR* gene along with ~500 bp of sequences downstream. The *salK* 5' end region was amplified using primers; 5'-SalK-del-seg 1 (CTT CGA TTA GGT CAA GTG AAC C) and 3'-SalK-del-seg1-Sal1 (ACG CGT CGA CAG CTG CTG TAT CAA TAA GCG GTC). The *salR* 3' end region was amplified using primers; 5'-SalKR-del-seg2-Sal1 (ACG CGT CGA CGT ATC CAA CTG TTA TTC CAA CAG) and 3'-SalKR-del-seg2 (GTC GTT TGA TTA TCT GCA ACT CAG). The PCR amplification products were digested and with SalI restriction enzyme (Invitrogen) and ligated together into the PCR 8/GW/TOPO vector and transformed into *E. coli* Top 10 cells (Invitrogen). The vector with sal sequences was then digested with SalI, ligated with a fragment containing a kanamycin containing SalI sites cut from vector pABG-5. The ligation product was transformed into *E. coli* cells to propagate plasmid vector containing kanamycin resistance cassette in between the 5'.end of *salK* and 3' end of *salR* resulting in psalKR-del. The plasmid then was isolated and used as a template in PCR amplification of the *salK*-Kan-*salR* sequence using primers; 5'-salK-BamHI

(CGC GGA TCC GAG AGA ACC TGT CTC TTC and 3'-SalKR-del-seg2-PstI (AAA ACT GCA GGT CGT TTG ATT ATC TGC AAC TCA G). The PCR fragment was digested with BamHI and PstI and ligated to the suicide streptococcal vector pJRS233 [85, 86] and initially transformed into *E. coli* cells for plasmid propagation. The pJRS plasmid was then isolated and transformed into WT *S. pyogenes* HSC5 strain. The recombination of the temperature sensitive pJRS vector resulted in replacing the WT *salKR* alleles during cycling of permissive to non-permissive temperature as described previously [86]. The *ΔsalKR* deletion was confirmed by PCR.

Zebrafish infection assays. Zebrafish infection was performed as described previously [16]. An inoculum of 1 x 10^5 CFU of mid-log phase bacteria was injected intramuscularly into the dorsal muscle using a U-100 ultrafine insulin syringe with a 29-gauge needle (BD scientific). Minimums of 6 fish were infected per bacterial strain. The infected fish were placed in 400 ml beakers containing 225 ml of sterilized double-distilled water with 60 mg/ml of aquatic salts (Instant Ocean). A ventilated plastic screen was also placed on the top of each beaker. The experiment was repeated at least 3 times.

Histology of *S. pyogenes* infected zebrafish. The infected zebrafish were euthanized 24 hours post-infection. Whole fish were fixed for 24 hours with 10% formalin in 15 ml tubes with rotation. Then the zebrafish were dehydrated by

several incubations with increasing concentrations of ethanol. After the final wash with 100% ethanol, the fish were incubated in toluene for 24 hours. Tissues were then placed in 60°C paraffin plus infiltration medium in tissue cassettes. Following a one hour incubation in a 60°C water-bath, the paraffin was changed and the fish was incubated for another 48 hours under the same conditions. The final incubation was done with paraffin only and then the cassettes were cooled. After solidification, the cassettes were stored in ice water containing a small amount of fabric softener. The fish were sliced into five-micron sections, and placed on microscope slides followed by H and E staining.

Protease activity assays. Proteolytic activity was examined by measurement of clearance zone on skim milk agar medium inoculated by stabbing the agar with 1 µl mid-log phase bacteria normalized to 1 × 10^8 bacterial cells/ml. The agar plates were incubated at 37°C in an anaerobic environment for 24 hours.

Dissemination of bacteria. Zebrafish were inoculated intramuscularly as described above. At 24-hour post infection, spleens were recovered from euthanized fish and aseptically homogenized. Homogenates were serially diluted and plated on THY plates to assess the number of live bacteria (CFU) from the spleen. A minimum of 8 spleens was tested per strain.

Whole Blood Assay. Heparinized blood was obtained from healthy volunteers by venapuncture and collected in BD vacutainers coated with 3.8% citrate. Mid-log phase bacterial cells were added to 1 ml of blood to a final concentration of approximately 1.0×10^2 CFU/ml. Each strain was tested in duplicate. The suspensions were incubated on a rotator at 37°C for 3 hours. To determine bacterial viability, serial dilutions of the samples was made in PBS (Phosphate Buffer Saline) and plated onto THY plates. The plates were incubated overnight in a CO_2 incubator at 37°C.

Isolation of neutrophils from human whole blood. Neutrophils were isolated from heparinized blood collected as described above. Erythrocytes were separated by sedimentation in 6% dextran (MP-biomedicals), followed by separation of neutrophils using Ficoll-Hypaque (Pharmacia) density gradient centrifugation [87, 88]. Contaminating red blood cells were lysed with ddH_2O for 28 seconds and immediately neutralized with 1.8% NaCl (Fluka). The purified neutrophils were collected by centrifugation and resuspended in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 1% fetal bovine serum (FBS; Invitrogen) at an approximate final concentration of 2.5×10^6 cells/ml.

Serum collection. Blood was collected from healthy volunteers using BD Vacutainer glass serum tubes with no additives and allowed to clot completely for

at least 30 min. Samples were then centrifuged at low speed for 5 min and serum was gently collected from the tubes.

Neutrophil killing assay. Extracellular neutrophil killing was analyzed by incubating mid-log phase bacterial cells with 10^6 neutrophils at an MOI of 1 in a total volume of 1 ml DMEM supplemented with 1% FBS. The mixtures were then incubated on a rotator at 37°C. Viable bacterial counts were determined by plating serial dilutions of aliquots collected at 0, 1, 2 and 3 hour time points. To determine intracellular neutrophil killing, DMEM was supplemented with 50% human serum. Percent bacterial survival was calculated as follows; (number of bacteria with neutrophils /number of bacteria without neutrophils) x 100. All assays were performed in duplicate for each time point and repeated at least 3 times.

Cytospin preparations of Neutrophil assays. After a 3 hour incubation of the bacteria with neutrophils in the conditions described above, 100 ul of the neutrophil assay was placed into a cytospin column with a glass microscope slide and centrifuged at 700 rpm for 5 min. Then the slides was visualized by staining using DiffQuik (Fisher).

Antimicrobial assay. Overnight cultures were transferred into fresh THY medium and allowed to grow to an approximate OD_{600} of 0.300. Normalized cultures (OD_{600} of 0.300) were collected and washed twice with PBS and

resuspended in 1ml PBS. Antimicrobial peptides were dissolved in the appropriate solvent at the following working concentration and then added to THY medium individually at the final concentration of Carbenicillin 1µg/ml, Erythromycin 1 µg/ml and Nisin 100 µg/ml. In a 96-well plate, 2-fold serial dilutions of the THY medium with antimicrobial peptides were made using THY medium. Then 4 µl of normalized cultures were inoculated into each well. Following overnight incubation in 5% CO_2 at 37°C, bacterial growth was determined by measuring OD_{600} using a plate reader (VersaMax).

Hydrogen Peroxide sensitivity assay. Bacterial cultures were grown in THY medium individually to mid-log phase. A 50-µl normalized culture ($1x10^6$ cells/ml) was spread evenly over the surface of a THY agar plate using a sterile cotton swab. Ten microliters of 3%, 0.3%, 0.03% or 0.003% H_2O_2 was added to the center of a 10mm diameter sterile disk made from blotting paper and placed at the center of each plate. After incubation at 37ºC for 16 hours in 5% CO_2, the zone of growth inhibition was measured. The experiment was repeated at least 3 times with duplicate plates of each H_2O_2 concentration and each bacterial strain. The bacterial growth in the presence of H_2O_2 was assessed in 96-well plate by inoculating 4 µl the normalized mid-log cultures in 196 µl of THY broth containing one of the H_2O_2 concentrations described above. After 3 hours incubation at 37ºC with 5% CO_2, the samples were serially diluted in PBS and plated to enumerate the colony forming units of bacteria.

In vitro Lysozyme sensitivity assay. Chicken egg white lysozyme (Sigma) was added to TE buffer at a stock concentration of 200 mg/ml. Bacterial cultures at mid-log phase were normalized to OD_{600} of 0.300 then inoculated 1:100 into fresh THY broth containing a concentration range of 5-25 mg/ml of lysozyme (range of 10,000-100,000 enzymatic units) and incubated at 37ºC with 5% CO_2. The OD_{600} was measured every hour for 8 hours to obtain a growth curve in the presence of lysozyme. A separate set of samples were serially diluted and plated to enumerate surviving bacteria (CFU) at the 3 hour time point.

Macrophage infection. Murine macrophage cells (RAW 264.7) were seeded at 1×10^6 cells/ml per well into a 24-well plate and allowed to adhere overnight in 5% CO_2 incubator at 37°C. The following day, cells were washed three times with PBS and incubated with log-phase bacteria cells in DMEM supplemented with 10% FBS at 1×10^6 CFU/ml per well, a multiplicity of infection (MOI) of 1. The plate was then incubated for 1 hour at 37°C in a 5% CO_2 incubator. Following incubation, each well was washed 5 times with PBS to remove non-adherent bacteria. The infected RAW cells were incubated for an additional hour in fresh DMEM plus 10% FBS with added 100 µg/ml gentamicin (Invitrogen) to eliminate extracellular bacteria. After the gentamicin treatment, each well was washed three times with PBS and fresh DMEM plus 10% FBS containing no antibiotic was added. At the 24-hour time point, aliquots were taken from the supernatants

and cells were lysed by the addition of 1 ml of sterile double-distilled water. The lysate was serial diluted in PBS and plated for determination of live bacterial cells after 24 hours in macrophages.

Cytochrome C binding assay. The cytochrome C binding assay was performed as previously described [77] with minor changes. Overnight cultures were diluted 1:100 into fresh THY medium and allowed to grow at 37ºC to OD_{600} ~ 0.7. After all cultures were normalized, 10 ml of each culture was centrifuged and resuspended in 1 ml of 20 mM MOPS buffer pH 7.0 with 0.5 mg/ml of cytochrome C. After incubation for 10 min at room temperature, the samples were centrifuged at 16,000 x g for 5 min. The supernatant was collected without disturbing the cell pellet and then cytochrome C absorption was measured at OD530. The percent cytochrome C captured was calculated by comparing to a cytochrome C absorption control without bacterial cells.

Dot plate method for bacterial viability. The Dot plate method was used as described previously [89] with minor modifications. One hundred microliters of sample was loaded into the first well of each row of a 96-well plate, and 10-fold serial dilutions were made using a multichannel pipette by transferring 10 µl from the first well (n) into 90 µl of medium in the next well (n+1) of each row, mixing by pipetting up and down 10 times, and repeating the process with pipette tips changed between dilutions. Following the dilution, 10 µl from each of the five

selected dilutions were plated in three replicates onto an agar plate using a multichannel pipette. The plates were allowed to dry completely before being incubated overnight in a 5% CO_2 incubator at 37°C. Colony counts were performed manually on the dilutions that showed individual separated colonies.

Neutrophil assay after the bacterial passage through macrophages. Mid-log phase cultures were normalized to an OD_{600} of 0.3 and used to infect macrophages (RAW) as described above. The bacterial cultures were also inoculated into DMEM supplemented with 10% FBS along with the macrophage infection as a control. The inoculums were also serial diluted and plated to enumerate the number of bacteria with a 6X6 dot plate method [89]. Following a 24 hour incubation, both the control macrophages and the infected macrophages were collected to use as a inoculum for the neutrophil assay. The bacterial cells were normalized to 1×10^6 CFU/ml and incubated with neutrophils as described in the neutrophil assay above.

RESULTS

The SalKR two component signaling system of the *sal* locus has an important role in virulence. One of the highly attenuated mutants isolated in a previous transposon mutagenesis screen [61] was found to have an insertion in the *salK* gene, encoding a putative histidine kinase, in the salivaricin locus (figure 1). When co-injected with the wild type strain, the competitive index (CI) for the *salK* (Ωtnp) mutant strain was 0.02. The LD_{50} of the *salK* insertion strain was $1x10^5$, which was approximately 2 logs higher than the wild-type strain. SalK is part of a putative two-component signaling system, which is coupled with its putative regulatory protein, SalR. To further investigate the role of SalKR in virulence, an in-frame deletion of the *salKR* genes in *S. pyogenes*-M14 was constructed using a streptococcal suicide plasmid, pJRS233 [86]. A non-polar kanamycin resistance cassette was left in place of the *salKR* genes. Analysis was performed to confirm that the *ΔsalKR* strain was not defective in growth in several media including THY, TP, DMEM+10%FBS and chemically defined media (CDM) (data not shown). In addition, the *ΔsalKR* strain displayed the same hemolytic phenotype as WT when grown on a blood agar plate (data not shown). However, similar to the *salK* transposon insertion strain, the *ΔsalKR* strain is 50% less virulent compared to the WT strain during a zebrafish infection assay (figure 2). The lesion observed in the zebrafish infected with the *ΔsalKR* strain was much smaller than that of wild-type and healed within 3 days (data not shown). The histological examination of the dorsal muscle tissue of fish at 24

hours post-infection showed similar destruction of tissue between the wild type and *ΔsalKR* infection (figure 3). The activity of the secreted SpeB protease produced by the bacteria was examined using skim milk agar medium. The measured zone of clearance showed no difference in the protease production between wild type and the *ΔsalKR* strain (figure 4). However, the tissue necrosis from the *ΔsalKR* infection seemed to be limited and slightly more infiltration of inflammatory cells was observed (figure 3). Analysis of dissemination to the zebrafish spleen at 24 hr post intramuscular (IM) injection showed that the *ΔsalKR* strain had 2 logs less colony forming units (cfu) compared to the WT strain (figure 5). The lower dissemination profile compared to WT suggested that the *ΔsalKR* strain may be unable to initiate a successful infection due to an inability to survive at the site of infection and/or in the blood stream.

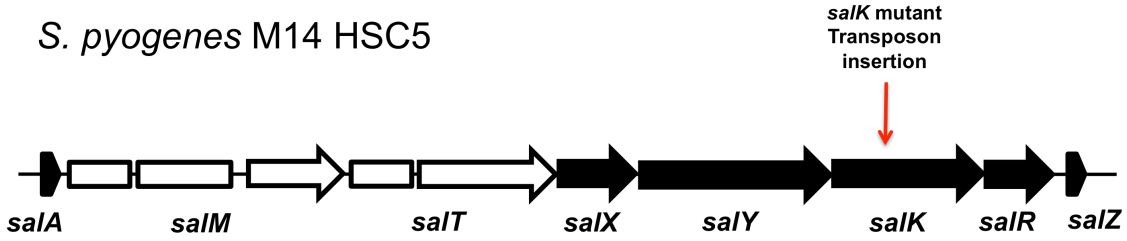


Figure 1. Sal locus gene structure and orientation of *S. pygenes* strain HSC5 serotype M14. The vertical arrow represents the transposon insertion region in *salK* (Ωtnp) mutant.

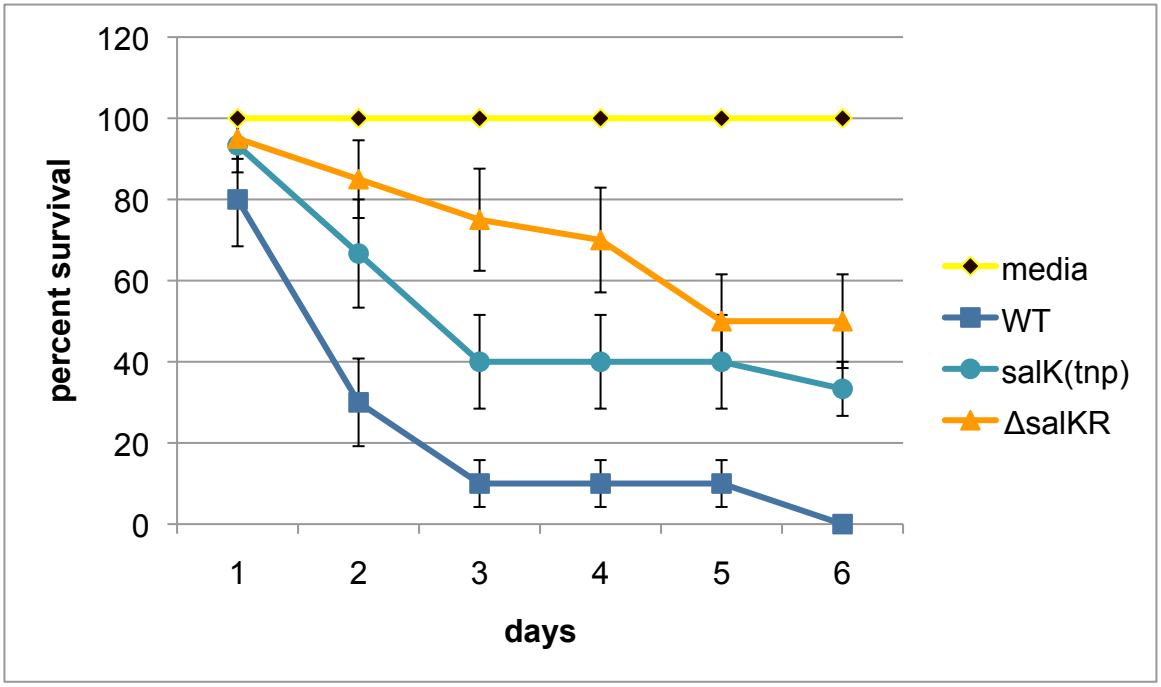


Figure 2. Survival curve of zebrafish infection. Attenuation of *ΔsalKR* in zebrafish infection assay. Percent survival of zebrafish infected with media (diamond), *ΔsalKR* mutant (triangle), salK (Ωtnp) mutant (circle) and WT (square) strains. Percent survival was calculated compared to mock infected fish.

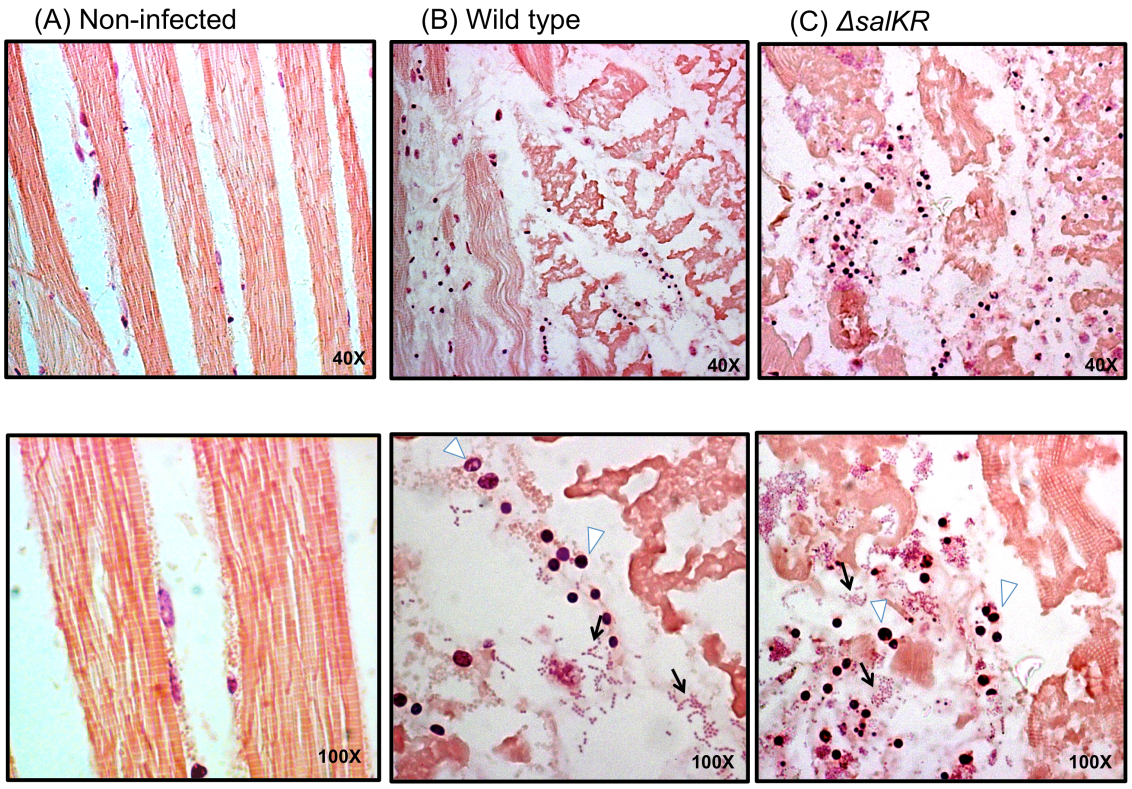


Figure 3. Histology of (IM) infected zebrafish dorsal muscle at 24 hours post infection. Images in column A were from the non-infected fish. Images in column B were from the fish infected with wild type *S. pyogenes*. Images in column C were from the fish infected with the *ΔsalKR* mutant. The white arrowheads indicate zebrafish immune cells. The black arrows indicate the bacteria cells. All samples were stained using a standard H and E staining protocol. The top row images are at 40X magnification and the bottom row images are at 100X magnification.

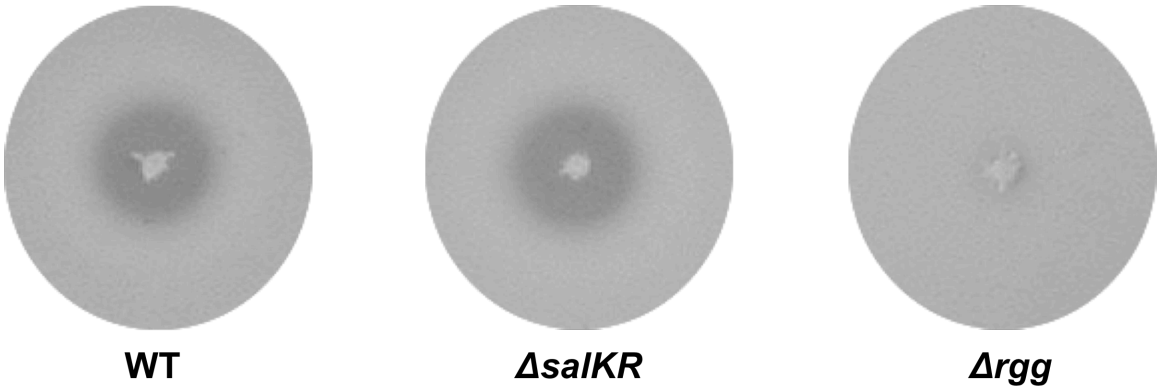


Figure 4. SpeB proteolysis assay. The zone of clearance detected on skim milk agar medium. Image A is the agar inoculated with wild type *S. pyogenes*, B is the *ΔsalKR* mutant and C is the *Δrgg* mutant that lacks SpeB cysteine protease activity.

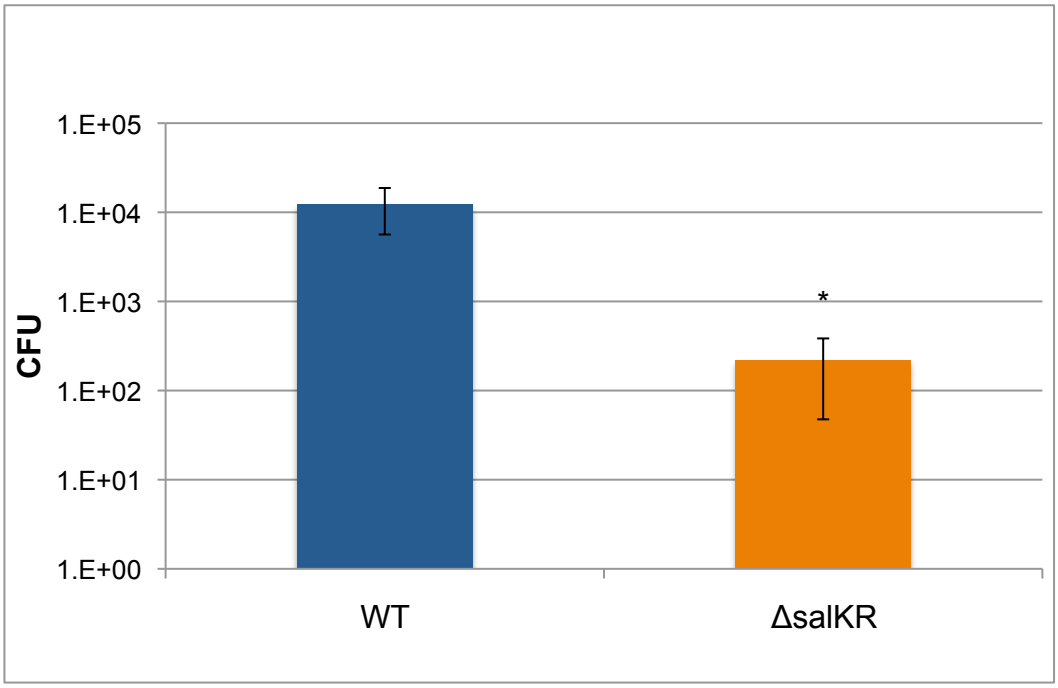


Figure 5. The dissemination of the bacteria to the spleen. Bacteria number (CFU) enumerated from the spleen of fish infected with the wild type or the *ΔsalKR* mutant strain. Columns represent the average CFU recovered from spleen with standard deviations of four independent experiments with 6 fish per strain per experiment. (*, *P value* < 0.05; two-tailed *t* test).

Attenuation of the *ΔsalKR* mutant strain in the presence of human immune factors. To test the ability of the mutant strain to survive in blood, we performed a human whole blood assay and observed that the *ΔsalKR* strain was attenuated for growth by 1 log compared to wild type growth in whole blood (figure 6). This suggests that the SalKR proteins play an important role in survival in the blood stream, which ultimately leads to successful dissemination. Since neutrophils are the predominant non-specific immune cells in the blood stream, the attenuation of the *ΔsalKR* strain could be due to an increased susceptibility to neutrophils. To test this hypothesis, neutrophils freshly isolated from human whole blood were used in a neutrophil killing assay at a 10:1 ratio of neutrophils:bacteria. Stained cytospin preparations made from the neutrophil killing assays show that in the presence of neutrophils with 50% human serum both the *ΔsalKR* and wild-type strains are intracellular in the neutrophils (Figure 7). However, intracellular survival of the *ΔsalKR* strain was 30% less than observed with the wild-type strain (figure 8) with 70% of the bacteria killed within 1 hour of incubation (data not shown). The serum conditions used in this assay (50% serum) mimic the conditions found in the bloodstream. Therefore, the SalKR proteins are playing a role in *S. pyogenes* survival in the bloodstream through protection from intracellular killing by neutrophils. In the absence of serum, similar to conditions found in the tissue, both the wild-type and *ΔsalKR* strains were found to be extracellular in the neutrophil killing assay (Figure 7). While the wild-type strain actually grew in this assay (>100% survival), the *ΔsalKR* strain survived to only

approximately 70% of the original inoculum, demonstrating extracellular killing by neutrophils (figure 8). This suggests that the SalKR proteins are also important in survival from extracellular killing by neutrophils, which is evidenced by the attenuation of the *ΔsalKR* strain at the site of infection. Collectively, these data demonstrate that the SalKR proteins are important in survival from both intracellular and extracellular killing by neutrophils. One of the extracellular killing mechanisms used by neutrophils is the release of chromatin in combination with azurophilic granule proteins that contain antimicrobial peptides and proteins, also known as Neutrophil Extracellular Traps (NETs) [71, 72]. The chromatin structure traps and prevents bacteria from spreading and also serves as a means to deliver concentrated microbicidal enzymes and peptides to the site of infection. DNases or endonucleases produced by Streptococcus species allow the bacteria to escape NETs killing [75, 90]. DNase was added to the neutrophil killing assays to determine if destruction of the chromation in the NETs would allow the *Δs**alKR* strain to escape and survive the extracellular killing to the level of the wild type strain. However, we observed no significant survival increase of the mutant (data not shown). Since the assays were performed in a small volume with rotation (see materials and methods) the neutrophil secreted antimicrobial peptides or enzymes would still come into contact with the bacteria and therefore may be responsible for the decreased percent survival of the *ΔsalKR* strain.

Neutrophils secrete a large number of enzymes and antimicrobial peptides [69, 70]. To determine if the SalKR proteins are involved in a mechanism that confers a non-specific resistance to antimicrobial peptides, the susceptibility of both the wild type and *ΔsalKR* strains to several bacterially derived antimicrobial peptides, nisin, gramicidin, carbenicillin and polymyxin B, was tested (data not shown). We found no significant difference in susceptibility to the antimicrobial peptides tested. Similarly, results from a hydrogen peroxide sensitivity test demonstrated that both the wild type and the *ΔsalKR* mutant are resistant to oxidative killing to the same level (data not shown). These results suggest that the attenuation of *ΔsalKR* strain is not due to an increased susceptibility to oxidative radicals or involved in a non-specific antimicrobial resistance mechanism.

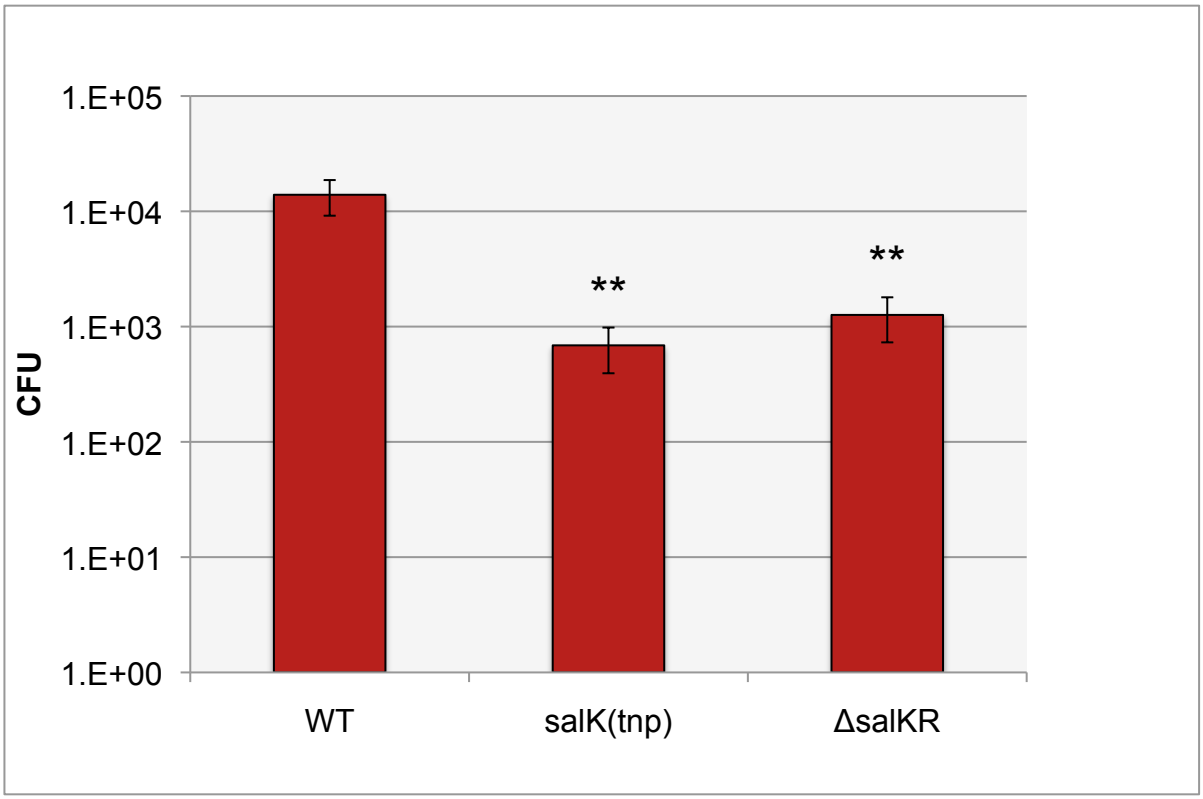


Figure 6. Whole blood assay with 100 bacterial cell inoculum. Data represent the average bacterial CFU grown in human blood for 3 hours with standard error. Average of six independent experiments. (**, *P value* < 0.001; two-tailed *t* test).

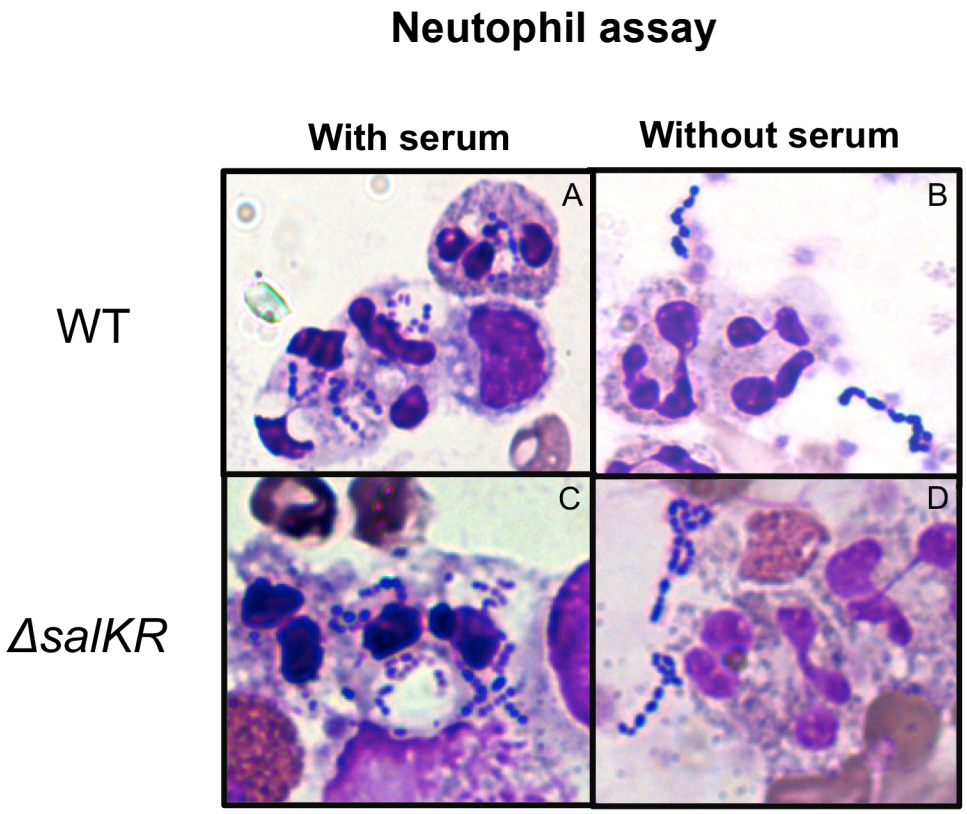


Figure 7. Cytospin preparations from the neutrophil assay. Images A and B are neutrophils with wild type, A) with serum and B) without serum. Images C and D are neutrophils with *ΔsalKR*, C) with serum and D) without serum. All Images are at 1000X magnifications.

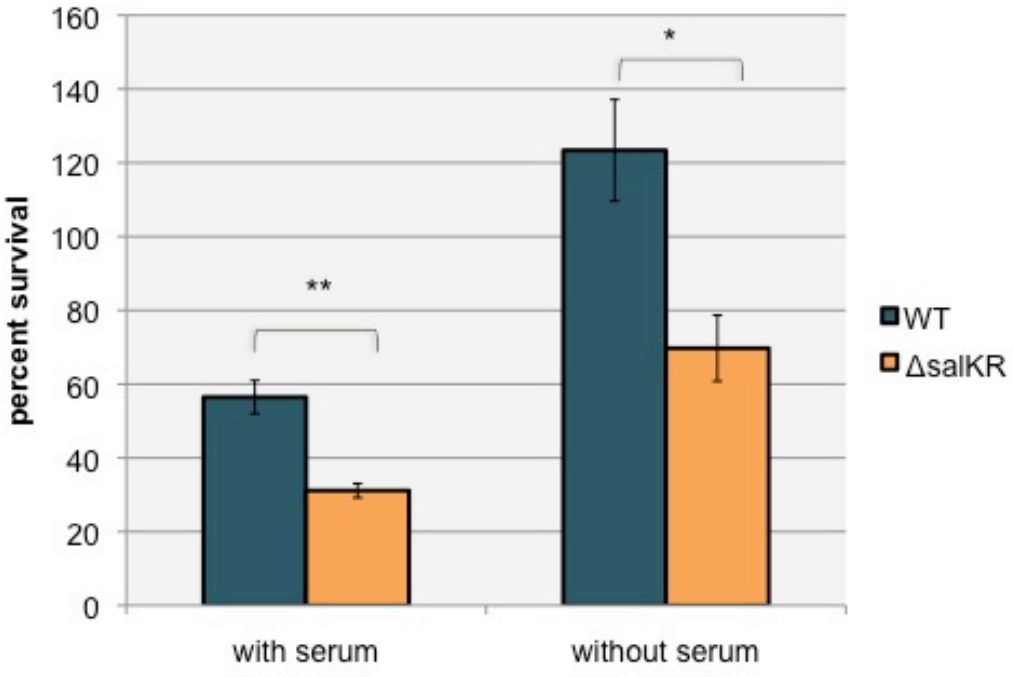


Figure 8. Neutrophil assay. Percent survival of enumerated bacteria recovered from 3-hour incubation calculated relative to control without neutrophils. The columns represent the mean value of percent survival of bacteria relative to control without neutrophils with standard deviation of three separate experiments. (*, *P value* < 0.05) (**, *P value* < 0.01; two-tailed *t* test).

The ∆*salKR* mutant strain is highly sensitive to lysozyme activity. Both intracellular and extracellular killing mechanisms of neutrophils involve degranulation and delivery of azurophilic granules. Lysozyme is the major microbicidal enzyme in the neutrophil granules [91]. Therefore the growth of the *Δs*a*lKR* and wild type strains was assessed in the presence of lysozyme by measuring the culture density of normalized mid-log phase cultures incubated with multiple concentrations of lysozyme for 16 hours. Results indicated that the *Δs*a*lKR* strain is highly sensitive to lysozyme treatment compared to the wild type strain (figure 9A). The *ΔsalKR* strain was growth inhibited starting at 50,000 lysozyme enzymatic units while the wild type strain was still able to grow at the highest concentration tested, 100,000 units of lysozyme. The viability of strains growing in lysozyme in the previous assay was tested by plating bacteria at the concentrations that showed little to loss of culture density, 40000, 50000, and 60000 units. The CFUs from the *Δs*a*lKR* strain were significantly (P value = 0.018, 0.0004 and 0.0001 respectively) less (~ 2 logs) at 30,000-lysozyme enzymatic units and more than 3 logs less at 50,000-enzymatic units (figure 9B).

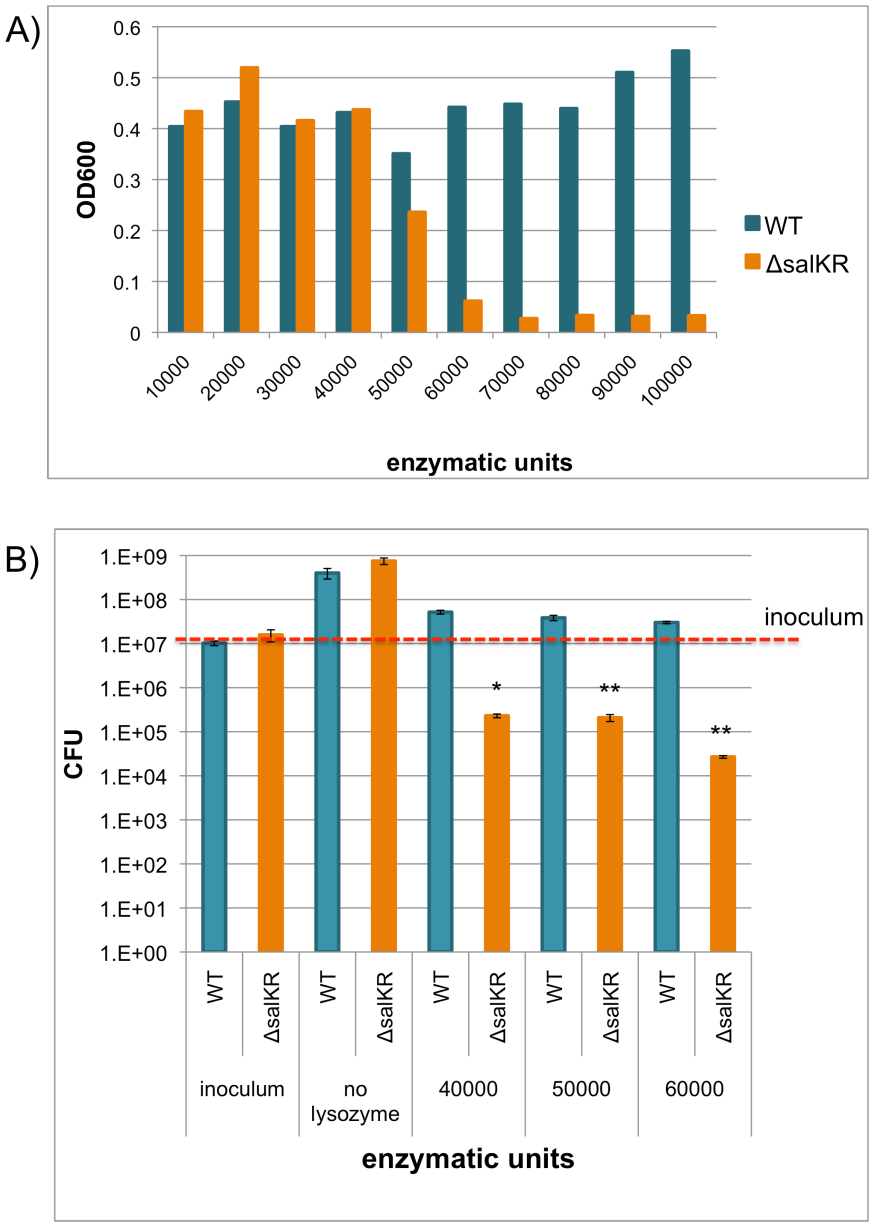


Figure 9. Lysozyme assay. Sensitivity of the *ΔsalKR* strain to lysozyme treatment. A) WT (blue bar) and *ΔsalKR* (orange bar) cells were grown in THY broth with increasing amounts of lysozyme enzymatic units. The OD_{600} was measured after 16 hours of incubation at 37ºC in 5% CO_2. B) The number of viable bacteria grown for 3 hours in THY broth in the presence of lysozyme at 40,000-60,000 enzymatic units. (*, *P value* < 0.05) (**, *P value* < 0.01; two-tailed *t* test).

The wild type and *ΔsalKR* strains differ in cell wall charge. Since an increased sensitivity to lysozyme can indicate a cell wall modification, the wild type and *ΔsalKR* strains were subjected to a cytochrome C binding assay to determine if the overall surface charge of the cell wall was the same between the two strains. Without cell wall modifications, the anionic property of lipoteichoic acid would allow more binding of the positively charged cytochrome C to bacterial cells. If the cell wall were modified, for example by D-alanylation, overall ionic charge would become more neutral resulting in less binding to cytochrome C. When grown in THY media, the *ΔsalKR* strain showed less cytochrome C bound than that of the wild type strain, implying that the cell wall of the *ΔsalKR* strain has less anionic charge than the wild type cell wall (figure 10). However, when the wild type and *ΔsalKR* strains were subjected to the cytochrome C assay after being collected from an infection of a murine macrophage cell line, the wild type strain showed a reduced anionic charge on the cell wall surface, as illustrated by binding less cytochrome C, while the *ΔsalKR* strain showed no change (figure 10). This suggests that the SalKR proteins may be involved in regulation of cell wall modifications.

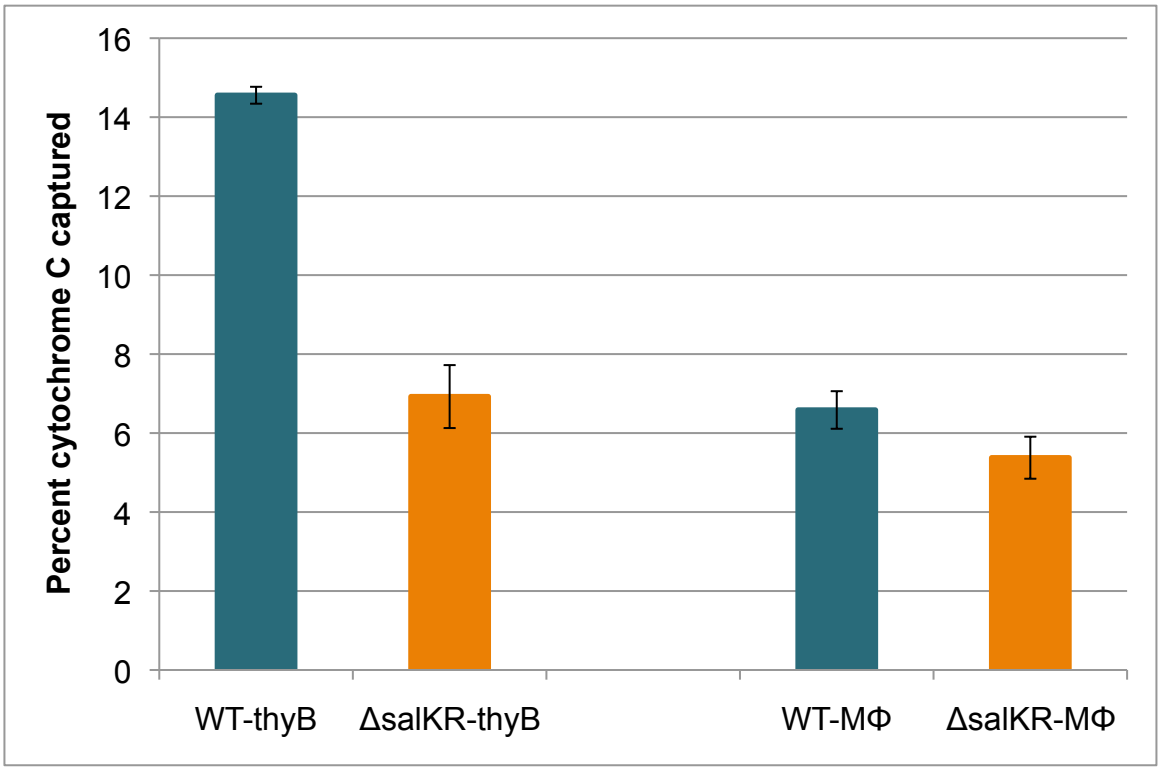


Figure 10. Cytochrome C binding assay. Binding of Cytochrome C to bacteria comparing WT (blue bar) to *ΔsalKR* (orange bar) that were grown in THY broth or murine macrophage cell line (MΦ). The data represent percent cytochrome C captured relative to control (THY control or MΦ cells control). The standard deviations were calculated from 3 independent experiments with triplicate samples per experiment.

Passage through macrophages increases resistance of *S. pyogenes* to Neutrophil killing. The cell wall modification of the wild type strain during macrophage infection suggested there may be an adaptation of the bacteria while residing intracellularly in macrophages. This modification was not observed in the *ΔsalKR* mutant strain. To test if the adaptation could result in increasing resistance of *S. pyogenes* to the host immune response, the neutrophil assay was performed using bacteria collected from a macrophage cell lysate after 24 hours of infection. The number of bacteria recovered from the in vitro macrophage infection was not different between the wild type and *ΔsalKR* strains (figure 11). However, after the neutrophil assay, the percent survival of the wild type strain from neutrophil killing increased to 19% compared to the control at 2%, whereas with the *ΔsalKR* strain, percent survival stayed the same as the control, ~2% (figure 12). As a control, the Ua1 mutant strain, which contains the same Kanamycin resistance cassette as the *ΔsalKR* strain, was shown to be similar to the wild type strain by an increase in resistance to neutrophils. The increase in resistance to neutrophil killing observed here was therefore SalKR dependent.

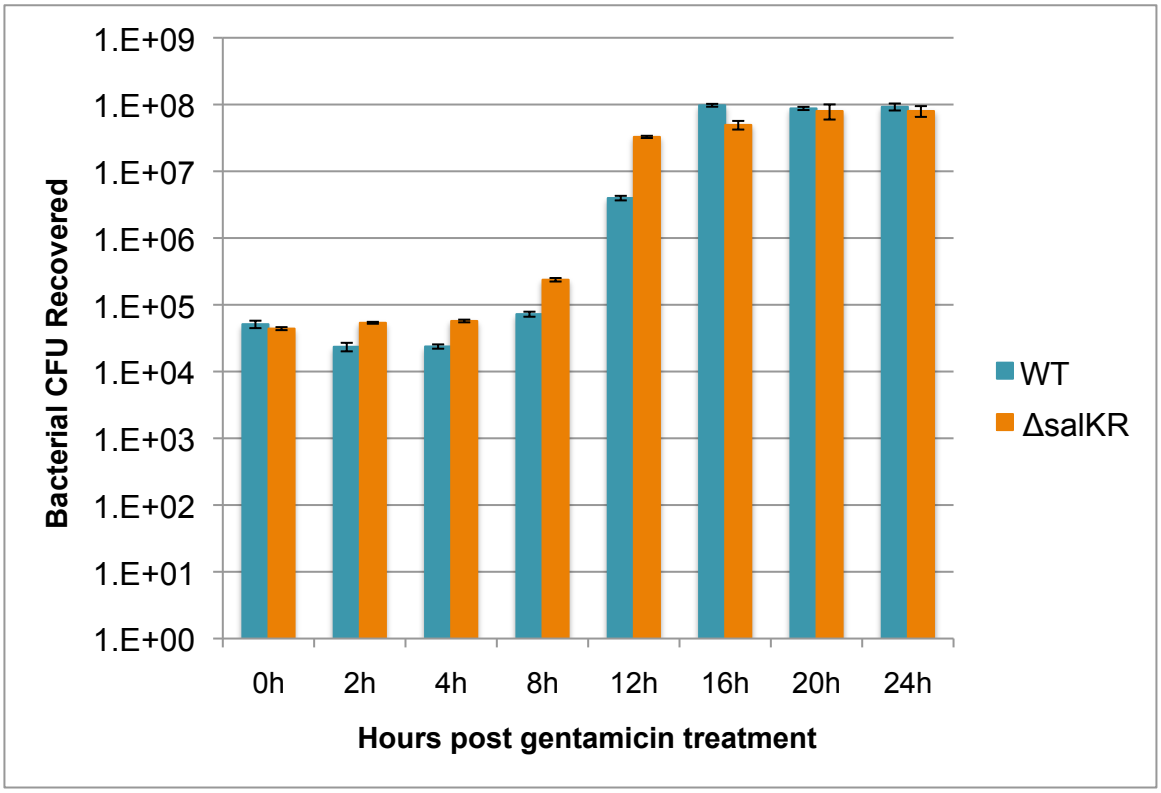


Figure 11. Macrophage infection. Bacteria recovered from murine macrophage tissue culture assay. At 24 hours post gentamicin treatment, the bacterial cell numbers recovered from macrophages of both WT and *ΔsalKR* are similar.

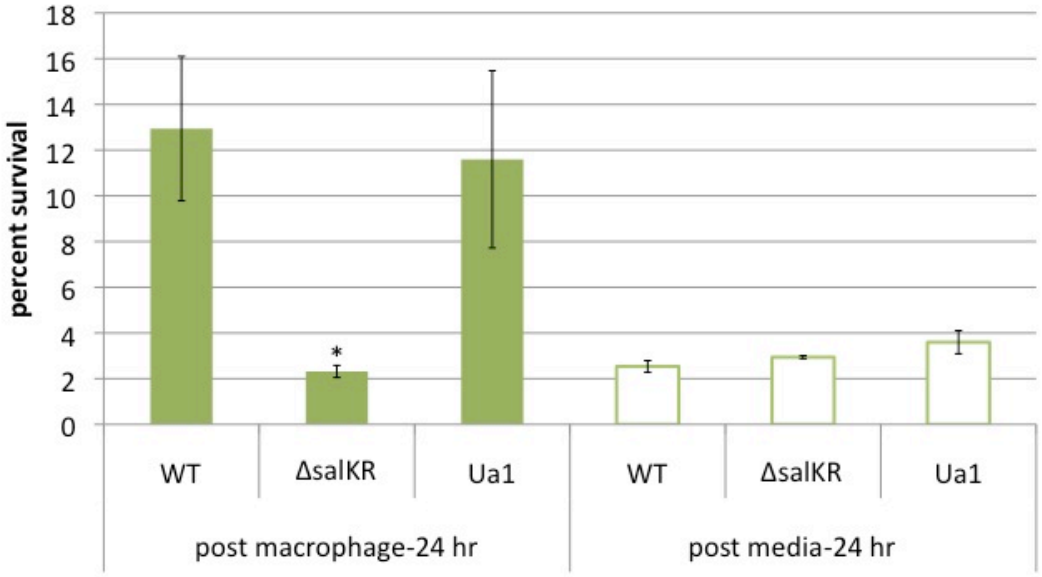


Figure 12. Neutrophil assay with serum. Percent survival of bacteria from neutrophil assay after the bacteria are passaged through the murine macrophage cell line. The open bars represent the neutrophil assay with bacterial cultures grown in DMEM + 10% FBS for 24 hours. The filled bars represent the neutrophil assay with bacterial cultures collected from 24-hour post gentamicin treatment of a macrophage infection. Percent bacterial survival was calculated as follows; (number of bacteria with neutrophils /number of bacteria without neutrophils) x 100. (*, *P value* < 0.05)

DISCUSSION

Bacterial pathogens must have mechanisms to respond quickly in order to adapt and survive the harsh environment created by the host immune response. The bacteria concomitantly adjust their growth to the nutrients present as well as their defense mechanisms to the host innate immune system. The coordinate changes in expression of metabolic and virulence genes in response to a drastic change of environment are orchestrated by two-component regulatory systems. During *S. pyogenes* interaction with human neutrophils, an immediate ~2 fold upregulation in expression of the Ihk-Irr two component regulatory system was observed within 30 minutes, with an overall differential gene expression resulting in changes in virulence, stress response and cell wall biosynthesis [45]. The *irr* mutant strain was severely attenuated in virulence in a mouse soft-tissue infection model. Several genes that are involved in cell wall modification were down-regulated in the *irr* mutant strain resulting in sensitivity to neutrophil antimicrobial peptides targeting the bacterial cell wall [44]. *S. pyogenes* CovRS is another two-component system that is involved in global gene regulation during host-pathogen interactions and contributes to bacterial pathogenesis [92-94]. The CovR response regulator was shown to modulate gene expression during bacterial growth in human blood and in a macaque pharyngitis model [95, 96]. Therefore multiple global regulators function to coordinate expression of required virulence factors during interactions with the host.

In this study, we identified the virulence function of SalKR, one of the 13 two-component systems identified in *S. pyogenes*. While the putative functions of SalK and SalR are a sensor kinase and a response regulator, respectively, involved in controlling the production of a lantibiotic, the *salK* transposon mutant (*salKΩ*) along with the *ΔsalK* and *ΔsalKR* deletion mutants were highly attenuated in the zebrafish infection model. The mutants were unable to cause the muscle necrosis observed during an infection with the wild type strain, suggesting that the mutants are attenuated for an invasive infection and therefore succumb to clearing by the host immune response. This was supported by the results of the dissemination assay in which the *salKR* mutant strain demonstrated ~2 logs less cfu recovered from the spleen at 24 hours post infection, suggesting that this strain was attenuated for an invasive infection.

Clinical features of invasive Group A streptococcal infection include destruction of tissue and rapid systemic spread, resulting in bacteremia and toxic shock [65, 97]. In this study, histology of infected tissue showed similar tissue destruction between the wild type and *ΔsalKR* infections with a slight difference in immune cell infiltration. Biopsies from patients with invasive streptococcal infection revealed high bacterial loads with a concomitant high expression of the SpeB streptococcal cysteine protease [65, 98]. Analysis of SpeB activity by casein proteolysis revealed no difference between the wild type and the *ΔsalKR* mutant strain. These data, along with the similar histology at the site of infection, suggest that the attenuation of the *ΔsalKR* mutant may be due to the inability of

the mutant to disseminate. Bacterial dissemination requires mechanisms of resistance to the host immune response both at the site of infection and in the bloodstream. In the present study, the *ΔsalKR* mutant cannot survive in a whole blood assay suggesting that the function of SalKR is required during exposure to whole blood. This hypothesis is supported by the microarray analysis of *S. pyogenes* during growth in human whole blood showing that the *salR* gene was upregulated ~50 fold as early as 30 min after exposure to blood [99]. While whole blood is a rich nutritive environment, immune components are also present including opsonizing antibodies and complement components that facilitate phagocytosis of the bacteria by neutrophils (predominant immune cells in the bloodstream). Previous reports demonstrate that *S. pyogenes* can survive intracellularly in macrophages and neutrophils, which facilitate systemic spread of the bacteria [65, 74, 80]. In this study, the *ΔsalKR* mutant was able to survive intracellular killing in murine macrophages but not intracellular or extracellular killing by neutrophils. Therefore the attenuation of the *ΔsalKR* strain is due to the inability to survive neutrophil killing, suggesting that the function of SalKR involves a protective response to host immunity. However, we have been unable to complement the deletion mutant with a plasmid containing the *salKR* genes expressed from a constitutive promoter, suggesting that *salKR* expression must be differentially regulated, which is further discussed in the following chapter.

In spite of effective bacterial killing from production of reactive oxygen species (ROS) by neutrophils, the H_2O_2 assay showed that the *ΔsalKR* mutant strain has the same level of sensitivity to ROS as that of the wild type strain. SalKR function therefore involves resistance to oxygen-independent effectors of neutrophils. The *ΔsalKR* mutant is attenuated in both intracellular and extracellular killing by neutrophils. In this study, we demonstrated that the attenuation of the mutant is due to sensitivity to lysozyme, which is present in all granules in neutrophils and is involved in both intracellular and extracellular killing [69, 71, 100].

Lysozyme has muramidase activity, which enzymatically hydrolyzes the β-1,4 glycosidic bond between N-acetyl glucosamine and N-acetyl-muramic acid of the peptidoglycan structure of the Gram-positive bacterial cell wall. The cleavage of the glycosidic backbone of peptidoglycan results in degradation of the cell wall, which leads to bacterial lysis. Moreover, lysozyme has a cationic anti-microbial-peptide (CAMP) function, similar to antimicrobial peptides that destabilize bacterial cell membranes without muramidase activity [101]. After testing the sensitivity to several cationic antimicrobial peptides and antibiotics, the *ΔsalKR* mutant exhibited the same level of resistance to cationic antimicrobial peptides as the wild type strain indicating that the function of SalKR may be involved in the resistance to muramidase activity. Despite the presence of lysozyme in macrophages, both the wild type and *ΔsalKR* strains are able to survive at the same level in our murine macrophage infection while the mutant is highly

sensitive to human-neutrophil killing and chicken egg-white lysozyme. The difference detected could be due to the differences observed between a macrophage and neutrophil infection. Previous analyses showed that the bacteria reside in the cytoplasm in macrophages [61] and may never be in a phagosomal compartment. In this study, we observe bacteria within phagosomal vacuoles in neutrophils. Since *S. pyogenes* are not in phagosomes in macrophages, they may never encounter lysosomal activity in this environment. In contrast the bacteria is most likely bombarded with lysozyme both intracellularly and extracellularly during a neutrophil infection.

Several Gram-positive pathogens have evolved resistance mechanisms to the muramidase activity of lysozyme. Bacterial cell wall modification that works to block lysozyme access to its target cleavage site appears to be a common theme among lysozyme resistant bacteria. N-acetylglucosamine deacetylase (PgdA) is an enzyme that functions in cell wall modification in *S. pneumoniae* [102]. O-acetylation at the C-6 position of N-acetyl muramic acid by *O*-acetyltransferase (OatA) is utilized by *S. aureus* [103, 104]. The same C-6 position can also be covalently linked to wall-teichoic acids (WTAs), which is facilitated by undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase (TagO) [105]. OatA and TagO are required for cell wall modification in *S. aureus* to become resistant to lysozyme [106]. In *S. pyogenes*, it has been shown that D-alanylation of teichoic acids increased bacterial resistance to antimicrobial peptides and contributes to neutrophil survival [77]. D-alanylation is a process

that reduces the negative charge of lipoteichoic acid, which is a component in the Gram-positive bacterial cell wall. Subsequent reduction of overall net charge on the cell surface can decrease the affinity for cationic antimicrobial peptides as well as the susceptibility to the muramidase activity of lysozyme [77]. The *S. pyogenes* M14 strain HSC5, used in our study, possesses all the genes necessary for the cell wall modifications mentioned above, suggesting that *S. pyogenes* is capable of cell wall modification to become resistant to lysozyme. In this study, we briefly investigated the overall net charge of the cell wall comparing the *ΔsalKR* mutant to the wild type strain and the difference observed indicates that there is a dissimilarity in cell wall modification between the mutant and the wild type strain. As a regulatory protein, SalR may be involved in this process by regulating the genes involved in physical changes on the cell surface. Further analyses are necessary to determine the role of SalKR in cell wall modification.

Notably, we also observed an alteration in overall net charge on the bacterial cell surface during *S. pyogenes* wild type *in vitro* infection of murine macrophages. However, the cell wall modification does not occur in the *ΔsalKR* mutant suggesting that SalKR may regulate the cell wall alteration in response to the host immune environment. This physical adaptation would allow the bacteria to become more resistant to host antimicrobial mechanisms. In our study, wild type *S. pyogenes* that had been passaged through murine macrophages for 24 hours increased its resistance to neutrophil killing while the *ΔsalKR* strain did not.

The Ua1 strain is also a mutant that contains the Kanamycin resistance gene cassette, similar to *ΔsalKR,* but behaved as the wild type strain in response to neutrophil infection. Therefore the adaptation observed with the wild type and Ua1 strain is SalKR dependent. There is also evidence of *S. pyogenes* adapting while residing intracellularly in neutrophils resulting in increased bacterial virulence [80]. One particular study showed that after 48 hours *in vivo* while intracellular in PMNs the bacteria undergo phenotypic switching and become more virulent with higher capsule production and better survival in blood [80]. Previous reports have demonstrated that bacterial virulence of *S. pyogenes* and *S. pneumoniae* can be enhanced by serial passage in animals, blood, organs or tissue cultures [107-109]. Notably, the percent survival in neutrophils observed in this study after passage through macrophages was relatively lower than the initial neutrophil assay. The dissimilarity may be due to the growth phase difference of the bacterial inoculum. The initial neutrophil assay uses mid-log bacteria grown in THY culture medium, but the bacteria are most likely in stationary phase after 24 hour infection of macrophages. *S. pyogenes* virulence during different growth phases is not well understood. Nevertheless, regardless of growth phase, there is a clear difference in survival in PMNs of the wild type strain compared to the *ΔsalKR* mutant strain after passage through macrophages, suggesting a clear role for SalKR in this process. Further analysis to determine the conditions in which the *salKR* genes are expressed in relation to growth phase is required for a better understanding of the SalKR role in bacterial adaptation.

In conclusion, the adaptation of *S. pyogenes* to the host environment is essential for its pathogenesis. The key players for this role are the two-component regulatory systems that control and regulate gene expression in response to environmental cues. In this study we demonstrated that the SalKR two component system plays an important role in *S. pyogenes* virulence through either direct or indirect regulation of bacterial protective response genes. As several lantibiotic and/or bacteriocin loci have been shown to be involved in bacterial virulence, this study provides another example of bacterial evolution through alteration of the original function of genetic loci to facilitate bacterial fitness in its environmental niche.

CHAPTER 2

TRANSCRIPTIONAL ANALYSIS OF THE SAL LOCUS AND THE REGULATORY ROLES OF SALKR AND SALA

ABSTRACT

The *sal* lantibiotic locus has an important role in virulence of *Streptococcus pyogenes.* Analysis of transcriptional regulation of this locus will provide a better understanding of the function of the *sal* locus in *S. pyogenes* pathogenesis. Transcriptional analysis of the *S. pyogenes sal* locus identified two possible promoters, one located upstream of the operon and another located upstream of *salKR* genes. The upstream *salA* promoter provided a read through transcript for the entire locus and was unaffected by the regulatory role of the SalR protein. The downstream *salKR* promoter had lower activity and different growth phase regulation compared to the *salA* promoter, suggesting regulation independent from the *salA* promoter. Use of a reporter construct to measure promoter activity revealed that the *salKR* promoter contains repression sites located upstream and downstream of the promoter elements, which are regulated by direct binding of the SalR protein as shown by EMSA analysis. Moreover, approximately two-fold higher expression of the s*alKR* promoter is also observed in the *salA* deletion strain indicating the SalA peptide may function as an intracellular signaling molecule or co-repressor on the *salKR* promoter. This is the first study to

demonstrate the repression role of SalR and SalA towards *salKR* promoter expression and the first to describe the exact promoter locations for the *sal* locus in *S. pyogenes.*

INTRODUCTION

Lantibiotics are produced exclusively by several species of Gram-positive bacteria. The mode of action of these peptides is to inhibit growth of other Gram-positive bacteria. Therefore it is crucial for the transcription of these loci to be tightly regulated to ensure the production of the peptides along with the self-immunity proteins to the cognate lantibiotic at the appropriate times. Regulation of lantibiotic production occurs at the transcriptional level through a regulatory protein that is activated by its cognate signaling kinase through phosphorylation as part of a two-component regulatory system. The genetic structure of a lantibiotic (lan) locus generally includes genes for the lantibiotic pre-peptide (*lanA*), posttranslational modification enzymes (*lanM*), a transport protein (*lanT*), an immunity protein(s) (*lanI* or lan*FEG*) and a two-component regulatory system (*lanKR*) [47, 110, 111]. The organization of the lantibiotic structural genes has no particular order or orientation, whereas the promoters that regulate the gene expression are commonly located upstream of *lanA* and *lanKR*, and in some cases, upstream of *lanI* or *EFG* [47, 110].

Biosynthesis of a lantibiotic locus is autoregulated by the cognate bacteriocin via interaction with LanK of the two-component signaling transduction system, resulting in transcriptional activation. The activation of the promoter is through direct DNA binding of the regulator LanR to the pentanucleotide-direct-repeat binding site located between -39 to -24 upstream of -10 promoter region

as described in the nisin and subtilin locus as the *nis* box [112] and the *spa* box respectively [51, 113]. Deletion of the genes encoding the two-component system negated the expression of the *lanA* promoter and the production of lantibiotic [51, 54, 114]. Mutation of the LanA peptide, as minimal as a 4 base pair deletion, conferred total loss of gene transcription and lantibiotic production suggesting that the lantibiotic peptides have dual function of as signaling molecule and a growth inhibitory molecule [54, 115, 116].

While most lantibiotic regulatory proteins perform a transcriptional activation role during lantibiotic production, the *Enterococcus faecalis* cytolysin regulator ($CylR_2$) acts in transcriptional repression on both the *cylL* and *cylR* promoters of the cytolysin operon [117]. The helix-turn-helix (H-T-H) domain of $CylR_2$ was shown to bind specifically to the two inverted repeats located between the intergenic region of the *cylL* and *cylR* promoters to repress transcription of the cytolysin genes [55, 118]. The accumulation of an active cytolysin, CylLs", signals the derepression of $CylR_2$, resulting in increased production of cytolysin. The CylLs cytolysin is a lantibiotic-family toxin that has activity against both prokaryotic and eukaryotic cells. Several Gram-positive bacteria are highly sensitive to bactericidal activity of the cytolysin giving *E. faecalis* a competitive advantage in its ecological niche [119]. The cytolysin also contributes to the virulence of *E. faecalis* as it is active against human erythrocytes and innate immune cells such as mouse neutrophils and macrophages [120].

The salivaricin locus is a lantibiotic locus found in *S. salivarius* and *S. pyogenes* with the structural genes of *salA* (pre-peptide lantibiotic), *salM* (lanthionine modification enzyme), *salT* (peptidase and transport protein), *salXY* (immunity proteins) and *salKR* (two-component regulatory system) [59, 121]. The promoters controlling this locus in *S. salivarius* were proposed to be located upstream of the *salA* gene and upstream of the *salY* gene [59]. However, the exact promoter locations have not been reported. The biosynthesis of the *sal* locus in *S. salivarius* depends on the autoregulation of an extracellular SalA that signals the activation of the two component system, SalKR [59]. The mutation of a structural gene, either *salM*, *salT*, *salA* or *salKR*, abrogated the transcription of *salA* in *S. salivarius* [59]. Exogenous addition of SalA could bypass the lack of modification and/or transport proteins and induce *salA* transcription. However, exogenous addition of SalA did not induce *salA* transcription in a *salKR* mutant. These results suggest the role of SalA as an inducing molecule and the role of SalKR in transcriptional activation of *sal* locus transcription.

The *sal* locus in *S. pyogenes* M14 strain HSC5 has approximately 85% homology to the *sal* locus in *S. salivarius*. However, the biosynthesis of an active salivaricin A is abolished in all *S. pyogenes* serotypes, except M4, possibly due to mutations resulting in early stop codons present in SalM (modifying protein) and SalT (transporting protein) [59, 61]. Previous reports demonstrate that *salA* transcription of an *S. pyogenes* M1 serotype strain can be induced by exogenous addition of SalA1 from an *S. pyogenes* M4 serotype. However, *S. pyogenes* does

not produce a functional salivaricin A and, unlike *S. salivarius*, does not exhibit specific immunity to the salivaricin A lantibiotic [56, 57] suggesting that the genes in the *sal* locus do not function in lantibiotic biosynthesis. Furthermore, the complete regulation and expression conditions, along with the functions of the proteins encoded by the *sal* locus genes in *S. pyogenes* are not known.

In the previous chapter, we reported a role for the *S. pyogenes sal* locus in virulence, especially the SalKR proteins that are required for resistance to PMN-mediated killing during host infection. Therefore, it is necessary to understand how this locus is regulated and under what conditions these genes are expressed, as well as the function of the encoded proteins in order to broaden the understanding of *S. pyogenes* pathogenesis during host-pathogen interactions.

In the present study, we analyzed the transcriptional regulation of the *sal* locus in *S. pyogenes* and the function of proteins encoded in this locus that are involved in this regulation. This is the first study to describe the exact *salKR* promoter location of the *sal* locus in *S. pyogenes*. Furthermore, this is the first study to demonstrate SalR as a transcriptional repressor of the expression of the *salKR* promoter, together with data, suggesting that SalA may be acting as an intracellular signaling molecule.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All plasmids were maintained in either *Escherichia coli* DH5α cells or *Escherichia coli* TOP10 cells (Invitrogen) and cultured aerobically in Luria-Bertani medium (BBL) supplemented with 25 μg/ml kanamycin, or with 20μg/ml chloramphenicol at 37°C. The *Streptococcus pyogenes* M14 serotype, HSC5 strain [82, 83], used in this study was cultured anaerobically in THY medium, Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (BBL), or TP medium, supplemented with 2% proteose peptone (BBL), and incubated in 15-ml conical tubes at 37°C under static conditions. Plasmids in *S. pyogenes* were maintained with media supplemented with 500 μg/ml kanamycin and 3 μg/ml chloramphenicol. *S. pyogenes* cultured on solid medium (THY supplemented with 1.4% agar) was incubated in an anaerobic gas chamber with GasPak (BBL) cartridges at 37°C. Mid-log phase cultures were grown by diluting overnight cultures of *S. pyogenes* 1:50 into fresh THY. When necessary, *S. pyogenes* mutants were selected on THY that contained chloramphenicol (Cam) (3 μg/ml), kanamycin (Kan) (500 μg/ml), or erythromycin (Erm) (1 μg/ml) as appropriate.

Manipulation of DNA. Chromosomal DNA was purified from *S. pyogenes* as described previously [84]. Plasmid DNA was isolated using Pure Yield Plasmid Mini Prep (Promega) and transformed into *E. coli* and *S. pyogenes* by

electroporation as described previously [84]. Restriction endonucleases, ligases, and polymerases were used according to the manufacturers recommendations. When required, DNA fragments were purified using a gel extraction kit (Fermentas).

RNA isolation. Ten milliliters of mid-log phase bacterial cultures were centrifuged and washed twice with 1xPBS, then the pellet was transferred into 1.5 ml microfuge tube. The pellet was resuspended in 100 µl TE buffer with 25 mg/ml lysozyme and 10 mg/ml mutanolysin and incubated at 37°C for 1 hour. One milliliter of TRIzol (Invitrogen) was added into the mixture and incubated at room temperature for 5 min. Then 200 µl chloroform was added, vortexed and incubated for 3 min at room tempertature. The mixture was centrifuged at 13,000 x g for 15 min at 4°C. The aqueous layer was then transferred into new microfuge tube containing 500 µl of isopropanol to precipitate the nucleic acid. The samples were then centrifuged at 13,000 x g for 15 min at 4°C. The pellet was washed with 70% ethanol and air-dried to evaporatee residual alcohol. The nucleic acid pellets were resuspended in 100 µl RNase-free water and subsequently treated with DNase and purified with RNeasy MinElute clean up kit (Qiagen).

RT-PCR expression analysis of genes in the *sal* locus. RNA was isolated from mid-log phase *S. pyogenes* cultures grown in THY medium as described above. The concentration of RNA was determined using NanoDrop (Thermo

Scientific). The RNA was first subjected to PCR amplification to check for possible DNA contamination. Initial cDNA synthesis was carried out using AMV transcriptase (Fermentas) with 1 µg of RNA sample and a reverse primer complementary to the 3' end of the *sal* locus, the *salZ* gene to make a single cDNA of the *s*al locus (salR-hyp-rev, 5'-CGT TCT GGG AGT TGT GAA GC-3'). Following incubation at 60°C for 1 hour, the cDNA was purified using the ChIP DNA Clean & Concentrator™ (Zymo Research). The cDNA was then used as a template for PCR amplification using the primers as described in Table 1. The PCR products were visualized on electrophoresis gel stained with SYBR safe stain (Invitrogen).

Table 1. Primers for RT-PCR.

PCR	Primer name	Sequence
1	**5' salA-N**	GAC TAA TGC TAT CGA AGA AGT TTC TG
	3' salA-N-2	GTA TCT AAT ATG TCG TAA TC
2	**5'sal-seq-5**	GAG GAT ATT CGT GAT GAT GC
	3'sal-seq-5	CTC ACC ACT GCT ACT ATT TGG
3	**5' salT-insert**	CAC CTT TGG GAG TTA TAT TGG
	3' salK-mid	CTT GAG GTG AGA GTA CAA AAG G
4	**5' Kan-insert-check**	GAT GGG AAA CTT CAC ATG GAG
	3' salY-N	GAT AAG TCC ATT TGT GAT GAC
5	**5'salKpro-del3**	CGC GGA TCC CAG TTT AGT ATG GTT CCT G
	3'salK-del-seq1-sall	ACG CGT CGA CAG CTG CTG TAT CAA TAA GCG GTC
6	**5' salK pro**	CGC GGA TCC CAT TGC GGT CAG TGA CAT CC
	3'salR-del-seq1-sall	ACG CGT CGA CTG CAA ATA GTC TGT GGT CAT C
7	**5' salR-del-seq1**	GAT ACT TAC CAC CCA ATA G
	5' salR-hyp-rev	CGT TCT GGG AGT TGT GAA GC

Promoter reporter construct. To measure expression of the *salA* and *salKR* promoters, a transcriptional fusion was constructed using an alkaline phosphatase gene (*phoZ*) placed downstream of each promoter. The promoter regions for *salA* and *salKR* were amplified using the primers listed in Table 2. The PCR products were gel isolated and digested with BamHI and EcoRI (invitrogen). The digested DNA fragments were then ligated using ligase (invitrogen) with the pMNN1 plasmid that was also digested with BamHI and EcoRI (Figure 13). The ligated products were subjected to butanol precipitation and resuspended in 10 µl of nuclease-free water and transformed into *E. coli* DH5α cells. Transformants were selected on LB agar plates containing 25 µg/µl Kanamycin and 20 µg/µl Chloramphenicol. The plasmids were propagated in *E. coli* grown in LB medium supplemented with 25 µg/µl Kanamycin and 20 µg/µl Chloramphenicol. Each plasmid construct was isolated and transformed into *S. pyogenes* wild type and mutant strains and maintained in THY medium containing with 500 µg/µl Kanamycin and 3 µg/µl Chloramphenicol. Promoter sequences were confirmed by sequencing.

Table 2. Primers for pMNN1- *phoZ* reporter constructs.

Plasmid constructs	Primers	Sequence
pMNN-salA pro	5' SalA-pro-BamHI	CGC GGA TCC GAA TGA TTT TGA GAT ACT TC
	3' SalA-pro-EcoRI	CCG GAA TTC CTT CTT CGA TAG CAT TAG TC
pMNN-salKpro	5' SalK-pro-BamHI	CGC GGA TCC CAT TGC GGT CAG TGA CAT CC
	3' SalK-pro-EcoRI	CCG GAA TTC GCC AGC CAA ATA AAA GCT G
pMNN-salKR-pro	5-salY-BamHI	CGC GGA TCC GAT AGA GGA GAG TAA TAT G
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G
pMNN-salKR-pro del1	5-salKpro-del1	CGC GGA TCC GTT ATC GCT AAT GGG TCA TTG
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G
pMNN-salKR-pro del2	5-salKpro-del2	CGC GGA TCC GAC TCC ATC TGC CAT AG
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G
pMNN-salKR-pro del3	5-salKpro-del3	CGC GGA TCC CAG TTT AGT ATG GTT CCT G
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G
pMNN-salKR-pro short	5-salKpro-del2	CGC GGA TCC GAC TCC ATC TGC CAT AG
	3-SalY-pro-short-EcoRI	CCG GAA TTC GTT TTT CCA AGG CTA ATT TCT G
pMNN-salKR-pro short-flip	5’ salKR pro-del2-EcoRI	CCG GAA TTC GAC TCC ATC TGC CAT AG
	3’ salK-pro short BamHI	CGC GGA TCC GTT TTT CCA AGG CTA ATT TCT G
pMNN-salKR pro del2A	5’salYK-pro del 2A	CGC GGA TCC GAG TTA ATG AAA GCT CAA GG
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G

Alkaline phosphatase filter lift assay. The bacteria that contained the pMNN1-*sal* promoter constructs were grown overnight on agar plates containing the appropriate antibiotics, LB medium with 25 µg/µl Kanamycin and 20 µg/µl Chloramphenicol and THY medium with 500 µg/µl Kanamycin and 3 µg/µl Chloramphenicol. To determine alkaline phosphatase activity of bacteria containing promoter constructs on solid medium, a colorimetric filter lift assay was used as described previously [122]. The colonies that were alkaline phosphatase positive were selected and stored in 40% glycerol at -80°C for later confirmation and quantification of alkaline phosphatase activity in the liquid assay described below.

Alkaline phosphatase liquid assay. *S. pyogenes* strains that contain the alkaline phosphatase reporter constructs were grown in THY medium with 500 µg/ml Kanamycin and 3 µg/ml Chloramphenicol overnight. Subcultures were made by transfer into fresh THY medium with the same antibiotics and grown for 3-4 hours. One milliliter of each log-phase culture was grown with treatment alongside an untreated culture for 3 hours at 37°C in the conditions described in Table 3 .

Table 3. Treatment conditions for *sal* promoter expression.

Media	THY medium and DMEM+10% FBS
Immune cells or immune factors	human serum or heat-inactivated serum, commercial FBS, and charcoal stripped human serum.

After treatment, all cultures were normalized to OD_{600} of 0.75 in PBS and 50 µl of culture was then added, in triplicate, into the wells of a 96-well plate containing 200 µl of 1-mg/ml *p*-nitrophenyl phosphate (Sigma) suspended in 1 M Tris (pH 8). Following incubation in the dark for 1 hour at room temperature, three optical densities (OD_{405}, OD_{550}, and OD_{600}) were measured using a spectrophotometric plate reader (VersaMax). Alkaline phosphatase activity was determined using the following formula: $[OD_{405} – (1.75 \times OD_{550})]/(\text{volume} \times \text{time} \times OD_{600}) \times 1{,}000$. Each assay was repeated at least three times.

DIG-labeled sequencing reaction. The fragment for the DIG-labeled sequencing template was PCR amplified using primers; 5'-salKpro-del2-CGC GGA TCC GAC TCC ATC TGC CAT AG and 5' phoZ-rev-GTT GCC TTC GCT TCA GCA ACC TCT G-3' with the reporter construct pMNN1-*salKR pro*-deletion2-short as template. The fragment was gel purified and the concentration was determined using NanoDrop (Thermo Scientific). The sequencing reaction was carried out using $Vent_R$ (exo-) DNA Polymerase (NEB) with 5' phoZ rev DIG labeled primer (DIG-5'-GTT GCC TTC GCT TCA GCA ACC TCT G-3'). The ratio of d/ddNTP mix is described in the Table 4 below. Following the PCR reaction,

the sequencing products were loaded directly onto the 8% Urea-acrylamide gel and ran with constant 45 mA for 3 hours using GIBCO BRL sequencing systems (Model S2). The DIG-labeled DNA was transferred onto a nylon membrane using capillary transfer method [123]. The labeled products were detected using Anti-DIG-AP antibody (Roche) and CDP-star (Roche) as a substrate following the manufacturer instructions and subsequently exposed to an autoradiograph film.

Table 4. dNTP and ddNTP ratio for sequencing reaction.

d/ddATP	**d/ddGTP**	**d/ddCTP**	**d/ddTTP**
100 mM ddATP + 0.5 mM dATP	25 mM ddGTP + 0.5 mM dGTP	50 mM ddCTP + 0.5 mM dCTP	150 mM ddTTP + 0.5 mM dTTP

Primer Extension. An overnight culture of *S. pyogenes* wild type expressing pMNN1-*salKR*pro-deletion2-short was transferred into fresh THY medium supplemented with 500 µg/µl Kanamycin and 3 µg/µl Chloramphenicol and grown for 4 hours. The cell pellet was collected for RNA isolation (see protocol above). Total purified RNA (5 µg) was used in primer extension with 5'-phoZ-rev-DIG labeled primer (DIG-5'-GTT GCC TTC GCT TCA GCA ACC TCT G-3'). RNA was heated at 90°C for 5 min and immediately cooled on ice. Reverse transcription was carried out using AMV reverse transcriptase (Fermentas) and incubated at 60°C for 1 hour. RNaseA (Invitrogen) was added to the reaction mixture and

incubated at 37°C for 1 hour to remove the remaining RNA due to its ability to interfere with electrophoresis mobility of labeled cDNA. The primer extension products were purified using a ChIP DNA Clean & Concentrator™ (Zymo Research) and eluted with 10 µl of nuclease-free water. The corresponding DNA sequencing reactions were performed using the same DIG-labeled primer and a PCR fragment containing the expected promoter region with Taq DNA polymerase sequencing grade (Promega). The extension product and sequencing ladder was resolved on an 8M urea-8% polyacrylamide gel. Electrophoresis was performed at 1500V, 45 mA for 3 hours. Transfer of labeled-DNA from the gel onto a Nylon membrane (Magna Charge) was done using the capillary transfer method [123]. The labeled products were detected using Anti-DIG-AP antibody (Roche) and CDP-star (Roche) as a substrate following the manufacturer instructions and subsequently exposed to an autoradiograph film.

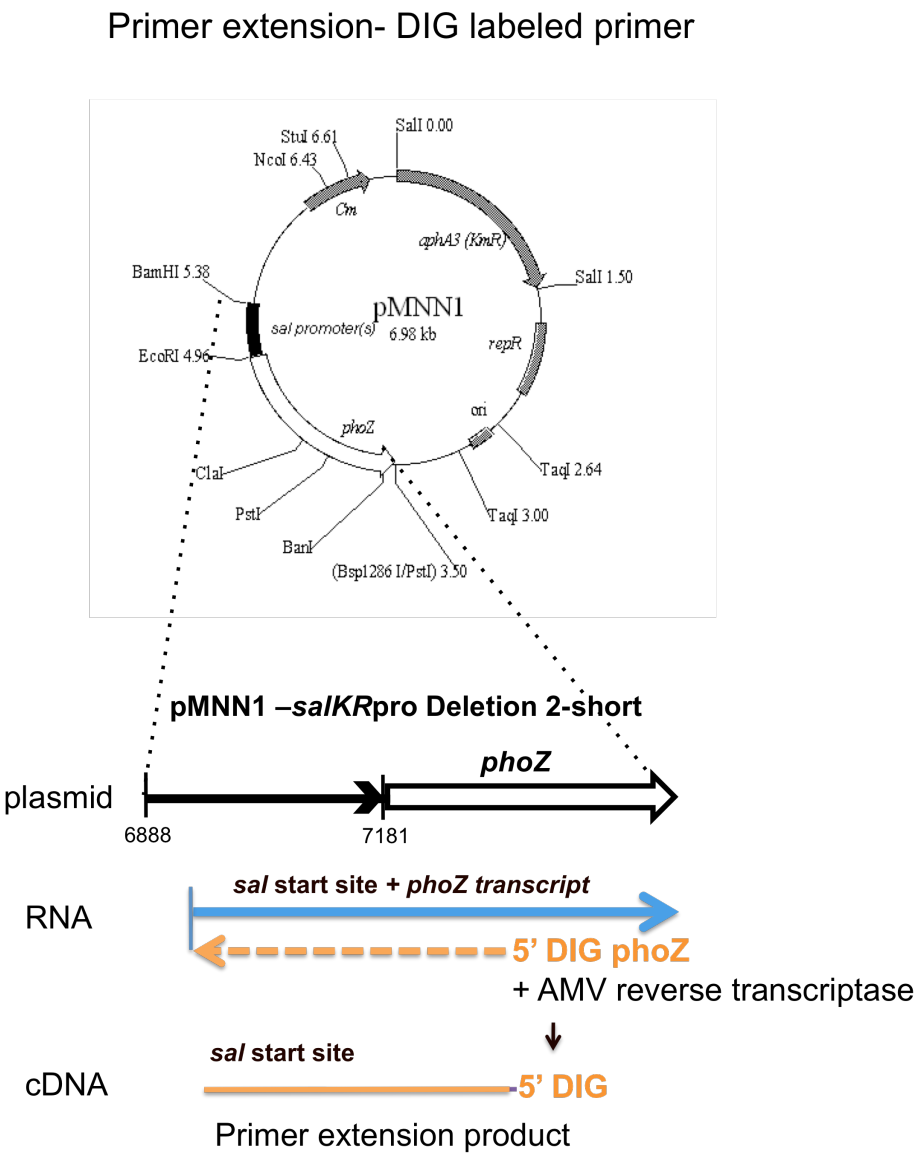


Figure 13. Illustration of the primer extension protocol using a universal primer, DIG labeled-5' phoZ-rev. The black line represents plasmid DNA. RNA was isolated from *S. pyogenes* WT HSC5 strain expressing the reporter plasmid. 5' DIG labeled-cDNA was synthesized using AMV reverse transcriptase.

Cloning of *salR* in to pMAL-c2X. The entire gene of *salR* was amplified using primers 5'-salR-SmaI (5'-TCC-CCC-GGG-ATG-AAG-ATT-TTA-TTA-ATT-GAT-GAC-C-3') and 3'-salR-PstI (5'-AAA-AACT-GCA-GGT-CGT-TTG-ATT-ATC-TGC-AAC-TCA-G-3'). The PCR products were gel isolated and digested with *SmaI* and *PstI*. After gel purification, the digested PCR products were ligated into the *SmaI* and *PstI* site on the pMAL-c2X vector, resulting in pMAL-*salR*, which subsequently was transformed into *E. coli* Top10 cells (Invitrogen).

SalR protein purification. SalR protein was purified using the pMAL protein purification system (NEB) with some modifications. *E.coli* Top10 cells with pMAL-*salR* was grown under shaking conditions at 37°C overnight in LB medium with 50 ug/ml ampicillin to maintain the plasmid. The overnight culture was then transferred into 100 ml of fresh LB media with 50 µg/ml ampicilin and incubated under the same growth conditions for 5 hours (OD_{600} approximately 0.5). SalR production was induced by addition of Isopropyl-b-D-thiogalactoside (IPTG) at 0.3mM concentration followed by incubation for an additional 2 hours. The cells were pelleted by centrifugation at 6500 rpm for 15 min. Cells were resuspended in column buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA) with Complete mini-protease inhibitor cocktail (Roche). The cell suspension was then frozen at -20°C overnight. The cells were thawed on ice and then lysed by sonication for 15 sec intervals for a total of 2 min. The crude cell lysate was collected by centrifugation at 9,000 x g for 30 min. The crude extract was diluted 1:10 with column buffer

with protease inhibitor added and passed through an amylose column prepared as per manufacturer's recommendation (NEB). The loaded column was then washed with 12 volumes of column buffer. The fusion protein was then eluted with 1 volume of column buffer supplemented with 10mM maltose and collected in 1.5 ml microfuge tubes. The concentration of each elution aliquot was measured using the BCA Protein Assay (Thermo Scientific). The highest concentration elution aliqouts were combined, desalted and concentrated using Amicon spin column with 10 KDa cut off. Collected purified protein was diluted with a 1:1 volume of 80% glycerol and stored in aliquots at -70°C.

Electrophoretic Mobility Shift Assay. The electro-mobility shift assay (EMSA) was carried out using the DIG gel shift kit 2nd generation (Roche) with minimal modification. Six probes were generated by PCR amplification using primers described in Table 5. The amplified products encompass DNA sequences within the *salY* region (figure 14). PCR products were gel purified from an 0.8% agarose gel using a gel purification kit (Fermentas). Quantification of purified PCR fragments were done by using fluorescent-based quantification method (Qubit, Invitrogen). Then the fragments were 3'-end labeled with DIG-dUTP using terminal transferase as specified in the DIG gel shift kit (Roche). Labeled probes were quantified using dot blot methods following the manufacturer's instructions. Binding reactions total volume was 25 µl. Each binding reaction contained 5 µl of 5X Binding buffer, ~3-6 ng of probe, 0.5 µl of 1mM $CaCl_2$, and 60 ng of Poly dI-C.

A variable amount of protein was added to the binding reactions. To confirm specific binding, 5X and 10X excess non-labeled specific probe was added. The binding reactions were incubated at room temperature for 45 min then 5 µl of loading buffer (kit) was added. Samples were mixed gently then loaded onto 8% TBE non-denaturing polyacrylamide gel. Initial voltage was set to 300V for 10 min to allow protein/probes and protein-probe complex to enter the gel. The gels were then run at 150V for 30-45 min. The reactions from the gel were subsequently transferred onto a positively charged nylon membrane (Magna charge) using a semi-dry electrotransfer apparatus (Bio-Rad). Probe mobility was then visualized with chemiluminescent detection as described in the DIG gel shift protocol. Membranes were exposed to X-ray film (Denville) and developed in an autoprocessor.

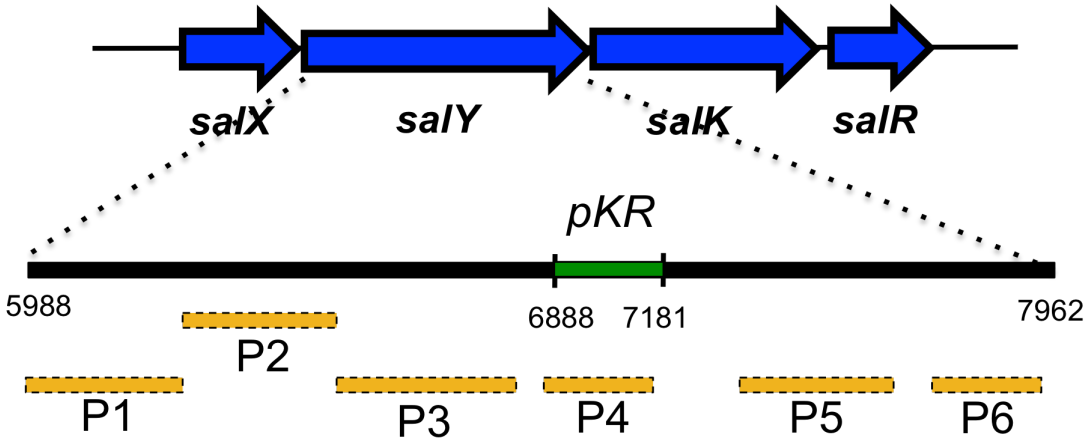


Figure 14. The location of PCR amplification products for DIG-labeled probes for EMSA. The yellow bars represent PCR products amplified using the primer sets described in the table 5 below.

Table 5. Primers for PCR amplification of DIG labeled probes

Probe number	Primers	Sequence
P1	5-salY-BamHI	CGC GGA TCC GAT AGA GGA GAG TAA TAT G
	3-salY pro#5	GAC TCC AAG CTC TTG ACT ACG
P2	5-salY-N	CGT AGT CAA GAG CTT GGA GTC
	3-salY-N	GAT AAG TCC ATT TGT GAT GAC
P3	5-salYK-pro del1A	CGC GGA TCC CAG CTA ATG AGA TTA TAG AGT C
	3-salY pro#4	GCA TAG AGC ATT ACC ATC
P4	5-salKpro-del2	CGC GGA TCC GAC TCC ATC TGC CAT AG
	3-SalY-pro-short-EcoRI	CCG GAA TTC GTT TTT CCA AGG CTA ATT TCT G
P5	5-salKpro-del3	CGC GGA TCC CAG TTT AGT ATG GTT CCT G
	3-salY pro#2	CAT TAT GAT ATT GCC TGC TAC C
P6	5-salK-pro-BamH1#2	CGC GGA TCC CCT GTA TTC ATT GGG ATT G
	3-salY-EcoRI	CCG GAA TTC CAT ATT ATT TAC TTA ATC G

Adherence and Internalization assay with macrophage cells. RAW 264.7 murine macrophage cells were seeded at 1 × 10^6 cells/ml per well into a 24-well plate and allowed to adhere overnight in a 5% CO_2 incubator at 37°C. The following day, cells were washed three times with PBS and incubated with log-phase *S. pyogenes* HSC5 in DMEM supplemented wit 10% FBS at 1 × 10^6 CFU/ml per well, a multiplicity of infection (MOI) of 1. To allow close contact of bacterial cells to macrophages, the 24-well plate was centrifuged briefly for 5 min at 1,000 rpm with a soft start and stop at room temperature. The plate was then incubated for 1 hour at 37°C in a 5% CO_2 incubator. Following the incubation, each well was washed 5 times with PBS to remove non-adherent cells. One set of the infected macrophages was then lysed, by the addition of 1 ml of sterile double-distilled water, serial diluted in PBS and plated to determine the number of adherent and internalized bacterial cells. The other set was incubated for an additional hour in fresh DMEM plus 10% FBS with 100 μg/ml gentamicin (Invitrogen) added to eliminate extracellular bacteria. Following the gentamicin treatment, the plate was washed three times with PBS. The 0 hour-time point aliquot was then collected and plated as described above for number of internalized bacteria cells at the 0 time point. Fresh DMEM plus 10% FBS containing no antibiotic was added to each remaining well. At the 24-hour time point, aliquots were taken from the supernatants and cells were lysed and plated for enumeration of viable bacterial cells after 24 hours in macrophages.

Construction of the *salA* overexpression (*salA* o/x) plasmid. The full length *salA* gene including the ribosomal binding site was PCR amplified using primers 5'-salA RBS BamHI-CGC GGA TCC CTG ATA GAA AGG AGA ATG AG and 3'-SalA stop PstI-AAA ACT GCA GTT AAC AAC AAA CGA ATA CTG using *S. pyogenes* wild type (HSC5) chromosomal DNA as a template. The PCR product was gel-purified from an 0.8% agarose gel with a gel purification kit (Fermentas). Following the restriction digest with BamHI and PstI, the PCR fragment was purified using a PCR clean-up kit (Fermentas) and cloned downstream of the Streptococcal *rofA* promoter on the pLZ12 vector [124, 125]. The expression plasmid was transformed and propagated in *E. coli* TOP10 cells (Invitrogen). The correct construct was confirmed using colony PCR with the primers mentioned above and sent for sequencing. The plasmid was isolated using a plasmid midi prep kit (QIAGEN) and transformed into *S. pyogenes* wild type conferring a *salA* overexpression strain.

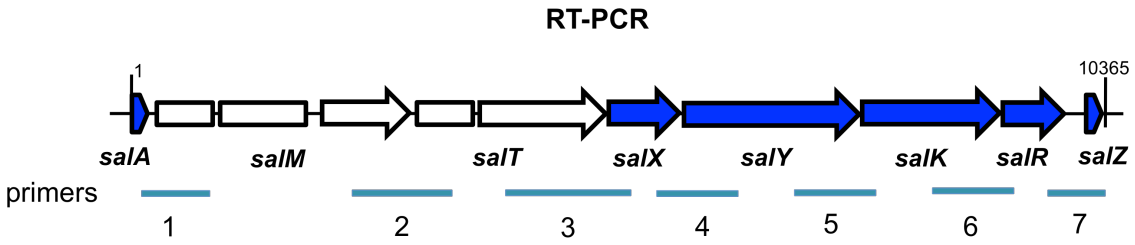
Determining whether the effect of SalA is intercellular or extracellular. Overnight cultures in THY medium of wild type, SalA-IFD (in-frame deletion) and salAo/x (WT expressing *p*LZ12-*rofA*-*salA*), were sub-cultured into fresh THY medium containing E-64 cysteine protease inhibitor. After the cultures were incubated at 37°C for 4 hours, they were centrifuged and bacterial cells filtered out using a 0.20-micron syringe filter (BD). The supernatant (spent media) was collected from the subcultures and fresh E-64 was added to the spent media to

inhibit protease activity, especially SpeB. *S. pyogenes* wild type and salA-IFD strains harboring pMNN1-*salKRpro-phoZ* were also subcultured into fresh THY medium and incubated 37°C for 4 hours. The cell pellets were collected by centrifugation at 6500 rpm for 10 min and the supernatant discarded. The cell pellets were then incubated with the spent media described above for another 3 hours at 37°C. After treatment, all cultures were normalized to OD_{600} of 0.75 and analyzed by the liquid alkaline phosphatase assay described above.

RESULTS

Analysis of *sal* locus gene expression. To determine whether genes in the *sal* operon of *S. pyogenes* M14 HSC5 strain are co-transcribed, we performed RT-PCR on mRNA isolated from the wild type strain during mid-log phase growth in THY media. A single primer that annealed to a sequence downstream of the *sal* locus in the reverse orientation was used to make a cDNA of the *sal* operon transcript. Fragments were amplified by PCR using the cDNA made from the *sal* transcript and primer pairs (see table 1) that overlap the 3' and 5' region of adjacent genes to confirm co-transcription. As shown in figure 15, we detected amplification products representative of a single transcript for all the genes in the *sal* locus including the newly identified hypothetical protein encoded by the *salZ* gene at the 3' end of the *sal* operon.

A)



B)

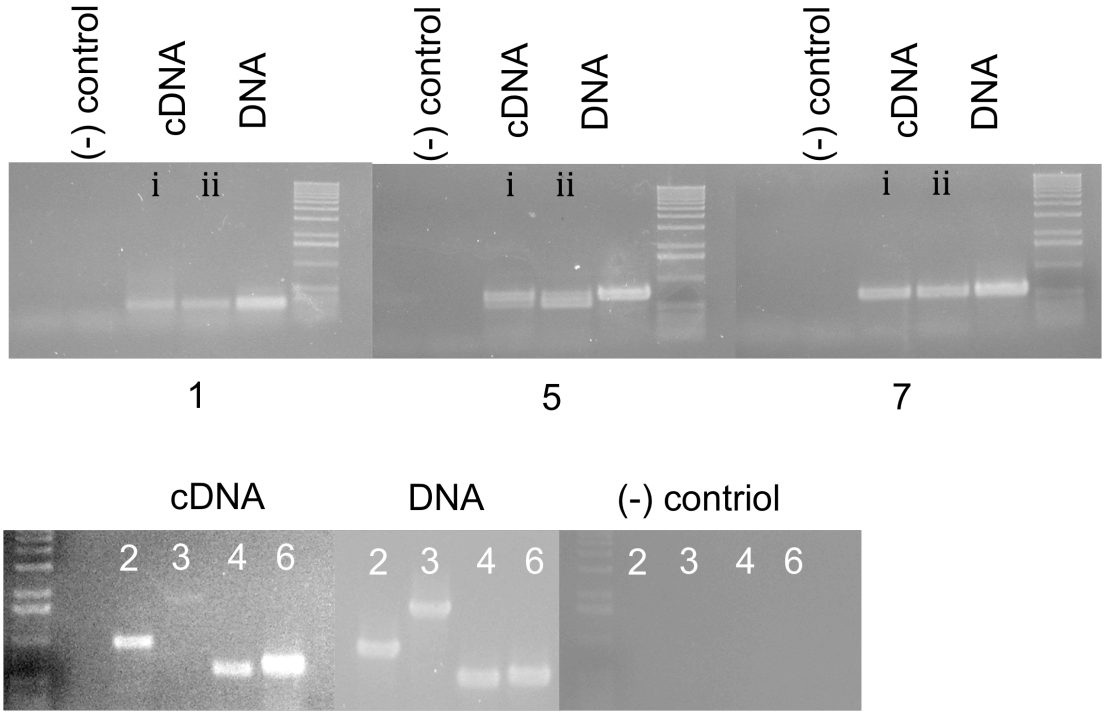
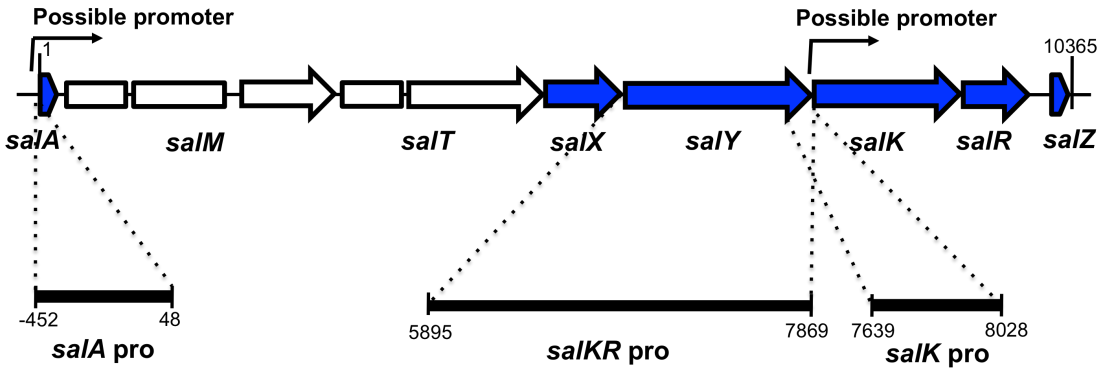


Figure 15. RT-PCR analysis of genes in the *sal* locus expressed in WT *S. pyogenes*. A) Illustration of the over lapping primer pairs 1-6 and their location on the *sal* locus. B) RT-PCR products from primers 1, 5 and 7, showing negative control using RNA sample with no RT in lane 1, cDNA from duplicated RNA sample i and ii. in lanes 2 and 3 and positive control with same primers and DNA. C) RT-PCR products using sample i as a template with primers 2, 3, 4 and 6. The negative control was done with no RT control.

Promoter identification. The promoter constructs for the putative *salA* and *salK* promoter regions were determined by the common organization of genes and promoters in lantibiotic loci [47]. The regions upstream of *salA* and upstream of *salK* were amplified by PCR as depicted in figure 16A. These regions were cloned individually upstream of the alkaline phosphatase reporter gene, *phoZ*, in plasmid pMNN1 and then transformed into the wild type *S. pyogenes* strain. Mid-log phase cultures of the wild type strain expressing pMNN-*salA*pro and pMNN-*salK*pro were tested for alkaline phosphatase activity. The strain expressing the *salA* promoter plasmid had 2470±128.84 units of alkaline phosphatase activity (figure 16B), whereas there was no alkaline phosphatase activity detected from the wild type strain expressing the *salK* promoter. This indicates that there is a promoter sequence within *salA* promoter construct region, while suggesting the absence of a promoter sequence within *salK* construct region.

To identify the region that contains a promoter for regulation of *salKR* expression, ~2000 bp upstream of the *salK* gene, encompassing the entire open reading frame of *salY*, was cloned into the pMNN1-reporter plasmid. This promoter construct was called *salKR* pro. Alkaline phosphatase activity of 985±177.60 units (figure 16B) was detected in the wild type strain expressing pMNN1-*salKR*pro indicating that an additional promoter of the downstream genes of *sal* locus is located within *salY* gene. Therefore two promoters were identified in the *sal* locus of *S. pyogenes* M14 HSC5 strain, one located upstream of the *salA* gene and the other located within the *salY* gene.

A)



B)

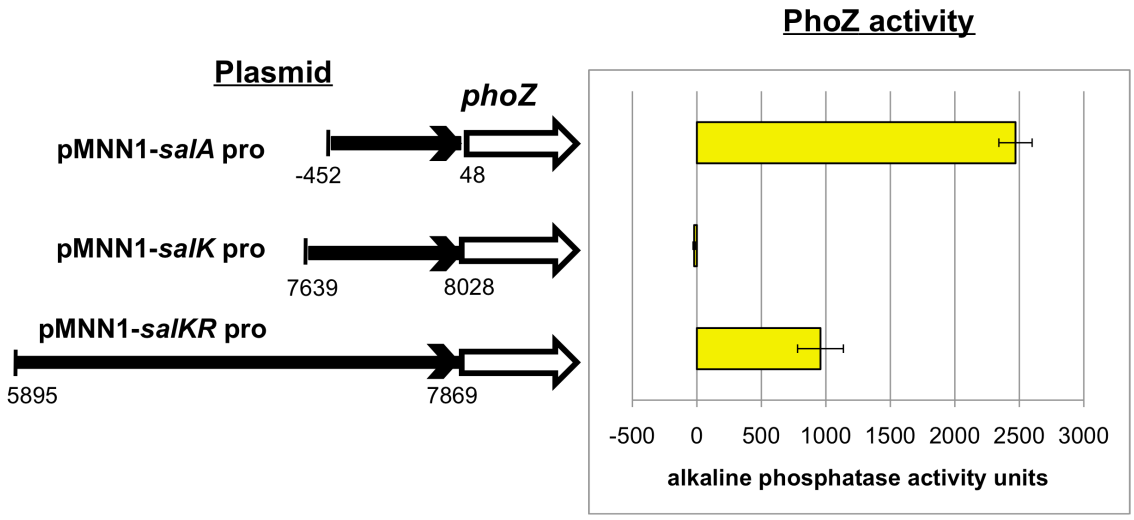


Figure 16. *sal* locus promoter identification. A) Illustration of the genetic map of the possible promoter locations suggested by the common organization of lantibiotic loci. The promoter regions were amplified using PCR (primers described in table 2). B) Alkaline phosphatase activity of WT *S. pyogenes* strain HSC5 harboring pMNN1-sal-promoter. Error bars reflect the standard errors.

Analysis of the *salKR* promoter. To narrow down the sequences required for expression of the *salKR* promoter, truncated forms of the *salKR* promoter region from plasmid pMNN-salKR with deletions from the 5' end were cloned into the same reporter plasmid as represented in figure 17. Truncation of 187 bp from the 5' end (deletion 1) resulted in an increased expression of *salKR* promoter activity. Further truncation of another 806 bp from 5' end (deletion 2) resulted in double the alkaline phosphatase activity compared to the full-lengh *salKR* promoter region. The increased promoter activity after deletion of the 5' sequences is indicative of a possible repression site(s). However, an additional deletion of 393 bp demonstrated a total loss of promoter activity, suggesting that promoter elements are contained within the 393 bp region.

A deletion from the 3' end of the deletion 2 construct, leaving only the 393 bp region also showed increased expression compared to the deletion 2 construct, suggesting that another possible repression site or alternatively, a terminator site is located downstream of the promoter region. To confirm that this region contained the -10 and -35 promoter elements, the 393 bp was cloned in the reverse direction (3' to 5') into the reporter plasmid, which resulted in no activity. Therefore the sequences required for the *salKR* promoter lie within the 393 bp region from 6888-7181 of the *salY* gene. (figure 17).

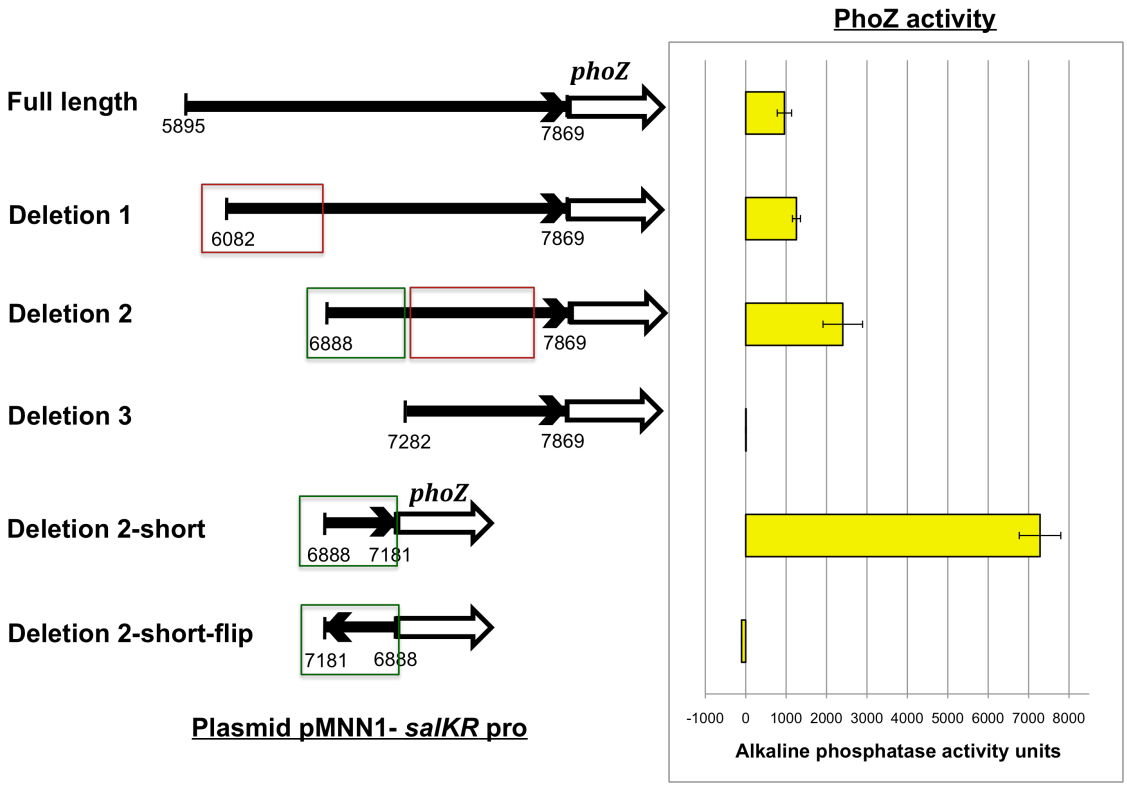


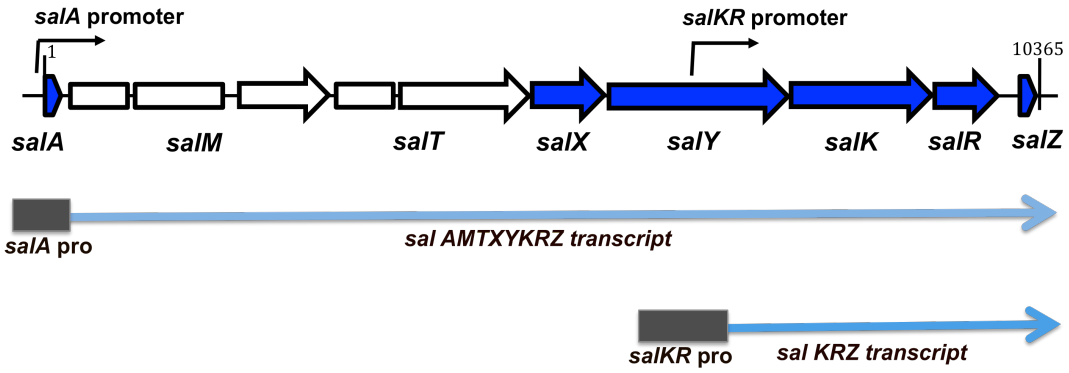
Figure 17. Analysis of the *salKR* promoter. Alkaline phosphatase activities were produced by *S. pyogenes* WT strain HSC5 expressing various truncations of *salKR* promoter reporter plasmid. The PhoZ activity was measured using a colorimetric assay with standard errors illustrated as error bars. The red boxes represent the DNA regions with possible repression sites. The green boxes represent the possible promoter region.

Mapping of the *salKR* transcription start sites. To confirm the read-through transcript provided by the *salA* promoter, RT-PCR was also performed with an RNA sample isolated from a *salY* in-frame deletion strain, which would result in a deletion of the *salKR* promoter. Results revealed that the *salK* transcript was still detected using primers for the *salK* gene (figure 18B) in the absence of the *salKR* promoter, indicating a read-through transcript from the *salA* promoter (figure 18A).

The exact location of *salKR* promoter elements was identified using primer extension. The protocol utilized a DIG-labeled universal primer that annealed to the reverse of the 5'end of *phoZ* gene as described in the figure 13. This protocol is designed to eliminate the interference of the long mRNA that is transcribed from the *salA* promoter.

The -10 and -35 promoter region was determined from identification of the transcription start site (+1 site) of the *salKR* transcript. A well conserved consensus -10 region (TATAAT) was identified as well as a degenerate -35 (TgGgCA) site (figure 19).

A)



B)

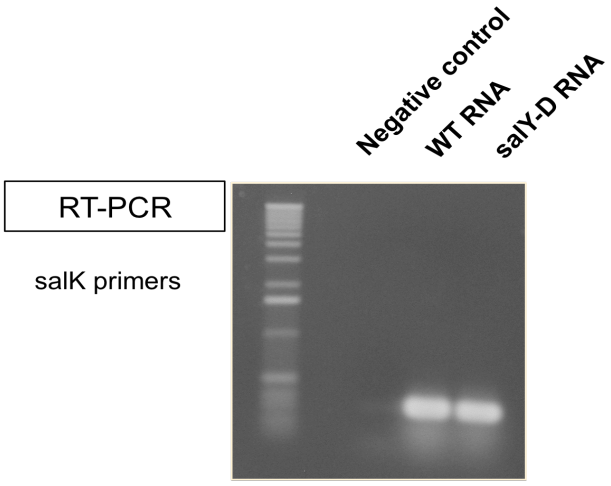


Figure 18. A) Illustration of the read-through transcript provided by the *salA* promoter. B) RT-PCR analysis of the WT strain and the *salY*-deletion strain RNA using one step-RT-PCR i(nvitrogen) with *salK* primers. Lane1. negative control; lane 2, WT RNA and lane 3, salY deletion strain RNA.

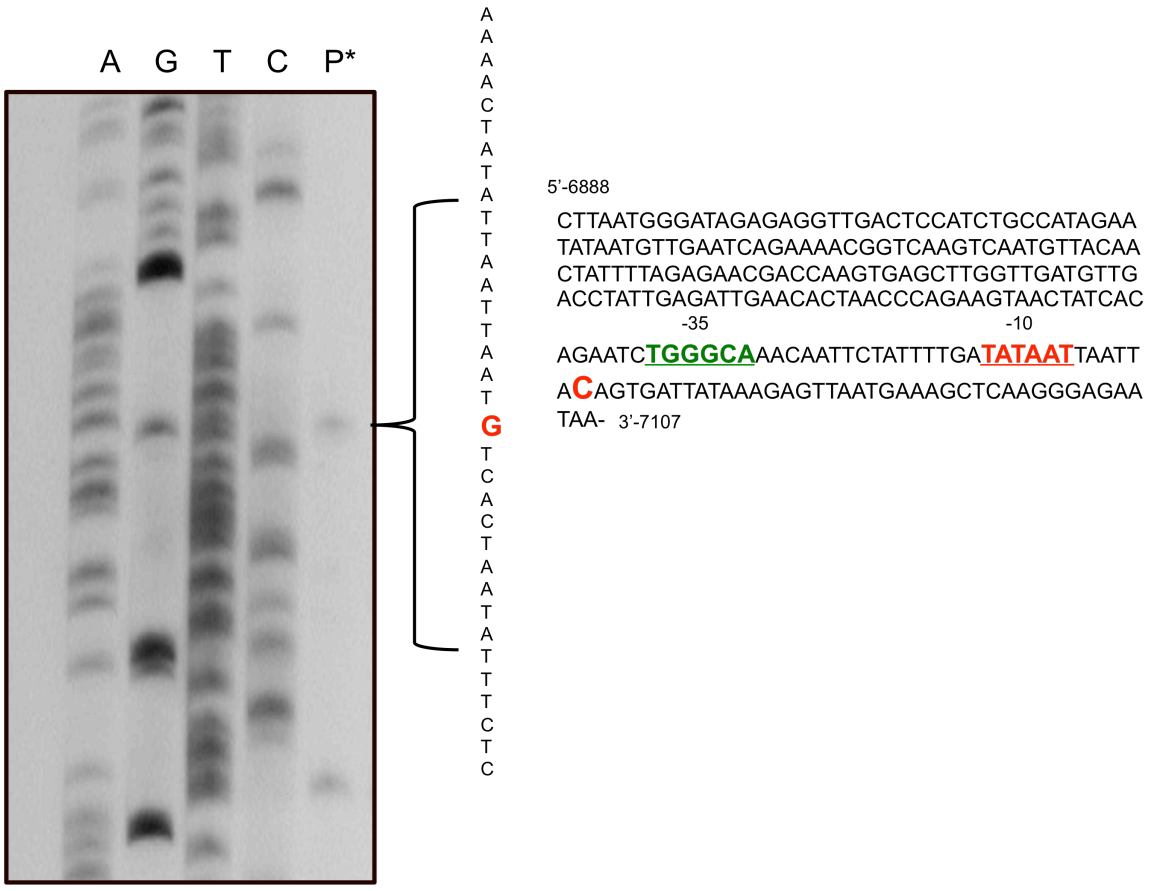


Figure 19. Identification of the 5' end of the *salKR* transcript that was transcribed from the *salKR* promoter expressed off the plasmid, pMNN1-salKR-deletion 2 short. The primer extension analysis was used to identify a transcriptional start site of *salKR* transcripts. The primer extension product is shown on the far right lane (P*). The nucleotide start site (red letter G) was compared to the sequencing ladder. The promoter region was identified from the transcription start site, -10 consensus region shown in red and a putative -35 shown in green.

SalR regulates *salKR* promoter expression. Based on homology to other two-component regulators, the SalK and SalR proteins comprise a putative two-component regulatory system. The putative regulator, SalR, has a conserved DNA binding domain that would allow it to bind directly to DNA resulting in regulation of gene expression. Most lantibiotic regulators control expression of their own promoters [52, 54, 115]. Therefore, to determine whether SalR has an effect on the *salA* and/or the *salKR* promoter expression, the reporter plasmid containing either the *salA* or *salKR* promoter was transformed into a strain containing a deletion of the *salKR* genes (*ΔsalKR*, construct details in methods). We compared *salA* and *salKR* promoter activity from the WT and the *ΔsalKR* deletion strain over time. Results showed that *salA* promoter expression was not affected by the absence of SalKR. In contrast, *salKR* promoter activity in the *ΔsalKR* strain was two times higher than in the wild type strain that expressed SalKR (figure 20). These results suggest that the SalR protein can act as a regulator to repress the expression of its own promoter.

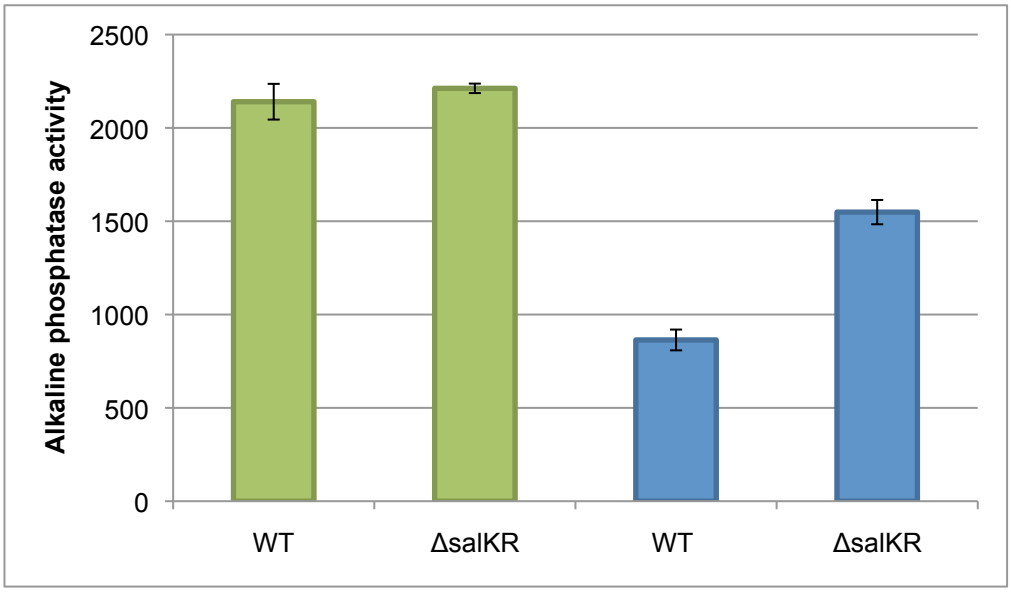


Figure 20. Alkaline phosphatase activity. The comparison of alkaline phosphatase activities produced by the WT and the *ΔsalKR* strains expressing the *salA* or the *salKR* promoter reporter plasmid. The alkaline phosphatase activities were measured using a colorimetric assay and error bars illustrate standard error.

SalR binds specifically to regions upstream and downstream of *salKR* promoter. To examine binding of the SalR protein to the *salKR* promoter region, we cloned the *salR* gene into the pMAL fusion vector and then expressed the pMAL-*salR* fusion in *E. coli* (Top10). The SalR protein was transcribed and translated as an MBP fusion protein, which was subsequently purified using an amylose column. Purified MBP-SalR was tested for the ability to bind to the *salKR* promoter region and areas surrounding the promoter region (figure 21A) using an Electrophoretic Mobility Shift Assay (EMSA). The probes for DNA binding assays were amplified using PCR (see methods) and were then 3' end-labeled with DIG and used as probes in the EMSA assays. Results demonstrated that the SalR protein binds specifically to probe 3, the region upstream of the *salKR* promoter, but did not bind directly to the promoter region, probe 4 (figure 21B). In addition to the upstream region, SalR also bound probes 5 and 6, which are regions downstream of the *salKR* promoter. We could compete for protein binding with excess un-labeled probe resulting in a shift in the probe to the unbound position (figure 21B). Moreover, the SalR protein showed differential binding affinity by comparing binding of the upstream region to the downstream region of the *salKR* promoter using an EMSA competition assay (figure 22). Probe number 3 showed the highest affinity binding to the SalR protein as it can completely compete with probe number 5 or 6. While probe 5 or 6 cannot fully compete probe 3 even when the un-labeled probe was added at ten times the concentration of the labeled probe.

Together, the data suggest that SalR regulates the expression of the *salKR* promoter by binding directly to sequences both upstream and downstream of the promoter.

Electrophoretic Mobility Shift Assay

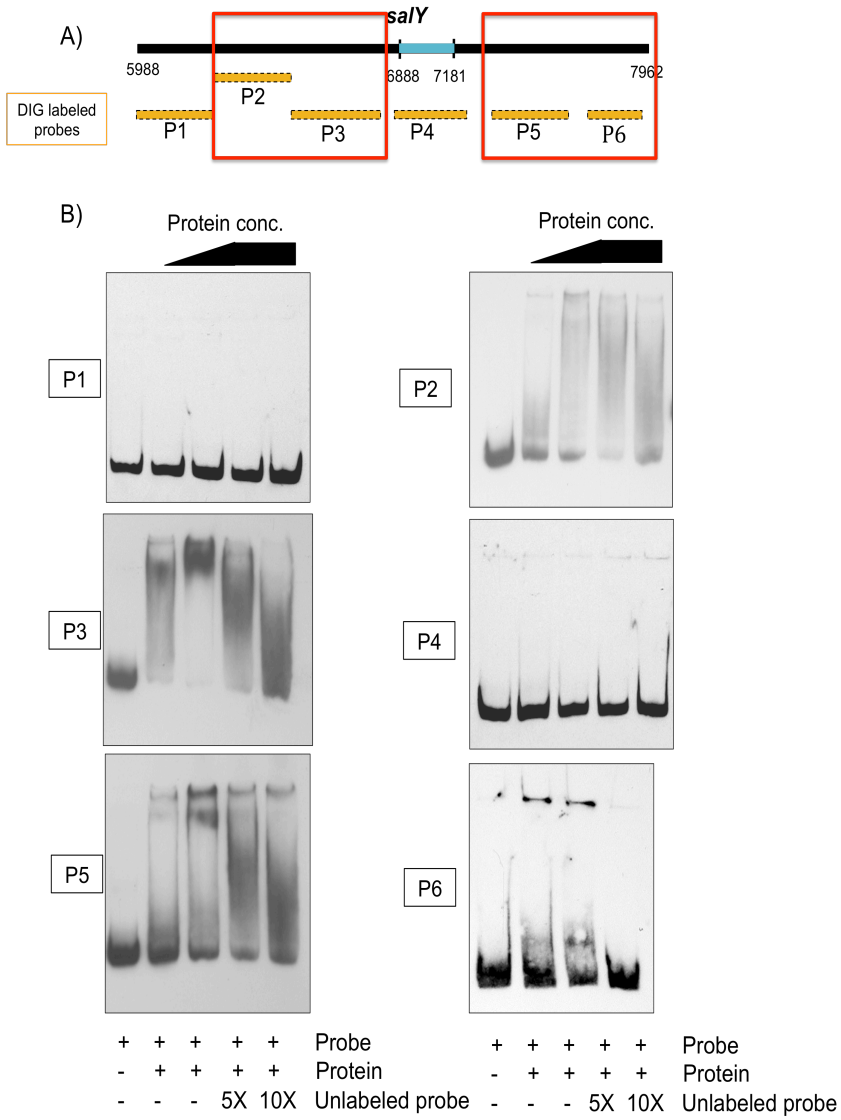


Figure 21. SalR binds to sequences upstream and downstream of the *salKR* promoter. A) The genetic map of the *salY* gene containing the *salKR* promoter depicted in blue, and the DIG labeled probes tested in the gel shift assay shown in yellow. B) Electrophoretic mobility shift assay showing binding of the SalR protein to the DIG-labeled DNA probe. Every lane contained 3-6 ng of probes. Lane 1 of each gel, probe alone; Lane 2 and 3, probe with an increasing amount of MBP-SalR protein, 25 ng- 50ng respectively. Lane 4 and 5, probe with 50 ng of MBP-SalR protein plus 5 fold and 10 fold of excess specific unlabelled probes.

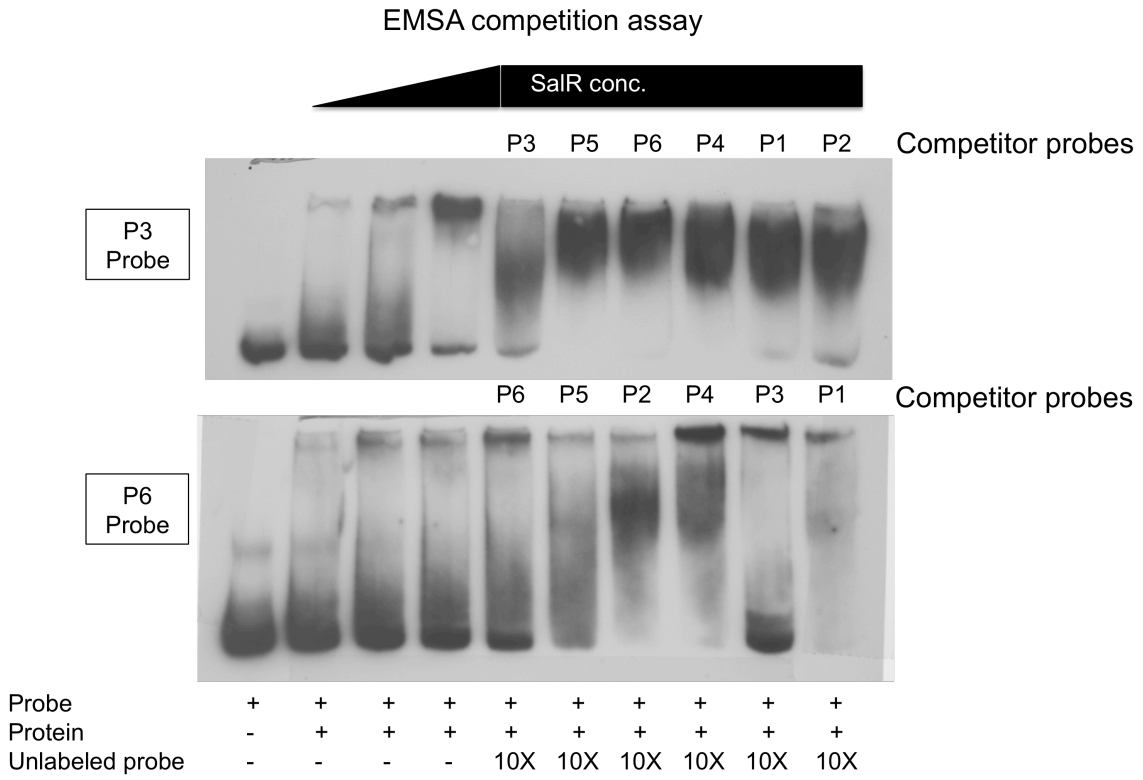


Figure 22. Affinity binding of MBP-SalR to the DIG-labeled DNA probes. The competitive assay was done using probes 3 and 6. Lane 1 contains 5-6 ng of probe only; lane 2-4, probe with increasing amount of MBP-SalR protein, 6.5 ng, 13 ng and 26 ng respectively; lane 5-10, probe with 26 ng of MBP-SalR and 10 fold excess amount of unlabeled competitor DNA.

The *salA*-IFD strain has a phenotype with enhanced adherence to macrophage cells. On a THY agar plate, the *salA*-IFD strain has a different colony morphology compared to the WT strain. The *salA*-IFD colonies appear larger and more mucoid. This suggested the possibility of an increased expression of capsule in the *salA*-IFD strain. However, RT-PCR analysis of *hasA* gene expression, which in *S. pyogenes* controls capsule expression, revealed no difference from the wild type hasA expression (data not shown). Moreover, buoyant density Percoll gradients demonstrated that the highly mucoid colony phenotype was not due to an increased production of capsule (data not shown).

Analysis of adherence and internalization of the salA-IFD strain using a macrophage infection assay demonstrated that the mutant strain bound and was internalized at a rate that was approximately two fold higher than that of WT (figure 23). This phenotype could be complemented with the addition of a plasmid that constitutively expressed the *salA* gene from the streptococcal *rofA* promoter. Interestingly, the same plasmid expressed in the *S. pyogenes* wild type strain (salA o/x) creating an overexpression of *salA* (plasmid and chromosomal *salA* expression), resulted in an adherence and internalization rate that was almost two fold less than the wild type strain alone (figure 23).

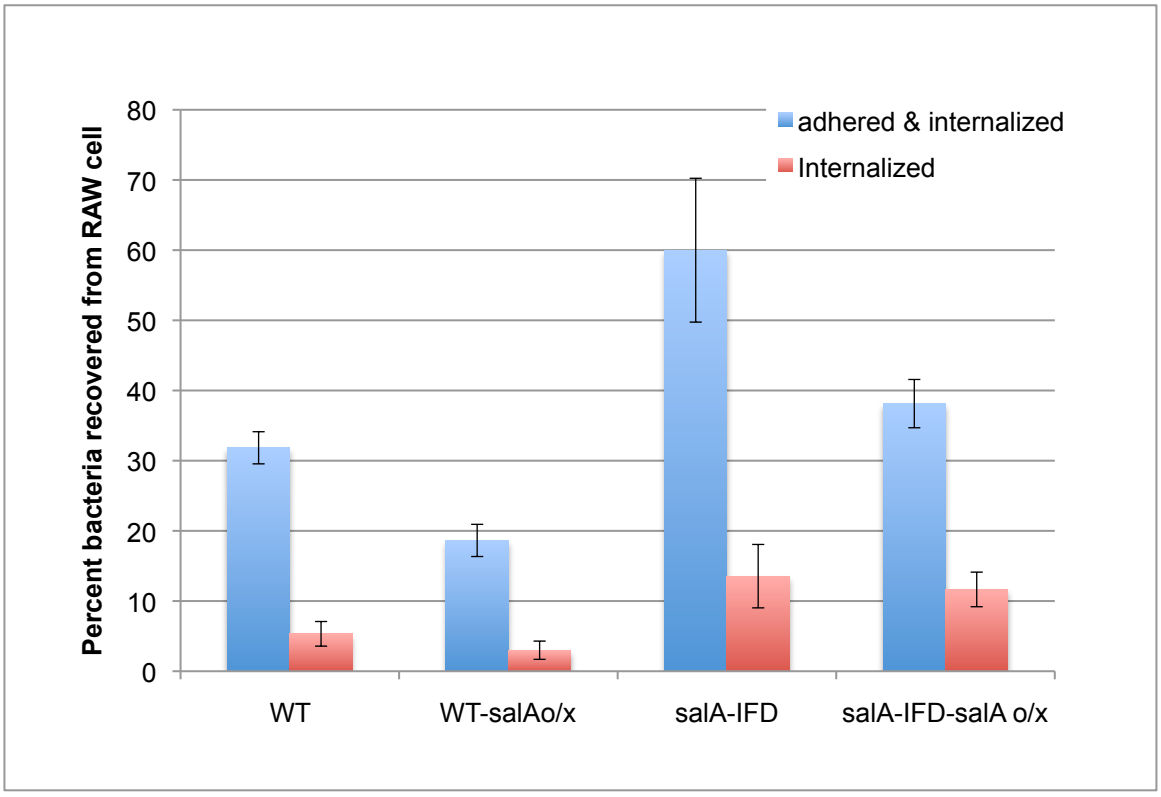


Figure 23. Adherence and internalization in of the bacteria in macrophage cells. Bacterial adherence and internalization at the initial interaction with murine macrophage cell line. The percent of bacteria recovered from the macrophage cells was calculated relative to the initial inoculum. The blue bars represent the percent of adhered and internalized bacteria and the red bars represent the percent of internalized bacteria. The errors bars represent standard errors.

SalA affects the *salKR* promoter expression. Most lantibiotic locus promoters are autoregulated and expression is induced when sensing the presence of the cognate lantibiotic peptide [47, 54]. While *S. pyogenes* M14 HSC5 strain does not produce detectable SalA lantibiotic activity, we did observe *salA* transcripts by RT-PCR. To investigate if SalA plays a role in expression of the *salA* promoter and the downstream *salKR* promoter, the *salA* and *salKR* promoter reporter plasmids were transformed separately into the wild type and a *salA* in-frame deletion (IFD) strain. Expression analysis revealed that the *salA* promoter was not affected by the absence of the SalA peptide, whereas *salKR* promoter activity in the *salA*-IFD strain was significantly (p value < 0.005) increased (figure 24) suggesting that SalA plays a role in *salKR* promoter repression.

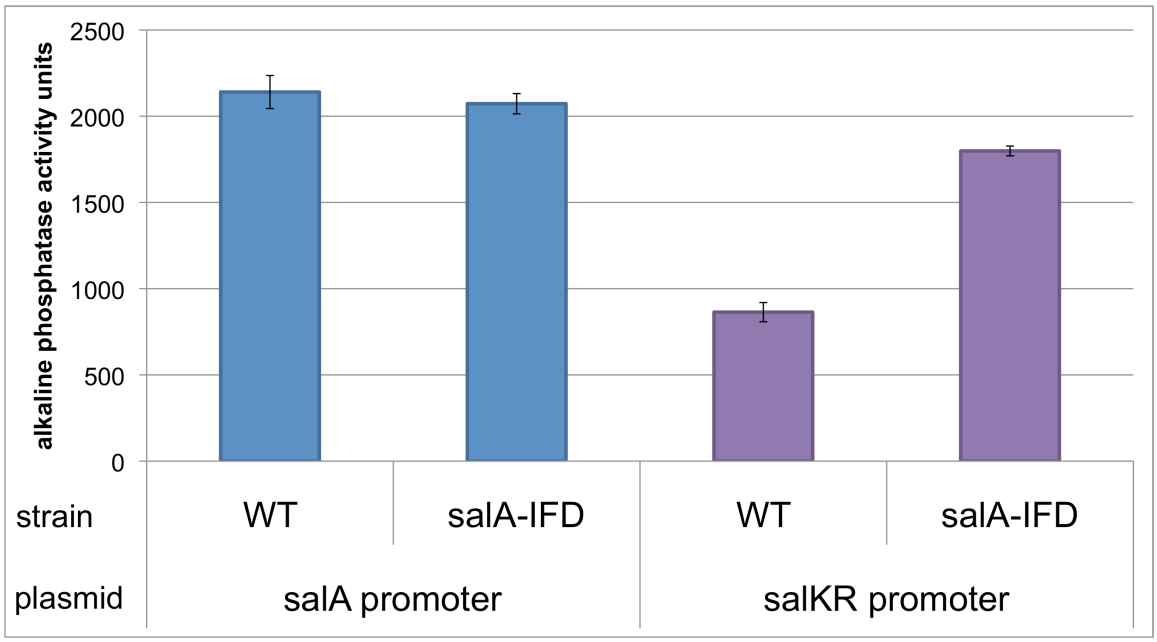
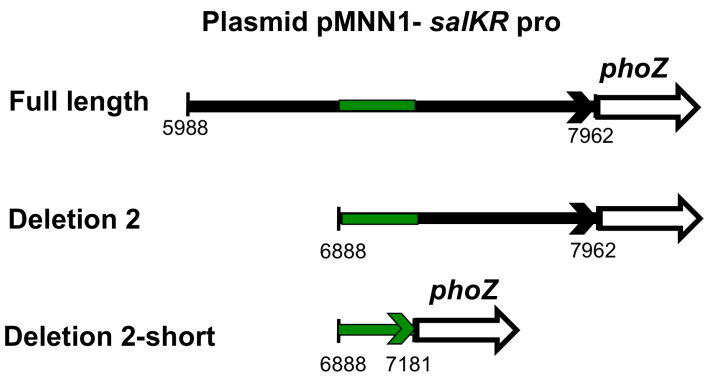


Figure 24. Alkaline phosphatase activity. Comparison of the alkaline phosphatase activities produced by the WT and the *salA*-IFD strains expressing pMNN1 s*alA* or *salKR* promoter plasmid.

SalA does not act extracellularly to affect *salKR* promoter expression. While the SalA peptide from *S. salivarius* is known to be secreted extracellularly to act as a signal for the histidine kinase in addition to its function as a lantibiotic, the cellular location of the *S. pyogenes* SalA peptide is not known. To determine if the *S. pyogenes* SalA peptide acts intracellularly or extracellularly to affect *salKR* promoter expression an alkaline phosphatase assay was performed on the wild type and the *salA*-IFD strains that contain the s*alKR* reporter plasmid. If SalA acts extracellularly, then the peptide would be secreted and found in the supernatant of cultures. As the *salA* promoter was found to be expressed at the highest level in late log phase growth (Figure 25) spent media was taken from cultures of the wild type, *salA*-IFD and *salA*o/x (a WT that constitutively expressed *salA* from a plasmid) strains grown to late log phase. The spent media was then used to treat wild type and *salA*-IFD cultures expressing the *salKR* reporter plasmid for 3 hours, after which an alkaline phosphatase assay was performed to measure promoter activity. All spent media cultures were treated with E64, a cysteine protease inhibitor, to prevent degradation of the secreted SalA peptide by SpeB. The results revealed that no changes in promoter activity were detected in either the wild type or the *salA*-IFD strains treated with spent media compared to untreated strains (data not shown), suggesting that the promoter expression difference observed between the strains may be due to the intracellular action of SalA.

The analyses of *salKR* promoter expression in the mutants revealed that the full-length promoter expressed higher in both *salA*-IFD and *ΔsalKR* mutants compared to that of WT. However, the shortest promoter construct, pMNN1-*salKR* pro-deletion 2 short, with minimal or non-repression sites, the promoter expression were highly expressed in all strains (figure 25).

<u>*salKR* promoter expression in mutants</u>



WT	ΔsalKR	salA IFD
958 ±177.60	1548 ±65.04	1798 ±128.35
1402 ±289.34.	2387 ±216.28	3486 ±374.29
6911 ± 513.66	5898 ±795.05	8031 ±825.11

Figure 25. Alkaline phosphatase activities measured in the WT, the *salKR* and the *salA*-IFD strains expressing various truncations of the *salKR* promoter reporter plasmid. The green regions represent the *salKR* promoter location. The values in the table show alkaline phosphatase activity units with standard errors.

Both the *salA* and the *salKR* promoters respond to human serum. Genes in the *sal* locus are important for full virulence of the *S. pyogenes* strain HSC5 [61]. To examine if the two promoters identified were affected by host factors, we tested the promoter expression of *salA* pro and *salKR* pro (full length-contains all the regulation sites) in the wild type strain, in the presence of human serum compared to fetal bovine serum. A significant (*P* value < 0.05) increase in expression of both the *salA* and *salKR* promoters was detected when exposed to human serum (figure 26). In an effort to determine what factors in the human serum were responsible for the increase in promoter activity, the serum was heat inactivated, which destroys complement factors found in the serum, or charcoal-stripped, which results in removal of hormones. Even under these conditions both promoters still expressed higher activity than with THY medium alone or with fetal bovine serum, suggesting that factors in the serum other than complement or hormones are responsible for the increased promoter activity.

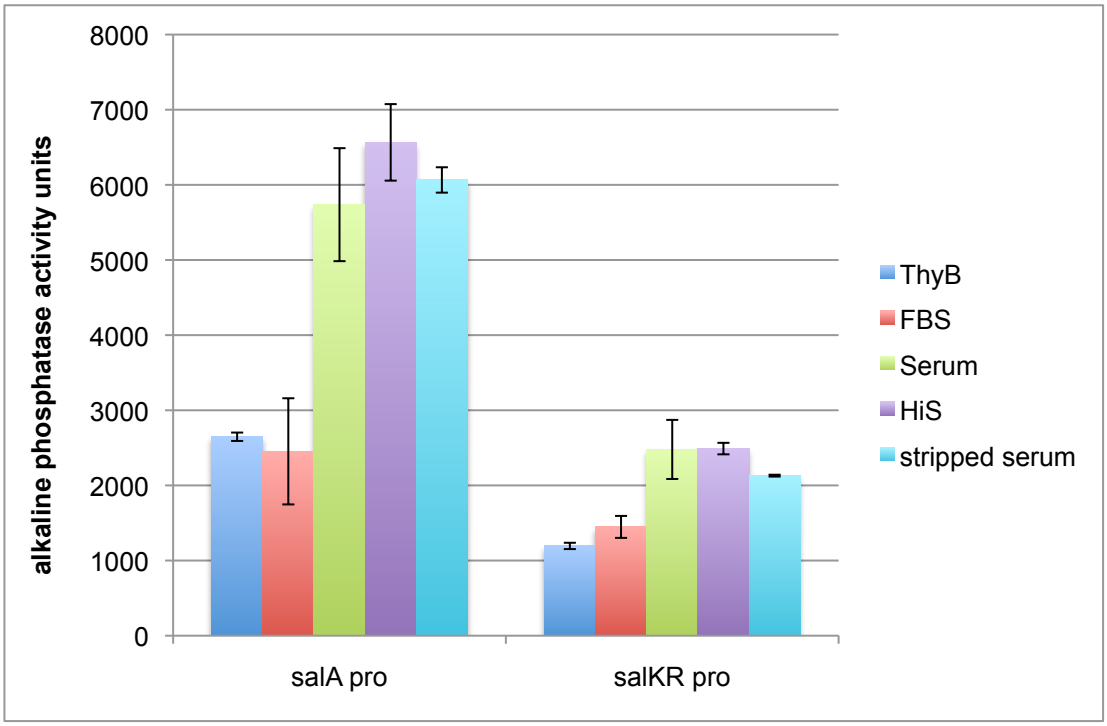


Figure 26. The *sal* promoter expression. Alkaline phosphatase activities produced by WT *S. pyogenes* expressing pMNN1-*salA*pro or pMNN1-*salKR*pro-full length after 3 hours of treatment with several conditions; ThyB medium, fetal bovine serum (FBS), human serum, heat-inactivated human serum (HiS), and charcoal-stripped human serum.

DISCUSSION

This study demonstrated that there are two promoters in the *sal* locus of *S. pyogenes*, one located upstream of the *salA* gene, controlling the expression of the entire locus and another promoter located within the *salY* gene, controlling the expression of the downstream *salKRZ* genes. This organization is similar to that found in other lantibiotic loci [59, 112, 113, 126, 127]. As shown by RT-PCR the *salA* promoter provides a read-through transcript, demonstrating that the *salAMTXYKRZ* genes are co-transcribed as a single transcript. The presence of the internal promoter suggests that the expression of *salKRZ* could be regulated independently from the *salA* promoter under different physiological conditions. These two promoters were found to have different expression profiles during growth. The *salA* promoter activity was detected strongly in early mid-log phase while the *salKR* promoter had much lower activity overall compared to the *salA* promoter with the highest expression detected in late-log phase indicating differential regulation for the *salKR* promoter.

Analysis of promoter activity in truncated versions of the *salKR* promoter suggested possible repression sites located both upstream and downstream of the promoter region. Deletion of both putative repression sites resulted in a 6-fold increase in *salKR* promoter expression. These results suggest that the *salKR* promoter requires tight regulation, indicating the importance of regulated expression of the downstream genes, *salKR*, encoding the two-component

signaling system. Previous reports demonstrate that the SalKR two-component system is required for full virulence of pathogenic bacteria like *S. suis* [128] and *S. pyogenes* [61].

As a transcriptional regulator, SalR represses its own *salKR* promoter by binding directly to the identified repression sites of the promoter. This feedback loop repression suggests that this promoter is only expressed transiently under certain conditions. Our finding correlates with the microarray data from *S. pyogenes* growth in human blood reporting that the *salR* transcript is increased 50 fold after only 30 min of exposure to blood, but reduced to 1.28 fold after 60 min and then upregulated again to 30 fold after 90 min of exposure [99]. The complex regulation of the *salKR* promoter is to ensure that the downstream genes, *salKRZ*, are only expressed at the appropriate time. Further investigation is required to identify possible global regulatory functions of SalR. We propose that SalR may also regulate genes other than those in *sal* operon. This hypothesis is supported by the microarray data from *S. suis* showing several genes encoding transporters and recombination proteins to be downregulated in the absence of *salKR* [128].

In most two component regulatory systems, the response regulator is required to be phosphorylated by the cognate histidine kinase to be active. The in vitro binding assays demonstrating the binding of SalR to regions surrounding the promoter occurred in the absence of SalR phosphorylation. A similar result was reported for the recently identified BovR response regulator encoded in the

bovicin lantibiotic locus of *S. bovis* that can bind to the *bovA* promoter region in the absence of phosphorylation [114]. Non-phosphorylated SpaR of the subtilin lantibiotic locus of *B. subtilis* binds to the *spaA* and *spaF* promoters at a conserved *spa-box*, a repeated pentanucleotide sequence (A/T)TGAT separated by six nucleotides [51]. Therefore, further investigation of the SalR DNA binding sequence could allow us to determine a consensus binding sequence, possibly leading to identification of other genes that are also regulated by SalR.

Unlike other lantibiotic operons where deletion of the two component regulatory system, *lanKR*, results in abrogated expression of the *lanA* promoter and loss of lantibiotic biosynthesis [52, 54, 59, 112, 114], the deletion of *salKR* in *S. pyogenes* HSC5 did not affect the activity of the *salA* promoter. Previous reports demonstrate that none of the *S. pyogenes* serotypes, except M4, produce an active Salivaricin A lantibiotic even though *salA* transcript was present [57, 59, 129], suggesting that the peptide is produced but not modified to the active lantibiotic. Our data suggest a role for the SalA peptide in *S. pyogenes*. As demonstrated above, SalR represses its own promoter, possibly in a feedback loop fashion, and generally, feedback loop repression, such as in the Lac operon, involves a cofactor or a co-repressor [130]. In our study, we hypothesize that the SalA peptide may play a role in co-repressing the *salKR* promoter as suggested by the results from the promoter expression analysis when in the absence of *salA, salKR* promoter expression was increased. This effect appeared to be additive as a double deletion strain of *salA* and *salKR* showed the highest

promoter expression (data not shown). For this to occur, localization of SalA should be intracellular as opposed to extracellular. However, we have not yet been able to detect SalA as either intracellular or extracellular using an antibody against a synthesized SalA peptide (data not shown). This suggests that SalA may be modified by *S. pyogenes* and therefore cannot be recognized by the antibody.

Mapping of the transcription start site of the *salKR* promoter revealed a well-conserved -10 region (TATAAT) with a putative (TgGgCA) -35 region. The promoter also contained the consensus sequence for an extended -10 promoter region (TNTGNTATAAT) with one underlined nucleotide mismatch to that in *S. mutans* but consensus to that in *S. pneumoniae* [131, 132]. The extended -10 promoter region is also found in front of other lantibiotic promoters, including the *mutR* promoter of the *S. mutans* mutacin II operon and the *nisR* promoter from the nisin locus in *Lactococcus lactis* [112, 133]. Interestingly, while the *nisR* promoter is constitutively expressed, the *mutR* promoter is controlled by an upstream repeat sequence that is a proposed consensus binding site for a LytR family regulatory protein [132]. The complex transcriptional regulation of lantibiotic two-component systems is also in evidence in the subtilin locus of *B. subtilis*. In this locus, the *spaKR* promoter is regulated by an alternative sigma factor H, which is in turn inhibited by a transition state regulator, ArbB [52]. Therefore, the regulation of *lanKR* promoter regions appears to be specific for each operon. Notably, the regulation of the *salKR* promoter is not affected by the

S. pyogenes global regulators CovR [95], Mga [134] or Rgg [38] as *salKR* promoter expression did not change when analyzed in strains carrying mutations in these regulators under the conditions tested (data not shown). However, this does not rule out a role for these regulators *in vivo*.

Both the *salA* and the *salKR* promoters show increased promoter activity when grown in media containing human serum. The signaling molecule(s) responsible for the increased expression are heat-stable, non-hormonal molecules that are unique to human serum, as the same result was not observed with fetal bovine serum. This response suggests that the *sal* locus in *S. pyogenes* is involved in sensing and responding to host-pathogen interactions in vivo, which is supported by the increased expression of the *sal* locus genes observed during an acute and chronic macaque pharyngitis infection model [96] as well as during growth in human blood [99].

In conclusion, this study demonstrated that even in the absence of production of a functional lantibiotic, the *sal* locus genes of *S. pyogenes* are expressed and differentially regulated. The complex regulation observed for the *salKR* promoter suggests the importance of the appropriate expression of the downstream genes, *salKRZ*. Several bacteriocin-like loci found in bacterial pathogens have been implicated in virulence, such as the *sag* operon in *S. pyogenes* [20], the *salKR* locus in *S. suis* [128], the Enterococcal cytolysin in *E. faecalis* [55, 135] and the Bsa bacteriocin locus in *S. aureus* [136]. Therefore,

further study of the role of the *S. pyogenes sal* locus in vivo is necessary to fully understand pathogenesis during host-pathogen interaction*s*.

CHAPTER 3

GENERAL CONCLUSIONS

In order for *S. pyogenes* to adapt, persist and disseminate to various sites of infection within the host requires an ability to regulate multiple virulence genes in response to changes of environmental signals. The role of two-component regulatory systems in responding to environmental stimuli to affect overall bacterial cell modification by global differential gene expression is well established. One of the mutants identified in a transposon mutagenesis screen [61] has a transposon inserted in the *salK* gene, encoding a sensor kinase of a two-component regulatory system. The *salK* transposon mutant (*salK*Ω) is highly attenuated in the zebrafish infection model. The *salK* gene is located within the salivaricin locus (*sal* locus), a lantibiotic locus encoding genes that regulate and function in lantibiotic production [59]. While the putative functions of the *sal* locus gene products have homology to lantibiotic producing proteins, *S. pyogenes* does not produce an active salivaricin A nor is it immune to salivaricin A produced by *S. salivarius* [59, 121]. Interestingly, even without the lantibiotic-producing functions, this locus is highly conserved among different *S. pyogenes* serotypes. Furthermore, the genes in the *sal* locus of *S. pyogenes* were highly expressed during an acute or persistent form of pharyngitis [96] as well as during exposure to host factors such as polymorphonuclear leukocytes (PMNs) [45] and

human blood [99]. This data, together with the observed attenuation of the *salKΩ* mutant in zebrafish, is consistent with a role for SalK in the virulence of *S. pyogenes*.

Two component systems generally are composed of a sensor kinase that interacts with a cognate response regulator. This is also true in the *S. pyogenes* salivaricin locus with the SalK sensor signaling kinase and the cognate regulatory protein, SalR. Previous to our STM screen, there are no reports of the *S. pyogenes* SalKR playing a role in virulence. Only one previous report showed that the SalKR homologs in *S. suis* play a role in resistance to PMN-mediated killing during a piglet infection [128]. Interestingly, *S. suis* carries only the *salKR* genes with no other homologous genes from the *sal* locus suggesting that it acquired the *salKR* genes horizontally and subsequently evolved a function in virulence. An in-frame deletion of the *salKR* genes in *S. pyogenes* conferred a similar attenuation as that observed in the *salKΩ* mutant. Therefore, the original attenuation was not due to a polar mutation on downstream genes. The data presented here are the first to report a virulence role for SalKR in *S. pyogenes*.

An *in vivo* infection in the zebrafish model resulted in a similar necrotic lesion developing at the site of injection between the wild type and the *ΔsalKR* strain. The two strains produced the same level of proteolytic enzyme, SpeB, which is responsible for the necrotic lesion. However, the mutant demonstrated an attenuated ability to disseminate to the spleen. Further investigations revealed that the attenuation of the *ΔsalKR* mutant strain was due to the inability to survive

in human whole blood because of its susceptibility to neutrophil killing, specifically to cell lysis by lysozyme. The major lysozyme resistance mechanism of some Gram-positive pathogens is bacterial cell wall modification. The sensitivity to lysozyme of the *salKR* mutant suggested that its cell wall modification process is different from the wild type strain. The cytochrome C binding assay showed that the overall net charge of the cell wall of the wild type and the *salKR* mutant strain were different, confirming the dissimilarity of their cell walls. *S. pyogenes* possesses the cell wall modification genes, *pgdA, tagO*, *oatA* and *dltA*, that contribute to lysozyme resistance in other Gram-positive bacterial pathogens [79, 103, 104, 106, 137]. Genes in the *dlt* operon of S. pyogenes, involved in D-alanylation, are concomitantly expressed with the genes of a two component system, *ihk/irr,* during phagocytosis by neutrophils [44]. The D-alanylation process was shown to increase *S. pyogenes* resistance to the neutrophil-derived cationic antimicrobial peptides, defensins, LL-37, CathepsinG and lysozyme [77, 78, 138]. However, the Ihk/Irr system has been proven to particularly respond to reactive oxygen species (ROS) produced by activated neutrophils. *ihk/irr* expression was induced by exposure to H_2O_2. The *irr* mutant exhibited sensitivity to ROS as well as cationic-antimicrobial peptides [44]. In addition, the genes that encode proteins involved in cell wall formation, which are regulated by the Ihk/Irr system may be directed towards resistance to ROS, which could also have an effect on the resistance to antimicrobial peptides. Conversely, the *salKR* mutant was not more sensitive to H_2O_2 and cationic-

antimicrobial peptides than the wild type strain. As a regulatory protein proven to specifically bind to DNA, SalR is most likely playing a role in differential gene expression that would result in cell wall modification directly towards a lysozyme resistance mechanism. Further analysis is required to determine the exact mechanisms regulated by SalKR. As dissemination to different sites of infection is a crucial step for systemic infection by *S. pyogenes*, the *salKR* mutant lacks the ability to survive in the presence of neutrophils, which results in a loss in the ability to disseminate and cause invasive disease.

Tight regulation of virulence gene expression is essential for a successful systemic infection when bacteria encounter the harsh environment created by the host immune response. In addition, an adaptive response triggered by specific host environments allows the systemic pathogen to be protected from subsequent insults. One important finding from this study was the onset of an intracellular adaptation process initiated by *S. pyogenes* while in macrophages, which allowed an increased resistance to subsequent *in vitro* neutrophil killing. This adaptation was shown to be SalKR-dependent. Of note in these assays was the observation that the percent survival of bacteria in neutrophils from the post-DMEM treatment was relatively lower than the previously described neutrophil experiments where cultures were first grown in THY media. This difference was possibly due to the growth phase of the inoculum as well as the nutritive environment. While the original neutrophil experiment was done with mid-log phase cultures grown in a rich medium, post-macrophage and post-DMEM

cultures required late stationary phase growth, ~20 hour after infection, in order to have the same inoculum. These results demonstrated that late stationary phase cultures do not survive neutrophil killing as well unless the culture has previously been passaged through macrophages. This may be due to several virulence factors having growth phase-dependent expression [139-141]. The mid-log phase culture of *S. pyogenes* has the highest expression of Mga, a 'stand alone' regulator, which was shown to regulate gene products related to adhesion and internalization. At this stage, the bacteria produce a high level of M-protein, capsule and C5a peptidase. Expression of Mga is shut off once the cell enters stationary phase and other growth phase regulators take over the control of gene expression, resulting in differential virulence factor production [140, 141]. Multiple *S. pyogenes* two-component regulatory systems, CovRS, Ihk/Irr and FasBCAX, are expressed during stationary phase of growth [141]. The expression of the *salKR* promoter tested in this study is not under the influence of either Mga or CovR, although the expression profile of the *salKR* promoter showed the highest expression at late log phase, going into stationary phase. The survival difference observed in the neutrophil assays is possibly due to differences in overall virulence gene production based on a particular growth phase. Resistance to the host immune response is not dependent on one particular virulence factor but the overall differential gene expression resulting in bacterial adaptation [141].

Gene regulation in the *sal* locus was also investigated in this study. There were two promoters identified, the *salA* and *salKR* promoters. We initially

detected expression of the *sal* locus gene transcripts during growth in THY media. The *salA* promoter provides a read-through transcript for the entire locus. Although *salKR* promoter activity was observed at a very low level, the *salKR* genes would still be expressed through the *salA* promoter allowing SalK to be produced and present on the cell surface to sense its cognate signal. The *salKR* promoter is located within the *salY* gene and upstream of *salKR* providing differential regulation separate from the *salA* promoter. Thus SalKR can be independently produced under different conditions regulated by its own SalR repression in a feedback loop fashion. SalR demonstrated DNA binding activity towards its own promoter by specific binding to both upstream and downstream regions of the promoter. Moreover, the finding that *salR* gene expression increased ~50 fold after a 30 min exposure to human blood followed by an immediate decrease in expression to ~1 fold after 60 min of exposure [99] supports our hypothesis of a feedback loop repression by SalR to the *salKR* promoter.

Notably, the identified SalR binding sites were not in close proximity to the -10 or -35 promoter regions of the *salKR* promoter. Therefore the repression was not due to interference with accessibility by RNA polymerase as described for other repressors such as LacI or LexA [142]. However, it has been shown that it is not necessary for repressors to bind within the promoter region to inhibit transcription. The CytR repressor binds at the -70 region upstream of the *E. coli deo* promoter and forms a complex with CRP resulting in inhibition of

transcription at this promoter [143]. The heat-stable nucleoid structure proteins (H-NS) were shown to form a tetrameric repression complex that causes DNA looping. At the *S. flexneri virF* promoter, H-NS dimerizes at the -1 bp and -250 bp, causing DNA bending through protein-protein interactions [144]. H-NS has also been shown to bind further downstream of the promoter creating a "Zip-up" mechanism of the DNA flanking the promoter and blocking transcription [145]. Furthermore, in the colicin bacteriocin locus, the Lex-A repressor binds directly to the operator region and represses transcription as a result of DNA bending [146, 147]. Therefore, it is possible that the repression mechanism used by SalR could be alteration of DNA topology in the regions around the promoter preventing transcription initiation through the bending of DNA. The sophisticated regulation of the *salKR* promoter showed that the expression of *salKR* is tightly regulated and crucial for virulence. This may explain why we are unable to complement the *salKR* mutant phenotype by simply expressing *salKR* from a constitutive promoter on a plasmid. In addition, expression from a plasmid could result in overexpression of the proteins, which could also be problematic for regulation. The native promoter, along with the regulatory sites, is necessary for the precise transcription of the *salKR* promoter. Further analysis is required to confirm that the mechanism used for repression by SalR is DNA bending through specific binding at identified sites on the *salKR* promoter.

Repression of the *salKR* promoter was also influenced by *salA*. Even though the function of SalA as a lantibiotic is lost in the salivaricin locus of *S.*

pyogenes, the data presented in this study suggest that SalA may function as an intracellular signaling molecule or possibly a co-repressor towards *salKR* promoter activity. Both SalA and SalKR have an additive effect on the repression of the *salKR* promoter. The absence of SalA in the *salA*-IFD strain increased adherence of *S. pyogenes* to macrophages suggesting that SalA, either directly or indirectly, also has an effect on the bacterial cell surface. SalKR was also shown to be involved in bacterial cell wall modification as demonstrated by the change in cytochrome C binding and resistance to lysozyme. Together, the data indicate the involvement of genes in *sal* locus in cell wall alteration in response to the host immune response.

The data from this study also emphasized the importance of resident tissue macrophages in *S. pyogenes* adaptation to the host response. Previous reports have shown that at the early stages of invasive Group A streptococcal infection, the majority of the bacteria were localized intracellularly in macrophages [65]. After the disease progressed, both intracellular and extracellular bacteria were found at the tissue infection site [65]. We propose the following model for the role of the *sal* locus in systemic infection by *S. pyogenes*. As the skin surface is a normal reservoir for Group A streptococcus, breakage of the skin barrier will allow the bacteria to enter into the underlying tissues. Normally, the bacteria at the skin surface are at the stationary phase of growth due to the limiting nutrients on the skin surface. SpeB is highly expressed at the site of infection as well as during stationary phase of *S. pyogenes* [65, 98]. Host

cell lysis by *S. pyogenes* cytolysins will allow bacteria access to nutrients. SpeB can also act as a protease to cleave host antimicrobial LL-37 [98] as well as bacterial virulence factors, like M-protein [148, 149], which may facilitate phagocytosis by resident macrophages. Because the bacteria are resistant to killing by macrophages, the intracellular environment provides a safe haven for the bacteria to multiply as well as the signals to trigger upregulation of virulence gene expression that is required for systemic infection. After this mechanism of adaptation, extracellular bacteria could be observed as early as 4 hours after macrophage infection. These extracellular bacteria are more resistant to neutrophil killing, which allow bacteria to travel freely in the blood stream and disseminate to different sites of infection. During exposure of *S. pyogenes* to human blood, a strong increase in *salR* gene expression suggests another stage of adaptation. Both SalA and SalKR are involved with a cell wall modification process, which may play a role in the bacteria becoming more resistant to lysozyme from neutrophils. Taken together, the results of this study suggest that the salivaricin locus plays a key role in responding to the host environment by regulating genes required for survival in the host. However, the role of SalKR as a two-component regulatory system requires further investigation to better understand the regulatory role of SalR during host-pathogen interactions and its contribution to the pathogenesis of *S. pyogenes*.

In conclusion, it is not a surprise to find that the *S. pyogenes* lantibiotic locus functions are involved in virulence. Several other Gram-positive bacterial

pathogens also exploit a lantibiotic or bacteriocin locus to survive in a competitive environment during host-pathogen interactions. For example, the *sag* operon in *S. pyogenes* [20], the *salKR* system in *S. suis* [128], the Enterococcal cytolysin in *E. faecalis* [55, 135] and the Bsa bacteriocin locus in *S. aureus* [136] all have homology to lantibiotic/bacteriocin loci and are all implicated in virulence. In this study we demonstrated that the lantibiotic locus in *S. pyogenes* has clearly functionally evolved towards surviving in a competitive environment created by the host immune response. These results provide further evidence of the plasticity of bacteria, which are able to adapt and evolve to their environmental niche. In keeping with the emerging antibiotic resistant superbugs, resulting in high mortality and morbidity, the emergence of severe invasive bacteria has also become a growing threat in this past decade. Therefore, we are pitted in a race against constant bacterial evolution for a better understanding of their pathogenesis in an effort to develop more effective treatments and preventative care.

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ABSTRACT

IDENTIFICATION OF THE ROLE OF THE SAL LOCUS IN *STREPTOCOCCUS PYOGENES* VIRULENCE DURING HOST-PATHOGEN INTERACTIONS

by

PHANRAMPHOEI NAMPRACHAN-FRANTZ

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Advisor: Melody N. Neely

Major: Immunology and Microbiology

Degree: Doctor of Philosophy

The pathogenesis of *Streptococcus pyogenes* is due to its ability to overcome and adapt to the harsh environment created by the host immune response. The focus of this project was the SalKR two-component regulatory system, which facilitates bacterial adaptation by responding to environmental signals during host pathogen-interactions. The first goal of this project was to determine a role in virulence for the SalKR regulatory system. The complete deletion of the *salKR* genes in the wild type *S. pyogenes* strain HSC5 produced a highly attenuated mutant in a Zebrafish infection model. The *ΔsalKR* mutant appeared to lose the ability to survive in the blood stream, which consequently inhibited systemic dissemination to the spleen. The attenuation of the *ΔsalKR*

mutant in the blood stream was due to its sensitivity to intracellular and extracellular killing by neutrophils. This was most likely a consequence of the high susceptibility of the *ΔsalKR* mutant to cell lysis by lysozyme, attributable to the differences observed in cell wall modification between the wild type and the mutant. Our data suggests that this modification is SalKR dependent. The SalKR system was also involved in a cellular adaptation process while residing intracellularly in macrophages resulting in greater resistance to neutrophil killing.

The second goal of this study was to identify the regulation of *salKR* gene expression. Transcriptional analysis of the *sal* locus revealed that expression of the *salKR* genes are governed by two promoters, the *salA* promoter and the *salKR* promoter. SalR regulates its own promoter by binding directly to repression sites identified both upstream and downstream of the *salKR* promoter. The SalA peptide, encoded by the *salA* gene at the 5' end of the *sal* locus also affected *salKR* promoter activity. Both the *salA* and *salKR* promoters showed increased promoter activity during exposure to human serum. Taken together, the data suggested the importance of the SalKR two component system in virulence and its requirement during host-pathogen interactions.

AUTOBIOGRAPHICAL STATEMENT
Curriculum Vitae

PHANRAMPHOEI NAMPRACHAN-FRANTZ

EDUCATION:

Bachelor of Science in Microbiology, March 2000
Chulalongkorn University
Bangkok, Thailand

Master of Science in Microbiology, December 2004
Michigan State University
East Lansing, Michigan

PhD. In Immunology and Microbiology, May 2011
Wayne State School of Medicine
Detroit, Michigan

HONORS/AWARDS:

2001-2004 The Royal Thai Government Scholarship, The Ministry of Science and Technology, Thailand

POSITIONS AND EMPLOYMENT:

2005 Research Assistant, Wayne State School of Medicine, Department of Physiology, Lab of Jeffrey L. Ram, Detroit, Michigan

2005-2011 Doctoral Candidate, Wayne State School of Medicine, Department of Immunology and Microbiology, Lab of Melody N. Neely, Detroit, Michigan

2011-present Research Scientist, The National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand