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Adaptive Mechanisms of Niche Remodeling in Streptococcus pyogenes

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ABSTRACT OF THE DISSERTATION

Adaptive Mechanisms to Niche Remodeling in *Streptococcus pyogenes*

By

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Professor Michael Caparon, Chairperson

The Gram-positive bacterium *Streptococcus pyogenes* is a remarkably successful pathogen, capable of infecting numerous tissue sites within its human host. The ability of *S. pyogenes* to invade these different niches is, in part, due to the species' ability to monitor various physical and chemical signals in its environment and alter its transcriptional profile in response to these differential conditions. As a member of the lactic acid bacteria, *S. pyogenes* has a simple fermentative metabolism and relies exclusively on a combination of homo-lactic and mixed acid fermentation as a means of generating energy in the cell. As a consequence of its fermentative metabolism, *S. pyogenes* produces several organic acid end products that, over time, accumulate in the surrounding environment, causing a substantial reduction in pH. Thus, growth of the bacterium itself results in a significant remodeling of its local tissue environment. It also indicates that over the course of infection, it must both adapt to its self-inflicted acid stress as well as exploit alternative carbon sources for survival. Although pH has been identified as a signal utilized by *S. pyogenes* to induce global transcriptional changes, the specific regulatory mechanisms behind this transcriptional remodeling have largely remained unclear. To further

characterize the process of *S. pyogenes*' pH adaptive response we have identified several novel pH-sensitive transcriptional regulators and analyzed their contribution to gene expression and *S. pyogenes* pathogenesis.

The malic enzyme pathway, which allows the cell to utilize malate as a carbon source for growth, consists of four genes in two adjacent operons, with the regulatory TCS MaeKR being required for the expression of the genes encoding a malate permease (*maeP*) and malic enzyme (*maeE*). Results show that expression of the *maePE* operon is influenced independently by external malate concentrations and pH in a MaeK-dependent mechanism. The ME genes are additionally regulated by a unique CcpA-independent form of catabolite repression which involves the PTS proteins PtsI and HPr. Furthermore, *in vivo* experiments demonstrate that loss of any individual ME gene has a significant effect on the outcome of a soft tissue infection.

The secreted toxins SPN and SLO have been shown to contribute to *S. pyogenes* cytotoxicity and virulence in multiple models of pathogenesis, however little information is known about the specific regulatory mechanism that control expression of these toxins. Our work has determined that the growth-phase pattern of expression of the *spn*/*slo* operon is regulated by environmental pH. Additionally, this regulation requires both the CovRS two-component system as well as an additional protein, RocA. Additional data suggests that RocA does not function as a traditional histidine kinase, despite high structural and sequence homology to known histidine kinases such as CovS. However, all three regulatory proteins are required for the pH-mediated regulation of this virulence operon.

Chapter I

Introduction

Overview of *Streptococcus pyogenes* and streptococcal diseases

Streptococcus pyogenes (GAS) is a Gram-positive, chain forming bacterium that is able to invade numerous different tissue sites within the host. *S. pyogenes,* a strict human pathogen, is responsible for numerous superficial and systemic diseases and is believed to cause a wider range of human diseases than any other bacterial pathogen (3-7).

S. pyogenes primarily causes superficial, self-limiting infections of the skin (impetigo) and throat (pharyngitis) (3, 7, 8). These diseases are typically limited to the initial site of infection and do not invade deeper tissues. *S. pyogenes* is the most common causative agent of pharyngitis, with approximately 600 million cases annually (11). The symptoms of pharyngitis include a sore throat and sudden onset fever. The sore throat is due to inflammation of the tonsils and pharynx, often with patchy exudates and enlarged lymph nodes (11). Pharyngitis is typically spread directly from person-to-person contact through nasal secretion or saliva droplets from infected individuals. Incidence of pharyngitis is highest in crowded places, such as schools, where approximately 15% of school children will contract the disease in developed countries (12). Impetigo is caused by a *S. pyogenes* infection of the skin, leading to the formation of large pustules that, once ruptured, will form thick, honey-colored scabs (11). This disease is spread by direct skin contact and is most often seen in children living in tropical or subtropical climates and in areas with poor hygiene (11). Despite the increasing numbers of antibiotic resistant pathogens, *S. pyogenes* remains sensitive to penicillin, and this is generally the first line of treatment for these superficial infections (13).

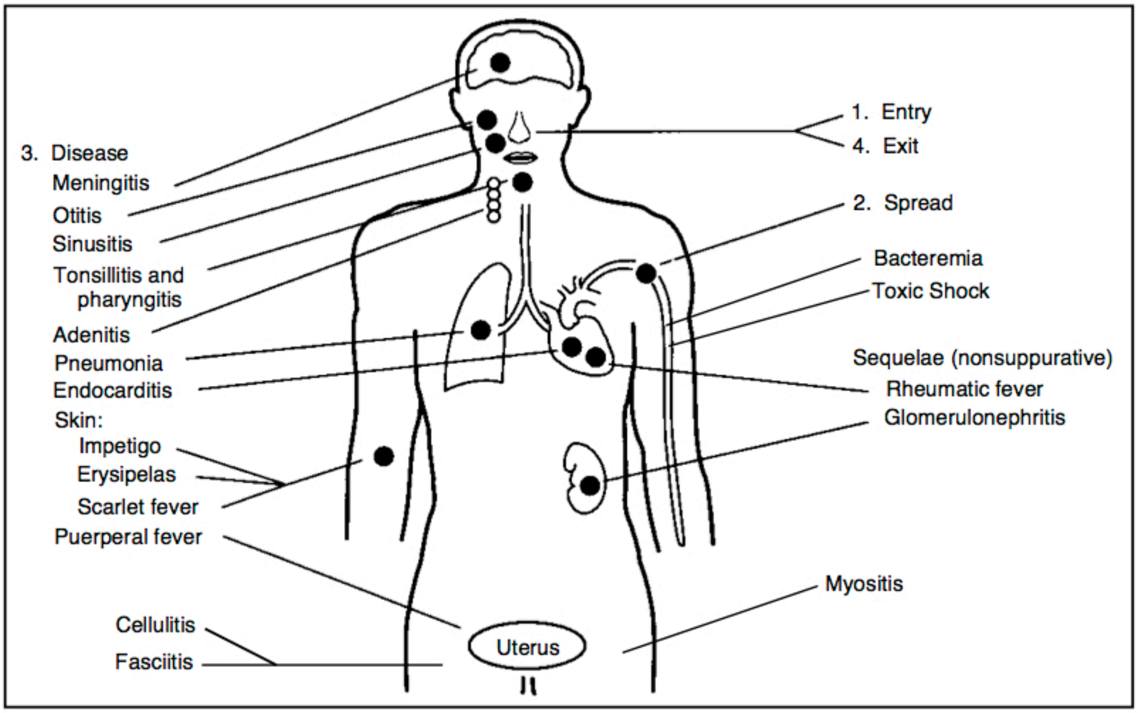

Figure 1. Pathogenesis of *S. pyogenes*. Diagram of host tissue sites and the resulting diseases caused. Adapted from (9).

Less frequent than the superficial infections of the skin and throat, *S. pyogenes* can also breach the epithelial barriers to cause a number of invasive diseases. These types of infections have a high morbidity and mortality rate, where approximately 8-23% of invasive infections lead to death within 7 days (14-16). The most common systemic diseases caused by *S. pyogenes* are cellulitis and bacteremia (11). Although less common, *S. pyogenes* can also cause necrotizing fasciitis and streptococcal toxic shock syndrome (STSS) (17). In the case of necrotizing fasciitis, antibiotic treatment with penicillin has little effect on the spread of the disease, suggesting that the release of bacterial toxins, not growth of the bacteria itself, is the main contributor of this disease (18). The main treatment for necrotizing fasciitis is surgical debridement of infected tissue, however mortality rates for this type disseminating disease is quite low (less than 20%) (11, 14-16).

Additionally, prior *S. pyogenes* infections can lead to a number of postinfectious sequelae, which include diseases such as acute rheumatic fever (ARF) and acute poststreptococcal glomerulonephritis (APSGN) (7, 11, 12). ARF, which can occur as a result of an untreated pharyngeal infection, can cause inflammation of the joints, heart, or neurological symptoms (17, 19). ARF is a major source of morbidity and mortality worldwide, causing long-term damage to the heart (rheumatic heard disease or RHD). As a result, RHD is the most common cause of pediatric heart disease worldwide, with over 2.4 million cases in children ages 5 to 14 (11, 19). APSGN results from an immune complex-mediated disorder that affects the kidneys. Symptoms of this disease include edema, hypertension, and urinary sediment abnormalities (11). Globally, there are over 470,000 cases diagnosed annually, with the highest rates seen in children in undeveloped countries (11). However, unlike ARF, with proper medical care, long-term damage from APSGN is rare (11).

Streptococcus pyogenes **Pathogenesis**

S. pyogenes' ability to successfully invade numerous tissue sites within its human host is, in part, due to its ability to produce a wide array of virulence factors throughout the infection cycle. These virulence factors, which include surface attached and secreted proteins, enable the bacterium to both inflict tissue damage to the host cells as well as evade the onslaught of immune factors produced by the host. The following sections will explore the function of several of the major virulence factors in *S. pyogenes* and their role in pathogenesis.

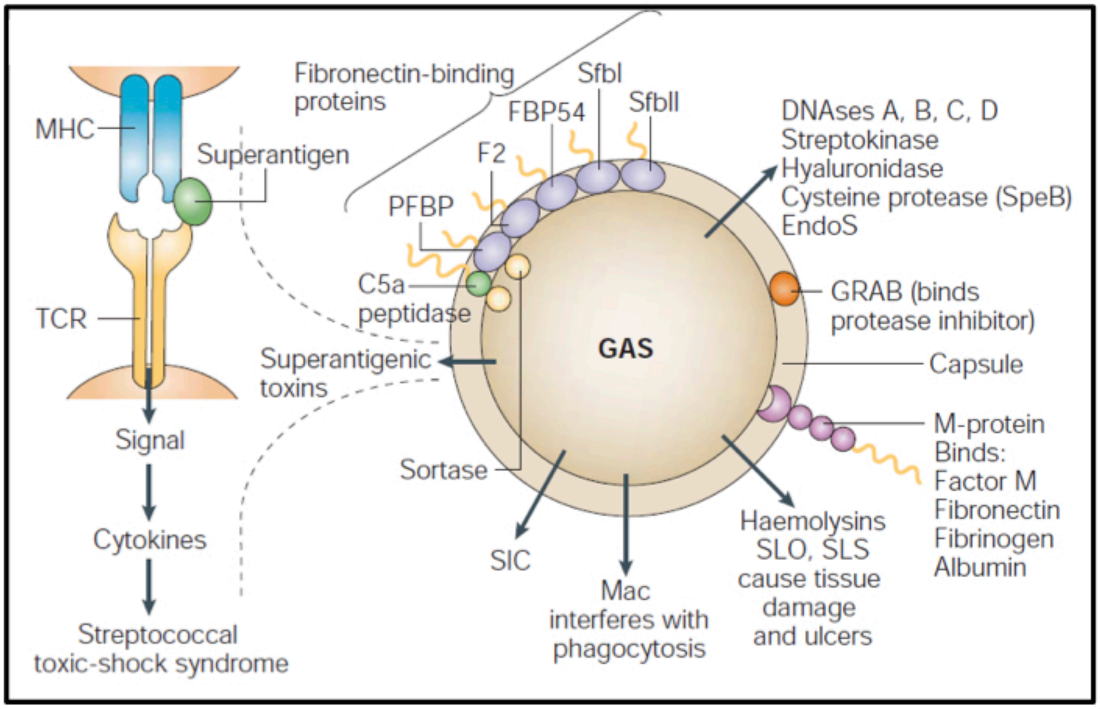

Figure 2. Virulence factors produced by *S. pyogenes*. The bacterium produces over 40 virulence secreted and surface exposed factors that contribute to adherence, tissue damage, and immune evasion. Adapted from (7).

Surface-associated virulence factors

Lipoteichoic acid (LTA)

The first step required for *S. pyogenes* to successfully invade host tissue is to adhere to human cells. Adherence is thought to be a two-step process, beginning with LTA. Bound to the surface of the bacterial cell, LTA is an amphiphilic polymer of glycerol phosphate containing glucose and D-alanine substitutes (10, 11, 20). It's thought that these polymers are involved in weak hydrophobic interactions with various host cell components. This initial interaction between the bacterial and host cell can then allow long-distance attachments and higher-affinity binding events (21).

M protein

The surface-attached M protein is one of the most well characterized virulence factors produced by *S pyogenes*. It is a fibrillar protein made up of α-helical coiled-coil dimers and is attached to the cell wall through the function of sortase and an LPXTG motif (21-24). *S. pyogenes* strains are classified by *emm* types, which are identified by the hypervariable region of the N-terminal sequence of the protein (21). Following the hypervariable region is a set of four repeat regions (A-D), where the A repeats are hypervariable and the B regions are semivariable (21, 22). Different hypervariable A regions from different M proteins have been shown to bind to C4b-binding protein (C4BP), plasminogen, IgA and IgG, and factor H (25). The B regions are necessary for binding to fibrinogen and IgG (22). The highly conserved C region can also bind factor H, as well as human serum albumin (HSA) and the host cell ligand CD46 (26, 27).

Due to its ability to interact with a number of human proteins, M protein contributes to *S. pyogenes* pathogenesis in multiple ways. Through binding to components of the extracellular matrix (ECM) such as fibronectin, it aids in adherence to epithelial cells and keratinocytes (28-33). In addition, it prevents phagocytosis by binding complement-inhibitory proteins C4BP, factor H, and factor H-like protein 1 (34-36). *In vivo* studies have shown that M protein is required for full virulence in subcutaneous mouse models of invasive disease (37).

Hyaluronic capsule

Encoded by the *hasABC* operon, *S. pyogenes* expresses a hyaluronic capsule, composed of polymers of glucuronic β-1, 3-*N*-acetylglucosamine (11). This capsule is structurally identical to the hyaluronic acid expressed on host cells and connective tissue, providing protection to the bacteria through molecular mimicry (17). Additionally, the thick capsule blocks immunological access to surface epitopes, inhibits host complement proteins and antimicrobial peptides, and prevents phagocytosis (38-41). *In vivo* studies have shown that *S. pyogenes* requires capsule for full virulence in both mouse and nonhuman primate models of invasive disease (42-46).

C5a peptidase

Encoded by the gene *scpA*, the C5a peptidase is a serine protease expressed on the surface of all GAS strains (47-51). ScpA, a subtilin-like protease, is produced as a 125 kD proenzyme, which is then cleaved to produce the active protease (11, 21, 52). The enzyme cleaves C5a, a chemotactic peptide of the complement system that is involved in neutrophil recruitment and stimulation (21, 53). In this manner, C5a peptidase interferes with the host phagocyte recruitment at the site of infection. Additionally, *in vivo* studies using a C5a peptidase loss of function mutant show that the mutant was deficient in colonization of the mouse nasopharynx compared to WT (10, 54).

Streptococcal inhibitor of complement (SIC)

SIC, one of the most polymorphic bacterial proteins known, is a 31 kDa protein that interferes with complement-mediated lysis by inhibiting the binding of the membrane attack complex (MAC) onto bacterial cell membranes (7, 55). Given that *S. pyogenes* is highly resistant to complement-mediated lysis due to its thick cell wall, the main contribution of SIC to *S. pyogenes* pathogenesis is likely not interference with MAC. Rather, SIC is able to disrupt other branches of the innate immune system including cathelicidin LL-37, α-defensins, and lysozyme (56-58).

Secreted virulence factors

<u>SpeB</u>

The cysteine protease SpeB is secreted as a 40kD zymogen, which is then autocatalytically cleaved into a 28kD active form. SpeB is one of the most abundantly produced virulence factors and its expression is regulated by numerous growth phase and nutritional cues, including carbohydrate availability, NaCl concentrations, and pH (59, 60). SpeB has broad-spectrum protease activity and has been shown to degrade a number of host proteins. Host targets of its protease activity include IgG, chemokines, complement protein C3b, and ECM components including fibrinogen (61-64). In addition, SpeB activity is responsible for cleaving several bacterial proteins, including other virulence factors such as SPN, SLO, M protein, and streptokinase, among others (61, 65, 66). For these reasons, the complex role of SpeB in promoting disease is unclear and varies by strain and by animal model.

<u>Streptokinase (Ska)</u>

Ska is a secreted enzyme that converts plasminogen (which is coated on the surface of the bacterial cell through the actions of several plasminogen binding M proteins (PAM)) to plasmin, the active form of the protein (67-71). Once active, plasmin functions as a broad-spectrum serine protease and is able to degrade blood clots, ECM components, and activate metalloproteases (72). As *S. pyogenes* is strictly a human pathogen, Ska is highly specific for human plasminogen. *In vivo* studies using humanized mice (transgenic for human plasminogen) have shown that Ska and acquisition of active plasmin is necessary for dissemination of the bacteria (73).

Superantigens

Different strains of *S. pyogenes* produce a variety of phage-encoded superantigens proteins called the streptococcal pyogenic exotoxins (Spe). This family of proteins includes SpeA, SpeC, SpeG, SpeH, SpeJ, SpeK, SpeL, streptococcal superantigen A (SSA), and the streptococcal mitogenic exotoxin Z (SmeZ) (7). Production of these superantigens is associated with severe bacterial diseases such as STSS and necrotizing fasciitis (7, 74). Superantigens bind to the β-chain of $CD4^+$ T cells and MHC class II molecules on B cells, monocytes, and dendritic cells (7, 75, 76), thereby resulting in an overstimulation of the host inflammatory response and production of large amounts of TNFα, IL-1β, IL-2, and IFNγ (7, 77). The release of these cytokines results in a drop in blood pressure and multi-organ failure, the classic hallmarks of STSS (7, 11).

Streptolysin S (SLS)

SLS is a β-hemolysin produced by the majority of *S. pyogenes* strains during stationary phase growth (78, 79) and is responsible for the beta-hemolysis seen on blood-agar plates, a classic marker for clinical identification. SLS is encoded in a highly conserved nine-gene operon comprised of genes *sagA-I* (78, 79). SLS contributes to *S. pyogenes* pathogenesis by lysing a large number of host cells, including lymphocytes and erythrocytes, among others (80). *In vitro* data suggests that SLS contributes to pathogenesis through cytotoxicity, stimulation of host inflammatory cells, and inhibition of phagocytosis (81). *In vivo,* SLS is required for full virulence in a murine model of necrotizing soft tissue infection, as infection with an SLS-deficient mutant resulted in decreases in bacterial burden, neutrophilic inflammation, and tissue necrosis (79).

SPN, SLO, and Cytolysin mediated translocation

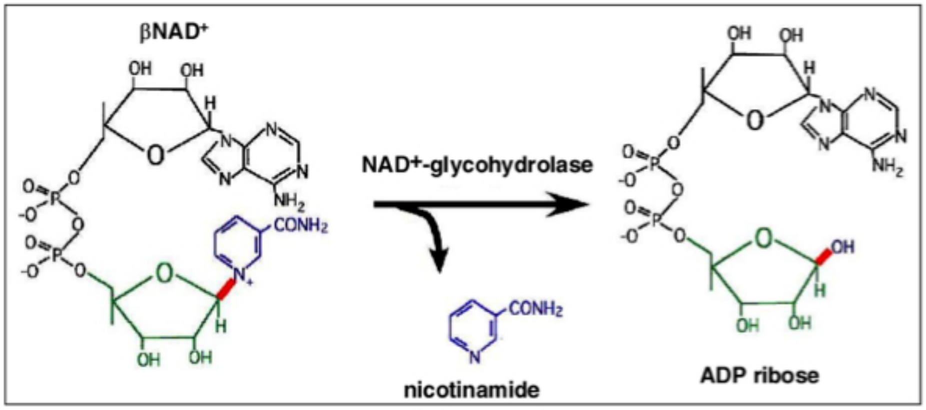

Figure 3. β-NAD+ cleavage. β-NAD+ is cleaved to form nicotinamide and ADP-ribose. Adapted from (1)

The streptococcal NAD+ glycohydrolase (SPN) is a 52kDa secreted protein that, when delivered into the host cell cytosol, cleaves β-NAD+ into nicotinamide and ADP-ribose (1, 82, 83). There are two important features that make SPN's enzymatic activity unique from other classes of NAD+ cleaving enzymes. First, SPN has been shown to be a strict NAD+ glycohydrolase and is unable to further catalyze the products from the initial reaction (1). Second, SPN is capable of cleaving β-NAD+ at an incredibly high rate, thus causing rapid depletion of β-NAD+ stores within the host cell (1, 82).

Recent studies analyzing the various alleles of *spn* have shown that this gene is evolving under positive selection, leading to a separation of two distinct subtypes, NADase positive which retains the glycohydrolase activity, and an NADase negative form (82, 84). Little is known as to the specific role of the NADase negative subtype in pathogenesis, but there is a correlation between SPN subtypes and tissue tropism. *S. pyogenes* primarily causes superficial infection of the skin or throat. Epidemiological evidence has shown that there are subpopulations of *S. pyogenes* that specialize in infections at only one of these two tissue sites (skin-specialists and throat-specialists) (85). In addition, there is a third subpopulation that can infect both tissue types (generalists) (85). An analysis of series of 113 clinical isolates demonstrated that skin or throat specialists were more likely to encode the NADase negative *spn* allele while generalist strains encoded the NADase positive *spn* allele (84). Recent work from the Caparon lab has also shown that both NADase active and inactive forms of SPN are cytotoxic to host cells, indicating that SPN's contribution to *S. pyogenes* pathogenesis involves a secondary mechanism beyond β-NAD$^+$ depletion (82).

The *spn* gene is the first gene in a 3-gene operon, which also includes the genes for immunity factor of SPN (IFS) and Streptolysin O (SLO). IFS is a small, cytosolic protein that binds to the active site of SPN, blocking its enzymatic activity while in the

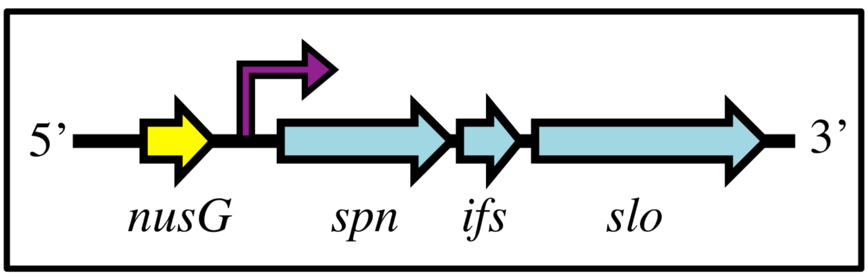

Figure 4. The *spn* operon. Organization of the *spn* operon in *S. pyogenes*.

bacterial cell (86). The third gene in this operon, *slo*, produces a cholesterol-dependent cytolysin SLO. This protein, when secreted, contributes to pathogenesis in several ways. First, SLO functions as a cholesterol dependent cytolysin. This class of proteins binds to cholesterol rich areas of host membranes, oligomerizes, and inserts itself into cell membranes to form pores (87). In this way, SLO contributes to cytotoxicity of host cells.

Secondly, a specific interaction between SPN and SLO allows for the translocation of SPN directly into host cell cytosols (83, 88-91). This process, termed cytolysin-mediated translocation (CMT), involves a complex series of interactions between SPN and SLO and a great deal of work in the Caparon lab has been performed to elucidate the mechanism behind this delivery system. From this work, several important details have been discovered about this process.

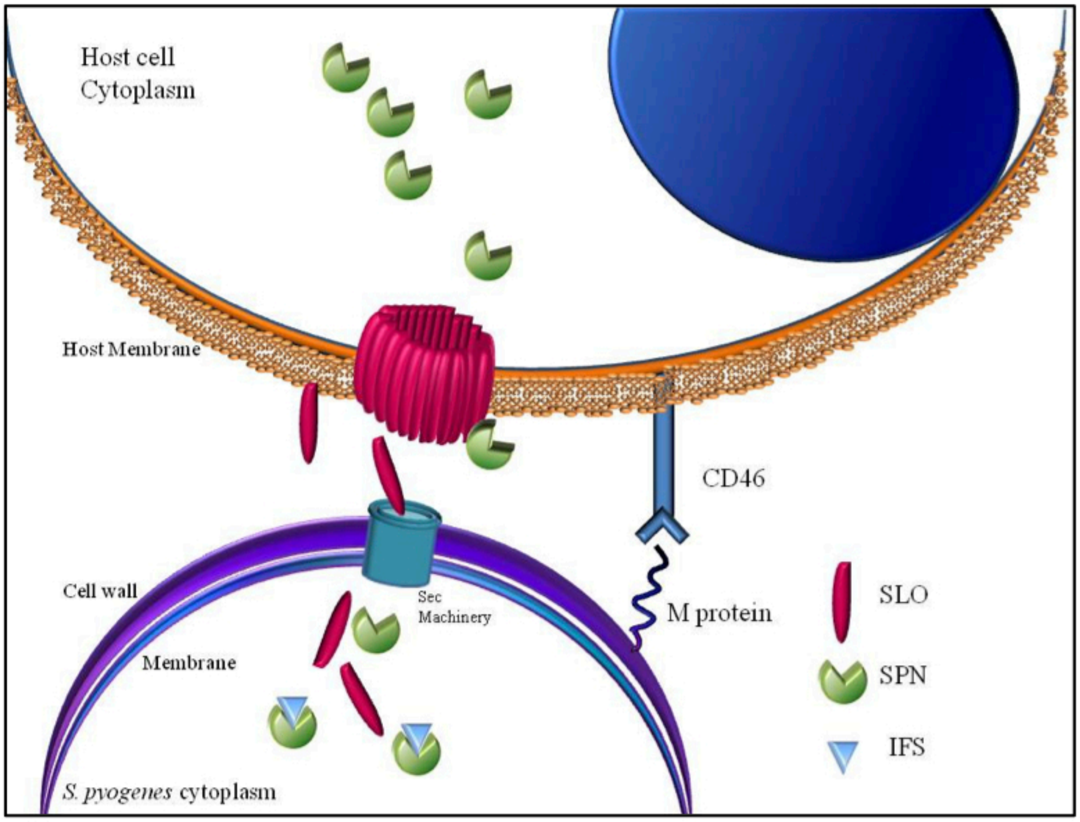

Figure 5. Cytolysin mediated translocation. SPN, IFS, and SLO are expressed during the exponential phase of growth. In the bacterial cytosol, IFS binds to the SPN active site, blocking its NADase activity. SPN and SLO are secreted through the sec machinery into the extracellular milieu. After the bacteria adhere to the host cell, SLO monomers oligomerize and form pores in the host cell membrane. The interaction between SPN and SLO at the membrane enables SPN to be translocated into the host cell cytosol. Image courtesy of S. Chandrasekaran.

First, CMT is highly specific for these two proteins. Replacement of SLO with the closely related cytolysin PFO does not allow SPN translocation (90). Second, SPN appears to be the only substrate involved in CMT (83, 89). Additionally, it has been shown that SLO pore formation is not necessary for SPN translocation to occur (90). Finally, recent work from our lab has established that SPN translocation can occur through a cholesterol-insensitive mode of membrane binding that requires both SPN and SLO for membrane binding (91). It has also been shown that both proteins play a role in cytotoxicity, as loss of either protein has reduced virulence in cultured epithelial cells and *in vivo* in a mouse model of soft tissue infection (83, 88). Taken together, these studies demonstrate that both SPN and SLO play an important role in the pathogenesis of *S. pyogenes*.

Metabolism of *Streptococcus pyogenes*

S. pyogenes is a member of the group *Lactobacillacea* or lactic acid bacteria (LAB). This group is characterized as lacking an electron transport chain (ETC) and TCA cycle. Instead, these bacteria rely solely on a mix of homolactic and mixed acid fermentation as a means of generating energy in the cell (92). *S pyogenes* is able to utilize a number of different carbon sources for growth, which can be obtained through several different pathways.

Carbohydrate Utilization

The majority of carbohydrates that *S. pyogenes* can utilize are transported into the cell through the actions of the phosphotransferase (PTS) pathway. Like most bacteria, the preferred carbohydrate for *S. pyogenes* is glucose, which can be brought into the cell and phosphorylated via the PTS system, where it then shuttles to the Embden-Meyerhof-Parnas pathway (92, 93). The breakdown of one glucose molecule through this pathway leads to the formation of two molecules of ATP, NADH and pyruvate (92). Further metabolism of pyruvate via homolactic and mixed acid fermentation allows for the re-oxidation of the NADH formed during glycolysis (92). In the absence of glucose, *S. pyogenes* is able to utilize alternative carbohydrates such as galactose. Similar to glucose, galactose utilization begins with uptake and phosphorylation through the PTS pathway. However, unlike glucose, the phosphorylated galactose molecule is broken down through the tagatose pathway, leading to formation of two three-carbon sugars, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (94). From there, these 3-carbon sugars are able to reenter the Embden-Meyerhof pathway for conversion to pyruvate.

Homolactic and Mixed Acid Fermentation

Lacking a complete TCA cycle and ETC, *S. pyogenes* relies exclusively on a simple fermentative metabolism as a means of regenerating NAD^+ necessary for additional rounds of glycolysis. The simplest and most well-known pathway for this is homolactic fermentation, the conversion of pyruvate to lactate via the enzyme lactate dehydrogenase (LDH) (95). In this pathway, each molecule of pyruvate is converted to lactate and one molecule of NADH is oxidized (92, 95). The enzymatic activity of LDH is influenced by the intracellular levels of fructose 1,6-bisphosphate, meaning that homolactic fermentation is generally only utilized when high levels of glucose are present (92, 95, 96).

As an alternative to homolactic fermentation, *S. pyogenes* can also undergo mixed acid

fermentation, a pathway that begins with the conversion of pyruvate into acetyl-CoA. In *S. pyogenes* this conversion is performed by the oxygen-sensitive enzyme pyruvate formate lyase (PFL), which converts pyruvate into acetyl-CoA and formate (92, 96). PFL, in addition to being sensitive to oxygen, is also inhibited by low intracellular levels of G3P and DHAP (92).

Therefore, homolactic fermentation is the predominant pathway utilized by *S. pyogenes* in high glucose environments. The pool of acetyl-CoA must be broken down further to regenerate NAD^+. This is achieved via the enzymes acetaldehyde dehydrogenase (ADH) and ethanol dehydrogenase (EDH), where ethanol is the end product of the pathway (92, 96). Alternatively, acetyl-CoA can be converted into acetate through the enzymes phosph otransacetylase (PTA) and acetate kinase (AckA). Although the PTA/AckA pathway does not allow for the oxidation of NADH, it does produce one molecule of ATP (92, 96). In this way, mixed acid fermentation enables the cell to balance its redox neutrality, as well as benefit from an additional source of ATP.

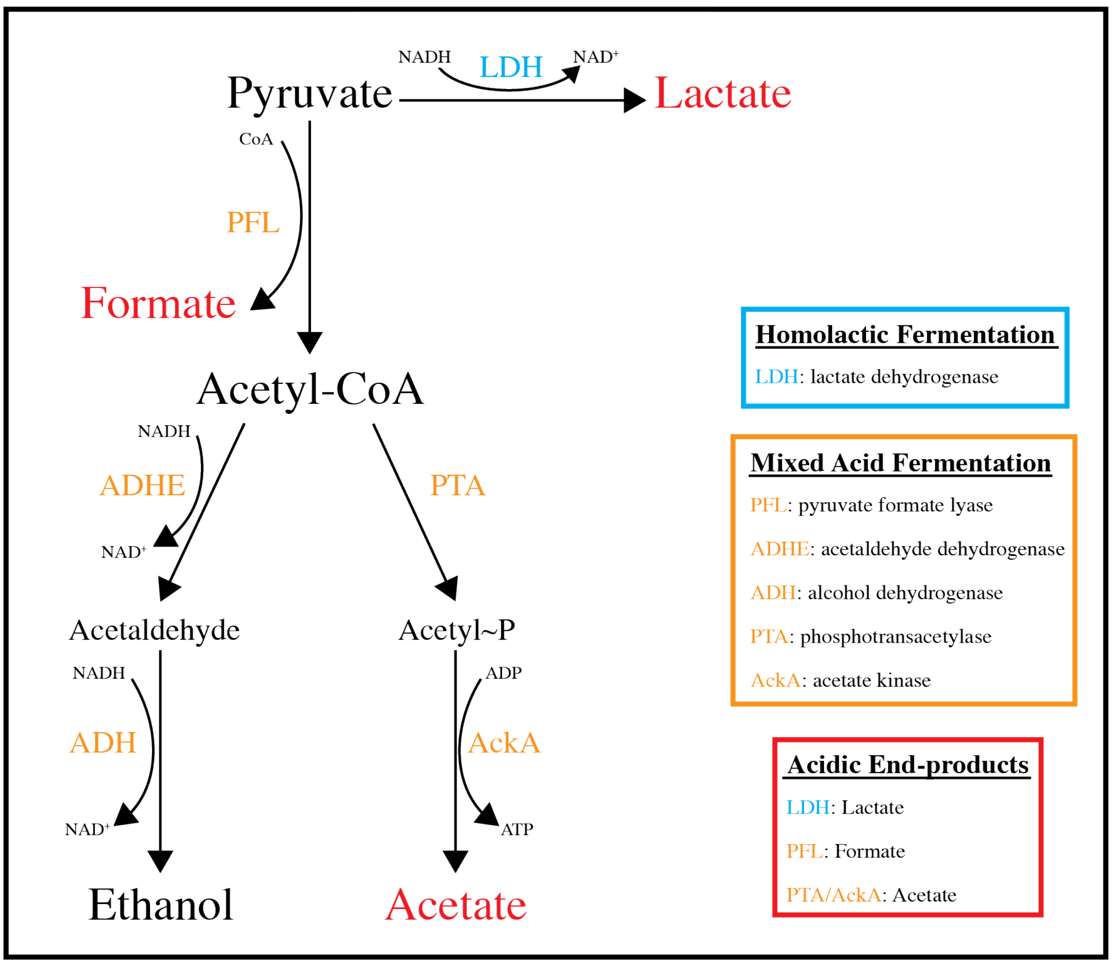

Figure 6. Fermentation pathways in *S. pyogenes*. Pyruvate, which is formed by the upper glycolytic pathway, is catabolized via homolactic or mixed acid fermentation.

Metabolism of Alternative Carbon Sources

Within the group of LAB, several pathways are present for the catabolism of various amino acids. In *S. pyogenes* the arginine deiminase (ADI) pathway has been shown to benefit the bacterium in several different capacities. This pathway enables the conversion of arginine to ornithine, ammonia, carbon dioxide, and one molecule of ATP (97, 98). Studies have shown that arginine can be utilized by the bacterium for growth, and that the production of ammonia acts as a buffering agent to counter the acid stress, which is a consequence of mixed acid fermentation (98, 99). Additionally, recent work from the Caparon lab has shown that the ADI pathway contributes to pathogenesis in a murine model of inflammatory infection of cutaneous tissue (99). It was shown that infection with *S. pyogenes* stimulates iNOS expression in cultured macrophages and that this innate immune response could be modulated by the availability of arginine. Therefore, the depletion of arginine via ArcA (the first enzyme involved in the ADI pathway) prevents production of NO□, allowing for enhanced virulence of *S. pyogenes*.

<u>Transcriptional regulation in *Streptococcus pyogenes*</u>

The ability of *S. pyogenes* to colonize and persist within its human host is dependent upon its capacity to acquire nutrients from the surrounding environment while evading host immune factors. The infection cycle of this pathogen begins with the initial colonization of the skin or throat, penetration into subcutaneous tissues, and, in the case of invasive disease, dissemination through the blood to secondary sites of infection (100). To survive and persist within each location the bacterium needs to adjust to numerous changes in the environment such as glucose levels, protein concentrations, pH, osmolarity, and temperature (100-102).

Throughout this infection cycle the bacterium must also negotiate a delicate balance between the damage inflicted on the host as a result of the virulence factors being produced and the immune response that this damage induces. Analysis of transcriptome changes of several bacterial pathogens throughout their infection cycle has suggested that careful spatial and temporal expression of virulence factors is important to the overall success of the pathogen's survival (103). Toxin production enables the bacterium to gain access to nutrients within the tissue, with cell damage being a side effect of their production. As a result, a certain level of tissue damage, and therefore immune stimulation, must occur, however, excessive toxin production could prove to be detrimental to the bacterium because of the immune response that is triggered.

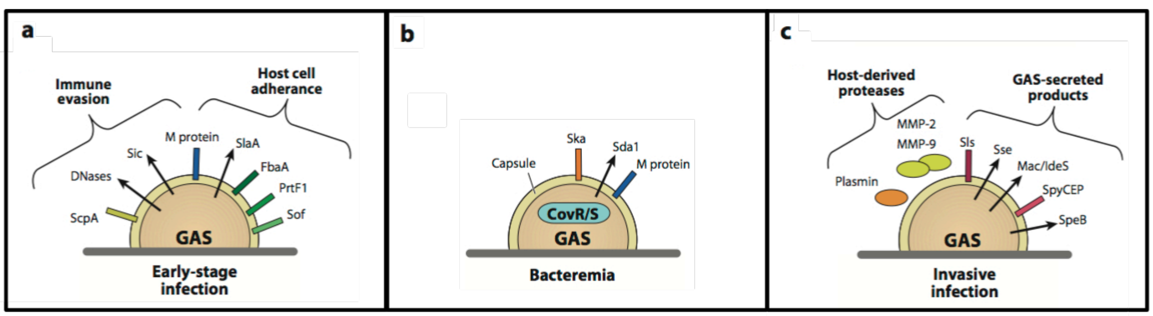

Figure 7. ***S. pyogenes*** **virulence factors are expressed in a tightly regulated temporal and spatial pattern.** **(A)** Virulence factors involved in adherence and host immune evasion are necessary during the initial stages of infection. **(B)** During the middle stages antiphagocytic factors such as M protein and hyaluronic capsule are highly expressed. **(C)** During an invasive infection numerous surface-bound and secreted virulence factors are expressed that cause direct damage to host tissue. Adapted from (10).

While *S. pyogenes* lacks alternative sigma factors, it encodes a number of two-component systems (TCS) and stand-alone response regulators that control global gene expression in response to numerous physical and environmental signals (4, 101, 104-111). The following sections will explore several of the most well characterized of these transcriptional regulators.

CovRS

The *S. pyogenes* genome contains 13 different TCSs (3, 11, 105) that are made up of a sensor histidine kinase (HK) and its corresponding response regulator (RR) (see Figure 8). The HK protein has an extracellular sensor domain and a cytosolic kinase domain, which are linked together by the protein's transmembrane domain. When a signal (or substrate) interacts with the sensor domain of the HK, this triggers activation of the autokinase domain, leading to autophosphorylation of the protein at a conserved histidine residue in the cytosolic domain. From there, the phosphate gets transferred directly to a conserved aspartate residue on the RR, leading to its activation as a DNA binding protein (112). This form of transcriptional regulation is very common in bacteria, as it allows for the cell to rapidly adapt to a particular environmental signal by altering the expression of a specific subset of genes.

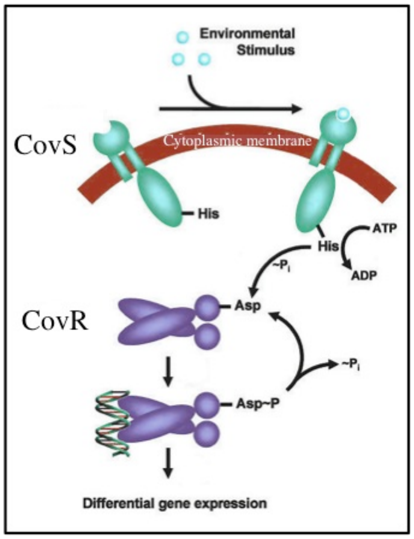

Figure 8. Bacterial two-component system (TCS).

Of the 13 identified TCS encoded by *S. pyogenes*, the control of virulence regulatory system (CovRS) is the best characterized. In this system, CovS acts as the sensor HK and CovR is its reciprocal RR (3, 11, 105). The function of the CovRS TCS is thought to mostly control genes involved in general stress response. Numerous environmental signals have been shown to trigger CovRS activation including increased temperature, low pH, high salt concentrations, high Mg^{2+} levels, LL-37, and iron starvation (105, 113-115). The *covRS* regulon includes up to 15% of the total genes in *S. pyogenes* and has been shown to be essential for survival in various stress conditions (2, 113, 116, 117).

CovR acts largely as a repressor of gene expression and is thought to bind and repress genes in both a phosphorylated and a nonphosphorylated state (118). It has been hypothesized that CovR acts on one subset of its regulon when phosphorylated and another subset of genes when in a nonphosphorylated state (118). The CovRS regulon includes a significant number of known virulence factors such as SpeB, Ska, SPN, SLO, Ig-degrading enzymes, and DNases, among others (116, 119). An important thing to note is that while CovRS acts as a repressor for most virulence factors, it is an activator for SpeB expression (11, 116).

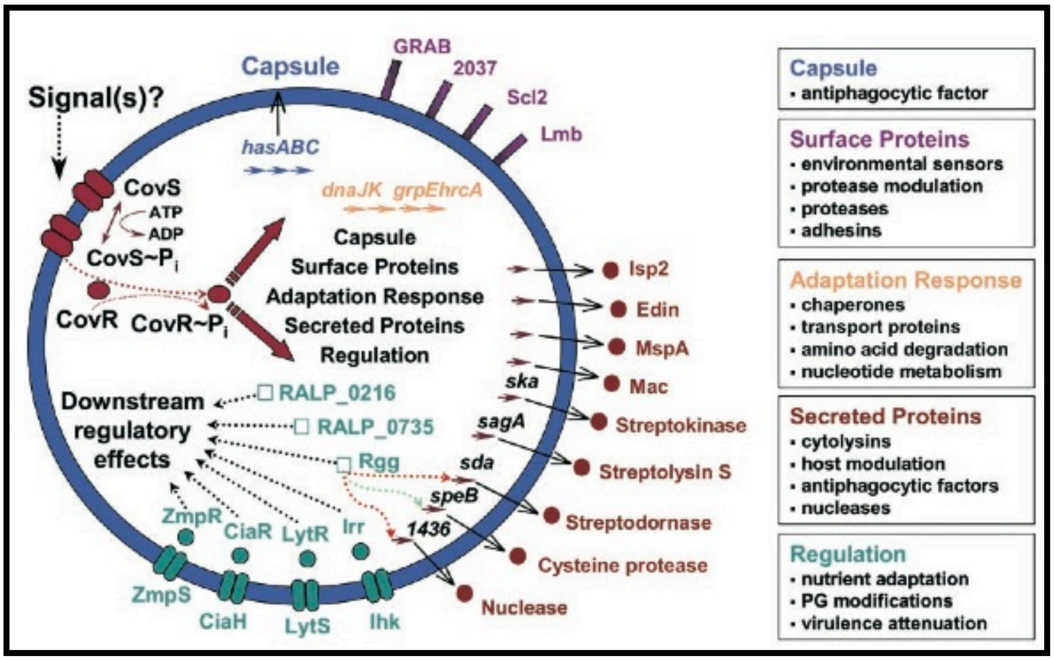

Figure 9. CovRS regulon. CovRS responds to multiple environmental signals and modulates expression of several virulence factors involved in growth and adaptation. Adapted from (2)

Strains incurring mutations leading to inactivation of either CovR or CovS are associated with hypervirulence in mice and invasive disease (11, 116, 120). This is likely due to the overexpression of numerous virulence factors that aid in tissue destruction and immune evasion (116, 121). In particular, *covRS* mutants are highly resistant to phagocytosis and neutrophil killing due to high productions of hyaluronic capsule, SIC, SpeA, Ska, and C5a peptidase (116).

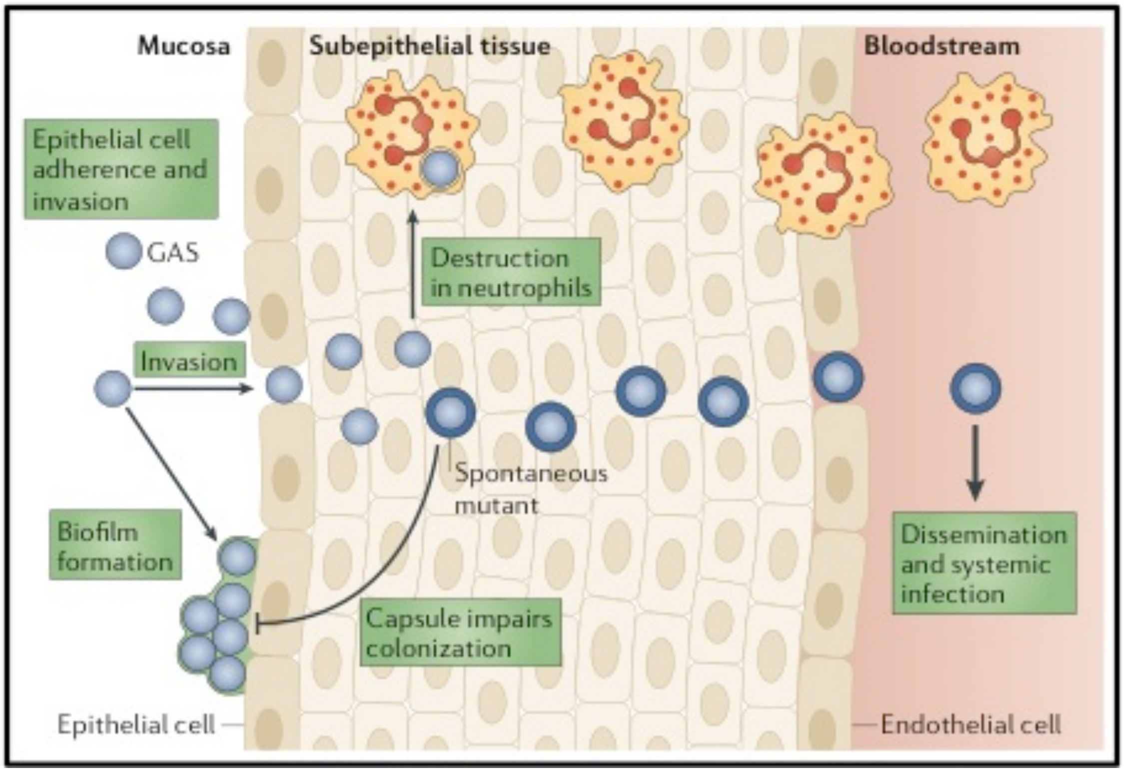

Figure 10. Model for dissemination of CovRS mutants. Spontaneous mutations in the *covRS* operon during invasion of subepithelial tissue can lead to enhanced resistance to neutrophil killing. These resistant mutants can then go on to invade deeper tissue, leading to systemic infection. Adapted from (3).

Mga

In addition to its 13 TCS, *S. pyogenes* encodes several stand-alone response regulators. One of the most well characterized of the stand-alone response regulators in *S. pyogenes*, Mga is a global transcriptional regulator and is responsible for positive regulation of target genes during exponential growth (11, 101, 109). Although the *mga* gene is present in all serotypes, there are two allelic variants of the gene. These variants have been linked to tissue tropism, where the *mga-1* allele is found mostly in throat-specialists and *mga-2* is associated with skin- specialists or “generalists” (85, 109).

Numerous growth phase and environmental signals are associated with Mga regulation including CO_2, temperature, and iron levels (122-124). Recent work has demonstrated that phosphorylation and inactivation of Mga can occur through interactions with proteins of the phosphotransferase system (PTS) (125, 126). This information provides a direct link between sugar metabolism and Mga activity.

Mga is associated with controlling expression of genes involved in colonization of host tissue and immune evasion. Genes directly activated by Mga (i.e. Mga directly binds to the promoters of these targets) are referred to as the "core" Mga regulon. Target genes within this group include adhesins (such as M protein, M-like proteins, fibronectin- and collagen-binding proteins), immune modulators (C5a peptidase, SIC, and Ig-binding proteins), and the *mga* gene itself (101, 106, 126). Beyond this core set, there are numerous other target genes whose expression is indirectly affected by Mga. These indirect targets can include virulence factors such as the *hasABC* capsule synthesis locus and *speB*, as well as genes involved in metabolism such as several PTS genes (106, 127). Additionally, there is a large amount of variation within the Mga regulon, indicating a significant amount of strain-specific regulation (109, 127). Since Mga is involved in the activation of several of the major S. pyogenes virulence factors, its role in virulence has been studied in detail. It has been shown that loss of *mga* results in defects in adherence to host cells, as well as an attenuation of virulence in murine models of invasive disease (128-131).

RopB

Another well-characterized stand-alone response regulator in *S. pyogenes* is RopB, first identified as a positive regulator of the secreted virulence factor SpeB (132). Regulation by RopB is linked to growth phase, controlling gene expression during stationary phase of growth (101, 133, 134). Analyses of the role of RopB in transcriptional regulation has shown that deletion of this regulator has a pleiotropic effect, altering the expression of genes involved in virulence, metabolism, and stress response among others (135-138). This varied response is likely, in part, an indirect effect, as RopB itself is a regulator of a number of uncharacterized transcriptional regulators (133, 139). Further complicating the role of RopB regulation in *S. pyogenes* is the fact that there is a great deal of strain specificity in the RopB regulon, with *speB* being one of the few genes that is consistently controlled by this regulator in multiple strains (133, 136).

Currently, the mechanism of activation for RopB is also unclear. RopB is classified as a member of the Rgg family of transcriptional regulators. In Gram-positive bacteria, this family is associated with quorum sensing via interactions with oligopeptide pheromones (133). However, no evidence has been reported to indicate that RopB is involved in a quorum sensing mechanism (133). Finally, the contribution of RopB to *S. pyogenes* pathogenesis is also unclear at this time. Despite both direct and indirect regulation of numerous virulence factors, there have been conflicting reports on the effects of RopB inactivation on virulence (135, 138, 140). These results are, at least partially, the result of the strain-specific nature of the RopB regulon (133).

CcpA

In Gram-positive bacteria, carbon catabolite repression (CCR) is largely under the control of the transcriptional repressor CcpA. CCR ensures that the bacterial cell maximize its fitness through the hierarchical utilization of carbon sources (141-143). In the presence of a preferred carbon source such as glucose, the molecule will be rapidly taken into the cell and degraded through the glycolytic pathway, leading to high intracellular concentrations of fructose-bisphosphate (FBP). The fluctuation of this metabolic intermediate affects the enzymatic activity of the protein HprK.

HprK, a protein found exclusively in Gram-positive bacteria, is a dual kinase/phosphatase whose role is to control the phosphorylation of the PTS protein HPr (142-144). In Gram-positives, HPr can be phosphorylated on either of two conserved residues. HprK controls phosphorylation of HPr on a specific serine residue, Ser46 in *S. pyogenes* (143, 145). At high FBP concentrations, HprK functions as a kinase, phosphorylating HPr at Ser46. This P~Ser-HPr acts as a cofactor for CcpA, binding to the CcpA dimer and inducing a structural change that is required for CcpA to bind target promoter DNA (146). When in its active conformation, CcpA will bind to catabolic-responsive elements (*cre*) sites, acting largely as a repressor of gene expression (144, 146).

CcpA has been shown to control up to 20% of the total genome of *S. pyogenes*. Global transcriptional analysis of the CcpA regulon has shown that, in addition to controlling alternative catabolic operons, it controls expression of a number of virulence factors including *speB*, *sagA*, and *cfa* (147, 148). Additionally, although a significant portion of the CcpA regulon includes glucose-regulated genes, there is a subset of genes that appear to be regulated by CcpA independently of glucose concentrations (148). This information suggests that there is a second, currently unknown, catabolite-sensing pathway involved in CcpA regulation.

LacD.1

LacD.1 was identified initially through a genetic screen as a negative regulator of the cysteine protease SpeB. It was determined that this protein repressed SpeB expression in response to neutral pH, high salt concentrations, and carbohydrate availability (60, 149). LacD.1 is annotated as a tagatose bisphosphate aldolase, an enzyme involved in the catabolism of lactose and galactose (150). In *S. pyogenes* there are two Lac loci, Lac1 (which includes LacD.1) and Lac2 (149, 151). The lac1 locus contains several truncated genes, making it unable to utilize lactose and galactose, but has evolved into a regulatory locus via LacD.1 (151). Conversely, the Lac2 locus has maintained full-length genes and is able to utilize lactose and galactose, but does not have any reported regulatory activity (151).

The specific mechanism that LacD.1 uses to regulate genes remains unclear, but it has been shown that this protein does not require its enzymatic activity for this regulation (149). It does, however, require the ability to bind to the glycolytic intermediates G3P and DHAP (149). This information has led to the hypothesis that LacD.1 functions to regulate genes in response to carbohydrate availability. Further indication of this includes the fact that a significant number of genes in *S. pyogenes* that are regulated by glucose levels are also part of the LacD.1 regulon (148). These include virulence genes like SpeB, as well as genes involved in various metabolic processes (148).

CodY

The global transcriptional regulator CodY is involved in controlling gene expression in response to amino acid starvation (102, 152, 153). CodY, which has been shown to control 17%

of the total genome through direct and indirect regulation, is activated by high levels of GTP and branched chain amino acids (BCAA) (102, 111). In the presence of high concentrations of these substrates, CodY is able to bind to DNA target promoters with high affinity, leading to repression of target genes. Conversely, when these substrates are present in low levels, as would be expected during starvation conditions, CodY is inactivated, leading to enhanced transcription of the CodY regulon. Genes identified as being regulated by CodY include transcriptional regulators such as *covRS, mga,* and *codY* itself (152). Additionally, numerous virulence factors are repressed by CodY including DNases, M protein, capsule synthesis, cytolysins SLO and SLS, and several proteases, among others (152, 153). This has led to the hypothesis that the main function of CodY is to alleviate starvation by allowing the bacterial cell to produce proteins that can aid in dissemination and macromolecular breakdown, thus providing the bacteria access to additional sources of nutrients during an infection (111, 153)

Aim and Scope of Thesis

The aim of this thesis was to provide insights into the convergence of metabolism and virulence in the pathogenic bacterium *Streptococcus pyogenes*. In particular, we sought to identify regulatory mechanisms utilized by *Streptococcus pyogenes* in response to remodeling of its local tissue environment during an infection. As a lactic acid bacterium, *S. pyogenes* utilizes a mix of homolactic and mixed acid fermentation to produce energy in the cell. As a result, several organic end products are produced and secreted, thus affecting the pH of the surrounding environment. In order for the bacteria to survive over time, it must adapt to late stage conditions of low pH and glucose depletion. Although it has been established that both carbohydrate availability and environmental pH are triggers for global transcriptome remodeling in this bacterium, the specific regulatory pathways controlling these transcriptional responses are largely unknown.

To that end, the work presented here will characterize several novel mechanisms by which *S. pyogenes* is able to adapt to its self-induced acid stress and carbohydrate depletion. The research in this thesis will describe two separate regulatory systems, one controlling an alternative catabolic pathway and one controlling an important pair of cytotoxic proteins, both of which are controlled by environmental pH. Taken together, this work provides greater insight into adaptive mechanisms utilized by *S. pyogenes* during late stages of growth.

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Chapter II

Streptococcus pyogenes Malate Degradation Pathway Links pH Regulation and Virulence

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SUMMARY

The ability of *Streptococcus pyogenes* to infect different niches within its human host most likely relies on its ability to utilize alternative carbon sources. In examining this question, we discovered that all sequenced *S. pyogenes* strains possess the genes for the malic enzyme (ME) pathway, which allows malate to be used as a supplemental carbon source for growth. ME is comprised of four genes in two adjacent operons, with the regulatory two-component MaeKR required for expression of genes encoding a malate permease (*maeP*) and malic enzyme (*maeE*). Analysis of transcription indicated that expression of *maeP* and *maeE* are induced by both malate and low pH, and induction in response to both cues is dependent on the MaeK sensor-kinase. Furthermore, both *maePE* and *maeKR* are repressed by glucose, which occurs via a CcpA-independent mechanism. Additionally, malate utilization requires the PTS transporter EI enzyme (PtsI), as a PtsI$^-$ mutant fails to express the ME genes and is unable to utilize malate. Virulence of selected ME mutants was assessed in a murine model of soft tissue infection. MaeP$^-$, MaeK$^-$, and MaeR$^-$ mutants were attenuated for virulence, whereas a MaeE$^-$ mutant showed enhanced virulence as compared to wild type. Taken together, these data show that ME contributes to *S. pyogenes'* carbon source repertory, that malate utilization is a highly regulated process, and that a single regulator controls ME expression in response to diverse signals. Furthermore, malate uptake and utilization contribute to the adaptive pH response and ME can influence the outcome of infection.

INTRODUCTION

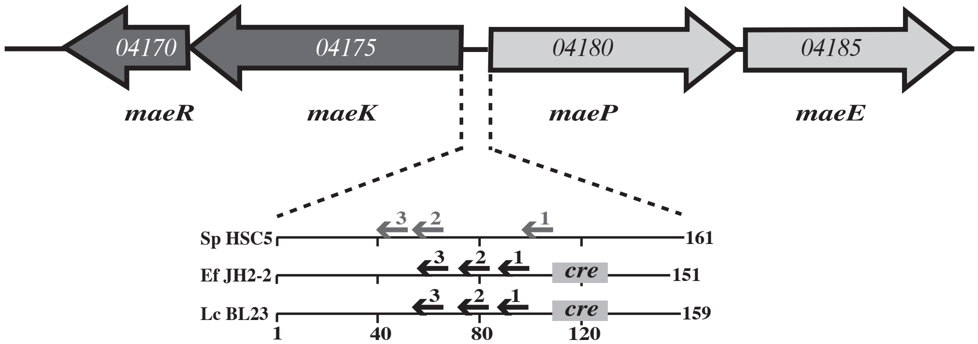
Although it has a relatively small genome (approx. 1.8 Mbp), the pathogenic Gram-positive bacterium *Streptococcus pyogenes* has a remarkable ability to adapt to a variety of human tissues. This trait allows it to cause numerous diseases ranging from superficial and self-limiting infections in soft tissues like the skin (impetigo) and pharynx (pharyngitis) to more problematic infections at a number of diverse anatomical sites (1). Understanding the complex regulatory interactions that allow it to adapt to these diverse environments provides a unique opportunity to gain insight into how a pathogen can efficiently employ a relatively limited genetic repertory to maximize its ability to cause disease.

An important question is how *S. pyogenes* uses its limited metabolic potential to grow efficiently in diverse tissues. Considerable evidence has accrued to suggest that the patterns by which *S. pyogenes* exploits available growth substrates are intimately associated with both temporal and compartment-specific patterns of virulence gene expression (2-4). As a lactic acid bacterium, *S. pyogenes* relies exclusively on fermentation via the homo-lactic and mixed acid pathways to generate energy (5-7). However, the specific carbon sources it preferentially utilizes in different tissues, the temporal patterns with which these are consumed, and how these patterns impact regulation of virulence gene expression are not well understood.

One approach to gain insight into conditions encountered during infection has involved comparison of the *S. pyogenes* transcriptome between organisms recovered from various models of infection to organisms cultured under different *in vitro* conditions. In general, these studies have revealed that at the latter time points of infection, patterns of gene expression most closely resemble those observed *in vitro* in environments of low pH (pH 6.0-6.5) and low concentrations of glucose (8-10). These two conditions are likely related, as the fermentation of glucose by lactic acid bacteria results in the highest rates of production of acidic end products including lactate, acetate and formate (6, 7). This suggests that *S. pyogenes'* choice of carbon source results in a significant remodeling of its local tissue environment. It also indicates that over the course of infection, it must both adapt to its self-inflicted acid stress as well as exploit alternative carbon sources. In this regard, transcriptome profiling revealed that one of the most highly differentially activated gene clusters under conditions of acid stress, glucose starvation, and in murine soft tissue encodes a putative operon of two genes predicted to function in the catabolism of malate (10), annotated as the malic permease (*maeP*) and the malic enzyme (*maeE*) (Fig. 1A).

The di-carboxylic organic acid malate is found in abundance in tissue and in the environment, so it is not surprising that numerous malate degradation pathways have been identified among both prokaryotic and eukaryotic organisms (11-17). In lactic acid bacteria, two distinct pathways have been identified that make very different contributions to physiology. The most common of these is malolactic fermentation (MLF), which allows for the conversion of malate into lactate through the function of the malolactic enzyme (MLE). Typically, MLF does not contribute to growth yields, but does play an important role in maintenance of ATP pools during starvation and in protection from acid killing (18-21). Since malate is a stronger acid than lactate, its decarboxylation by MLF results in alkalization of the cytoplasm and the resulting pH gradient drives the malate/lactate antiporter coupled to ATP synthesis (7, 18-21).

A.



B.

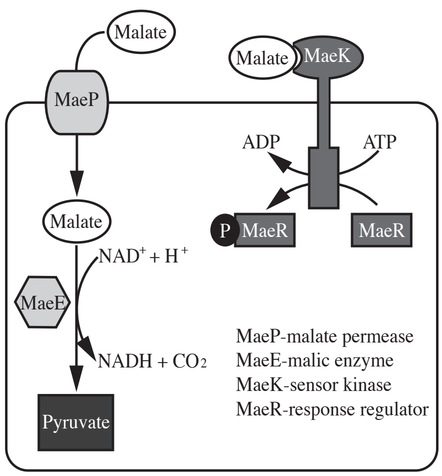


Figure 1. The malic enzyme (ME) pathway in *S. pyogenes*. (A) The arrangement of the open reading frames that comprise the ME locus of *S. pyogenes* are shown by large arrows. Gene names are shown below and the genomic loci listed within the open reading frames are based on the genome of *S. pyogenes* HSC5 (23). Known (black font) and predicted (grey font) regulatory elements of the intergenic region of *S. pyogenes* and *Enterococcus faecalis* JH2-2 (Ef JH2-2, (58)) and *Lactobacillus casei* BL23 (Lc BL23, (15)) are shown below. Arrows indicate sites in DNA bound by MaeR, while sites bound by CcpA are boxes labeled "*cre*" (catabolite regulatory element). Numbers below indicate intergenic distances in numbers of base-pairs. **(B)** Schematic of the ME pathway. The subcellular localization, function and reactions catalyzed by the various components of the ME pathway that are listed in the Figure are shown.

Less commonly found is an alternative degradation pathway that converts malate to pyruvate and carbon dioxide (18) that is known as the malic enzyme (ME) pathway (Fig. 1B). A unique feature of ME is that, unlike MLF, it enables cells to utilize malate as a carbon source for growth (16, 18). However, while the MLF system has been extensively studied, the regulation and physiological significance of the ME pathway is not as well understood. Studies in several lactic acid species, including *Enterococcus faecalis, Streptococcus bovis,* and *Lactobacillus casei* (13, 15, 17, 22), have indicated that ME requires 4 genes organized into two adjacent operons (Fig. 1A). These include the *maePE* operon that encodes the transmembrane permease (*maeP*) and cytosolic malic enzyme (*maeE*). Expression of these genes is dependent on the adjacent two-component system (TCS), which includes a sensor histidine kinase (*maeK*) and response regulator (*maeR*) (15, 17, 18). This similar organization is observed in the *S. pyogenes* chromosome (Fig. 1A), and as noted above, *maePE* is upregulated by acid stress and infection in S. *pyogenes*. In addition, examination of the *S. pyogenes* profiling data shows that both the *maePE* operon and the adjacent TCS had similar patterns of regulation, suggesting that these two systems function together (10).

Interestingly, while other ME operons are activated by malate (15, 17, 18) and repressed by glucose (15, 17), regulation by pH has only been described for *Lactobacillus casei* (18). Whether this system is regulated by pH in other bacterial species that contain a functional ME pathway, and the physiological role of this regulation is not understood. Rather, pH regulation is more commonly associated with the MLF pathway, where it is associated with acid resistance (20, 21). Examination of the *S. pyogenes* genome has not revealed the presence of MLF genes (23) so the significance of pH regulation of the ME pathway and whether it compensates for MLF in acid tolerance is not clear. In this study, we examined the contribution of malate catabolism and its unique pattern of regulation to *S. pyogenes* physiology and virulence. This analysis revealed that *S. pyogenes* has a functional ME pathway, that catabolism of malate contributes to growth and that its regulation shares some similarities with other lactic acid bacteria, but also has several unique features. Finally, we show that the presence or absence of ME genes can influence virulence in a murine model of soft tissue infection.

RESULTS

ME is necessary for *S. pyogenes* malate-enhanced growth. It is unclear why the ME pathway in *S. pyogenes* is regulated by pH, as MLF and not ME is typically associated with acid-stress resistance in other lactic acid species (20, 21). However, *S. pyogenes* lacks the genes necessary for MLF (23), so the contribution of the ME pathway to streptococcal physiology was investigated. The malic enzyme uses NAD^+ to oxidize malate to produce CO_2, NADH and pyruvate (Fig. 1B). Since pyruvate can be further metabolized to produce ATP, the signature function of ME is to allow cells to utilize malate as a carbon source for growth (16). To test this growth phenotype, *S. pyogenes* HSC5 was cultured overnight in a carbohydrate-reduced medium (C medium) in the presence or absence of 0.5% malate. Although cultures had comparable growth rates in both conditions ($t_{1/2}$ = 56 min. and 63 min., respectively), 0.5% malate enhanced growth yields by approximately 50% (Fig. 2A). Additionally, pH measurements of cell-free supernatants taken throughout growth indicate that malate utilization does not alter the pH of the media compared to unmodified C medium (Fig. S2A). This is due to the fact that, unlike when grown in media supplemented with glucose, when grown on malate, the bacteria utilize mixed acid fermentation, producing large amounts of formate, which has a much higher pKa than the lactate commonly produced (Fig. S2B).

In-frame deletion mutants in *maeP* (malate permease), *maeE* (malic enzyme), *maeK* (malate sensor kinase), and *maeR* (malate response regulator) were constructed and were found to have identical growth characteristics to wild type in unmodified C medium. However, all mutants failed to shown an increased growth yield the in the presence of malate (Fig. 2B). Malate concentrations were measured from cell-free

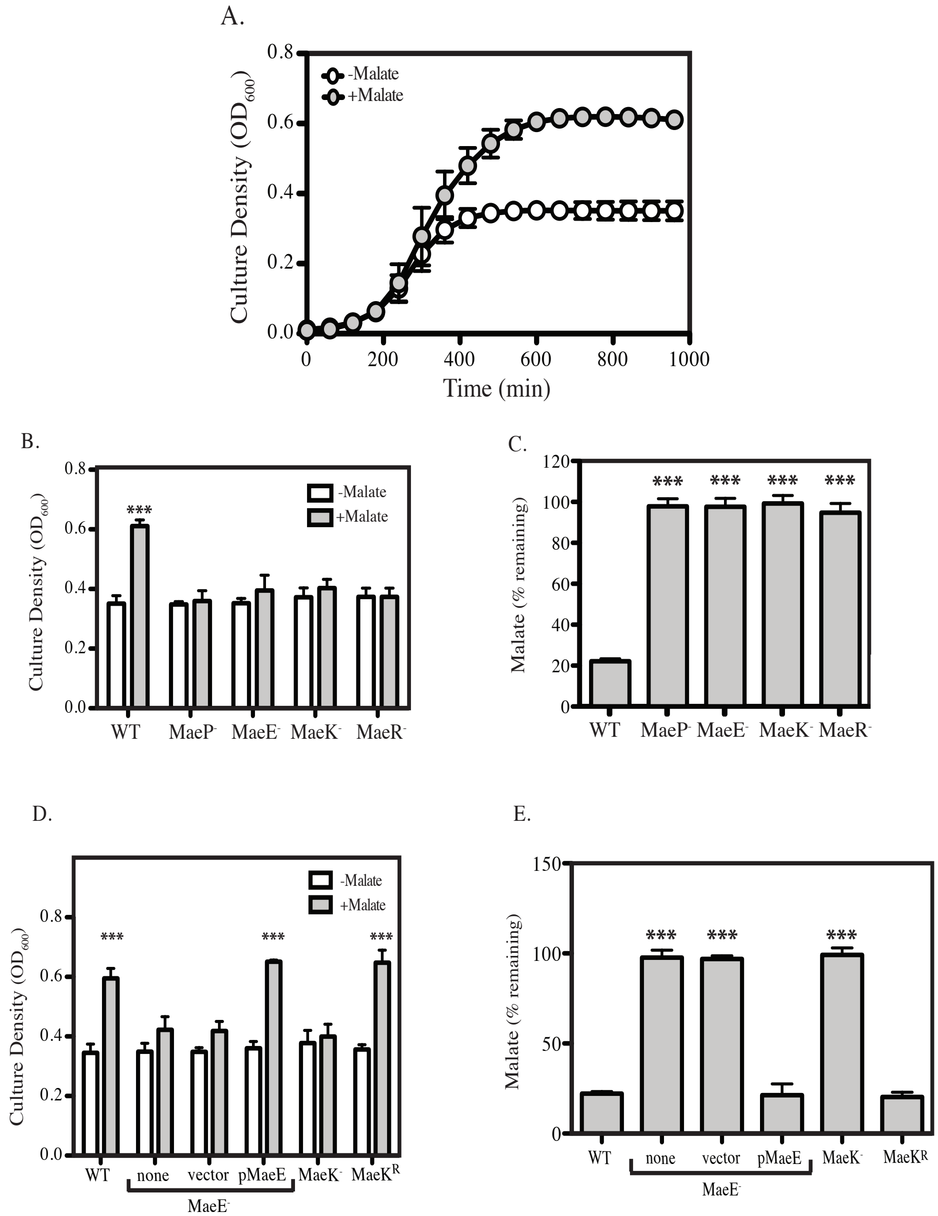


Figure 2. ME mutants are deficient in malate catabolism. (**A**) WT bacteria were tested for malate utilization by measuring growth over the course of 16 hrs of cultures grown in either unmodified C medium or C medium supplemented with 0.5% malate. Data are presented as means and standard deviations from 3 independent experiments. (**B** and **D**) WT and ME mutants were grown in unmodified C medium or C medium supplemented with 0.5% malate. Following 16 hrs of incubation, growth yields were measured by OD_{600}. Data are presented as the means and standard deviations from 3 independent experiments. (**C** and **E**) Malate concentrations from cell-free culture supernatants from over-night cultures of WT or ME mutants grown in C medium supplemented with 0.5% malate were measured (see Methods). Data are presented as percent remaining (compared to initial concentration) and are represented as means and standard deviations from 3 biological samples analyzed in duplicate. Asterisks indicate significant differences (***, $P < .001$) compared to WT in C medium.

supernatants of overnight cultures grown in 0.5% malate to determine malate consumption by wild type (WT) and the four ME mutants. WT cultures exhibited an approximately 80% reduction from the initial concentration of 37.3 mM, while malate concentrations were unchanged by growth of the mutants (Fig. 2C). With the exception of *maeE*, it was not possible to express the ME genes from a plasmid for complementation. As an alternative, allelic replacement was used to restore the full-length *maeK* gene in a ΔMaeK mutant background to make the reversion strain $MaeK^R$. In this way, we were able to complement at least one gene from each operon. Complementation of *maeE* and reversion of *maeK* restored both enhanced growth yields in the presence of malate (Fig. 2D) and consumption of malate (Fig. 2E).

Expression of ME genes is dependent on malate and requires MaeK. In other lactic acid bacteria, expression of ME requires both the presence of malate and the ME TCS (15, 17, 18). To determine if this common regulatory mechanism is also utilized in *S. pyogenes* an analysis of transcript levels of ME genes using real time RT-PCR was performed. The results indicated that during the exponential phase of growth (OD_{600} = 0.2), *maeE* and *maeP* were highly upregulated in the presence of malate by 100- and 200-fold, respectively (Fig. 3A). This response was dependent on MaeK, as transcript levels were equivalent in the presence or absence of malate. Restoration of the protein in a $MaeK^R$ reversion strain also restored malate induced transcription (3A). Transcription of *maeK* and *maeR* was also increased in the presence of malate in WT cells, with both genes showing an approximately 3-fold increase compared to unmodified media (Fig. 3B).

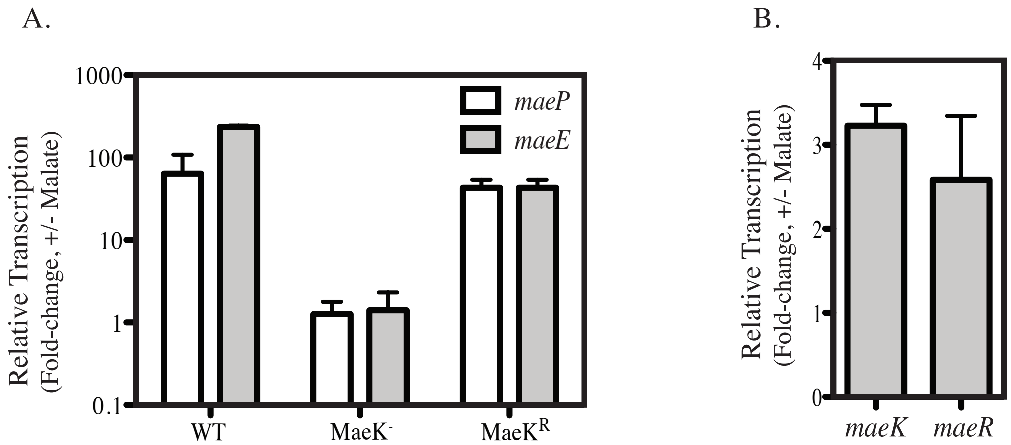


Figure 3. MaeK regulates malate-dependent expression of *maePE*. (**A**) WT, MaeK⁻, and MaeK^R strains were grown in C medium supplemented with 0.5% malate until exponential phase (OD_{600} of 0.2). Total RNA was isolated and used for real time RT-PCR analysis of *maeP* and *maeE* transcripts. (**B**) WT bacteria were grown in C medium supplemented with 0.5% malate as described before. Total RNA was isolated and used for real-time RT-PCR analysis of *maeK* and *maeR* transcripts. Data presented for all genes are the ratios of transcript abundance in modified medium to that of unmodified C medium and represent the means and standard deviations of 3 biological samples, each analyzed in triplicate.

Glucose regulation of ME is CcpA-independent. Malate catabolism in other lactic acid bacteria is repressed by glucose, indicating this pathway is regulated through a mechanism of carbon catabolite repression (CCR) (15, 17). CCR allows the bacteria to metabolize preferable carbon sources in the environment, usually through transcriptional repression of genes involved in the processing of alternative, and less favorable carbon sources (reviewed in (35-37)). A key transcriptional regulator of global CCR in Gram-positive bacteria is CcpA (35), which has been shown to regulate ME in response to glucose in both *Lactobacillus casei* and *Enterococcus faecalis* (15, 17). To test for CCR regulation of the ME pathway in *S. pyogenes*, transcription of the four ME genes was analyzed in the absence or presence of glucose (0.2%) by real time RT-PCR. Results showed a significant repression of 4-6-fold ($\log_2$ scale) for all four ME genes (Fig. 4), consistent with observations in other lactic acid species (15, 17). However, in contrast to these other species, repression occurred independently of CcpA, as the addition of glucose still repressed expression of all ME genes in a CcpA$^-$ mutant (Fig. 4) (38). Repression does have the characteristics of CCR, as glucose was repressive even in the presence of malate (Fig. 4), indicating that *S. pyogenes* has adopted a CcpA-independent CCR mechanism for regulation of malate catabolism.

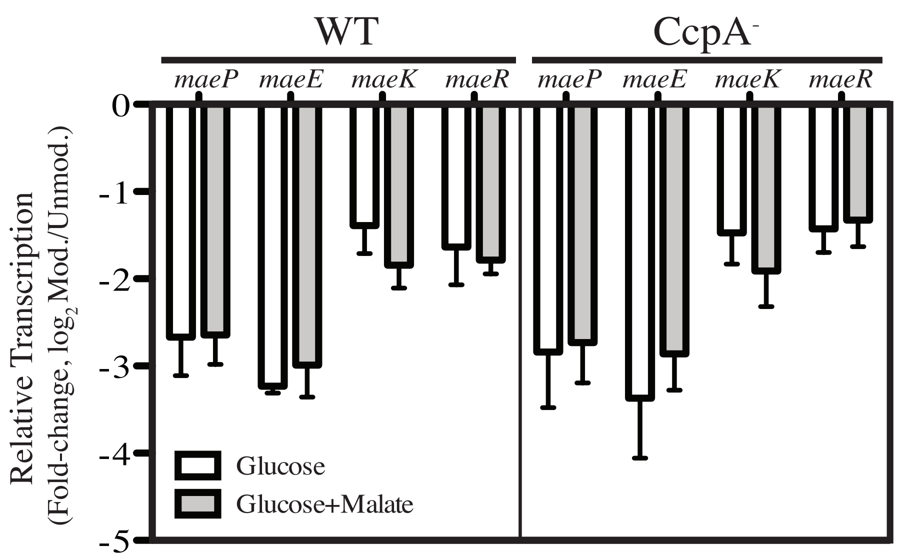


Figure 4. Carbon catabolite repression of ME genes is CcpA independent. WT and CcpA$^-$ bacteria were grown in C medium supplemented with 0.2% glucose until exponential phase (OD_{600} of 0.2). Total RNA was isolated and used for real-time RT-PCR analysis of the individual *mae* transcripts. Data are presented as the ratios of transcript abundance in modified media to that in unmodified C medium and represent the means and standard deviations derived from 4 biological samples, each analyzed in triplicate.

Malate catabolism is regulated by PTS-mediated phosphorylation. An alternative mechanism of CCR in bacteria is known as induction prevention, which is dependent on the sugar phosphotransferase (PTS) system and the phosphorylation state of a conserved histidine residue of the phosphocarrier protein HPr (36), which in the case of *S. pyogenes* is His15 (39). If the ME loci are controlled by a mechanism similar to induction prevention, then cells unable to produce P~His-HPr should be unable to utilize malate. To test this hypothesis, two mutants were constructed. The first is an allelic exchange mutant with a swap of a chloramphenicol cassette with *ptsI*, which encodes EI, the enzyme responsible for phosphorylation of the His15 site within the HPr protein. The second mutant contains a single amino-acid substitution in HPr, replacing His15 with alanine, which has been shown to maintain HPr capability to be phosphorylated at Ser46 and is functional for sugar transport, but lacking in the ability to participate in regulation ($HPr^{H15A)}$ (36, 40, 41).

When grown in the presence of malate, both the $PtsI^-$ mutant and the HPr^{H15A} mutant have a significant growth defect compared to WT, resulting in a reduced growth rate and lower final culture density (Fig. 5A and 5B). To verify that this growth defect was specific for malate utilization and not a general defect in all conditions, strains were also grown in unmodified C medium, as well as in C medium supplemented with 0.2% maltose (a non-PTS sugar) (34). Comparisons of final yield from overnight cultures demonstrate that growth of both the $PtsI^-$ and HPr^{H15A} mutants are similar to WT in unmodified media (Fig. 5B). In addition, upon supplementation of maltose, all three strains showed an identical increase in growth (Fig. 5B). Thus, mutations that block formation of P~His-HPr are deficient in malate utilization, but are still able to utilize non-PTS carbon sources.

Expression of the ME genes was then examined in the presence of malate and it was discovered that when compared to WT, the HPr^{H15A} mutant had a substantial reduction in transcript levels for all four ME genes (Fig 5C). Taken together, these data demonstrate that the ME pathway in *S. pyogenes* is repressed by glucose through a mechanism similar to induction prevention.

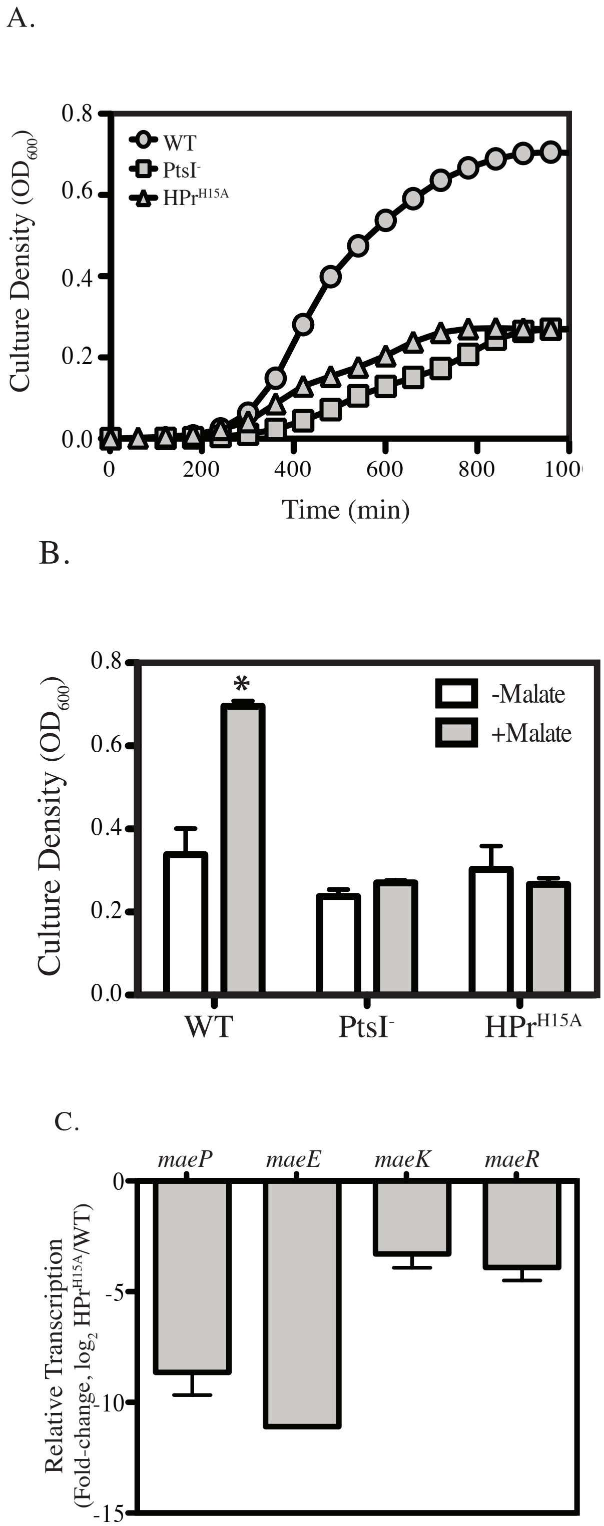


Figure 5. Carbon catabolite repression of ME genes controlled by P~His-HPr. WT, PtsI$^-$, and HPr ^{H15A} strains were grown in unmodified C medium or C medium plus 0.5% malate. (**A**) Growth of WT and PTS mutants in malate-supplemented medium was measured by OD_{600} over the course of 16 hrs. Data presented is from a representative experiment. **(B)** WT and PTS mutants were grown in unmodified C medium or C medium supplemented with 0.5% malate. Following 16 hrs of incubation, growth yields were measured by OD_{600}. Data are presented as the means and standard deviations from 3 independent experiments. Asterisks indicate significant differences (*, $P < .05$) compared to WT in C medium. (**C**) WT and HPr^{H15A} strains were grown in C medium supplemented with 0.5% malate until exponential phase (OD_{600} of 0.2). Total RNA was isolated and used for real-time RT-PCR analysis of transcript abundance of the individual *mae* transcripts. Data are presented as ratios of transcript abundance of HPr^{H15A} to that of WT and represent the means and standard deviations derived from 3 biological samples, analyzed in triplicate.

pH regulation of ME is independent of malate, but dependent on *maeK*. Prior transcriptional profiling revealed that *maeP* and *maeE* are among the genes most highly regulated by pH in *S. pyogenes* (10). Additionally, growth of WT cells in acidified media was enhanced with the addition of malate, demonstrating that malate catabolism can occur in a low pH environment (Fig. S3). To further characterize the role of environmental pH on the ME pathway, WT *S. pyogenes* was grown in C medium buffered to either low (pH 6.0) or neutral (pH 7.5) pH and transcription of the ME genes was analyzed by real time RT-PCR. When compared to unbuffered medium and in the absence of the addition of malate, growth at low pH, but not neutral pH, enhanced abundance of the *maeP* and *maeE* transcripts by approximately 10- and 20-fold, respectively (Fig. 6A). Neither low nor high pH environments altered expression of *maeK* or *maeR* when compared with unmodified media (Fig. 6B). However, MaeK itself was required for the enhanced expression of *maeP* and *maeE*, as the abundance of these transcripts did not increase in the $MaeK^-$ mutant during growth at low pH, but regulation was restored in the $MaeK^R$ strain (Fig. 6C). Finally, to address the hierarchy of stimuli between malate and pH, quantitative RT-PCR was performed on cells in the presence of both high malate (0.5%) concentrations and neutral pH and compared to malate alone. Results show that, for both *maeP* and *maeE*, transcription is dramatically increased in the presence of 0.5% malate, and that this enhanced expression is unaffected by the pH of the media (Fig. 6D). Overall, this data shows that *mae* gene expression is regulated by environmental pH, and that this regulation is mediated through MaeK and is independent of malate regulation.

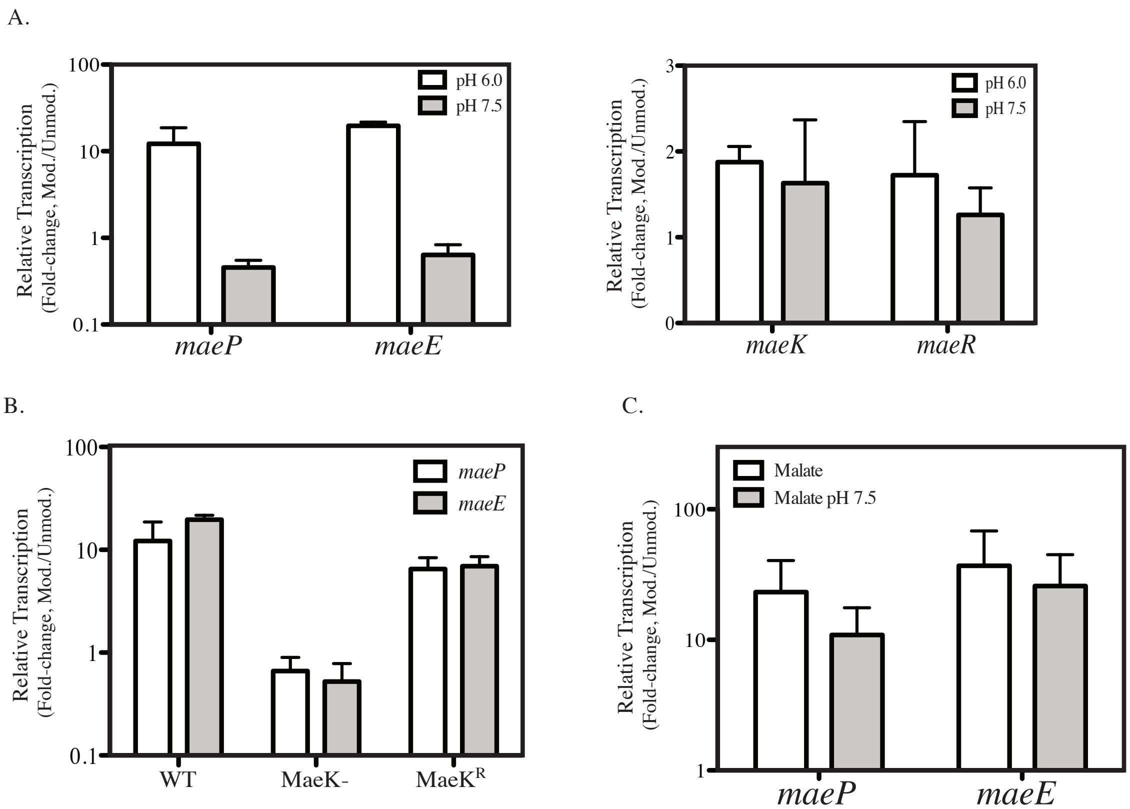


Figure 6. pH regulation of ME is malate-independent, but requires MaeK. (**A**) WT bacteria were grown in C medium buffered to pH 6.0 or pH 7.5 until exponential phase (OD_{600} of 0.2). Total RNA was isolated and used for real-time RT-PCR analysis of the individual *mae* transcripts. Data are presented as the ratios of transcript abundance in buffered media to that in unmodified C medium and represent the means and standard deviations derived from 3 biological samples, each analyzed in triplicate. (**B**) WT, MaeK^{-}, and MaeK^{R} strains were grown in C medium pH 6.0 and total RNA was isolated as described before and used for real-time RT-PCR analysis of *maeP* and *maeE* transcripts. Data are presented as ratios of transcript abundance in buffered media to that in unmodified C medium and represent the means and standard deviations derived from 3 biological samples, each analyzed in triplicate. (**C**) WT bacteria were grown in C medium plus 0.5% malate or C medium plus 0.5% malate buffered to pH 7.5 and total RNA was isolated as described before and used for real-time RT-PCR analysis of *maeP* and *maeE* transcripts. Data are presented as ratios of transcript abundance in modified medium to that in unmodified C medium and represents the means and standard deviations derived from 3 biological samples, each analyzed in triplicate.

Loss of MaeE causes enhanced virulence *in vivo*. The *in vitro* experiments presented in this work were done with bacterial cultures grown in C medium, which is characterized as having low carbohydrate concentrations and high salt and peptide levels (10). It has been demonstrated previously that these conditions are highly analogous to the *in vivo* milieu of carbon sources within the murine soft tissue environment (10). Additionally, malate, being one of the intermediate products of the citric acid cycle, is abundant in host tissue (42). Therefore, the fact that *S. pyogenes* is able to utilize malate *in vitro* when added to C medium lends strong support that it can also be utilized *in vivo* during host tissue infections.

Thus, it was of interest to determine if malate utilization and the ME pathway play a role in virulence. To assess this, a murine soft tissue model was used. Briefly, approximately 10^7 bacteria were injected subcutaneously into the flank of immunocompetent hairless mice. Infection of WT *S. pyogenes* HSC5 produces a localized necrotic lesion and formation of an escher within 24 hours, but does not cause a systemic infection (33). Lesion size increases over time, peaking in size at day 3 post-infection (32). Measurement of the lesion area over time is therefore used as a marker for virulence in this model. For this analysis, mice were infected with WT, $MaeP^-$, $MaeE^-$, $MaeK^-$, or $MaeR^-$ strains and lesion areas were compared 3 days post-infection. Mice infected with strains $MaeK^-$, $MaeR^-$, and $MaeP^-$ all formed lesions that were significantly smaller than WT (Fig. 7). Conversely, mice infected with the $MaeE^-$ strain formed lesions that were significantly larger than WT (Fig. 7). These results demonstrate that malate catabolism is an important factor during a soft tissue infection, and that the loss of individual ME genes can have differential effects on the outcome of an infection.

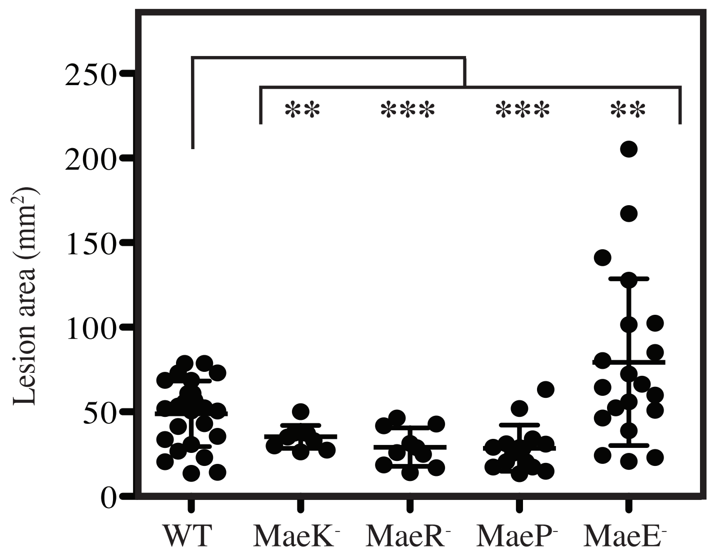


Figure 7. Loss of MaeE causes hypervirulence *in vivo*. Hairless SKH1 mice were infected subcutaneously with WT or individual ME mutant strains and the resulting lesions formed at day 3 post-infection were measured. Each symbol plotted represents the value derived from an individual animal. Data shown are pooled from at least 2 independent experiments with the mean and standard deviation indicated. Differences between groups were tested for significance using the Mann-Whitney U test (* $P < 0.05$, ** $P < 0.01$).

DISCUSSION

In this study we have shown that the ME genes of *S. pyogenes* allow the bacterium to use malate as a carbon source for growth. Additionally, we have shown that this pathway is subjected to regulation by both positive and negative signals, including glucose, malate, and pH. The former of these is via PTS-mediated phosphorylation, while the latter two signals are recognized by the MaeKR regulatory system. Finally, these data show that loss of any individual *mae* gene can alter the outcome of a soft tissue infection in mice, suggesting that the ability to transport and utilize malate are both key processes in pathogenesis.

Regulation of ME gene expression in *S. pyogenes* was found to share features in common with other bacterial species (15, 17, 18). Most notably is that they are induced by malate and that induction requires the MaeKR TCS. A prior analysis of the *maePE* and *maeKR* promoter regions in *L. casei* identified the DNA-binding site for MaeR as a series of direct repeats and a similar site is shared among other lactic acid bacteria, including *S. pyogenes* (Fig. S1) (15, 17). Another common feature to ME pathway regulation is that all species repress ME gene expression in the presence of glucose, a regulatory mechanism known as carbon catabolite repression (CCR) (36, 37, 43). However, in *S. pyogenes*, glucose-mediated regulation of the ME loci functions independently of the major carbon catabolite protein CcpA. In support of this finding is the fact that, unlike the other characterized lactic acid bacteria, the promoter region in *S. pyogenes* lacks any identifiable *cre* sites (Fig. 1, Fig. S1) (15, 35, 43, 44).

Instead, an alternative method of CCR, induction prevention, is likely regulating ME genes in *S. pyogenes*. Evidence to support this idea includes the fact that multiple genetic strains that are unable to form P~His-HPr (either through loss of the EI enzyme or direct mutation of HPr) are likewise deficient in ME transcription and malate utilization. This method of regulation, therefore, enables the cell to activate ME genes only in the presence of a high concentration of P~His-HPr. During normal growth utilizing preferred carbohydrates, levels of intracellular P~His-HPr would likely be low (36, 45). This is due to either rapid accumulation of P~Ser-HPr or transfer of the phosphate group on P~His-HPr to downstream PTS transporters to allow uptake of PTS sugars. In this way, the bacterium is able to preferentially utilize a number of available PTS sugars before turning on the alternative ME pathway.

In order for this form of regulation to be controlling ME expression, it requires a transfer of the phosphate from P~His-HPr to an ME regulatory protein. Phosphorylation of non-PTS protein by P~His-HPr is known to occur in a variety of species (for review see (36)) and often involves a PTS regulatory domain (PRD)- containing protein. Currently, the only non-PTS protein shown to act as a phosphate acceptor from P~His-HPr in *S. pyogenes* is the transcriptional regulator Mga (46). This protein has been characterized as containing several unique, but related, PRD domains (PRD_Mga) and previous work has shown that P~His-HPr is able to phosphorylate specific residues within these domains *in vitro* (46, 47). Although Mga has been shown to regulate a large number of target genes (3, 4, 47, 48), the ME cluster has not yet been identified as part of its regulon. Alternatively, there are two additional transcription regulators in *S. pyogenes* HSC5 predicted to include a PRD_Mga domain (Paluscio and Caparon, unpublished), both within the RofA family of regulators (46, 49-51). It remains possible that one of these proteins may be necessary for ME gene expression. One likely mechanism for this regulation would be that the phosphate from the P~His-HPr gets transferred to one of the PRD transcriptional regulators, which then allows this protein to bind to the promoter region of *maeKR* and induce its expression. Further evidence to support this hypothesis is the presence of several putative regulatory elements within the *mae* promoter region of *S. pyogenes* (Fig. S1), which are absent from the promoters of the other lactic acid bacteria. These sequences may serve as binding sites for one of the PRD-containing regulatory proteins mentioned above.

This work also demonstrated that the *maePE* operon is regulated by a pH-dependent mechanism, whereby acidic pH induces transcription of these genes and neutral pH is inhibitory. In *S. pyogenes*, it appears that, in addition, the MaeKR TCS was necessary for this regulation, as the loss of MaeK prevents the pH-dependent expression of *maePE* seen in wild type cells. Interestingly, this work is the first to identify a signal other than malate that is recognized by the MaeKR regulatory system. Though uncommon, MaeK is not the first transcriptional regulator identified that is able to respond to multiple extracellular signals. In *Escherichia coli,* the *cad* operon is regulated by CadC, which recognizes both acidic pH and lysine to induce transcription of the *cad* genes (52, 53). In *Streptococcus mutans* the AguR protein, which controls the expression of the agmatine deiminase system (AgDS), recognizes acidic pH and agmatine (54, 55). An important distinction between CadC, AguR, and MaeK is that former two proteins require both signals to be present in order to allow for activation and transcription of their target genes. This work has shown, however, that MaeK functions in the presence of either signal and does not require both for transcriptional activation. Nonetheless, given that all three proteins respond to multiple signals, one of which is low pH, they may all share some similar mechanisms of activation. It is hypothesized that for both CadC and AguR the acidic pH environment induces a conformational change in the protein, and this change then allows for binding of the substrate (lysine and agmatine, respectively) (53, 54). Likewise, in the presence of acidic pH, MaeK may undergo a conformational change that induces activation of the protein. However, unlike CadC and AguR, the MaeK protein likely has a separate malate sensor domain that can bind malate in the presence or absence of acidic pH. This mechanism would predict that

MaeK has two distinct regions required for signal recognition and that either can control the activity of the protein.

The identification of a pH-dependent response for ME expression is of particular interest due to the metabolism of the bacterium. *S. pyogenes* is a member of the lactic acid bacteria, a group that relies on a mix of homolactic and mixed acid fermentation as a means of generating energy in the cell (6, 56). Over time, in the presence of rapidly metabolized carbohydrates such as glucose, high concentrations of organic acid end products will accumulate (Fig. S2) (5, 6, 56, 57). In this way, there is a direct link between carbohydrate availability and pH, with depletion of glucose leading to a corresponding reduction of the surrounding pH. In this respect, low pH could function as an early warning signal for changes in carbohydrate availability. Thus, low pH may function as an inducer of expression for multiple alternative catabolic operons, and is likely not exclusive to malate catabolism alone. Additionally, the MaeKR TCS may be necessary for controlling this pH adaptive response for these other catabolic operons.

Taken together, this work demonstrates that under conditions of low glucose or acidic pH, malic acid catabolic genes are highly expressed. Given that these signals are amongst those that *S. pyogenes* encounters at specific points in a soft tissue infection (8-10, 57), the question of whether this alternative metabolic pathway was important for virulence was of particular interest. Although all of the ME mutants have similar phenotypes *in vitro*, a loss of growth on malate, the mechanism to cause this deficiency is different for each strain. All three attenuated strains are unable to transport extracellular malate into the cell (either through deletion of the malate transporter gene or loss of *maeP* expression in an MaeK$^-$ or MaeR$^-$ mutant). Alternatively, a MaeE$^-$ mutant is able to transport malate into the cell, but cannot convert this molecule into pyruvate. This differentiation may, in part, explain the *in vivo* phenotypes observed.

In this case, it remains possible that malate may serve a secondary, as yet unknown, benefit to the bacterial cell independent of increased pyruvate concentrations. Thus, the ability of a MaeE$^-$ mutant to allow uptake of malate may allow for this unused malate to be shuttled into an alternative pathway, ultimately serving to benefit *S. pyogenes* during infection. Alternatively, MaeP$^-$, MaeK$^-$, and MaeR$^-$ mutants would be depleted of any internal malate accumulation, and this loss would ultimately decrease fitness for the cells compared to a WT or a MaeE$^-$ mutant. In this case, it remains possible that malate may serve a secondary, as yet unknown, benefit to the bacterial cell independent of increased pyruvate concentrations. Thus, the ability of a MaeE$^-$ mutant to allow uptake of malate may allow for this unused malate to be shuttled into an alternative pathway, ultimately serving to benefit *S. pyogenes* during infection. Alternatively, MaeP$^-$, MaeK$^-$, and MaeR$^-$ mutants would be depleted of any internal malate accumulation, and this loss would ultimately decrease fitness for the cells compared to a WT or a MaeE$^-$ mutant. Another possibility is that the accumulation of intracellular malate in the MaeE- mutant may cause the mis-regulation of virulence factor expression, leading to enhanced virulence. In preliminary studies, we have found that while expression of the SpeB cysteine protease does not differ between WT and the MaeE$^-$ and MaeP$^-$ mutants (Fig. S4A), the addition of malate alters the temporal pattern of SpeB expression in both mutants as compared to WT (Fig. S4B). Since this alteration in SpeB expression is similar between the two mutants, it cannot explain the enhanced virulence of the MaeE$^-$ mutant. However, it does support the possibility that alterations to malate metabolism can result in changes in patterns of virulence factor expression. Further analyses of virulence factor expression will be required in order to determine is specific factors are specifically mis-regulated in the MaeE$^-$ mutant and whether these factor are responsible for hypervirulence. This work does, however, provide novel insights into the unique regulatory mechanisms utilized by *S. pyogenes* for malate degradation, as well as demonstrate for the first time the importance of this alternative metabolic pathway on influencing pathogenesis.

ACKNOWLEDGEMENTS

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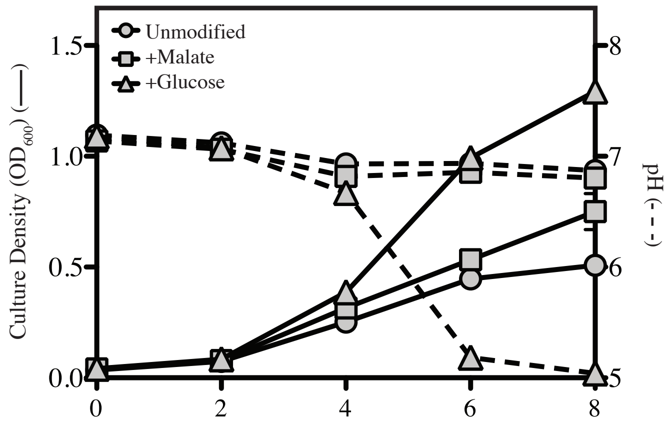
Figure S1. Schematic representation of ME promoter regions in lactic acid bacteria. Predicted functional domains of the *S. pyogenes* **(A)** promoter are compared to those of *Enterococcus faecalis* **(B)** and *Lactobacillus casei* **(C)** [1, 2]. Predicted elements are shown in grey, while those elements that have been confirmed experimentally are shown in black. Sites are as follows: (+1), transcription start site; (SD), Shine-Delgarno; (-10), the -10 promoter region; (mbs), MaeR-binding sites. Degenerate binding sites are indicated by dotted arrows. Translational start codons are shown in bold font. Predicted *cre* sites are highlighted in gray.

A.



B.

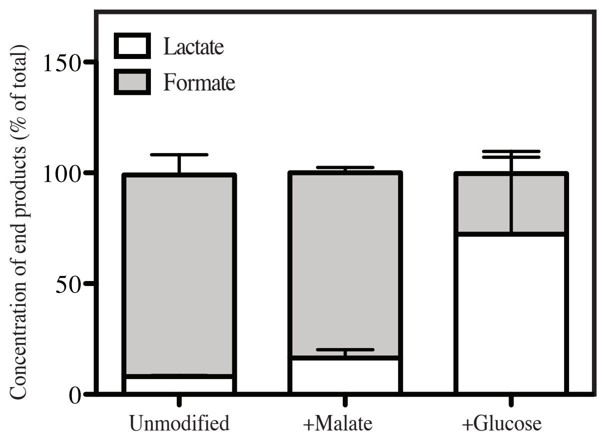


Figure S2. Additional characterization of growth in malate-supplemented media. **(A)** WT bacteria were grown in unmodified C medium, C medium plus 0.5% malate, or C medium plus 0.2% glucose over the course of 8 hrs. Samples were removed every 2 hrs and analyzed for growth (OD_{600}, left axis) and pH was determined in cell-free supernatants (right axis). Data are presented as means and standard deviations from 3 biological samples. **(B)** Lactate and formate concentrations from cell-free culture supernatants from over-night cultures of WT cells grown in C medium, C medium supplemented with 0.5% malate, or C medium supplemented with 0.2% glucose were measured (see Methods). Data are presented as percent of total organic acid concentrations measured and are represented as means and standard deviations from at least 2 biological samples analyzed in triplicate.

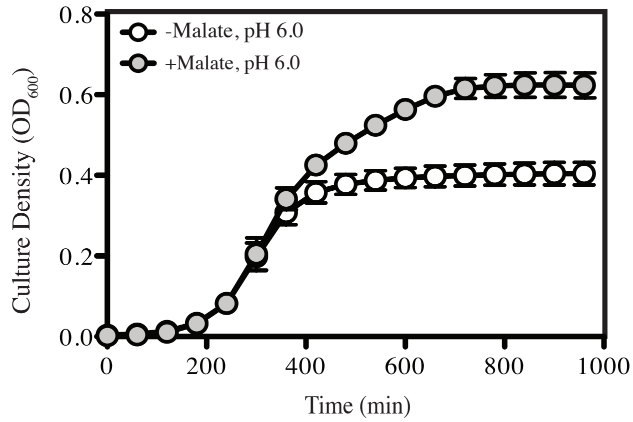
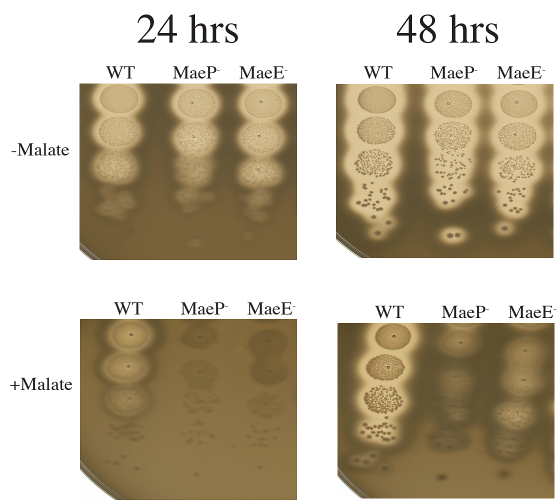


Figure S3. Growth at low pH. WT bacteria were tested for malate utilization by measuring growth over the course of 16 hrs of cultures grown in either unmodified C medium or C medium plus 0.5% malate, both buffered to pH 6.0. Data are presented as means and standard deviations from 3 independent experiments.

A.



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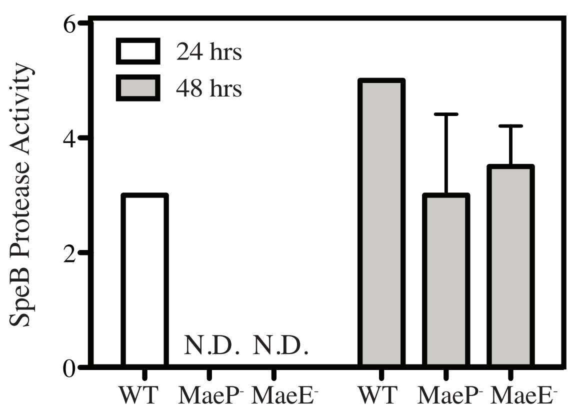

Figure S4. SpeB activity of ME mutants. (A) SpeB activity of WT and ME mutants grown in the presence or absence of 0.5% malate. Protease activity is apparent as a zone of clearance around colonies plated on protease indicator plates. **(B)** Quantification of SpeB activity of WT and ME mutants grown in the presence of 0.5% malate. Data presented are the means and standard deviations from 2 independent experiments as determined in (8).

Table S1. Strains used in this study

Strain	Relevant Genotype	Mutated Loci[a]	Plasmid	Description[b]	Reference
S. pyogenes					
HSC5	wild-type		none	wild-type	Port, et al. (2013)
CKB206	Δ*ccpA*	02310	none	in-frame deletion of *ccpA*	Kietzman, et al. (2010)
EP184	Δ*maeP*	04180	none	in-frame deletion of *maeP*	This work
EP120	Δ*maeE*	04185	none	in-frame deletion of *maeE*	This work
EP121	Δ*maeK*	04175	none	in-frame deletion of *maeK*	This work
EP122	Δ*maeR*	04170	none	in-frame deletion of *maeR*	This work
EP212	Δ*maeP*, Δ*maeE*	04180, 04185	none	in frame deletion of *maeP* in Δ*maeE* strain	This work
EP92	Δ*ptsI*::*cat*	05585	none	Cam, allelic replacement of *ptsI* with *cat*	This work
EP132	$ptsH_{H15A}$	05590	none	allelic replacement of WT *ptsH* with $ptsH_{H15A}$	This work
EP160	Δ*maeE*		pEP66	Complementation of *maeE*	This work
EP161	Δ*maeE*		pABG5	empty vector	This work
EP181	$MaeK^R$		none	restoration of Δ*maeK*	This work

a. Loci are based on the genome HSC5 (Port et al. 2013) and follow the formate L897_xxxxx, where xxxxx are numbered
b. antibiotics are abbreviated as follows: chloramphenicol (Cam)

Table S2. Primers used in this study

Name	Sequence[ab]	Template	Plasmid	Description
maeP 5' F (BamHI)	GGCGGATCCGAGAGGTTGTGACAACAATCATAGATACC	HSC5	pEP78	primers for in-frame deletion of *maeP*
maeP 5' R	AAATAGCTGTTTGAGGCATTTTCTTGCTTATTGTTT	HSC5		
maeP 3' F	AATGCCTCAAACAGCTATTTTAAGAATTATTTTTAGTTAATCCAA	HSC5		
maeP 3' R (EcoRI)	GGCGAATTCCGCAGTACCATCACTGATAACTGC	HSC5		
maeE 5' F (BamHI)	GCCGGATCCTTAACATGAACCCAGTGGAAGCAGC	HSC5	pEP50	primers for in-frame deletion of *maeE*
maeE 5' R	ACCTGACGCTAAGGGCAAGTTGACCTAATTGATTTTTCAT	HSC5		
maeE 3' F	ACTTGCCCTTAGCGTCAGGTCGGTAGTCCTTAAATCATAG	HSC5		
maeE 3' R (EcoRI)	GCCGAATTCGAAAACTGGAAAGATTGACGCAACAAGAC	HSC5		
maeK 5' F (BamHI)	GCCGGATCCTATTTTGCCATGCTTTTGAATGCTC	HSC5	pEP51	primers for in-frame deletion of *maeK*
maeK 5' R	GTTGCCCTCCAACTGGCCCATAAACGTAGTGGTTTTTCAT	HSC5		
maeK 3' F	ATGGGCCAGTACACCTTATGTAGGAGGGCAACATGAACGT	HSC5		
maeK 3' R (EcoRI)	GCCGAATTCCTTCGCAAGGAATATGCTGAGCACG	HSC5		
maeR 5' F (BamHI)	GCCGGATCCTTCAACATGATCAGAATCATTTAATCAGCC	HSC5	pEP52	primers for in-frame deletion of *maeR*
maeR 5' R	GACGGCCAACGGGATCATCTTCAATGATTAAAACGTTCAT	HSC5		
maeR 3' F	AGATGATCCCGTTGGCCGTCCCTATCGCGTG	HSC5		
maeR 3' R (EcoRI)	GCCGAATTCGCCAAGGCCTTACTTCCAGATGAGA	HSC5		
maeE F (EcoRI)	GCCGAATTCTCCAAACCAATAAAAAGAGAGGTATTTCCT	HSC5	pEP66	primers for complementation of *maeE*
maeE R (ClaI)	GCCATCGATGCTTCTACTTATTTTGTGATTTAAAGTATAACTTTCTC	HSC5		
HPr F (BamHI)	GCGGATCCGCTCTAGCGTTTTGGTGATAGAAGC	HSC5	pEP56	primers for His15 to Ala point mutation in *ptsH*
HPr R (BamHI)	GCGGATCCCGTCTTGTGCAGCTTGGAGTG	HSC5		
HPr H15A F	TCACATTGTTGCAGAAACAGGTATT***GCT***GCGCGTCCAGCG	HSC5		
HPr H15A R	CGCTGGACGCGC***AGC***AATACCTGTTTCTGCAACAATGTGA	HSC5		
maeP RT F	CCGATGTTACTTGGAATGGTTTGTG			RT primers for *maeP*
maeP RT R	CGACTCCTGTAATGGCACTGTAACC			
maeE RT F	TCATATTCCTGTTTTCCATGACGATC			RT primers for *maeE*
maeE RT R	CTGTCACTTTCGTAGCACCTGCTG			
maeK RT F	CGTCAAAACGCAATTATACCCTGTCT			RT primers for *maeK*
maeK RT R	CCAGCTTTATCTACCACAAATACGGC			
maeR RT F	GGCAATGGGATTCAATTTTTGGAG			RT primers for *maeR*
maeR RT R	CTGTTGGATGCTTTCTTGAAAGCG			

a. Engineered restriction sites are underlined
b. Engineered mutation sites are in bold italics

Table S3. Plasmids used in this study

Plasmid (Resistance)[a]	Description	Reference
pGCP213 (Erm)	temperature-sensitive shuttle vector, used for allelic replacement	Nielsen et al. (2012)
pCRK (Kan)	temperature-sensitive shuttle vector, used for allelic replacement	Le Breton et al. (2013)
pABG5 (Kan, Cam)	shuttle vector, used for ectopic expression	Meehl et al. (2005)
pEP78 (Kan)	pCRK::Δ*maeP*	This work
pEP50 (Erm)	pGCP213::Δ*maeE*	This work
pEP51 (Erm)	pGCP213::Δ*maeK*	This work
pEP52 (Erm)	pGCP213::Δ*maeR*	This work
pGCP793 (Erm, Cam)	pGCP213::Δ*ptsI::cat*	Port et al. (2014)
pEP56 (Erm)	pGCP213::$ptsH_{H15A}$	This work
pEP66 (Kan, Cam)	pABG5:: *maeE* (for MaeE complementation)	This work
pEP74 (Kan)	pCRK::*maeK* (for MaeK restoration)	This work

a. Antibiotics are abbreviated as follows: kanamycin (Kan), chloramphenicol (Cam), erythromycin (Erm)

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *Escherichia coli* strain TOP10 (Invitrogen) was used for cloning using standard molecular biology techniques. The *Streptococcus pyogenes* strain HSC5 (23) and mutant derivatives were utilized in this study. Strains were grown in Todd Hewitt broth (THYB) with 0.2% yeast extract (Difco) or C medium (24). C medium was adjusted to pH 7.5 as described previously (24). Routine growth conditions utilized sealed culture tubes incubated at 37°C under static conditions. Streptococcal strains grown on solid medium containing 1.4% Bacto agar (Difco) were cultured in a sealed jar with a commercial gas generator (GasPak catalogue no. 70304, BBL). For experiments utilizing malate supplementation, filter sterilized 5% (w/v) stock solution buffered to pH 7.0 with NaOH (Sigma) was used to add malate (Sigma) to a final concentration of 0.5% to the media. For experiments utilizing glucose or maltose supplementation, filter sterilized 20% (w/v) stock solution was used to add glucose or maltose (Sigma) to a final concentration of 0.2% to the media. For experiments utilizing buffered media, 1M stock solutions of HEPES (pH 7.5) or MES (pH 6.0) (both obtained from Sigma) were added to a final concentration of 0.1M to the media. All media used were sterilized in an autoclave prior to supplementation. When appropriate, antibiotics were added at the following concentrations: erythromycin 1 mg/mL, kanamycin 250 mg/mL, chloramphenicol 3 mg/ml.

Construction of mutants. All references to genomic loci are based on the genome of HSC5 (23). In-frame deletion mutations in the genes encoding MaeP (L897_04180), MaeE (L897_04185), MaeR (L897_04170), and MaeK (L897_04175), as well as the modified allele for HPr (L897_05590) (Table S1) were generated using allelic replacement and the PCR primers listed in Table S2. The deletion alleles were transferred to the HSC5 chromosome using the allelic replacement vector pGCP213 (25) as described (26) and listed in Table S3. Each deletion allele was obtained through overlap extension PCR (27) using the primers listed in Table S2. All molecular constructs and chromosomal structures of all mutants were verified using PCR and DNA sequencing (Genewiz, South Plainfield, NJ) using oligonucleotide primers (IDT, Coralville, IA) of the appropriate sequences.

Complementation of ME mutants. To complement the *maeE* in-frame deletions, DNA fragments containing *maeE* from HSC5 in the absence of its promoter was amplified using the primers listed in Table 2 and inserted under control of the *rofA* promoter in pABG5 as previously described (28). The resulting plasmid, pEP66, was then used for ectopic expression of MaeE, (Table S3). For complementation of the *maeK* in-frame deletion, a reversion strategy was used to restore the wild-type locus in the MaeK^{-} mutant background. A DNA fragment containing the *maeK* open reading frame and flanking regions was amplified from HSC5 using primers listed in Table S2 and inserted into the plasmid pCRK (29). The resulting plasmid, pEP74, was then used to create the strain MaeK^{R} as described previously (30) (Table S1).

Metabolic assays. Malate, lactate, and formate concentrations were measured using commercially available kits (Sigma). Briefly, cultures of each individual strain tested were grown overnight in C medium with the appropriate supplement added (see text). Cultures were then subjected to centrifugation, filtered through 0.22 μm filters (Millipore), and then assessed per manufacturer's protocol. Data shown are the means and standard deviation from duplicate determination of three separate biological samples prepared from at least 2 independent experiments.

Isolation of RNA and transcript analysis. Transcript abundance of selected genes was analyzed as previously described (31). Briefly, overnight cultures were diluted 1:25 into fresh C medium with the appropriate supplement added (see text) and harvested at mid-log phase (OD_{600} 0.2). Total RNA was isolated using Qiagen RNeasy Mini kit per the manufacturer's protocol. RNA was subjected to reverse transcription (RT) using iScript (Bio-Rad) per manufacturer's protocol. RT-PCR analysis of cDNA samples were performed using iQ SYBR Green Supermix (Bio-Rad) and the primers listed in Table S2. Relative transcript abundance was determined using the $\Delta\Delta C_t$ method using *recA* transcript as a standard and are presented in comparison to unmodified C media or in comparison to wild type. The data shown are the means and the standard deviation from triplicate determinations of at least two separate biological samples prepared from at least two independent experiments.

Infection of mice. As previously described (32, 33), 5-to-6-week-old female SKH1 hairless mice (Charles River Labs) were injected subcutaneously with approximately 10^7 CFU of *S. pyogenes* of the strains indicated in the text. Following infection, the resulting ulcers formed were documented over a period of several days by digital photography and lesion areas measured as previously described (32). Data presented is pooled from at least two independent experiments with at least 10 mice per experimental group.

Growth rate calculations. Indicated bacterial strains were back-diluted 1:50 into 1 mL of fresh C medium (unmodified or altered as indicated in text) and their growth monitored at 37°C using a Tecan Infinite M200 Pro plate reader. During growth the plate was shaken every 10 minutes for 30s, followed by a 5s wait period and measurement of the OD_{600}. Data was normalized relative to uninoculated media and growth rates calculated as described previously (34). Growth rates are reported as doubling time ($t_{1/2}$) and were determined from a series of 7 time points collected over a 60 minute period that defines the peak rate of growth, which typically occurred prior to the culture reaching 15-30% of max OD_{600}. Growth yields were calculated from the maximum OD_{600} reached by the culture and are expressed as a percentage relative to the wild type strain under identical conditions. The average doubling time and percent growth yield was calculated from each replicate from at least three independent experiments.

Statistical analyses. Differences between mean values obtained for wild type and mutant strains in *in vitro* assays were tested for significance using the Student's t-test. For infection of mice, differences in lesion area between wild type and individual mutants were tested for significance using the Mann-Whitney U test. Computation of test statistics utilized Instat (version 3.1) and Prism (version 6.0) from Graphpad Software (San Diego, CA). For all tests, the null hypothesis was rejected for $P < 0.05$.

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Chapter III

RocA is Required for Growth-Phase Expression of Virulence Factors SPN and SLO in *Streptococcus pyogenes*

SUMMARY

The interaction between the NAD$^+$ glycohydrolase SPN and the cholesterol dependent cytolysin SLO allows for delivery of SPN directly into host cells by a process known as cytolysin-mediated translocation (CMT). It has also been shown that both proteins play a role in cytotoxicity, as loss of either protein has reduced virulence in cultured epithelial cells and *in vivo* in a mouse model of soft tissue infection. Taken together, these studies demonstrate that both SPN and SLO play an important role in pathogenesis of *S. pyogenes*. And although these secreted proteins have been shown to be involved in virulence of *S. pyogenes*, little information is known about how the bacterium regulates their expression. Analysis of expression patterns for these genes demonstrated that both are growth-phase regulated, with peak expression at exponential phase. Additionally, this temporal expression pattern is controlled by environmental pH, where acidic pH has a repressive effect on expression of both genes. Through a transposon mutagenesis screen a novel regulator of the *spn* operon, RocA, a predicted histidine kinase, was identified. An analysis of the RocA protein revealed that, although it does function as a transcriptional repressor of the *spn* operon, it is not a histidine kinase. Finally, RocA, along with the two-component system CovRS, is shown to be essential for the pH regulation of the *spn* operon. Taken together, this work sheds light on a regulatory mechanism utilized by *S. pyogenes* during adaptation to acid stress.

INTRODUCTION

An important factor in the colonization of host tissue by a pathogen is the ability to monitor changes in their surrounding environment and adapt as needed for survival. This adaptation is often seen at the level of transcription, resulting in careful spatial and temporal expression patterns of a number of proteins, including virulence factors. Identifications of conditions that control gene expression *in vitro* is, therefore, a useful tool for understanding the potential regulatory cues sensed by the bacteria during an infection *in vivo*.

The Gram-positive bacterium *Streptococcus pyogenes* is an incredibly versatile pathogen, capable of colonizing numerous sites within the human host and causing a variety of clinical diseases. These diseases range from mild, self-limiting infections such as impetigo and pharyngitis, to invasive and systemic diseases including toxic shock and necrotizing fasciitis (1-3). The ability of *S. pyogenes* to successfully infect multiple niches in the human body is the result of careful monitoring of variations in environmental stimuli, which, in turn, lead to global transcriptional changes in the bacterium (4-6). The regulatory cues that can lead to this transcriptional remodeling include temperature, pH, osmolarity, and nutrient availability, among others (5-8)

As a lactic acid bacterium (LAB), *S. pyogenes* is solely dependent on a simple fermentative metabolism to generate energy in the cell (9, 10). A by-product of this metabolism is the production of several organic acid end products that, over the course of growth, accumulate in high concentrations in the surrounding area (9, 10). This autoacidification process is significant for several reasons. First, it has been established that pH is a signal used by the bacterium to induce global transcriptional changes (7, 11, 12). Second, transcriptome studies have shown that the local tissue environment during later stages of infection is both low in glucose and low in pH (7). Therefore, understanding the regulatory mechanisms utilized by *S. pyogenes* in response to variations in environmental pH will provide insight into important adaptive strategies that the bacterium must use *in vivo* in infected tissue.

S. pyogenes is known to produce a large number of secreted virulence factors that can affect host cellular functions in numerous ways (1-3, 13-17). One important secreted factor is the NAD^+ glycohydrolase, SPN. Upon delivery into the host cell cytosol, SPN is able to cleave β-NAD^+ into nicotinamide and adenosine diphosphoribose (ADPr) (14, 18, 19). The process by which SPN is able to gain access to the host cytosol is a complex process known as cytolysin-mediated translocation (CMT) (20-22). CMT also requires the cholesterol dependent cytolysin Streptolysin O (SLO) for the direct translocation of SPN across the host cell membrane (20-22). It has also been established that both proteins play a role in pathogenesis, as loss of either protein has reduced virulence in cultured epithelial cells and *in vivo* in a mouse model of soft tissue infection (20, 23, 24). While a significant amount of work has been done in understanding the complex process of CMT, as well characterizing SPN's effects on the host cell (14, 18, 20-22, 25), little is known about how the bacterium regulates the expression of these important toxins.

While *S. pyogenes* lacks alternative sigma factors, it encodes a number of two-component systems (TCS) and stand-alone response regulators, many of which are influenced by both growth phase and environmental conditions (5, 6, 26-32). One of the most well characterized transcriptional regulators in GAS is the two-component system (TCS) CovRS. This system exerts its effect during late exponential and stationary growth, functioning mostly as a repressor of a number of surface-adhered and secreted virulence factors in response to multiple environmental stimuli including Mg^{2+}, temperature, pH, and the cathelicidin LL-37 (27, 33-38).

CovRS is one of the most well studied TCSs in *S. pyogenes* and is known to control up to 15% of the total genes in this bacterium, including *spn* and *slo* (33, 35, 38).

spn is the first gene in a 3-gene operon that includes *ifs* (immunity factor for SPN) and *slo* (19, 25, 39). While both SPN and SLO are secreted, IFS is a bacterial cytosolic protein that binds to SPN's active site to block its enzymatic activity within the bacterial cell (39). From few global transcription studies it has been established that expression of the *spn*/*slo* operon is associated with exponential phase of growth (4, 30), indicating that it is growth-phase regulated, however, the signal controlling this expression remains unclear. Additionally, while it is known that *spn* and *slo* are repressed by CovRS, it is not known if this is through direct or indirect regulation. Moreover, it remains unknown if any additional transcriptional regulators control the *spn* operon.

In this study we examined the regulatory mechanism controlling the growth phase regulation of the *spn*/*slo* operon. The analysis revealed that expression of the *spn* operon is controlled by pH and that this regulation requires both the CovRS system as well as a stand-alone transcriptional regulator, RocA.

RESULTS

Expression of *spn* and *slo* is controlled by pH. As a lactic acid bacterium, *S. pyogenes* growth in broth culture can have a significant effect on the pH of the medium. Growth in a glucose-rich medium such as THY results in a substantial reduction in environmental pH from a starting point of 7.4 to a low of approximately 5.4 (Fig. 1A).

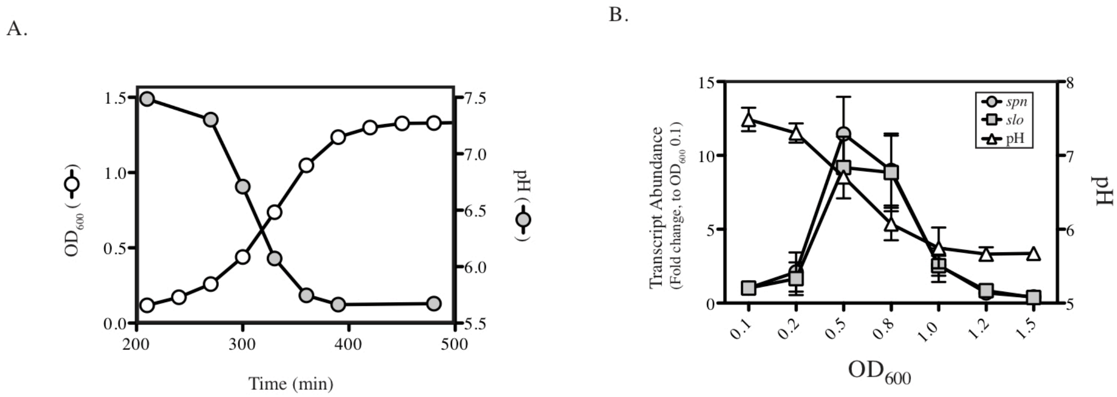

Figure 1. Growth-phase expression of *spn* and *slo*. (A) WT *S. pyogenes* was grown in THY medium over the course of 8 hours. Samples were removed at various time points and analyzed for growth (OD_{600}, left axis) and pH of cell-free supernatants were measured (right axis). **(B)** Transcript abundance of *spn* and *slo* (left axis) and pH of cell-free supernatants (right axis) were measured at specified stages of growth (OD_{600}, x-axis). Results are presented as mean and standard deviations from at least 2 biological samples analyzed in triplicate.

Transcriptional expression of the *spn*/*slo* operon is associated with the exponential phase of growth (4, 30), when culture pH is still near neutral (Fig. 1B). To provide more cumulative data on the pattern of transcriptional expression of this operon, WT cells were inoculated into fresh THY medium and samples were collected at seven distinct time points throughout the growth cycle and transcript abundance of *spn* and *slo* were measured (Fig. 1B). Both *spn* and *slo* had similar patterns of expression, with induction beginning during early exponential phase (OD_{600} of 0.2), peaking at mid-exponential phase (OD_{600} of 0.5), and then rapidly turned off as cells entered late exponential/stationary phase (OD_{600} of 1.0). This late phase in the growth cycle correlated with the culture pH dropping to an acidic level of approximately 6.0-6.5 (Fig. 1B).

These data led to the hypothesis that transcription of the *spn* operon is regulated by environmental pH, where acidic pH is a repressive signal on transcription of these genes.

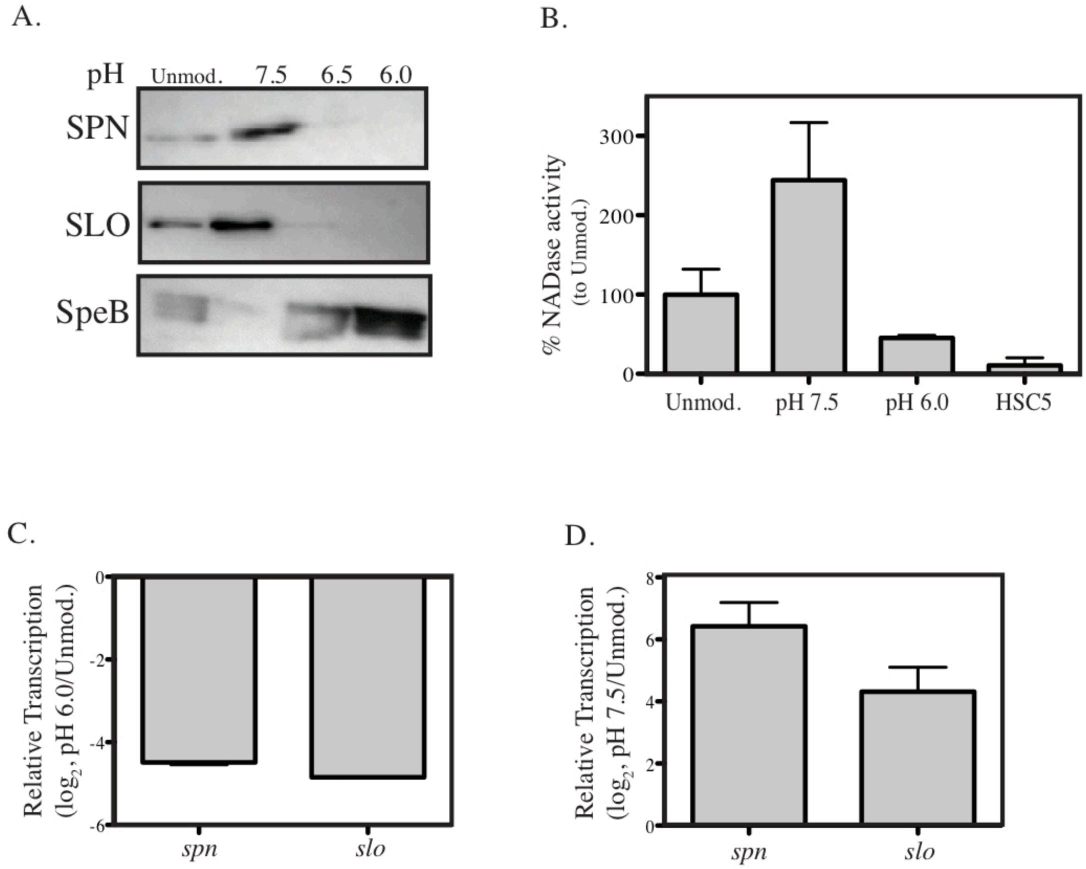

Figure 2. Expression of *spn* and *slo* is regulated by pH. (A and B) WT *S. pyogenes* was grown in THY buffered to specified pH and SPN and SLO protein was measured from cell-free supernatants by Western blot **(A)** or NADase activity **(B)**. **(C and D)** Transcript abundance of *spn* and *slo* was measured from WT cells grown in THY buffered to pH 6.0 **(C)** or 7.5 **(D)**. For analysis total RNA was isolated from cultures at exponential phase **(C)** or stationary phase **(D)**.

To test this, WT cells were grown in THY medium buffered to a range of pHs from 7.5 to 6.0. Cell-free supernatants from these overnight cultures were collected and analyzed by Western blot for expression of both SPN and SLO protein levels (Fig. 2A). Supernatants from cultures buffered to pH 7.5 had substantially more SPN and SLO than samples from cultures grown in unmodified THY. Conversely, growth in THY medium buffered to pH 6.5 or 6.0 repress protein expression to undetectable levels. As a control to show that general protein secretion is not affected, supernatants were also tested for levels of SpeB protein, which has been shown to be positively regulated by low pH (Fig. 2A) (7). As a second measure of SPN production, cell-free supernatants from cultures grown in unmodified THY or THY buffered to neutral (7.5) or acidic (6.0) pH were collected and analyzed for β-NAD$^+$ glycohydrolase (NADase) activity (Fig. 2B). Results of this analysis show that, compared to unmodified THY, neutral buffered media enhanced NADase activity 2- to 3-fold, while acidic buffered media reduced NADase activity 2-fold. As a negative control, overnight supernatants of strain HSC5 (which produces an NADase negative version of SPN) (18). All together, this data demonstrates that low pH is a repressive signal for SPN and SLO expression and that production of these proteins can be enhanced by buffering the medium to a neutral pH.

To determine whether the pH regulation of SPN and SLO was occurring at a transcriptional or post-transcriptional level, WT cells were grown in buffered THY medium and samples were collected at a specific stage of growth and transcript abundance of the *spn* and *slo* genes were measured (Fig. 2 C and D). WT cells were grown in either acidic buffered THY (pH 6.0) to mid-exponential phase (OD_{600} of 0.5) or in neutral buffered THY to late exponential/stationary phase (OD_{600} of 1.2). Results of this analysis show that growth in acidic media represses transcription of both *spn* and *slo* at exponential phase approximately 4-fold (log_2 value) compared to cells grown in unmodified THY (Fig. 2C). Additionally, late exponential/stationary phase cells grown in neutral buffered media had 4- to 6-fold (log_2 value) increased transcript abundance compared to cells grown in unmodified THY (Fig. 2D). Taken together, these results demonstrate that pH is an environmental signal controlling the growth phase expression of the virulence genes *spn* and *slo*, where low pH is a repressive signal for transcription of this operon.

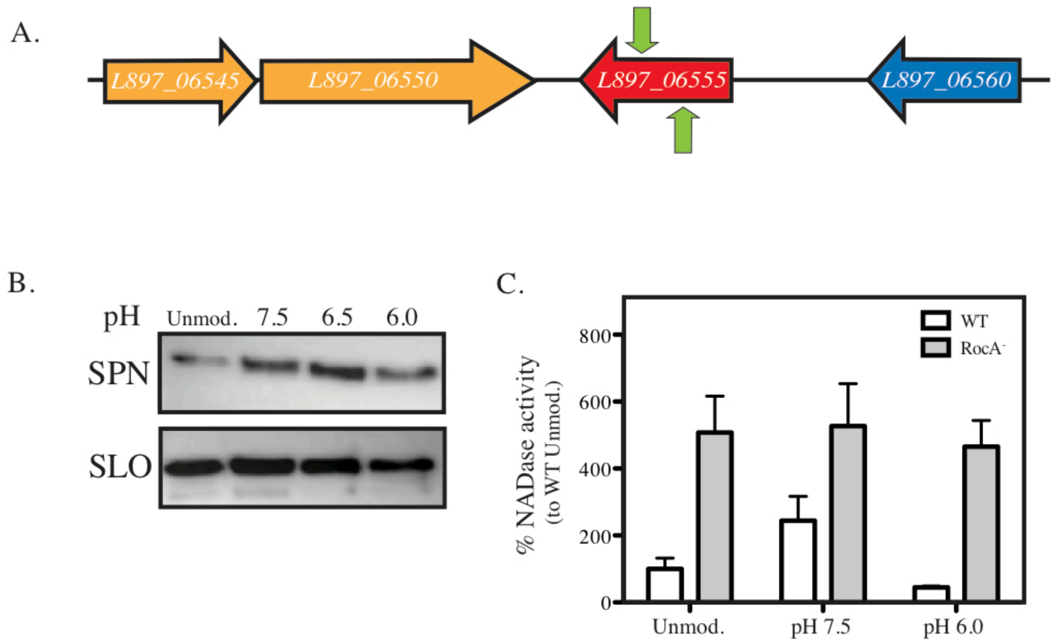

Figure 3. Deletion of RocA uncouples *spn* and *slo* from pH regulation. (A) Schematic of the genomic region containing the *rocA* gene (L897_06555) in *S. pyogenes*. Green arrows indicate location of transposon insertion sites. **(B and C)** The *S. pyogenes* RocA⁻ mutant was grown in THY buffered to specified pH and SPN and SLO protein was measured from cell-free supernatants by Western blot **(C)** or NADase activity **(D)**.

Deletion of RocA uncouples *spn* and *slo* from pH regulation. A random mutagenesis screen was performed in order to identify potential regulators involved in controlling the expression of the *spn*/*slo* operon in response to low pH. We screened approximately 2000 transposon mutants for production of SPN, measured by NADase activity of culture supernatants, after growth in THY pH 6.0. Within the mutant library, two independently derived transposon mutants were discovered to have high NADase activity in acidic THY. These mutants both had a transposon disruption in a gene (L897_06555) annotated in the HSC5 genome as a putative histidine kinase (Fig. 3A) (40). A BLAST search for related genes in *S. pyogenes* lead to the identification of the unknown kinase as the gene *rocA*, a highly conserved gene present in all sequenced strains of *S pyogenes*. RocA was first identified as a transcriptional regulator and was shown to act as a positive regulator for the *covRS* operon in JRS4 (40). Examination of the genomic region around *rocA* is that it appears to be a stand-alone histidine kinase (Fig. 3A), which is unusual as these proteins are usually part of a two-component system where both genes are encoded in a single operon (41). However, this is not the case for this gene of interest.

To verify that the *rocA* disruption is responsible for the SPN over-expression phenotype, a strain was made with an in-frame deletion of *rocA* in a WT background. This strain, RocA$^-$, was then tested for altered expression patterns for both SPN and SLO. RocA$^-$ cells were grown overnight in THY unmodified or buffered to pH 7.5, 6.5, or 6.0 and supernatants were collected for Western blot analysis. The results show that, under all conditions tested, RocA$^-$ causes overproduction of both SPN and SLO protein (Fig. 3B). As a second measure of SPN expression, WT and RocA$^-$ strains were grown overnight in THY, THY pH 7.5, or THY pH 6.0 and supernatants were collected and measured for NADase activity. The results demonstrated that the RocA$^-$ strain had consistently high NADase activity compared to WT under all pH conditions (Fig. 3C). Thus, loss of the RocA protein uncouples SPN and SLO from pH regulation. To restore pH repression of SPN and SLO the *rocA* gene was expressed ectopically on a plasmid under a constitutive promoter. This plasmid (pRocA) was then used to transform both WT and a RocA$^-$ mutant and NADase activity of supernatants from overnight cultures grown in unmodified THY medium was measured. Surprisingly, over-expression of RocA repressed SPN production by 80% in unmodified media (Fig. 4A). Thus, over-expression of RocA can repress SPN in the absence of any pH signal.

RocA is a transcriptional repressor of *spn* and *slo*. As a putative histidine kinase, RocA would be predicted to be part of a TCS and, therefore, is likely a transcriptional regulator. To test this, total RNA from WT and RocA⁻ strains grown in unmodified THY was collected and transcript abundance of *spn* and *slo* were measured by quantitative RT-PCR (Fig. 4B). The results show that loss of RocA causes an increase in transcript of both genes approximately 10-fold compared to WT. Additionally, when RocA was then ectopically expressed on a plasmid (pRocA) in either a WT or a RocA⁻ background, transcript levels were dramatically reduced nearly 10-fold compared to the non-complemented strains (Fig. 4B). Additionally, it is important to note that this experiment was performed with cells grown in unmodified THY, indicating that overexpression of RocA alone is sufficient to repress transcription of *spn* and *slo* and does not require a strong acidic pH signal to induce this repression. Taken together, this data

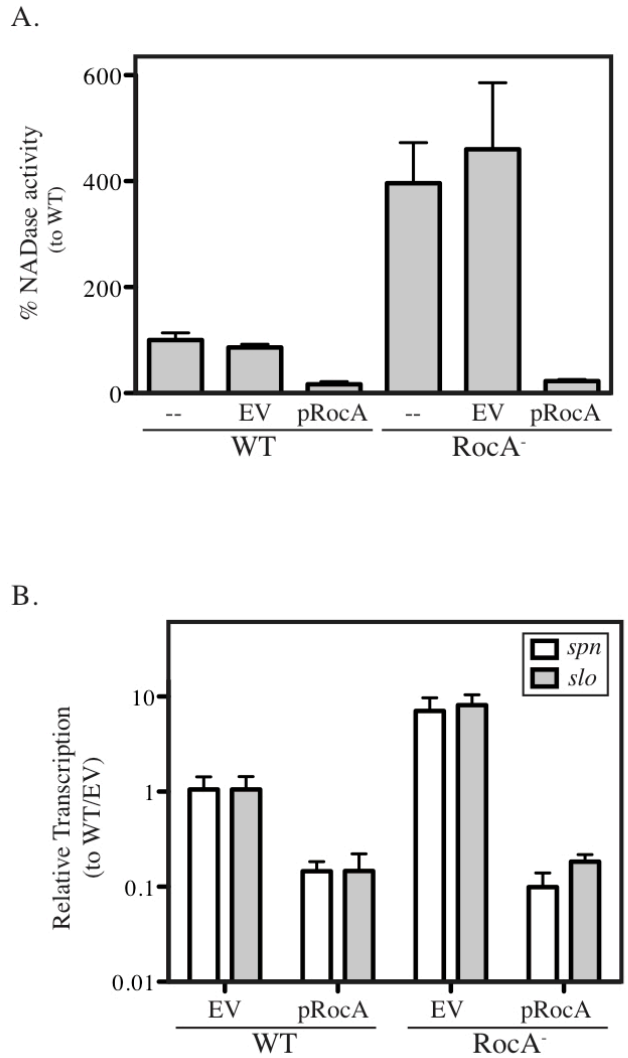

Figure 4. RocA is a transcriptional repressor of *spn* and *slo*. (A) NADase activity of cell-free supernatants from WT and RocA⁻ cultures containing indicated plasmid was measured. (EV, empty vector) **(B)** Total RNA from exponential phase cultures *slo* of WT and RocA⁻ strains containing indicated plasmid was isolated and transcript abundance of *spn* and was measured. Data presented is means and standard deviation from 3 biological replicates measured in triplicate.

shows that RocA is a transcriptional repressor of *spn* and *slo* and can function independently of a pH signal.

RocA is not a histidine kinase. Given how little this protein has been studied at this point, we sought to further characterize its functional activity. Previously, it has been hypothesized that RocA is a histidine kinase (42). Using structural prediction software (43), we obtained a putative structure of the cytoplasmic domain of the RocA protein. Histidine kinases (HK) are characterized as having three domains, the extracellular sensor domain (which vary among the different proteins), a transmembrane domain, and a cytosolic enzymatic domain. These last two domains are highly conserved among HKs (44). An examination of the cytoplasmic domain of RocA revealed that there was four histidine residues scattered throughout this domain (H247, H315, H387, H437) (Fig. 5A). To identify the key histidine residue necessary for RocA's function, single point mutations were made by directed mutagenesis using the pRocA plasmid, converting His to Ala independently at all four positions. These new constructs (pRocA^{H247A}, pRocA^{H315A}, pRocA^{H387A}, pRocA^{H437A}) were transformed into the RocA^{-} mutant and tested for protein expression and NADase activity (Fig. 5 B and C). The results show that all four pRocA mutant were able to repress SPN and SLO expression to similar levels as the WT pRocA, indicating that all four histidine mutants are dispensable for RocA regulatory function.

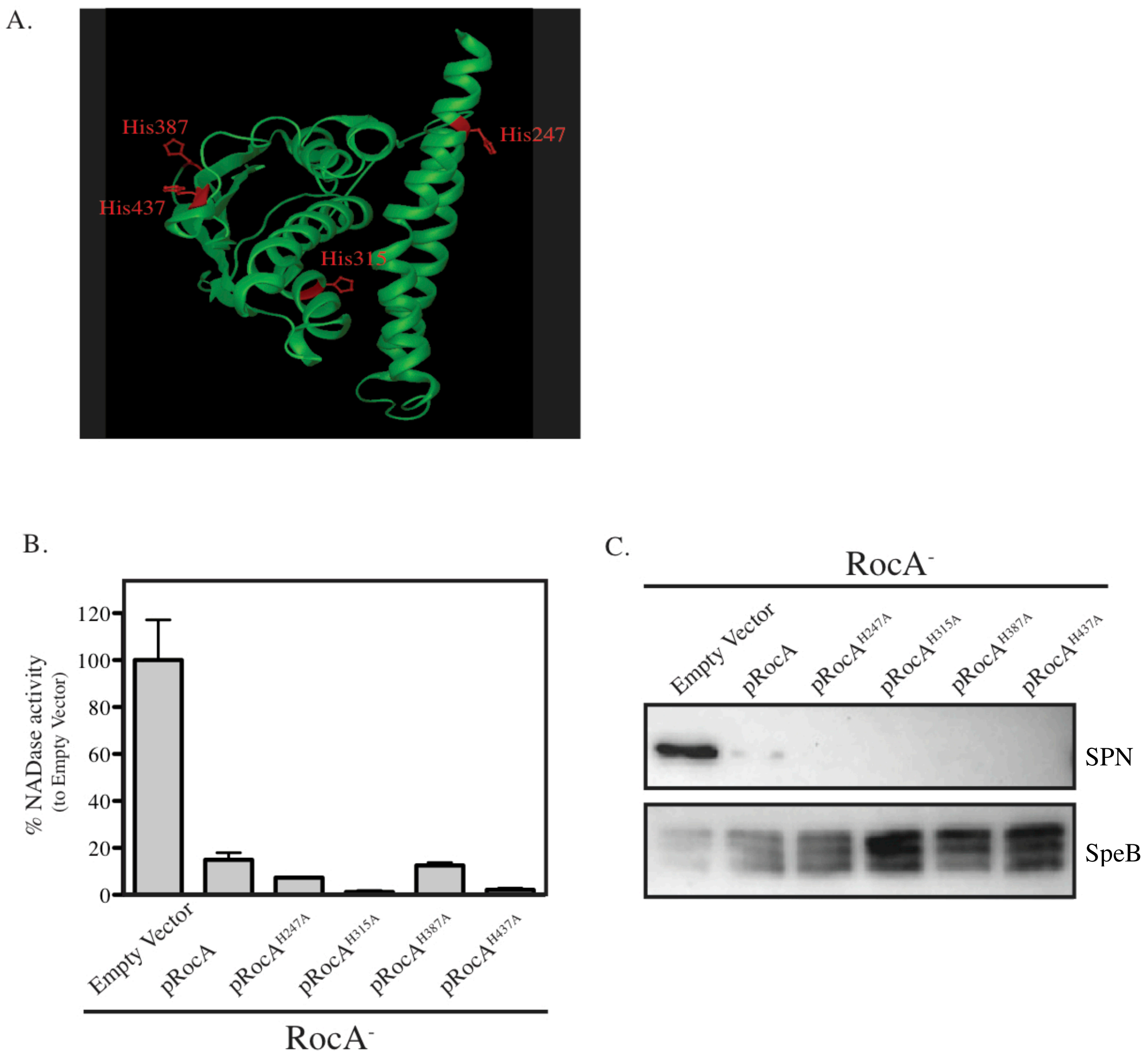

Figure 5. RocA is not a functional histidine kinase. **(A)** Structural prediction of RocA's cytoplasmic domain with individual histidine residues highlighted in red. **(B and C).** pRocA mutant constructs were tested for regulation of SPN by NADase activity **(B)** and Western blot **(C).**

RocA repression of SPN and SLO requires CovRS. Given that RocA was initially identified as a regulator of the *covRS* operon (40), and that the CovRS TCS is a known regulator of the *spn*/*slo* operon (33, 35, 38), it was of interest to investigate if RocA-mediated regulation of *spn* and *slo* involved CovRS. To test this, in-frame deletion mutants of *covR* or *covS* were made individually in a WT background and these mutants were tested for altered SPN production by measuring NADase activity of culture supernatants (Fig. 6). Results showed that deletion of either gene lead to significantly higher levels of NADase activity than WT, indicating that both CovR and CovS are required for repression of SPN. This finding was in agreement with previous reports of CovRS regulation of the *spn*/*slo* operon and appeared identical to a RocA$^-$ mutant (33, 35, 38). Lastly, to evaluate the contribution of either protein in the RocA regulatory pathway, the plasmid pRocA was transformed into both CovR$^-$ and CovS$^-$ and these strains were tested for SPN production. NADase activity of overnight supernatants from cultures grown in unmodified THY broth from strains RocA$^-$, CovR$^-$, CovS$^-$, plus their pRocA complemented strains. The results show that over-expression of RocA on pRocA can only complement the RocA$^-$ mutant, but neither of the Cov deletion mutants (Fig. 6). Thus, all three components are required for repression of the *spn*/*slo* operon.

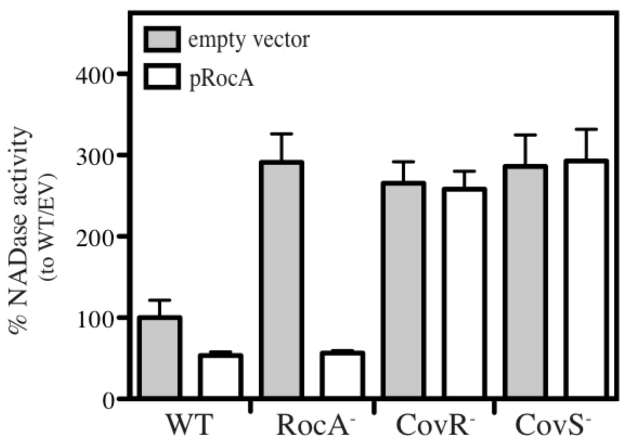

Figure 6. RocA repression of SPN requires CovRS NADase activity of cell-free supernatants from WT and mutant and complemented strains was measured.

DISCUSSION

In this study we have shown that the virulence factors SPN and SLO, which are co-transcribed together with the protein IFS in a single operon, are expressed in a growth-phase pattern of expression, with their peak transcript abundance occurring during exponential phase. In addition, we have shown that this pattern of regulation is controlled by environmental pH, where exposure to acidic conditions can repress transcription of this operon and, conversely, maintenance of a neutral pH can extend transcription through to stationary phase. Furthermore, we have identified the transcriptional regulator RocA, in addition to the global regulatory TCS CovRS, as being required for this pH-mediated response. Finally, we have shown that while RocA is, in fact, a transcriptional regulatory protein, it is likely not a functional histidine kinase as previously reported. Taken together, these data shed light on the specific growth-phase regulation of several important virulence factors and further characterizes the contribution of RocA in *S. pyogenes* pathogenesis.

Growth-phase expression of virulence factors is a common regulatory strategy used by multiple pathogenic bacterial species (10, 31, 45). Often times this growth-phase regulation is linked to metabolism. For example, in *S. pyogenes,* the catabolite control protein CcpA regulates a number of virulence factors including the cysteine protease SpeB and the lactate oxidase gene *lctO* in response to carbohydrate availability (46). The transcriptional regulator CodY, which recognizes branched chain amino acids, is responsible for the growth-phase regulation of the virulence factors *pel* and *sagA* (6). We can now add the genes *spn* and *slo* to this group. As a lactic acid bacterium, *S. pyogenes* ferments glucose, leading to the formation of multiple organic acids which, when secreted into the environment, cause a significant drop in pH (9, 10, 31, 45, 47). Thus, as glucose is consumed, there is a corollary drop in environmental pH. In this way, pH is an indirect metabolic signal for the bacterial cell.

This work also provides new information on the role of RocA and its involvement with the CovRS TCS. Although RocA is a highly conserved protein present in all sequenced *S. pyogenes* strains, few studies have been done to characterize this protein and it's role in virulence regulation and pathogenesis. Previous studies have identified RocA as a regulator of *hasA*, which is necessary for capsule synthesis (40, 42), although this is attributed to the fact that RocA is a regulator of CovR, which is known to repress capsule synthesis genes (27). There is, however, data that suggests that RocA's role in transcriptional regulation extends beyond interactions with CovRS. A proteomics study to identify regulatory targets of RocA identified approximately 30 proteins whose expression were significantly altered in a RocA null mutant compared to WT (42). Of these 30 targets, only one third of them were determined to be part of the CovRS regulon. In addition, the majority of these RocA targets, most of which are repressed by the protein, are involved in metabolism (42). Thus, RocA's main contribution to the cell may be as a key metabolic regulator. As previously mentioned, there are a few well-characterized metabolic regulators in *S. pyogenes* known to respond to various nutritional cues such as glucose, carbohydrates, and amino acid starvation (5, 6, 28, 46). An important next step in the study of RocA would be to determine what conditions or signals it responds to. The work presented here suggests that pH may be one of those signals, but, like CovRS (34, 36, 38), RocA may recognize multiple environmental cues.

In addition to identifying the conditions that trigger RocA activation, there is also the question of how RocA is functioning as a transcriptional regulator. The work presented here demonstrates that RocA is not a functional histidine kinase, as all histidine residues within the cytoplasmic domain of the protein are dispensable for function. This conclusion is further supported by previous findings that the RocA protein sequence lacks several key residues necessary for function in other known histidine kinases (40). Additionally, analysis of the sequence of the predicted ATPase domain of the protein also revealed mutations to key residues necessary for enzymatic activity (E. Paluscio and M. Caparon, unpublished). Taken together, this information suggests that RocA may not possess any enzymatic activity. Additionally, no DNA binding domains have been identified in RocA, yet there is strong evidence from this work and other that indicate this protein is involved in transcriptional regulation of multiple target genes (40, 42).

Given that RocA maintains strong homology to other bacterial histidine kinases, but lacks the specific residues necessary for phosphorylation, a possible hypothesis is that RocA is a functional pseudokinase. Pseudokinases can be described as proteins that are classified as part of a specific enzyme group based on sequence or structural homology, but lack any enzymatic activity (48). Pseudokinases, which are found in all domains of life, are thought to function in signaling pathways in several specific ways, including as modulators of kinase and phosphatase activity (49-52). In the case of RocA and regulation of the *spn* operon, we hypothesize that this protein is affecting the phosphorylation state of CovR through modulation of CovS's enzymatic activity.

CovS has been shown to maintain both kinase and phosphatase activity (53), thus altering the phosphorylation state of CovR. Additionally, it has been suggested that CovR's phosphorylation state determines its regulatory function, including which target promoters it can bind to and whether it functions to enhance or repress transcription of its target genes (27, 53, 54). It is possible that for regulation of *spn*, and possibly additional genes, the interaction between RocA and the CovRS system is necessary for sufficient transcriptional repression. The concept of a TCS requiring auxiliary proteins for signal transduction is a common mechanism among both Gram-positive and Gram-negative species and has been associated with a variety of cellular processes from cell division to virulence factor regulation (50, 51, 55-57). The precise mechanism by which this is occurring to regulate the growth phase expression of the *spn/slo* operon remains unclear. However, the work presented here has established that this regulation is controlled by environmental pH and provides new insights into virulence gene regulation in *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *Escherichia coli* strain TOP10 (Invitrogen) was used for cloning using standard molecular biology techniques. The *Streptococcus pyogenes* strain JOY3 (18), which is an NADase positive version of the strain HSC5 (58), and mutant derivatives were utilized in this study. Strains were grown in Todd Hewitt broth (THYB) with 0.2% yeast extract (Difco). Routine growth conditions utilized sealed culture tubes incubated at 37°C under static conditions. Streptococcal strains grown on solid medium containing 1.4% Bacto agar (Difco) were cultured in a sealed jar with a commercial gas generator (GasPak catalogue no. 70304, BBL). For experiments utilizing buffered media, 1M stock solutions of HEPES (pH 7.5) or MES (pH 6.0) (Sigma) were added to a final concentration of 0.1M to the media. All media used were sterilized in an autoclave prior to supplementation. When appropriate, antibiotics were added at the following concentrations: erythromycin 1 mg/mL, kanamycin 250 mg/mL, spectinomycin 100mg/mL.

Mutagenesis strategy. Transposon mutagenesis utilized a modified version of Tn*4001* containing a spectinomycin resistance cassette (59). Construction of a transposon mutant library was conducted as described previously (59). For mutants of interest, the transposon insertion site was mapped by arbitrary PCR as described (60). Briefly, DNA flanking the transposon insertion site is enriched through two rounds of amplification using primers specific to the 5' end of the transposon and nonspecific primers that can anneal to random sites within the bacterial chromosome. The first round of PCR was performed using primers ARB1 (5'-GCCGACCGCTGGACTGTACG-

NNNNNNNNNNGTAGC) and OUT3 ((5'- GCGTGCCTACACGTGTCG). The second round of PCR utilized 5μL of the first-round product as the template and PCR primers ARB2 (5'-GCCGACCGCTGGACTGTACG) and OUT1 (5'-GTCCTCCTGGGTATGT-TTTT). Second round PCR products were purified and sequenced using primer OUT1.

Directed mutagenesis and complementation. All references to genomic loci are based on the genome of HSC5 (58). In-frame deletion mutations in the genes encoding RocA (L897_06555), CovR (L897_01565), and CovS (L897_01570) (Table 1) were generated using allelic replacement and the PCR primers listed in Table 2. The deletion alleles were transferred to the HSC5 chromosome using the allelic replacement vector pGCP213 (61) as described (62) and listed in Table 3. Each deletion allele was obtained through overlap extension PCR (63) using the primers listed in Table 2. All molecular constructs and chromosomal structures of all mutants were verified using PCR and DNA sequencing (Genewiz, South Plainfield, NJ) using oligonucleotide primers (IDT, Coralville, IA) of the appropriate sequences. To complement the *rocA* in-frame deletions, DNA fragments containing *rocA* from HSC5 in the absence of its promoter and including an HA tag at the C-terminal end of the protein was amplified using the primers listed in Table 2 and inserted under control of the *rofA* promoter in pABG5 as previously described (39). The resulting plasmid, pEP85, was then used for ectopic expression of RocA-HA (Table 3). The four RocA mutants (H247A, H315A, H387A, H437A) were made from the WT *rocA* sequence in pEP85 using the Quikchange XL II mutagenesis kit (Agilent Technologies).

Analysis of protein expression. SPN production was measured by a fluorometric assay measuring β-NAD^+ glycohydrolase activity of cell-free supernatants as described (20). Specific activity of each strain is reported relative to wild type as described (19). Expression of SPN and SLO protein was measured from cell-free supernatants as described (22). For all experiments, samples were normalized to OD_{600} of overnight cultures.

Isolation of RNA and transcript analysis. Transcript abundance of selected genes was analyzed as previously described (64). Briefly, overnight cultures were diluted 1:25 into fresh THY medium with the appropriate supplement added (see text) and harvested at the OD_{600} indicated in the text. Total RNA was isolated using Qiagen RNeasy Mini kit per the manufacturer's protocol. RNA was subjected to reverse-transcription (RT) using iScript (Bio-Rad) per manufacturer's protocol. RT-PCR analysis of cDNA samples were performed using iQ SYBR Green Supermix (Bio-Rad) and the primers listed previously (7). Relative transcript abundance was determined using the $\Delta\Delta C_t$ method using *recA* transcript as a standard and are presented in comparison to unmodified THY media or in comparison to wild type. The data shown are the means and the standard deviation from triplicate determinations of at least two separate biological samples prepared from at least two independent experiments.

Table 1. Strains used in this study

Strain	Relevant Genotype	Mutated Loci[a]	Plasmid	Description[b]	Reference
S. pyogenes					
JOY3	SPN$_{J4}$	00945	none	wild-type	Chandresakaran, et al. (2013)
HSC5			none	NAD+ glycohydrolase negative	Port, et al. (2013)
EP154	Δ*rocA*	06555	none	in-frame deletion of *rocA*	This work
EP155	Δ*covS*	01570	none	in-frame deletion of *covS*	This work
EP156	Δ*covR*	01565	none	in-frame deletion of *covR*	This work
EP177	SPN$_{J4}$	00945	pABG5		This work
EP180	Δ*rocA*	06555	pABG5		This work
EP178	Δ*covS*	01570	pABG5		This work
EP179	Δ*covR*	01565	pABG5		This work
EP181	SPN$_{J4}$	00945	pEP85		This work
EP182	Δ*rocA*	06555	pEP85		This work
EP183	Δ*covS*	01570	pEP85		This work
EP184	Δ*covR*	01565	pEP85		This work
EP185	Δ*rocA*	06555	pEP86		This work
EP186	Δ*rocA*	06555	pEP87		This work
EP187	Δ*rocA*	06555	pEP88		This work
EP188	Δ*rocA*	06555	pEP89		This work

a. Loci are based on the genome HSC5 (Port et al. 2013) and follow the formate L897_xxxxx, where xxxxx are numbered
b. antibiotics are abbreviated as follows: chloramphenicol (Cam)

Table 2. Primers used in this study

Name	Sequence[ab]	Template	Plasmid	Description
rocA 5' F (BamHI)	GGCGGATCCTCTTTTAAGCTGTAGAAACCTTTGC	HSC5		primers for in-frame deletion of *rocA*
rocA 5' R	CTATTAACTGTCCTAAAAATTGAAGAAAATCTTCTAACAT			
rocA 3' F	ATTTTTAGGACAGTTAATAGAAATAGCTAAGCCTGACTGA			
rocA 3' R (EcoRI)	GGCGAATTCCAGACGATCAAGTCGCTCTAACAG			
covR 5' F (BamHI)	GCCGGATCCGCAAGGGTTGTTTGATGAATAAAACT	HSC5		primers for in-frame deletion of *covR*
covR 5' R	ATCCCATGCCATCTTCAATAATTAAAATTTTCTTTGTCATTTATA			
covR 3' F	TATTGAAGATGGCATGGGATACGTTATTCGTGAG			
covR 3' R (EcoRI)	GCCGAATTCGTCATCAATTCTCAGATGTGTTTTATCG			
covS 5' F (BamHI)	GCCGGATCCAATCCACAAAACCGTTCAGTTAATCG	HSC5		primers for in-frame deletion of *covS*
covS 5' R	GGTTTCCTTATTTCTTCTGTTTTTGTTTCTGATTTTCCAT			
covS 3' F	ACAGAAGAAAATTCCTTTGGCCCAGTCTAAAGAGAG			
covS 3' R (EcoRI)	GCCGAATTCGCCGTCTTCAGCTTGTCTACTGTCA			
rocA F (EcoRI)	GCCGAATTCCGAGATGTGATAACATATTTTGAACGAG	HSC5		primers for complementation of *rocA*-HA
roA R (PstI)	GCCCTGCAGTTAAGCATAATCTGGAACATCATATGGATAGTCAGGCTTAGCTATTTCTATTAACTGG			
rocA H247A F	GAGCTGGTTAATTATAGTCAG***GCC***CTTGGATTGCTGTATCAAGA	HSC5		primers for His247 to Ala in *rocA*
roA H247A R	TCTTGATACAGCAATCCAAGG***GCC***TGACTATAATTAACCAGCTC			
rocA H315A F	TTAGATAAATTACAAGTTGAAGCAATCAGA***GCT***ATTGTTTTAGCTAAATTAATTGAGGCAAAA	HSC5		primers for His315 to Ala in *rocA*
roA H315A R	TTTTGCCTCAATTAATTTAGCTAAAACAAT***AGC***TCTGATTGCTTCAACTTGTAATTTATCTAAA			
rocA H387A F	TTATCAATAGCATTTCTGGATCAAAAC***GCT***AAACTTATCATAGTCATTCAAAGCAG	HSC5		primers for His387 to Ala in *rocA*
roA H387A R	CTGCTTTGAATGACTATGATAAGTTT***AGC***GTTTTGATCCAGAAATGCTATTGATAA			
rocA H437A F	ATGACTATCTCACAATTAGCTC***GCA***GATTGCTGATGGCATTTTAACC	HSC5		primers for His437 to Ala in *rocA*
roA H437A R	GGTTAAAATGCCATC***AGC***AATCTGCGAGCTAATTGTGAGATAGTCAT			

a. Engineered restriction sites are underlined
b. Engineered mutation sites are in bold italics

Table 3. Plasmids used in this study

Plasmid (Resistance)[a]	Description	Reference
pGCP213 (Erm)	temperature-sensitive shuttle vector, used for allelic replacement	Nielsen et al. (2012)
pABG5 (Kan, Cam)	shuttle vector, used for ectopic expression	Meehl et al. (2005)
pMGC57-Spc	TnSpc plasmid	Lyon et al. (1998)
pEP85 (Kan, Cam)	pABG5::*rocA-HA*	This work
pEP86 (Kan, Cam)	pABG5::*rocA*$_{H247A}$-HA	This work
pEP87 (Kan, Cam)	pABG5::*rocA*$_{H315A}$-HA	This work
pEP88 (Kan, Cam)	pABG5::*rocA*$_{H387A}$-HA	This work
pEP89 (Kan, Cam)	pABG5::*rocA*$_{H437A}$-HA	This work

a. Antibiotics are abbreviated as follows: kanamycin (Kan), chloramphenicol (Cam), erythromycin (Erm), spectinomycin (Spc)

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Chapter IV

Alterations of CcpA Activation has Significant Effects on the Outcome of a *Streptococcus pyogenes* Infection

SUMMARY

Streptococcus pyogenes can infect a number of different tissue types within the human host. Successful adaptation to these varying environments is, in part, due to the bacterium's ability to sense changes in environmental signals and rapidly alter its gene expression profile in response to these changes. Carbon catabolite repression (CCR) allows the bacteria to metabolize preferable carbon sources in the environment, usually through transcriptional repression of genes in the processing of alternative, and less favorable, carbon sources. The key transcriptional regulator of CCR in *S. pyogenes* is CcpA. This protein has been shown to be a global regulator of gene expression, affecting transcription of genes involved in both carbon metabolism as well as a number of known virulence factors. The majority of the identified CcpA-regulated genes are upregulated in the absence of the protein, demonstrating that the main role of CcpA is in repression of target genes. A great deal of work has been done to characterize the role of CcpA in *S. pyogenes* pathogenesis, however, all of these previous studies utilize CcpA null mutants. But, given the fact that CcpA acts mostly as a repressor of target genes, these previous studies are unable to demonstrate the significance of CcpA function on metabolism and pathogenesis. To provide a more complete analysis of CcpA function, we have designed a constitutively active CcpA protein, $CcpA^{T307Y}$. Both a $CcpA^{-}$ and $CcpA^{T307Y}$ mutants were then tested for altered virulence in a soft tissue infection and in a vaginal mucosal colonization model. Both CcpA mutants displayed altered virulence phenotypes in both models of infection. Further characterization of the effects of these mutants on pathogenesis may lead to new insights into the role of CcpA regulation in *S. pyogenes*.

INTRODUCTION

Carbon catabolite repression (CCR) is the mechanism by which bacteria preferentially metabolize one carbon source over another from their environment, primarily through transcriptional repression of genes necessary for processing alternative, and less favorable, carbon sources. The key transcriptional regulator of CCR in low G+C Gram-positive bacteria is the catabolite control protein A (CcpA) (1-4). In addition to controlling metabolic genes, CcpA has been identified as a regulator of several virulence factors in a number of pathogenic bacterial species (2, 5-8). This indicates that nutrient availability is linked to virulence factor production and pathogenesis.

In the bacterium *Streptococcus pyogenes,* CcpA has been shown to be a global regulator of gene expression, affecting transcription of approximately 20% of the total genome. *S. pyogenes* is known to cause a wide variety of infections at numerous different tissue sites within the human host (9-13). The diseases caused by *S. pyogenes* range from mild and self-limiting infections such as impetigo and pharyngitis, to systemic and life threatening diseases such as cellulitis and necrotizing fasciitis, as well as serious postinfection sequelae such as rheumatic fever and glomerulonephritis (9, 10, 12, 14). The ability of *S. pyogenes* to infect a number of different tissue types within the human host is, in part, due to the bacterium's ability to sense changes in environmental signals and rapidly alter its gene expression profile in response to these changes (15-21). Several studies have demonstrated the ability of *S. pyogenes* to integrate various environmental cues as a mechanism for global gene expression changes. These signals include temperature, osmolarity, pH, and nutrient availability (17, 22-24).

Lacking functional alternative sigma factors, *S. pyogenes* transcription is under the control of a number of two component systems and stand-alone response regulators to control gene expression in response to various signals (17, 22, 25-31). Several of these regulators are known to control gene expression in response to specific nutritional cues. RelA, CodY, and RopB are all global transcription regulators whose function is linked to amino acid catabolism (29, 30, 32-34). However, in addition to regulating a number of metabolic genes, all three regulators control a number of virulence factors (29, 30, 32, 33). LacD.1, which was first identified as a regulator of the cysteine protease SpeB, functions in response to the levels of the glycolytic intermediates glucose-6-phosphate and dihydroxyacetone phosphate, indicating that it too functions as a regulator of carbon catabolic control (35, 36). Additionally, it's been shown that glucose concentrations can have a global effect on gene expression in *S. pyogenes*, affecting both alternative catabolic operons and numerous virulence factors, and this response largely controlled through CcpA (5, 7, 8, 37). Taken together, this data indicates that nutrient availability and virulence factor production are intimately linked through the actions of multiple global regulatory pathways.

Numerous studies have been undertaken to assess the global effects of CcpA regulation and its contributions to pathogenesis (2, 5-8). From these studies, several important pieces of information have been derived. First, microarray analysis has shown that, although a significant number of CcpA-regulated genes are also glucose-regulated genes, there is also a subset of genes regulated by CcpA only, demonstrating that CcpA function is controlled by glucose-dependent and -independent signals (5). Second, although CcpA primarily acts as a transcriptional repressor, expression of a small subset of genes, including the major virulence factor SpeB, is positively regulated by CcpA (2, 5). Finally, loss of the *ccpA* gene results in an attenuated virulence phenotype in a murine model of soft tissue infection (2, 5). Transcriptional analysis of CcpA-regulated genes during the course of a 7-day infection with either WT or the CcpA$^-$ mutant showed temporal misregulation of targets in the mutant strain including both metabolic and virulence genes. In particular, patterns of misregulation were most strongly associated with earlier time points, suggesting that CcpA regulation is crucial during the early stages of colonization (5). Additionally, the $CcpA^-$ mutant has also been shown to be defective in asymptomatic mucosal carriage using a murine vaginal colonization model, and this defect was primarily attributed to the dysregulation of the lactate oxidase gene *lctO* (13). Taken together, these data demonstrate the significant and complex role that CcpA regulation has in controlling the outcome of infection in multiple tissue environments.

A caveat to these data, however, is that these studies were limited to utilization of a *ccpA* null mutant for analyses, which provides information exclusively on the effects of the loss of CcpA function. Therefore, to further analyze the effect of CcpA activation and its role in pathogenesis, we designed and tested a constitutively active mutant allele of CcpA, $CcpA^{T307Y}$. The constitutively repressive activity of the $CcpA^{T307Y}$ mutant was verified by analyzing transcript levels of a series of known CcpA targets *in vitro*. The $CcpA^{T307Y}$ over-activation mutant was then tested *in vivo* using two different mouse models of disease, subcutaneous soft tissue infection and vaginal mucosal colonization and compared to WT and $CcpA^-$. The results indicate that over-activation of CcpA causes attenuation of virulence in soft tissue, but allows for extended carriage during asymptomatic mucosal colonization.

RESULTS

Construction of CcpA "super repressor". In WT *S. pyogenes* CcpA functions as a repressor of gene expression in the presence of high glucose concentrations (Fig. 1). When glucose is abundant, it is rapidly taken up into the cell and metabolized through the glycolytic pathway (38). During this process there is a high intracellular concentration of the glycolytic intermediate fructose-bis-phosphate (FBP). FBP levels in the cell influence the enzymatic activity of the protein HprK, which can act as both a kinase and a phosphatase to control the phosphorylation state of the phosphocarrier protein HPr (38, 39). At high FBP levels, HprK functions as a kinase to phosphorylate HPr at the serine 46 residue (38, 39). This serine-phosphorylated form (P~Ser-HPr) then functions as a cofactor for CcpA activation. The binding of two molecules of P~Ser-HPr to the CcpA dimer induce a conformational change, shifting the two CcpA molecules from

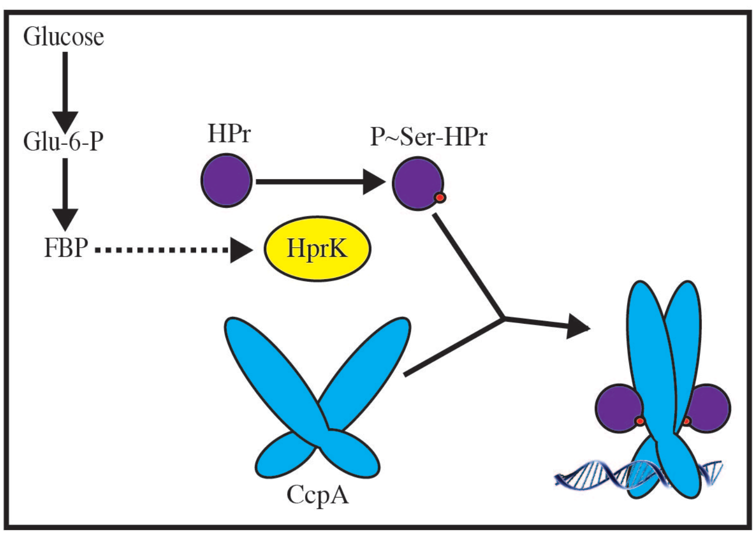


Figure 1. CcpA and carbon catabolite repression. Glucose is rapidly metabolized, resulting in high levels of the glycolytic intermediate FBP. FBP stimulates the kinase activity of the protein HprK, leading to formation of P~Ser-HPr. The interaction of the CcpA dimer with P~Ser-HPr induces a conformational change in the CcpA dimer, allowing CcpA to bind DNA promoters at *cre* sites.

an open and inactive conformation to a closed conformation, which can then bind to DNA at specific promoter sites (Fig. 1, 2A) (40).

We sought to create a constitutively active form of CcpA through the mutation of a single amino acid, Thr 307. Previously, the crystal structure of CcpA from the Gram-positive bacterium *Bacillus megaterium* was solved (40). That work identified a series of amino acids found at the

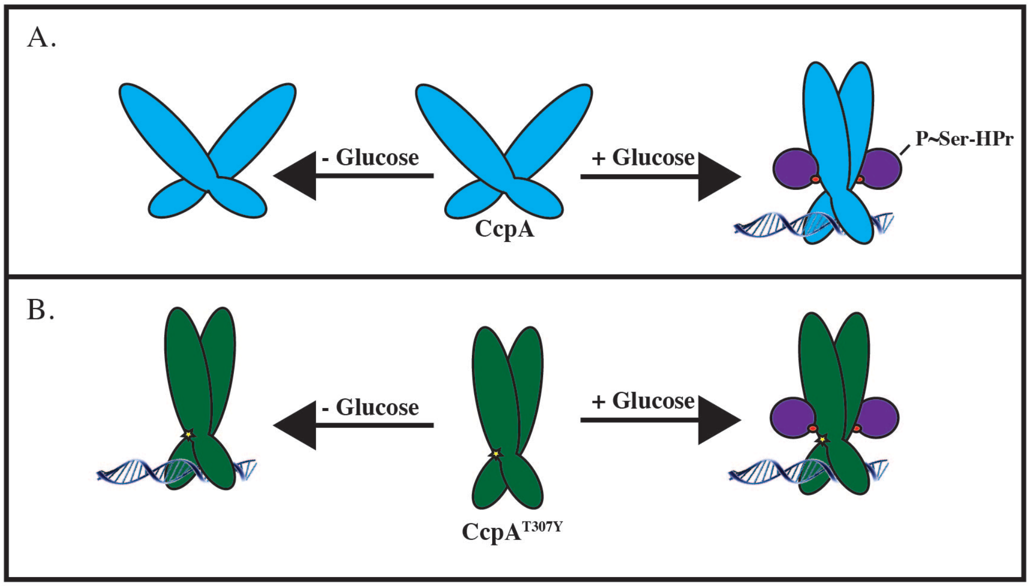


Figure 2. Design of constitutively active CcpA. (A) WT CcpA, in the absence of glucose, remains in an open conformation and is unable to bind DNA. In the presence of glucose, a high quantity of serine phosphorylated HPr is formed. The interaction of P~Ser-HPr with CcpA switches the regulator into the closed conformation, which can then bind DNA. **(B)** Mutation of the Thr 307 residue of CcpA to Tyr forces the CcpA dimer into its closed conformation, mimicking P~Ser-HPr cofactor binding. $CcpA^{T307Y}$ functions independently from glucose concentrations and is active under all conditions.

dimer interface that were involved in the structural rearrangement of the CcpA dimer upon P~Ser-HPr binding. In particular, they found that replacing the Thr with an amino acid with a bulky side group would mimic P~Ser-HPr binding and force the CcpA dimer into its active, closed conformation absent any cofactor binding (40). In *S. pyogenes* this key residue is Thr307.

Therefore, through directed mutagenesis the threonine residue was replaced with a tyrosine to create the mutant CcpA^{T307Y} (Fig. 2B).

CcpA^{T307Y} is constitutively active in the absence of glucose signal. To test for growth defects of the CcpA^{-} and CcpA^{T307Y}, both strains plus WT were cultured overnight in both a glucose rich media (THY) (Fig. 3A) and a low carbohydrate media (C medium) (Fig. 3B). Growth in THY medium was identical to WT for both CcpA^{-} and CcpA^{T307Y}, indicating that in glucose rich conditions these mutants have no growth defects. When comparing growth in C medium, both WT and CcpA^{-} mutant strains grew at a similar rate and reached a final OD_{600} of 0.40, however the CcpA^{T307Y} mutant had a slight growth defect and reached a final OD_{600} of 0.35.

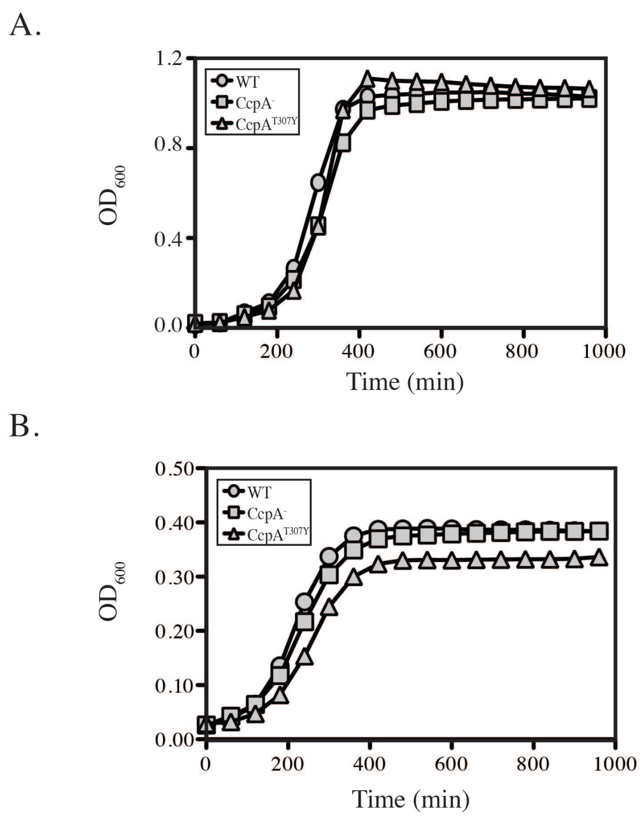
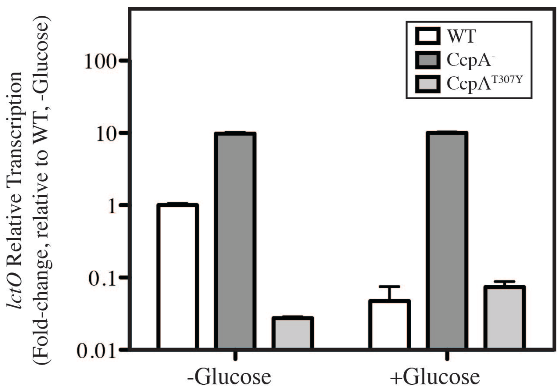


Figure 3. Growth of CcpA mutants *in vitro*. Growth of WT and CcpA mutants were grown in **(A)** rich media (THY) or **(B)** minimal medium (C medium).

To test for the functionality of the CcpA^{T307Y} mutant, real time RT-PCR was performed on a series of known CcpA target genes (2, 5). Previously published work identified the lactate oxidase gene *lctO* as being repressed by CcpA in response to glucose (5). For this analysis WT, CcpA^{-}, and CcpA^{T307Y} strains were grown in unmodified C medium or C medium supplemented with 0.2% glucose. Transcript levels were measured from cells grown to exponential phase and normalized to WT in unmodified C medium (Fig. 4A). The addition of glucose resulted in a 10-fold reduction in transcript abundance in WT cells, yet had no effect in a CcpA- strain, indicating that glucose-dependent CcpA-activation is responsible for *lctO* repression.

A.



Furthermore, the CcpA^{T307Y} strain, displayed constitutively low *lctO* transcript abundance compared to WT regardless of glucose content, demonstrating that this CcpA^{T307Y} mutant is able to repress genes in the absence of a glucose signal.

Although CcpA primarily acts as a repressor of gene expression, in some cases it can enhance transcription (2, 5). One gene that is positively regulated by CcpA is *speB*, the gene that encodes the SpeB cysteine protease (2). WT and the two CcpA strains

B.

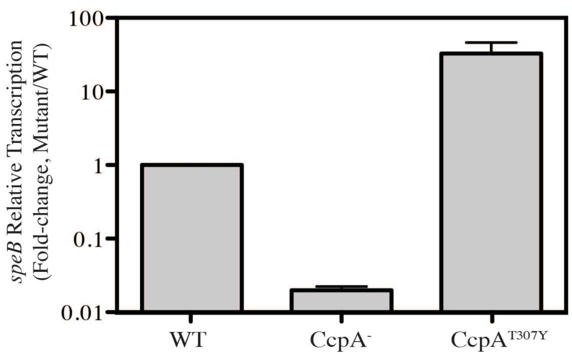


Figure 4. CcpA^{T307Y} is constitutively active in the absence of glucose signal. WT, CcpA^{-}, and CcpA^{T307Y} strains were grown in C medium with or without glucose to exponential phase (OD_{600} of 0.2). Total RNA was isolated and used for real-time RT-PCR analysis of *lctO* **(A)** or *speB* **(B)** transcript.

were grown in C medium and transcript levels were measured from cells at stationary phase, when *speB* is maximally expressed (Fig. 4B). As previously published, the CcpA^{-} strain displayed reduced transcript levels compared to WT. Interestingly, the CcpA^{T307Y} mutant displayed an approximately 50-fold increase in *speB* transcript compared to WT. Taken together, these data indicate that the CcpA^{T307Y} mutant is constitutively active, and depending on the target gene, it induces either hyper-repression or hyper-activation.

CcpA^{T307Y} is attenuated in a murine soft tissue infection. Loss of CcpA is associated with attenuation in a murine soft tissue infection model (5). Therefore, we sought to investigate the effects of constitutive CcpA repression on virulence in this mouse model. For this analysis mice were infected with WT, CcpA^{-}, or CcpA^{T307Y} and lesion areas were compared at day 3-post infection (Fig. 5A and B). Comparison of lesion sizes shows that infections with either CcpA mutants formed significantly smaller lesions than a WT infection. Additionally, lesions were dissected at day 3 and tissue was plated to count bacterial CFU (Fig. 5C). Although the CcpA^{-} mutant had significantly reduced CFU, the CcpA^{T307Y} mutant, however, had similar CFU to WT at day 3 despite the strong attenuation phenotype seen with the lesion data. Taken together, this data shows that although both the CcpA^{-} mutant and the CcpA^{T307Y} mutant are attenuated in lesion formation, however, only the CcpA^{-} strain has reduced CFU, suggesting that the cause of the attenuation of these two strains is unique for each strain.

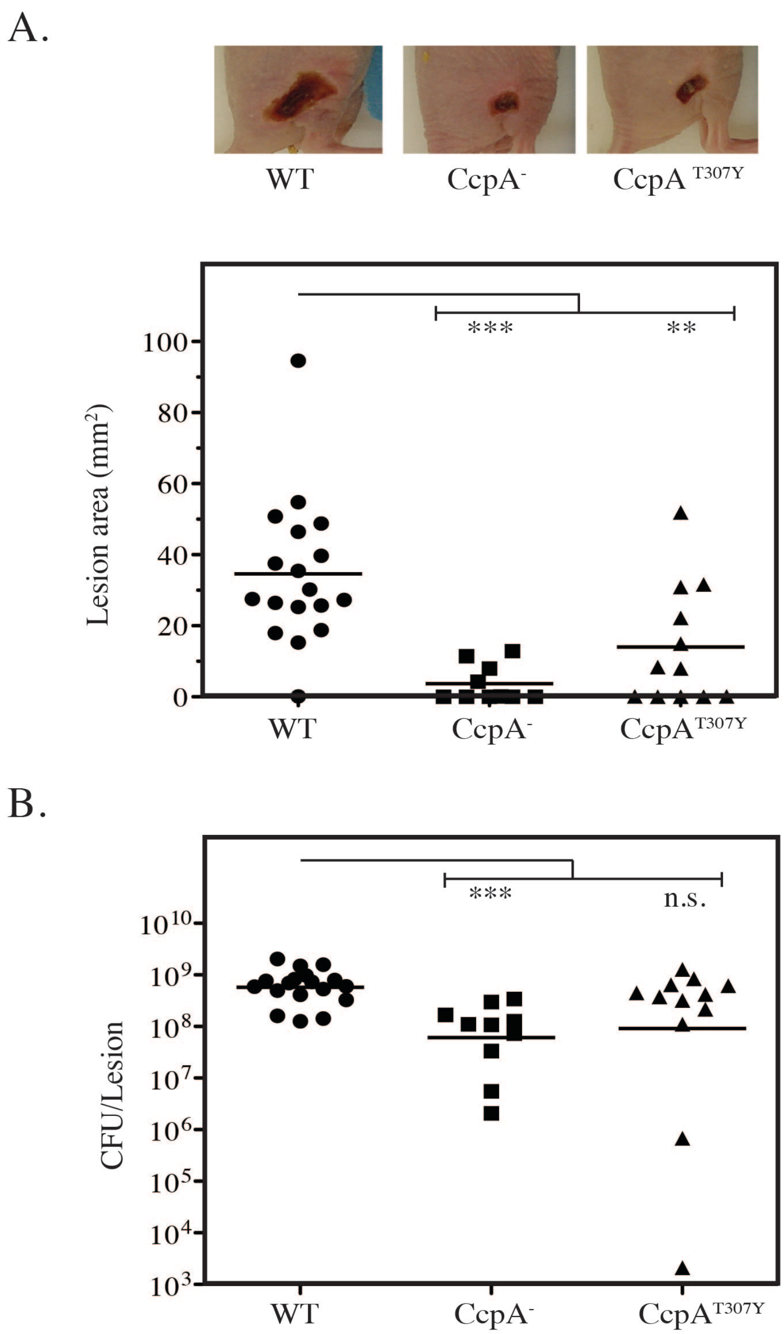


Figure 5. CcpA mutants have reduced virulence in soft tissue infections. Hairless SKH1 mice were infected subcutaneously with WT, CcpA$^-$, or CcpA^{T307Y} and the resulting lesions formed **(A)** and CFU **(B)** were measured at day 3 post-infection. Data shown are pooled from 2 independent experiments. Differences between groups were tested for significance using the Mann-Whitney U test (** *P* < 0.01, *** *P* < 0.001).

CcpA mutants have differential phenotypes in murine mucosal colonization model. In addition to causing inflammatory infections of the skin, *S. pyogenes* can asymptomatically colonize mucosal tissue (13). Recently, a model of asymptomatic carriage in the murine vaginal mucosa was developed and it was shown that a CcpA$^-$ mutant was attenuated in this system (13).

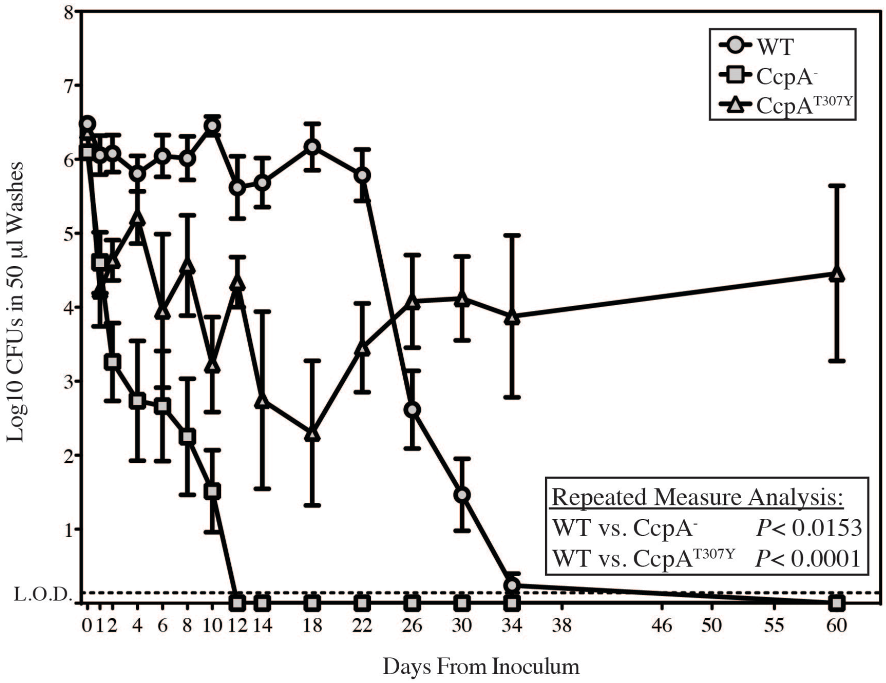


Figure 6. CcpA mutants have differential phenotypes in mucosal colonization model. Estrogenized C57BL/6J mice were vaginally inoculated at day 0 with 1x10^6 CFU of streptomycin-resistant WT, CcpA$^-$, or CcpA^{T307Y} mutants. Vaginal washes were collected at the time points indicated and processed for determination of CFU. Each symbol represents the mean and standard error of the mean derived from at least 5 mice per group. A repeated measure analysis was used to compare mutants to WT.

This finding demonstrated that CcpA regulation was essential for long-term mucosal colonization of *S. pyogenes*. Given this information, the next question to address is what effects a CcpA^{T307Y} mutant will have on mucosal colonization. WT, CcpA$^-$, or CcpA^{T307Y} strains were vaginally inoculated into pre-estrogenized C57BL/6J mice and colonization was monitored by counting viable CFU from vaginal washes collected over the course of 60 days. WT *S. pyogenes* maintained a high level of colonization through day 22, after which there was a rapid drop in CFU (Fig. 6). The $CcpA^-$ mutant displayed an immediate drop in CFU, leading to complete clearance by day 12 (Fig. 6). Conversely, the $CcpA^{T307Y}$ mutant had a distinct phenotype than either WT or $CcpA^-$. CFU from the $CcpA^{T307Y}$ mutant dropped several logs over the first 14 days, but maintained higher numbers than the $CcpA^-$ mutant. Interestingly, the $CcpA^{T307Y}$ mutant displayed extended carriage, with approximately 10^5 CFU detected at day 60 post inoculation, long after WT was cleared (Fig. 6). This data indicates that enhanced CcpA activity is beneficial to the bacteria during mucosal colonization.

DISCUSSION

For a pathogenic bacterial species such as *Streptococcus pyogenes,* precise spatial and temporal expression of virulence factors is essential for the pathogen to achieve maximum fitness in host tissue. Disruption of various regulatory pathways, leading to misregulation of numerous target genes, has been shown to be detrimental for the bacterium (2, 13, 18, 41, 42). In *S. pyogenes*, the catabolite repressor protein CcpA has been established as an important growth phase-dependent regulator that is responsible for controlling a large number of metabolic and virulence genes (2, 5). Although multiple studies have explored the role of CcpA through loss of function mutants, this study sought to characterize the effects of CcpA activation on pathogenesis.

In this study we have developed a constitutively active form of CcpA to use as a tool to explore the role of catabolite repression on virulence in multiple tissue environments. *In vitro* transcription levels of two known CcpA target genes demonstrated that the mutant, $CcpA^{T307Y}$, is a functional protein, capable of either constitutive repression or constitutive activation of gene expression, depending on the target gene. *In vivo* experiments demonstrate that misregulation of the CcpA regulon, in either a $CcpA^{-}$ or $CcpA^{T307Y}$ mutant, alters the outcome of both the soft tissue infection and mucosal carriage. In the case of the inflammatory soft tissue model, both CcpA mutants displayed reduced virulence, but only $CcpA^{-}$ was impaired in growth. In the vaginal mucosal, $CcpA^{-}$ displayed reduced carriage compared to WT. Conversely, the $CcpA^{T307Y}$ mutant had an extended carriage phenotype, indicating that excessive catabolite repression may be beneficial for the bacterium in this environment.

The finding that both CcpA mutants are strongly attenuated in a soft tissue infection demonstrates the importance of proper temporal control of gene expression to maximize fitness of the bacteria. CcpA-mediated regulation is a dynamic process, linking gene expression to the constant changes in nutrient availability. Loss of this regulatory system, either through loss of or hyper-activation of CcpA, appears to be equally detrimental to pathogenesis of *S. pyogenes*. However, the specific causes of this attenuation appear to be distinct for each of the CcpA mutants.

The $CcpA^-$ infection lead to reduced lesion areas and fewer recoverable CFUs from lesion tissue compared to WT. This finding indicates that the $CcpA^-$ mutant displayed a growth defect *in vivo* that was not observed *in vitro,* and this defect most likely contributed to the attenuation phenotype. One possible cause of this defect is that a $CcpA^-$ infection induces an altered host response, leading to more efficient clearance of the bacteria. Preliminary investigations into host immune response during infection have found that the loss of CcpA resulted in a significant alteration in cytokine response during infection (C. Kietzman and M. Caparon, unpublished). In particular, TNFα was significantly upregulated in a $CcpA^-$ infection compared to WT (C. Kietzman and M. Caparon, unpublished). This finding suggests that a $CcpA^-$ mutant may be deficient in producing an as-yet-unknown immune modulating virulence factor, resulting in a robust TNFα response and more efficient bacterial clearance.

Conversely, an infection with $CcpA^{T307Y}$ created smaller lesions than WT, but had equivalent amounts of recoverable bacteria from lesion tissue as WT. In this case, the observed attenuation may be due to repression of one or more virulence factors necessary for the tissue damage and necrosis that occurs when skin lesions develop. One possible candidate responsible for this is the cytolysin Streptolysin S (SLS), which is both repressed by CcpA and is associated with tissue damage and lesion formation (43-47). In a $CcpA^{T307Y}$ infection SLS would theoretically be constitutively repressed, and without this key virulence factor, less tissue damage would likely occur. It also remains possible that the attenuation of the $CcpA^{T307Y}$ mutant is the result of excessive repression of multiple virulence factors, including SLS.

Similar to skin infections, the two CcpA mutants displayed distinct phenotypes when infecting the murine vaginal mucosa. The $CcpA^{-}$ mutant's rapid depletion and clearance is similar to what has been seen previously for this mutant in mucosal tissue (13). In that work the authors demonstrate that the lack of successful colonization is due to over-production of LctO, leading to toxic levels of hydrogen peroxide production (13). The $CcpA^{T307Y}$ mutant, however, displayed an initial loss of CFU early in colonization, but had an extended carriage greater than both the $CcpA^{-}$ strain and WT. This pattern suggests that CcpA-mediated repression may be detrimental during early stages of mucosal colonization, but beneficial for long-term colonization. It has been suggested that the bacterial cells could be in a metabolically inactive state during long-term carriage (13) and, if this were the case, repression of transcription for a large set of genes would allow the cell to conserve energy and possibly persist longer in the tissue.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Standard molecular cloning techniques utilized the *Escherichia coli* strain TOP10 (Invitrogen). Cultures were grown in Luria-Bertani medium at 37°C. *Streptococcus pyogenes* strain HSC5 (48) and mutant derivatives were utilized in this study. Strains were grown in Todd Hewitt broth (THYB) with 0.2% yeast extract (Difco) or C medium, adjusted to pH 7.5 as described previously (49). Routine growth conditions utilized sealed culture tubes incubated at 37°C under static conditions. Streptococcal strains grown on solid medium containing 1.4% Bacto agar (Difco) were cultured in a sealed jar with a commercial gas generator (GasPak catalogue no. 70304, BBL). For experiments utilizing glucose supplementation, filter sterilized 20% (w/v) stock solution was used to add glucose (Sigma) to a final concentration of 0.2% to the media. All media used were sterilized in an autoclave prior to supplementation. When appropriate, antibiotics were added at the following concentrations: erythromycin 1 mg/mL.

Construction of mutants. All references to genomic loci are based on the genome of HSC5 (48). The mutant strain $CcpA^{-}$ was described previously (2). The modified allele for CcpA (L897_02310), $CcpA^{T307Y}$, was generated using the Quikchange XL II mutagenesis kit (Agilent Technologies) and the PCR primers CcpA T307Y F (5'-GTGCTGTTAGCATGCGGATGTTGTATAAAATCATGAACAAAAGAAGAGT) and CcpA T307Y R (5'-ACTCTTCTTTGTTCATGATTTTATACAACATCCGCATGCT- AACAGCAC) to create plasmid pEP44. The modified allele was transferred to the HSC5 chromosome using the allelic replacement vector pGCP213 (50) as described (51). All molecular constructs and chromosomal structures of all mutants were verified using PCR and DNA sequencing (Genewiz, South Plainfield, NJ) using oligonucleotide primers (IDT, Coralville, IA) of the appropriate sequences.

Isolation of RNA and transcript analysis. Transcript abundance of selected genes was analyzed as previously described (52). Briefly, overnight cultures were diluted 1:25 into fresh C medium with the appropriate supplement added (see text) and harvested at mid-log phase (OD_{600} 0.2). Total RNA was isolated using Qiagen RNeasy Mini kit per the manufacturer's protocol. RNA was subjected to reverse-transcription (RT) using iScript (Bio-Rad) per manufacturer's protocol. RT-PCR analysis of cDNA samples were performed using iQ SYBR Green Supermix (Bio-Rad). RT-PCR primers for *lctO* and *speB* reported previously (2). Relative transcript abundance was determined using the $\Delta\Delta C_t$ method using *recA* transcript as a standard and are presented in comparison to wild type. The data shown are the means and the standard deviation from triplicate determinations of at least two separate biological samples prepared from at least two independent experiments.

Infection of mice. Infection of murine subcutaneous tissue was conducted as described previously (53, 54). Briefly, 5-to-6-week-old female SKH1 hairless mice (Charles River Labs) were injected subcutaneously with approximately 10^7 CFU of *S. pyogenes* of the strains indicated in the text. Following infection, the resulting ulcers formed were monitored over a period of several days by digital photography and lesion areas measured as previously described (53). Data presented is pooled from at least two independent experiments with at least 10 mice per experimental group. The ability of strains to maintain asymptomatic colonization of the murine vaginal mucosa was measured in C57BL/6 mice, as previously described (13). Colonization was assessed at selected time points over the course of 60 days by monitoring recoverable CFUs in a 50μL vaginal wash. Data presented was collected from a single infection of 3-5 mice per strain.

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Chapter V

Conclusions

CONCLUSIONS

Streptococcus pyogenes is an extremely versatile bacterium, as it can colonize numerous different tissue types within the human host as well as induce an inflammatory or noninflammatory infection (1-3). A key factor for this versatility lies in the bacterium's ability to monitor various environmental signals in the surrounding tissues and rapidly alter its global transcriptome to adapt itself for survival in a particular niche (4-8).

Through comparisons of gene expression patterns observed in infected tissue to expression patterns from various *in vitro* growth conditions, it was previously determined that the infected tissue environment is low in glucose and is low in pH (10). Additionally, these two metabolic cues are linked due to the simple fermentative metabolism present in *S. pyogenes* (11-14). Glucose fermentation results in the production of a large amount of organic acid end products, which accumulate in the local environment and cause a significant drop in pH of the surrounding tissue (11-13). Additionally, both pH and glucose levels have been shown to be signals utilized by the bacterium to induce global transcriptional changes (10, 15), however the specific mechanism by which these signals are sensed by the various regulatory pathways remains largely unclear. The aim of this work was to identify these regulatory pathways and elucidate their role in *S. pyogenes* pathogenesis. The major findings of this work include: 1) *S. pyogenes* is able to utilize malate as an alternative carbon source through the malic enzyme pathway, 2) regulation of the ME pathway in *S. pyogenes* is distinct from other LAB in that it is positively regulated by low pH and is controlled by a CcpA-independent form of catabolite repression, 3) loss of any ME genes can alter the outcome of an infection, 4) temporal expression of the virulence factors SPN and SLO is controlled by environmental pH, 5) pH regulation of *spn* and *slo* require the two-component system CovRS and the protein RocA, 6) RocA is not a histidine kinase as previously reported in the literature, but is involved in a transcriptional regulatory pathway that includes CovR and CovS, and 7) constitutive activation of CcpA alters the outcome of disease in two different mouse models, leading to a unique phenotype distinct from either a WT or CcpA$^-$ infection.

These studies provide new insights into the relationship between metabolism and pathogenesis in *S. pyogenes*. In particular, it explores the effects of carbon source utilization on local tissue remodeling and the regulatory mechanisms that the bacterium uses to adapt to these specific changes.

During the initial stages of a soft tissue infection, glucose levels are at their highest. The available glucose will be rapidly taken into the bacterial cell and metabolized, leading to repression of alternative catabolic operons through both CcpA-dependent and –independent pathways. Also during the early stages of infection the bacterial cells will begin to produce and secrete toxins SPN and SLO, among others, to induce local tissue damage and cytotoxicity. Over time, as glucose continues to be metabolized, both the concentration of glucose as well as the pH begins to decrease. In response to these signals, alternative catabolic genes, such as the malic enzyme genes, are expressed, as the cell needs to scavenge for alternative carbon sources. At this point, malate is abundant at the site of infection, likely being released from host cells due to the expression of several cytotoxic proteins, including SPN and SLO. While the acidic pH induces expression of the ME genes, it simultaneously acts as a signal to turn off expression of *spn* and *slo*, and this regulation is carried out via RocA and CovRS. Finally, upon depletion of glucose, CcpA repression is relieved, allowing for expression of genes necessary for late stages of colonization and dissemination.

FUTURE DIRECTIONS

Determine the regulatory pathway involved in activation of malic enzyme expression

The work presented here has established that the malic enzyme genes are under a form of catabolite repression known as induction prevention, and this process is mediated through a phosphorelay system that includes the general PTS proteins EI (encoded by the gene *ptsI*) and HPr. What is currently unknown is the intermediate step between formation of P~His-HPr and expression of the *maeKR* and *maePE* operons. Determination of the missing link in this regulatory pathway will provide new insights into alternative carbon catabolite repression pathways beyond the heavily studied CcpA side of catabolite repression.

One possible scenario is that the phosphate from P~His-HPr would be transferred to another, currently unknown, transcriptional regulator, which then leads to activation of transcription of the MaeKR regulatory operon. It has been shown in *S. pyogenes* and a number of other bacteria that the phosphate from P~His-HPr can be directly transferred to regulatory proteins containing a conserved PRD domain (16, 17).

In *S. pyogenes* there are three transcriptional regulators predicted to contain PRD domains: Mga, RofA, and an uncharacterized RofA-like protein (RALP). Preliminary data indicates that neither Mga nor RofA are required for ME expression or malate utilization (data not shown). Work is currently in progress to investigate the third RALP protein as the possible intermediate regulator in this regulatory pathway.

An alternative possibility is that a different, non-PRD regulator, or a PRD regulator not identified via genome annotation or BLAST searches is involved in malate catabolism. We currently plan to employ a transposon screen to unbiasedly identify mutants that are unable to utilize malate. We predict to find known genes including all four malate utilization genes (*maeE*, *maeP*, *maeK* and *maeR*), as well as HPr and PtsI, and any novel genes, including possible transcriptional regulators. A more direct way to identify this potential regulator would be to perform a pull-down experiment using the *mae* promoter region to see what binds to the DNA sequence, followed by mass spectrometry analysis to identify these proteins.

A final possible hypothesis is that the transcriptional regulator in question is MaeK itself. I find this unlikely due to the lack of a PRD domain. However, this is still a possibility that should be tested. The most direct way to do his would by an *in vitro* kinase assay to look for the direct transfer of the phosphate from P~His-HPr to MaeK.

Determination of RocA's functional activity

There has been limited work done on RocA and its role in transcriptional regulation and virulence. The work presented here refutes the previously published data suggesting that RocA is a functional histidine kinase, but supports the findings that RocA interacts with the CovRS TCS. The next step in understanding this regulatory circuit would be to determine what, if any, enzymatic activity the RocA protein has. Structural prediction software (18) categorizes RocA as a histidine kinase, based in part on the presence of a putative ATPase domain, among other features. Data presented here has established that this protein is not functioning as a histidine kinase, as mutating all histidine residues within the predicted cytoplasmic domain does not affect RocA's regulatory activity on SPN and SLO expression. It is also unlikely that the predicted ATPase domain is functional, as several key residues necessary for its enzymatic activity are absent in the sequence of RocA. However, these findings have not yet been verified biochemically. What is clear is that RocA is a necessary part of the regulatory system controlling the *spn* operon in response to pH.

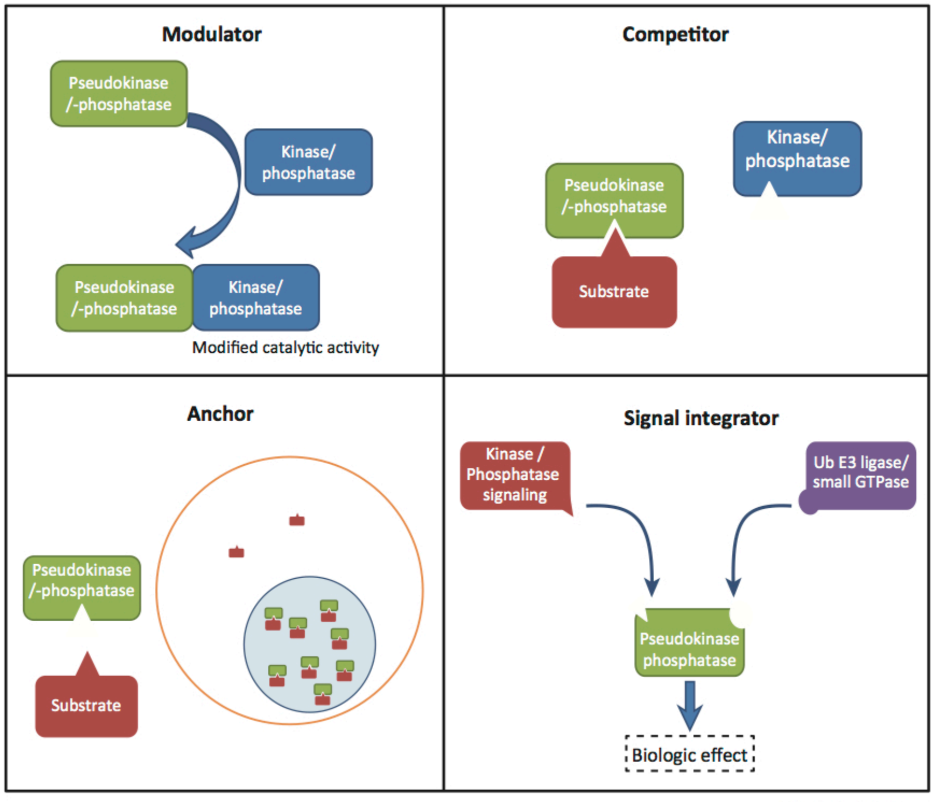

Figure 1. Mechanism of action for pseudokinases. All identified pseudokinases have been associated with at least one of the four activities: Modulation of kinase/phosphatase activity, competitive inhibitor for substrate binding, anchor protein for substrate localization, or scaffold protein for signal integration. Adapted from (9).

The next step in this process will be to determine how RocA, CovR, and CovS work together to coordinate the regulation of this virulence operon. One possibility that I find most intriguing is that RocA functions as a pseudokinase, a protein that structurally resembles a kinase but is lacking any enzymatic activity. Pseudokinases are present in all domains of life (9, 19-24) and all known pseudokinases function in at least one of four mechanisms to modulate cellular activity (see Fig. 1). Perhaps the most likely mechanism for RocA's function is modulation of the phosphorylation of CovR (Modulator). It's been shown in previously published work that CovR's activity is dependent on its phosphorylation state, where different subsets of genes are repressed by CovR depending on its phosphorylation state (6, 25). Additionally, CovS is known to maintain both kinase and phosphatase activity (6, 25). Therefore, the next step in this work should be to analyze the phosphorylation state of CovR in the presence or absence of RocA.

Finally, it is highly probable that RocA controls the expression of numerous genes in *S. pyogenes*. Recent work on RocA function suggests that this protein may also function as a regulator of a subset of genes independent from the CovRS regulon (26). Additionally, many of the genes that were identified as being part of the RocA regulon are metabolic genes (26), suggesting that RocA may be an as-yet unidentified metabolic regulator. To verify this and to characterize the complete RocA regulon, a microarray or RNA-Seq experiment would provide an in depth analysis of RocA's contribution to

transcriptional regulation and virulence. Of particular interest would be to test a WT and RocA mutant grown in buffered media, either neutral or acidic. If, in fact, RocA is a key regulator for acid stress response there is likely many more genes being regulated by this protein. I think this is just the beginning of the RocA story and it has the potential to provide some really interesting and novel findings, particularly in relation to CovRS.

Analyzing the effect of CcpA activation on fitness and pathogenesis

One avenue to explore with the CcpA mutants is to study the effects of CcpA-mediated regulation on growth and metabolic fitness. CcpA is a global regulatory protein and is the primary regulator for most of the proteins in the main glycolytic pathway (15) and yet growth of either of the CcpA mutants was unaffected *in vitro* under the conditions tested. An interesting follow-up to those initial experiments would be to test WT and CcpA mutants in a range of conditions and with several different carbohydrate substrates. Multiple aspects of cell growth could be measured under these conditions such as final yield, growth rate, metabolic intermediates, and fermentation end products. Evaluating these different measurements could provide insights into CcpA regulation and how this control is affecting the overall fitness of the bacteria.

When considering the effects of CcpA regulation on virulence, the data presented in this work establishes that both the $CcpA^-$ and $CcpA^{T307Y}$ mutants were severely attenuated in a mouse model of soft tissue infection. It's been shown previously that CcpA regulates a number of known virulence factors, some of which have immune modulatory function (15, 27). *S. pyogenes* has been shown to induce a number of inflammatory cytokines within the host during an infection, and work from the Caparon lab has shown that the loss of CcpA results in a significant alteration in cytokine response during infection. In particular, TNFα was shown to play an important role in controlling virulence in a $CcpA^-$ infection (C. Kietzman and M. Caparon, unpublished). The previous studies indicate that the loss of CcpA repression has a measurable effect on transcriptional regulation and host immune response throughout the course of an infection. An important next step in understanding CcpA and its role in pathogenesis will therefore be to measure host immune factors during a soft tissue infection with the super-repressor $CcpA^{T307Y}$. In particular, we will look for alterations in inflammatory cytokines by ELISA or real time RT-PCR, as a more robust immune response may be responsible for the attenuation in the $CcpA^{T307Y}$ mutant.

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