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The Convergence of Metabolism and Virulence Factor Regulation on Streptococcus pyogenes Pathogenesis

Zachary Thomas Cusumano
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Biochemistry

Dissertation Examination Committee
Michael Caparon, Chair
Daniel Goldberg
Jeffery Henderson
Timothy Lohman
Christina Stallings
Joseph Vogel

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by

Zachary Thomas Cusumano

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ABSTRACT OF THE DISSERTATION

The Convergence of Metabolism and Virulence Factor Regulation on *Streptococcus pyogenes* pathogenesis

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Zachary Thomas Cusumano

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Professor Michael G. Caparon, Chairperson

Nutrient acquisition is a strong driving force in the evolution of pathogenesis. For many pathogens such as *Streptococcus pyogenes*, the coordination of metabolism with the expression of virulence factors is necessary for colonization. Previous research examining the regulation of virulence factors in *S. pyogenes* identified a unique catabolite sensing regulatory pathway composed of the tagatose bis-phosphate aldolase LacD.1. Examination of LacD.1 has determined that while it still maintains its ancestral enzymatic activity its regulation occurs via a mechanism independent of catalysis. Our approach to understanding how *S. pyogenes'* metabolism contributes to pathogenesis involves both understanding the adaptation of LacD.1 as a novel metabolic regulator as well as investigating the role of LacD.1-regulated genes in pathogenesis.

Analysis of LacD.1's ancestral enzymatic activity and comparison to a paralogous protein encoded in the genome, LacD.2, suggested that the adaptation of LacD.1 required a decrease in enzymatic activity as compared to LacD.2. This decrease in activity is largely due to an increase in K_m, and suggests an optimization in affinity for the signaling

substrate(s) necessary for LacD.1's regulation. Additionally the regulatory function of LacD.1 was found to be essential for the fitness of bacteria when cultured in media that mimicked a soft tissue environment. The selective advantage observed for bacteria in this environment can likely be attributed to LacD.1's positive regulation of the arc operon, which encodes genes necessary for arginine catabolism and contributes to both the production of ATP and protection from acid stress.

To further investigate the arc operon's contribution to the fitness of bacteria in a soft tissue environment we examined the influence of arginine catabolism on pathogenesis. Analysis of several mutants defective in catabolism revealed that both the utilization of arginine as well as the novel utilization of citrulline aids in *S. pyogenes* pathogenesis. This influence on pathogenesis included contributions to both the cellular metabolism of *S. pyogenes* as well as the modulation of the host immune response. Taken together this work demonstrates that LacD.1's adaptation to a regulator and its coordination of metabolism and virulence factor expression was essential to increase the fitness of *S. pyogenes* as a pathogen.

CHAPTER I

Introduction

Streptococcus pyogenes and Streptococcal Diseases

Streptococcus pyogenes (group A streptococcus) is a strict human pathogen believed to cause the widest range of diseases compared to any other bacterium (130). This variety of diseases caused by *S. pyogenes* includes infection in a number of different tissues through out the body (Figure 1(104)). While the clinical symptoms of these diseases can vary greatly, they can loosely be classified into three main categories: superficial, invasive, and post-streptococcal diseases.

Superficial group A streptococcal infections

Superficial infections are the most common form of *S. pyogenes* infections and include pharyngitis and pyoderma. Pharyngitis often occurs in children between the age of 5-12 years and presents with fever, tender and enlarged anterior cervical nodes along with tonsillopharyngeal erythema and exudate (42). Although it is believed treatment with antibiotics does not drastically reduce the duration of the infection they are recommended in most cases(130).

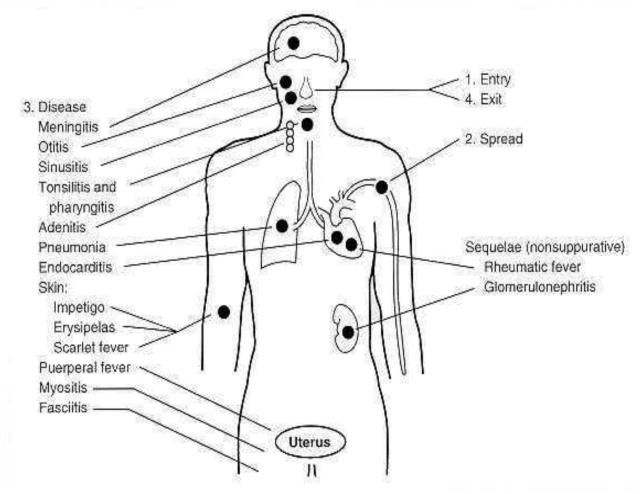

Figure 1: Pathogenesis of *S. pyogenes* infections. Diagram of host tissues and resulting diseases associated with infection

Pyoderma refers to purulent skin infections and includes non-bullous impetigo typically caused by *S. pyogenes*. Impetigo is common in children and is often found in more tropical climates as well as developing countries. Infection usually follows an initial

trauma, allowing introduction of the bacteria. Initial presentation usually occurs as a small pimple or scratch that gradually evolves to a purulent lesion. These lesions tend to be highly contagious thus spreading quickly to other regions of the body or to other individuals in close proximity (48).

Diagnostic assays for these diseases often involve the culturing of the bacteria from the site of infection, followed by observation of beta-hemolysis by streptococci on sheep-blood agar plates. Additionally a rapid antigen test has been developed and is believed to have a high degree of specificity and sensitivity (50). While there is a growing rise of antibiotic resistance occurring in most pathogens, *S. pyogenes* remains sensitive to penicillin, the first-line agent commonly recommended for treatment (12). In 2005 it was estimated that there was 111 million existing cases of pyoderma and 616 million new cases of pharyngitis, demonstrating the frequency of these superficial, less invasive diseases (21).

Invasive group A streptococcal infections

Despite the prevalence of superficial group A streptococcus infections there are still a large number of invasive cases, 663,000, that result in nearly 170,000 deaths a year (21). Invasive cases of *S. pyogenes* are described as infection of normally sterile sites and include infections of soft tissue, pneumonia, and meningitis.

Necrotizing fasciitis is one of the most well known invasive diseases due to its high morbidity and mortality, and involves infection of the muscle fascia and epidermis. The severity of this infection is amplified by the rate at which disease progresses, with 90% of deaths in children occurring within 48 hrs after presentation (26, 76). Initial

diagnosis requires detection of infection past the dermis and typically involves disproportionate pain and tenderness around the site of infection. Once diagnosed surgery and amputation are often required for survival.

In addition to necrotizing fasciitis other invasive diseases including pneumonia and meningitis require aggressive treatments. Pneumonia accounts for 10% of invasive *S. pyogenes* diseases, and often requires antibiotic treatment that can last for three weeks or longer (96). Meningitis caused by *S. pyogenes* is fairly rare in the first world but still occurs in developing countries today and is often lethal (125).

Additional complications of invasive streptococcal infections can arise as a result of the large number of superantigens produced by *S. pyogenes* and can culminate in streptococcal toxic shock syndrome. Superantigens are known to activate T-cells by linking Class II molecules on antigen-presenting cells to the T-cell receptor. This non-specific interaction results in a much larger stimulation of T-cells and the production of a large amounts of cytokines (80, 109). This "cytokine storm" results in a fever, rash, and hypotension followed by shock and organ failure (132).

Post-streptococcal diseases

While post-streptococcal diseases are the least understood they account for the most morbidity and mortality of all streptococcal infections, with 233,000 deaths caused by rheumatic heart disease alone (21). Other post-streptococcal diseases include acute rheumatic fever, acute glomerulonephritis, and reactive arthritis. Additionally other immunopathologcial syndromes such as PANDAS (pediatric autoimmune

neurophsychiatric disorders) have been identified as being associated with streptococcal infections (37, 126).

Pathogenesis of *Streptococcus pyogenes*

Given the lack of any environmental reservoir, *S. pyogenes* has evolved a number of strategies to allow for the efficient colonization of the skin and mucous membranes of humans. Infection of colonization sites often occurs in the superficial tissue, and while self-limiting can result in transmission of the bacteria followed by colonization of a new host. This infection cycle of *S. pyogenes* requires a number of virulence factors that have evolved specialized function within the human host (Figure 2 (140)).

Adhesion

The initial step in a *S. pyogenes* infection begins with attachment to a cell receptor or surface structure. A proposed model of adherence suggests that attachment occurs by a two-step mechanism (34, 58). The first step involves the binding of the bacteria cell wall component, lipoteichoic acid (LTA) to the host protein fibronectin(5, 127). This initial interaction is believed to be weak and transient and serves to allow for a second more specific interaction to occur between an additional *S. pyogenes* adhesin and a host ligand. These additional adhesins provide tissue specificity

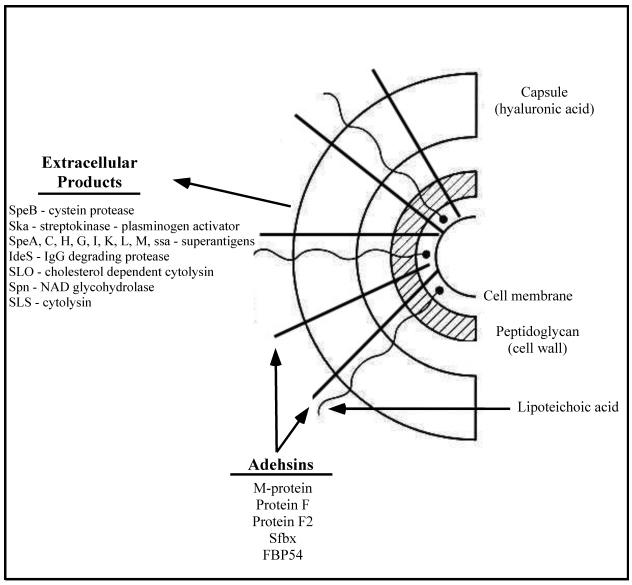

Figure 2. Main virulence factors in *Streptococcus pyogenes.* A schematic diagram of cell wall and secreted virulence factors produced by *S. pyogenes* Adapted from (140).

and increased adherence, and include the hyaluronic acid capsule, M protein, and Protein F.

The hyaluronic acid capsule of *S. pyogenes* is composed of repeating units of glucuronic acid and N-acetylglcosamine(133) and is synthesized by the three *S. pyogenes* genes *hasA*, *hasB*, and *hasC* that form a polycistronic transcript(41). Investigation into an acapsular mutant demonstrated that capsule aids in adhesion by directly interacting with host cells that express CD44 (38). However, an inverse correlation was demonstrated with CD44 deficient cells, demonstrating an inhibition of binding to CD44 negative cells (35). In addition to playing a critical role in adhesion, capsule has also been demonstrated to protect the bacteria from phagocytosis by host immune cells (148).

Probably the most studied virulence factor of *S. pyogenes* is M protein, a major surface protein that is anchored in the membrane by the C-terminus and extends from the cell wall surface. This protein forms a coiled-coil dimer and is composed of a conserved domain located in the C-terminus, and a variable domain in the N-terminus (34, 37). Previously the variable region of M-protein, encoded in the *emm* gene, had been used diagnostically to classify *S. pyogenes* strains through serological typing. Recently this serological classification has been replaced with sequence typing of the *emm* gene, and to date over 200 *emm* types have been indentified (9). In addition to serving as a diagnostic tool, it is believed that both domains of M-protein play key roles during an infection with specific interactions with host cell structures. The variable domain has been demonstrated to bind fibronectin, however given the location of this activity in the variable region it has been determined to be dependent on the type of M protein expressed (36, 118). Conversely the C-terminal region contains repeating units that have

been shown to interact with the host receptor CD46 (100), and while it has not been tested it is believed this interaction is conserved among all M-proteins. In addition to its function as an adhesin, M-protein also prevents phagocytosis (119), contributing to its role in colonization and pathogenesis (2, 33).

In addition to the adhesins described above, *S. pyogenes* relies on a number of additional adhesins, including: protein F, protein F2, PFPB, Sfbx, and FBP54 to target binding to host fibronectin. The targeting of fibronectin appears to occur not only in *S. pyogenes*, but also through out nature, with a number of pathogens evolving mechanisms to bind fibronectin (58). This convergence on a single target strongly suggests that interactions with fibronectin play a key role in bacterial pathogenesis. Overall the variety of adhesins expressed by *S. pyogenes* aids in its ability to colonize a number of different tissues and cause disease through out the body.

Colonization

Following adherence, colonization of the skin or nasopharynx requires a number of virulence factors that allow the bacteria to replicate while remaining protected from the host immune system. This involves the coordinated expression of a number of secreted bacterial proteins that function in eliciting host cell death, degradation of host cytokines, and evasion of host defenses.

Streptolysin O & Streptococcus NAD Glycohydrolase

Two major virulence factors expressed by *S. pyogenes* include the cytolysin Streptolysin O (SLO) and the NAD glycohydrolase (SPN). These two virulence factors are encoded in the same operon, and are expressed as a single transcript. As a cytolysin,

SLO is known to bind to host membranes and oligomerize to form large pores (11, 88). This pore formation, serves multiple purposes including the induction of apoptosis in host cells (14), as well as the translocation of SPN into the host cell (88). The translocation of SPN has been demonstrated to be specific, suggesting a direct interaction between these two proteins (53). Once inside the cell, the function of SPN is still unknown, but detailed biochemical experiments have identified that it is a potent NAD glycohydrolase, which upon entry into host cells results in increased cellular death (88) (52). Interestingly, additional variants of SPN that lack this glycohydrolase activity have been identified, but proven to be equally virulent (52), suggesting an additional role for SPN in the host cell. Additionally, variants of SPN have also been shown to correlate with strains associated with a specific tissue tropism (114); which may indicate the separate functions of these two variants of SPN are required for infection of different tissues. Nevertheless, the combination of SLO and SPN result in increased host epithelial and immune cell death aiding in nutrient acquisition and protection from killing, respectively (54, 99).

Interleukin-8 protease (SpyCEP), C5a peptidase (ScpA), Streptolysin S (SagA)

S. pyogenes' propensity to colonize and survive within the host stems from the number of virulence factors produced that are dedicated to the evasion of host defenses. This evasion is accomplished by several proteins that inactivate or block specific chemokines that result in the recruitment of neutrophils. This includes the surface peptidases SpyCEP and ScpA that inactivate IL-8 and C5a, respectively, inhibiting the chemotaxis of neutrophils to the site of infection (43) (149). Disruption of either of these virulence factors, results in an influx of neutrophils to the site of infection and ultimately a decrease in bacterial survival (68, 151). Finally streptolysin S or sagA is a small,

modified peptide that is secreted by *S. pyogenes* and aids in pathogenesis through a number of different mechanisms. One of sagA's most notable roles is as one of the two toxins that result in the lysis of red blood cells that is used diagnostically to identify *S. pyogenes* through β-hemolysis. In addition to the lysis of red blood cells, sagA is able to help evade the host immune response through direct lysis of neutrophils (95) as well as through inhibition of neutrophil recruitment to the site of an infection(78).

(IdeS) Immungolobulin G endopeptidase & Streptococcal inhibitor of complement (SIC)

In addition to inhibiting the recruitment of host immune cells, *S. pyogenes* has also evolved a number of mechanisms to resist phagocytosis and killing. IdeS another peptidase, can cleave IgG bound to the surface of *S. pyogenes* thus preventing Fc-mediated opsonophagocytosis(77, 144). M protein described above is able to bind the complement regulatory protein Factor H, thus inhibiting phagocytosis (65). Additionally, protection of *S. pyogenes* from the host can occur through the secretion of a 31-kDa protein, SIC. This protein is believed to protect the bacteria from the antimicrobial peptides LL-37 and α-defensins produced by the host (45, 46, 49). All together these virulence factors serve an essential role, allowing *S. pyogenes* to both evade and protect itself from the host so that it may replicate and successfully colonize host tissue.

Dissemination

While *S. pyogenes* can be carried asymptomatically, with one meta-analysis finding 12% of children examined colonized (120), typically colonization by group A streptococcus results in an acute infection, transmission to a new host, followed by clearance. Less frequently *S. pyogenes* can disseminate to deeper tissue causing severe,

life-threatening diseases. While dissemination of *S. pyogenes* requires a number of virulence factors, some discussed above, there are a select few that have been associated with increased invasiveness and may be leading determinants.

Streptococcal exotoxin B (SpeB)

SpeB is a cysteine protease secreted by *S. pyogenes* as a 40 kDa zymogen that is autocatalytically processed to the 28 kDa active form(59). This virulence factor is highly conserved among strains of *S. pyogenes* (70), and is among the most abundant protein secreted by most strains (51). While there are extensive studies demonstrating the ability of speB to cleave a number of different host proteins, including but not limited to: vitronectin, fibronectin (70), activating IL-1b precursor to the active form (69), activating an endothelial cell matrix metalloprotease (18), and releasing kinins from their precursor (60), the role of speB in invasive disease is still debated. A study looking at antibodies in patients with streptococcal infections found that during invasive disease antibodies are made against speB, with fatal cases associated with lower amounts of antibodies compared to milder infections (63). Additionally, several studies have demonstrated a major role of speB in pathogenesis in a number of different mouse and tissue models of infection, suggesting a direct link between speB and virulence (67, 84-86). However in contrast, recent studies have demonstrated that comparison of isolates from patients with invasive disease versus pharyngeal disease have revealed a global shift in their transcriptional profile due to a mutation in a two-component sensor kinase *covS*. This mutation has resulted in the mis-regulation of a number of virulence factors including a reduction in speB production in invasive isolates (134). In addition, to increasing the transcripts of certain virulence factors, the down regulation of *speB* leads to an increase

in stability of other secreted or surface associated virulence factors that are typically degraded by speB's protease activity (4). These two conflicting roles of speB in invasive *streptococcal* infections underscores the complexity of *S. pyogenes* infections, and suggests that invasive disease may involve the confluence of a number of different factors.

Streptodornase D (Sda1) and Streptokinase (Ska)

Mutation of the two-component regulator *covS* found in invasive isolates leads to the down regulation of *speB* as well as the up regulation of other secreted virulence factors, including streptodornase or Sda1, a DNase up-regulated five fold (145). Sda1 is encoded in a prophage that has been acquired by certain *S. pyogenes* strains, and thus is unique to certain M types. It is believed secretion of Sda1 aids in *S. pyogenes*' ability to escape the neutrophil extracellular traps (NETs), that are composed of DNA and are important for containing bacteria at the initial site of infection (15, 16). Up-regulation of *sda1* in invasive isolates is associated with an increase in NET clearance, and reduction in neutrophil mediated killing (145). In addition to the up-regulation of known virulence factors, the down-regulation of *speB* results in the increased stability of secreted virulence factors as well as surface associated adhesins and proteins. These surface associated proteins include plasminogen-binding group A streptococcal M-like protein (PAM), PAM-related protein (Prp), streptococcal enolase, and glyceraldehyde 3-phosphate dehydrogenase, that have all demonstrated the ability to bind human plasminogen (8, 102, 103, 117). The binding of plasminogen to *S. pyogenes'* surface, is followed by the conversion of the pro-enzyme plasminogen to the active plasmin by streptokinase (13, 31). Plasmin is a human serine protease that is known to degrade

blood cots, extracellular matrix components, and activate additional metalloproteinases (147). The accumulation of plasmin on the surface of *S. pyogenes* is believed to be an important step required for the dissemination of *S. pyogenes* from the initial infection site to deeper tissues (30, 135).

Virulence factor regulation in *Streptococcus pyogenes*

The variety and severity of diseases that *S. pyogenes* can cause is likely a direct result of the quantity and variety of virulence factors produced by group A streptococcus. However, this large arsenal of virulence factors are not all expressed at once, or at the same level, but rather tightly controlled by a number of transcriptional regulators. These regulators look to coordinate the expression of virulence genes at the appropriate time so as to allow for the efficient colonization and infection of the host. Additionally, they also serve to respond to changes in environment so as to protect the bacteria from any stress encountered.

Two-component signal transduction systems

One of the most common methods of sensing and responding to the environment is through a two-component signal transduction system (TCS). TCSs are composed of a transmembrane bound sensor histidine kinase, and a DNA binding response regulator. The sensor histidine kinase is often embedded in the bacteria membrane and includes both intracellular and extracellular regions. Upon recognition of a signaling molecule outside the cell, the sensor kinase will typically dimerize resulting in the autophosphorylation of the intracellular domain. The phosphate on the sensor kinase is then transferred to the cytoplasmic response regulator, activating it as a transcriptional

regulator. This system thus allows the cell to sense a specific signal, and respond appropriately by activating the transcription of a specific set of genes (61). To date an average of 13 TCSs have been identified in *S. pyogenes* with the most notable being the TCS CovR/CovS (7, 47, 128).

CovRS or control of virulence TCS is the most characterized TCS in *S. pyogenes,* and has been determined to regulate roughly 15% of its genome including a number of secreted and cell wall associated virulence factors (55). This regulation includes the control of capsule synthesis as demonstrated by the additional name of this system CsrRS (capsule synthesis regulator). While still unclear, it is believed activation of this pathway occurs in response to Mg^{2+}(57), but also may occur in response to general stress (39). Additional genes regulated by CovRS include the known virulence factors: *sagA*, *sda1*, *ska*, and *speB* (28). Regulation of CovRS acts primarily through repression, where phosphorylated CovR acts to prevent transcription thus explaining the up-regulation of virulence factors experienced in a *covR* mutant (28). In addition to the phosphorylation of CovR, CovS can also function as a phosphatase resulting in the removal of the phosphate on CovR and the de-repression of genes regulated by CovR. This activity was determined to be important for survival in stress caused by increased temperature, salt concentration, or a decrease in pH (39). This complex network is further complicated by the recent findings that CovR can still function as a regulator in the absence of CovS (141). This regulation in the absence of CovS is still not clearly understood, but is believed to occur by phosphorylation of CovR through some unidentified mechanism and perhaps includes low-molecular-weight compounds such as acetyl phosphate. Additionally, it is believed that nonphosphorylated CovR is capable of binding promoters

in the absence of CovS(141). The importance of CovRS regulation is highlighted by the recent findings that more invasive strains have mutations in this TCS, resulting in global changes in protein expression. As discussed above, the mis-regulation of specific virulence factors can result in more virulent bacteria that are able to disseminate causing severe disease. However, disruption of CovRS regulation can also negatively influence growth in saliva, suggesting that tight regulation of virulence genes is required for pathogen fitness (141).

In addition to CovRS other TCS have also been identified and shown to be important for adaptation to specific environmental conditions. SptRS is a TCS identified for its importance in growth in saliva, and is believed to regulate the expression of a number of different metabolic transporters and pathways required for growth and persistence in saliva (121), while the TCS Ihk/Irr has been shown to be important for survival and protection against killing by neutrophils (44).

Stand-Alone Response Regulators

In addition to TCS, modulation of gene expression in response to the environment can also occur by "stand-alone" response regulators. These cytoplasmic transcriptional regulators are not associated with a sensor kinase, and thus are believed to regulate gene expression by responding to an intracellular signal. While there are a number of these regulators encoded in the genome of *S. pyogenes* the best characterized include Mga and RopB.

Multiple gene regulator of Group A Streptococcus (Mga)

Mga was initially identified as the regulator of the important virulence factor and adhesin, M protein (20). Further characterization of Mga has revealed conservation in all serotypes, and an important role in regulating genes during exponential growth or initial colonization (10, 74). This regulation includes a number of cell-wall associated adhesins and virulence factors involved in evading the host immune response and include in addition to M protein: streptococcal collagen-like protein, serum opacity factor, C5a peptidase, and SIC (37, 112). Transcriptome analysis of the Mga regulon in three different serotypes have demonstrated that 10% of *S. pyogenes'* genome is regulated by Mga, and in addition to identified virulence factors include a number of genes involved in the transport and utilization of sugar (113). This activation of operons involved in sugar utilization are important for *S. pyogenes* growth as demonstrated by a growth defect of a Mga mutant in chemically defined media containing different sugar sources (113). Examination of a *mga* mutant *in vivo* revealed attenuation in a number of different animal models as well as a growth defect in biofilms, demonstrating the importance of this regulation during an infection. (73, 136) (27).

Despite an understanding of the genes regulated by Mga, it remains unclear as to the signals that trigger expression of Mga's regulon. Regulation appears to be linked to growth phase with increased expression of Mga-regulated genes in the presence of elevated CO_2, temperature, sugar sources, or increased iron (19, 93, 105, 107). Analysis of Mga's protein sequence reveals a conserved phosphotransferase system (PTS) regulation domain (PRD) in the center of the protein, and suggests an interaction with the PTS system in *S. pyogenes* (64). The PTS system functions to transport sugar into the cell and might explain the regulation of sugar metabolism by Mga. Direct

phosphorylation of Mga by the PTS system has yet to be detected, but does raise interesting questions regarding the direct link between sugar metabolism and virulence gene regulation.

Regulator of SpeB (RopB)

RopB was first identified as being required for activation of the virulence gene *speB*, which encodes a secreted cysteine protease (87). It has also been termed Rgg due to its homology to proteins in the Rgg family, these proteins function by controlling the expression of a number of secreted proteins in other *Streptococcus* species (110, 111, 116). Regulation by RopB was demonstrated to be growth phase dependent with activation of *speB* occurring as the bacteria enter stationary phase (97). Identification of additional genes regulated by RopB also included a number of virulence factors as well as genes involved in amino acid metabolism (25). However comparison of RopB's regulon between different strains has revealed a divergence in RopB function, with only the conservation of *speB* regulation between strains (40).

Investigation into the mechanism by which RopB activated transcription identified two binding sites within the 1 kb region found between *speB* and *ropB* (97). However, ectopic expression of *ropB* failed to change the growth phase dependent expression of *speB,* suggesting additional levels of regulation (97). Recently, it has been discovered that a number of proteins in the Rgg family found in other *Streptococcus* species are activated by small, secreted peptides that function in a quorum-sensing manner (24, 90). It is believed that small propetides are produced in the cell, secreted, processed to an active form and then transported back into the cell where they activate

transcription through a putative interaction with the Rgg protein (24, 90). While RopB is similar in structure to other Rgg proteins, no signaling peptide has been discovered for it to date, leaving the mechanism of RopB activation unclear. However, identification of a novel regulator of *speB*, LacD.1, has provided interesting insight into *speB* regulation and may explain the growth phase dependent expression (82).

Metabolic Regulators

Virulence regulation can occur by a number of different mechanisms, and includes both TCS as well as the "stand-alone" response regulators described above. However, for pathogens such as *S. pyogenes* that is not found in the environment, and has evolved to colonize and survive on human hosts, common metabolic regulators that typically function in controlling cellular metabolism have evolved to also regulate virulence gene expression. This coordination of virulence factor expression and metabolism is suggestive of the role of virulence factors in nutrient acquisition, and illustrate the importance of nutrient availability in pathogenesis.

CcpA

Carbon catabolite control in *S. pyogenes* is regulated by the transcriptional factor CcpA. This regulator functions to ensure the cell efficiently utilizes the energy sources available in a hierarchical fashion, often by repressing metabolic pathways involved in the catabolism of secondary substrates (146). In the presence of a primary substrate, often glucose, CcpA will bind to catabolite-responsive elements (*cre)* sites preventing transcription. This binding is augmented by interaction with the phosphoprotein HPr, a component of the PTS system. Phosphorylation of HPR at a conserved serine residue by

HPR kinase, is believed to increase the affinity of HPR for CcpA, and thus enhance binding to *cre* sites. HPR kinase activity is regulated by the metabolite, fructose bisphosphate, thus linking the phosphorylation of HPR to the metabolic state of the cell (146). In addition to regulating cellular metabolism in *S. pyogenes,* CcpA has also been identified as a regulator of key virulence genes including *speB*, *sagA,* and *slo* (124). This regulation can occur via direct interaction with virulence gene promoters or through the influence of additional regulatory pathways (72). While glucose often represses genes via CcpA it has been found that glucose can repress virulence genes in a number of pathogens as well as *S. pyogenes* independent of CcpA, suggesting the presence of additional catabolite sensing pathways (6, 72).

LacD.1

An additional catabolite-sensing pathway recently indentified in *S. pyogenes* involves the protein, LacD.1, annotated as a tagatose bisphosphate aldolase. LacD.1 was initially identified in a transposon screen looking for the de-repression of *speB* under conditions identified to inhibit transcription of this gene, in the presence of salt, neutral pH, or glucose (82). Regulation by LacD.1 has been determined to require key amino acid residues in the active site necessary for the binding of key metabolic substrates: fructose bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate (82). While the binding of one or more of these metabolic substrates is believed to be required for regulation, disruption of catalysis does not impede LacD.1's ability to function as a regulator, suggesting LacD.1's enzymatic activity is not necessary for controlling the transcription of *speB* (82). One proposed model of regulation, includes a direct or indirect interaction with RopB, resulting in its inability to activate *speB*

transcription. Modulation of this interaction is believed to occur by a change in concentration of metabolite(s) recognized by LacD.1. Thus in conditions when the metabolite(s) is abundant LacD.1 is able to sequester RopB, preventing it from activating the transcription of *speB*. This model is supported by the early activation of SpeB expression when *ropB* is ectopically expressed in a *lacD.1* mutant (82).

This novel regulator was initially discovered to control the expression of the virulence factor *speB*, however it has also been found to regulate additional genes including those involved in arginine catabolism (71, 72). The adaptation of LacD.1 as a novel catabolite-sensing regulator was unique given its similarity to the paralog, LacD.2, also encoded in the genome of *S. pyogenes*. Comparisons of LacD.1 and LacD.2 have found that despite being highly similar in amino acid sequence their functions in the cell are divergent and unique to each protein (83).

CodY

In response to amino acid starvation, *S. pyogenes* undergoes what is known as the stringent response, resulting in the production of the alarmone pppGpp by RelA followed by pppGpp-mediated inhibition of RNA synthesis protecting the cell from starvation (94). Additionally, *S. pyogenes* can respond to amino acid starvation in a RelA independent manner and involves the transcriptional regulator, CodY (131). CodY is involved in controlling a number of genes in response to amino acid availability, and include: transporters of amino acids and peptides, enzymes involved in amino acid metabolism, additional regulators such as *mga*, *ropB*, and *covRS*, as well as a number of virulence genes including *saga* and *scpA* (89). CodY regulation is controlled by GTP and branched

chain amino acids (BCAA) and has been demonstrated to bind DNA with higher affinity in the presence of GTP or BCAA (92). While in most bacteria the response to starvation includes inhibition of protein synthesis, it is believed that in pathogenic bacteria this response has been co-opted to also include production of virulence factors involved in the destruction of eukaryotic cells. Thus, attempting to liberate the necessary metabolic substrates required for growth. Additionally these virulence factors can also aid in the spread and dissemination to other tissues that may contain more nutrients (129). This link between the bacteria's metabolism and virulence gene expression highlights the importance of metabolism and its contribution to pathogenesis.

Metabolism of *Streptococcus pyogenes*

Streptococcus pyogenes belongs to the family *Lactobacillacea* or lactic acid bacteria, a wide family of bacteria grouped together by their common metabolic end product. Lactic acid bacteria include species from the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Enterococcus*, *Lenconstoc*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (3), and are best known for their involvement in the food industry. Additionally lactic acid bacteria are believed to be common components of human's microbiota and play a beneficial role in immunomodulation, intestinal integrity, and pathogen resistance (142), benefits that have resulted in the use of these bacteria in probiotics (79). The exploitation of these bacteria in the food industry as well as in probiotics has resulted in an increase in research examining their physiology and metabolism. Thus in comparison to *S. pyogenes* much more is known regarding their cellular metabolism resulting in them often being used as surrogates for understanding metabolic pathways present in *S. pyogenes*.

Carbohydrate Utilization

The preferred carbohydrate energy source of lactic acid bacteria is glucose, typically transported through the phosphotransferase systems (PTS), glucose can also be brought into the cell by additional permeases and ATP-binding cassette (ABC) transporters (Biotechnology of Lactic Acid Bacteria ref)(138, 139). Glucose transport through the PTS occurs with phosphorylation of the glucose moiety, which is then shuttled into the Embden-Meyerhof-Parnas pathway (138)(Figure 3). Catabolism of glucose by this pathway results in the formation of two molecules of ATP, two molecules of NADH, and two molecules of pyruvate. In the absence of a respiratory chain, which lactic acid bacteria lack, for glycolysis to continue the bacteria must re-oxidize NADH so that it may be recycled and enter the pathway again. This oxidation of NADH occurs via the metabolism of pyruvate, which can occur by a number of different mechanisms, the most common occurring through the utilization of the enzyme lactate dehydrogenase (Ldh). Ldh functions through the conversion of pyruvate to lactate with the concomitant oxidation of NADH (29). The production of lactate as the major end product is descriptive of homolactic fermentation as opposed to heterolactic fermentation that typically occurs under glucose-limiting conditions, and involves the production of acetate, formate, and ethanol (150). Production of these end products requires additional enzymes, pyruvate formate lyase in anaerobic conditions and pyruvate dehydrogenase complex in aerobic conditions, to oxidize NADH with the concomitant production of acetyl-CoA and formate. Production of acetyl-CoA can be further converted to acetate by acetate kinase with the formation of a molecule of ATP (1, 98). Additionally, the

acetyl-CoA can also be converted to acetaldehyde and finally ethanol by the acetalaldehyde dehydrogenase and alcohol dehydrogenase, respectively.

In addition to glucose, lactic acid bacteria can readily metabolize additional monosaccharides including galactose. Galactose metabolism can occur by two main pathways, the most common being the Leloir pathway, involving the isomerization of galactose 6-phosphate into glucose 1-phosphate, followed by conversion to glucose 6-phosphate and reentry into the Embden-Meyerhof-Parnas pathway (62). In *Streptococcus pyogenes,* galactose metabolism occurs by the less common, tagatose pathway (Figure 3). This pathway involves the isomerization of galactose 6-phosphate to tagatose 6-phosphate by LacAB, which is then phosphorylated by LacC to produce tagatose bisphosphate (TBP). This product is then cleaved by an aldolase, LacD, to create the two three carbon sugars: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate(115). These three carbon sugars can go on to renter the Embden-Meyerhof pathway and continue through pyruvate metabolism, which have been shown to involve heterolactic fermentation in other lactic acid bacteria (137). Examination of the genome of *S. pyogenes* has revealed that it encodes two Lac operons dedicated to the metabolism of galactose (83). However, research in to the function of these two operons has revealed that while the Lac.2 operon is required for metabolism of galactose, the Lac.1 operon has evolved to function in the regulation of transcription (83). Specifically, the aldolase in the Lac.1 operon, LacD.1, has been discovered to function as a novel regulator of virulence (82). This divergence in function suggests a duplication of the Lac operon followed by adaptation, and presents itself as an interesting case to study the evolution of novel protein function.

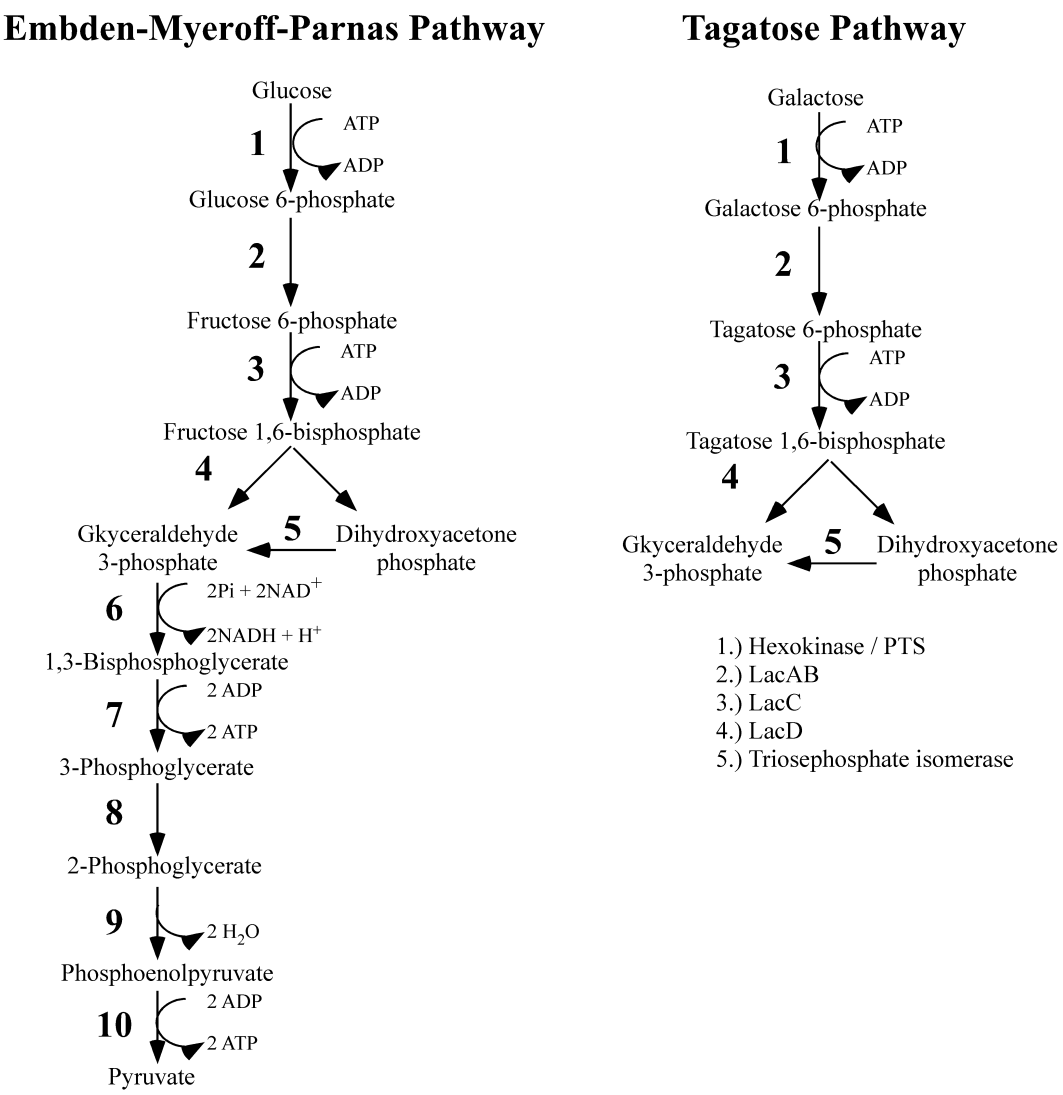

Figure 3. Metabolism of glucose and galactose in *S. pyogenes.* A schematic diagram of the metabolism of glucose in the Emden-Myeroff-Parnas pathway and metabolism of galactose through the tagatose pathway. The enzymes utilized by the pathway are represented by numbers and described below each pathway..

While simple sugars are the preferred choice for energy, for most of the pathogens in this family they must rely on complex carbohydrates for energy *in vivo* (56, 101, 143). Colonization of a human nasopharynx by *S. pyogenes* likely involves utilization of complex maltodextrins as demonstrated by a decrease in colonization of a mouse oropharynx by a MalE mutant that lacks the ability to transport maltodextrins, into the cell (122). In *S. pneumoniae,* disruption of the transport of sucrose by either an ATP-binding cassette transport system or a sucrose PTS, disrupt the ability to cause pneumonia or colonization of the nasopharynx, respectively (66). In addition, endoglycosidase and exoglycosidase play important roles in pathogenesis through both immune evasion mechanisms as well as nutrient acquisition (17, 123). The utilization of these complex sugars suggest that the *in vivo* environment encountered by most pathogenic bacteria is in stark contrast to the conditions experienced during *in vitro* growth in a laboratory setting.

Amino Acid Metabolism

Lactic acid bacteria are commonly thought of as nutritionally fastidious given their lack of an electron transport chain, an incomplete TCA cycle, and their inability to synthesize many biomolecules, including amino acids. Thus to grow, they must scavenge their environment for available carbon sources along with the amino acids necessary for life. Often these amino acids are imported in the cell as dipeptides or short oligonucleotides. Production of these peptides, and processing of them require a number of different peptidases with a variety of specificity and functions. While location of these peptidases in most lactic acid bacteria are believed to occur intracellular (75), *S. pyogenes* is known to express and secrete a cysteine protease (SpeB) in high abundance during entry into stationary phase (97), and during an infection of mouse soft tissue (81). While

a direct link between nutritional acquisition and the expression of SpeB has never been found, it is theorized that expression of this protease may aid in accumulating the essential amino acids required for cellular growth. A theory supported by association between *speB* expression, the nutritional environment (108), and the expression of the dipeptide permease, *dpp* (106).

While accumulation of these amino acids are required for the biosynthesis of proteins, amino acid catabolism can also aid in the ability to obtain energy in nutrient-limiting environments. Catabolism of these amino acids can include among others glutamate, aspartamate, and histidine, although the metabolic pathways of these amino acids are not all characterized or conserved in every genus of lactic acid bacteria. One of the most well characterized catabolic pathways is the arginine deiminase pathway (ADI) that provides energy via substrate level phosphorylation. This pathway involves three enzymes: 1) ArcA, an arginine deiminase (EC 3.5.3.6), liberates an ammonia molecule from arginine to create citrulline, 2) ArcB, an ornithine carbamoyltransferase (EC 2.1.3.3) converts citrulline into carbamoyl phosphate and ornithine, and 3) ArcC, a carbamate kinase (EC 2.7.2.2) generates ATP by transferring the phosphate moiety from carbamoyl phosphate to ADP. The ornithine produced by ArcB is further utilized by the antiporter ArcD to transport a new arginine molecule into the cell in an ATP-independent manner. In addition to the production of ATP, this pathway has been demonstrated to be important for protection against acid stress as well as aid in survival in stationary phase (22, 23, 32, 91).

Furthering studying the evolution of LacD.1 as a novel regulator

The coordination of metabolism and virulence gene regulation highlights how important the ability to sense the nutritional environment is to a pathogen. While the onslaught of virulence factors produced by *S. pyogenes* are directly involved in the tissue damage and disease caused by this bacteria, the expression of these virulence factors is often in response to the nutrients available, as well as other important environmental signals the bacteria can sense. To gain insight into *S. pyogenes'* pathogenesis, it is important to determine what signals are being sensed by *S. pyogenes* and how these signals influence virulence gene expression.

Previous research has identified LacD.1 as an important regulator of virulence gene expression, demonstrating its ability to act as a repressor of the virulence gene *speB* as well as an activator of the metabolic pathway involved in arginine catabolism. This evolution of a metabolic enzyme to a regulator of transcription provides a unique example to probe how novel protein functions evolve, as well as examine how metabolism and virulence gene expression are coordinated in *S. pyogenes*. Furthermore, understanding the adaptation of LacD.1 can serve as a model system for examining how proteins evolve novel functions.

This works main focus was to investigate the function of LacD.1 in *S. pyogenes.* Through the direct comparison between LacD.1 and LacD.2 we have discovered unique regions that are important for their respective function, and have gained insight into the mechanism of LacD.1 regulation. Additionally, through a number of competition experiments we have identified that the regulatory function of LacD.1 is essential for the

fitness of *S. pyogenes.* In addition to examining the evolution of LacD.1, an essential aspect to understanding LacD.1's function is investigating the contribution of its regulon to virulence. The identification of LacD.1's regulation of the arginine catabolism operon provides an opportunity to understand how changes in expression of this metabolic pathway may influence pathogenesis. Through a series of in-frame deletions in the ADI pathway we have identified the importance of arginine and citrulline catabolism in pathogenesis, as well as discovered its novel contribution to the modulation of the host innate immune response.

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CHAPTER II

Adaptive Evolution of the *Streptococcus pyogenes* Regulatory Aldolase LacD.1.

SUMMARY

In the human pathogenic bacterium *Streptococcus pyogenes*, the tagatose bis-phosphate aldolase LacD.1 likely originated through a gene duplication event and was adapted to a role as a metabolic sensor for regulation of virulence gene transcription. Although LacD.1 retains enzymatic activity, its ancestral metabolic function resides in the LacD.2 aldolase, which is required for the catabolism of galactose. In this study, we compared these paralogous proteins to identify characteristics correlated with divergence and novel function. Surprisingly, despite having identical active sites and 82% similarity in amino acid sequence, LacD.1 was less efficient at cleaving both fructose and tagatose bis-phosphates. Analysis of kinetic properties revealed that LacD.1's adaptation was associated with a decrease in k_{cat} and an increase in K_m. Construction and analysis of enzyme chimeras indicated that non-active site residues previously associated with the variable activity of human aldolases isoenzymes modulated LacD.1's affinity for substrate. Mutant LacD.1's engineered to have LacD.2-like levels of enzymatic efficiency lost the ability to function as regulators, suggesting that an alteration in efficiency was required for adaptation. In competition under growth conditions that mimic a deep tissue environment, LacD.1 conferred a significant gain in fitness that was associated with its regulatory activity. Taken together, these data suggest that LacD.1's adaptation represents a form of neofunctionalization where duplication facilitated the gain of regulatory function important for growth in tissue and pathogenesis.

INTRODUCTION

Adapted metabolic enzymes provide interesting models for examining the role of gene duplication in driving the process of protein evolution. Derived from metabolic enzymes, these proteins have been adapted by evolution to perform novel functions that are not directly associated with catalysis, often functioning as components of transcriptional regulatory complexes (18). Many of these enzymes are multifunctional, where the same protein entity participates in both catalysis and regulation (18, 36). However, others are divergent in function, participating in regulation while no longer retaining a clear metabolic role (24, 35). The members of this latter class are often the products of an apparent gene duplication event where the ancestral protein is retained as a component of the original metabolic pathway. Since the duplicate is initially redundant, how it avoids the accumulation of deleterious mutations while undergoing adaptation is not well understood (10). However, the fact that adaptation often results in only minor alterations to the primary amino-acid sequence and its three-dimensional structure, suggests that careful comparison of the adapted enzyme and its ancestor can provide insight into the evolutionary forces driving adaptation. A challenge then becomes the identification of those specific amino-acid residues whose divergence is associated with the acquisition of novel functionality.

In *Streptococcus pyogenes* (group A streptococcus), LacD.1 is an adapted metabolic enzyme implicated in the transcriptional regulation of several virulence factors, including the secreted SpeB cysteine protease (26). This Gram-positive bacterium is a human pathogen responsible for a large number of diseases ranging from largely superficial infection of the skin and mucous membranes (impetigo, pharyngitis), to highly invasive

and life-threatening diseases (necrotizing fasciitis), as well as serious post-infection sequelae (rheumatic fever, glomerulonephritis) and immunopathological syndromes (PANDAS: pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection) (reviewed in (8)). Deletion of the gene encoding LacD.1 has been associated with de-repression of transcription of virulence genes regulated by several different environmental cues, including pH, salt and carbon source (25). Functionally, LacD.1 has the characteristics of a tagatose 1,6 bis-phosphate (TBP) aldolase, a component of the tagatose 6-phosphate pathway used by Gram-positive bacteria to metabolize lactose and galactose (38). However, the ability to metabolize these carbohydrates in *S. pyogenes* resides not in the Lac.1 operon of which LacD.1 is a member, but rather the nearly identical Lac.2 operon (27). In fact, the Lac.1 operon is defective, lacking functional copies of the genes encoding several key enzymes, which instead reside in the operon as pseudogenes. The high degree of homology between the two operons suggests that the Lac.1 operon arose as a duplication of the Lac.2 operon and subsequently, the LacD.1 TPB aldolase was adapted for a regulatory role that specifically required the loss of function of other enzymes in the pathway. This hypothesis is supported by the fact that restoration of LacC.1 pseudogene function phenocopies deletion of LacD.1 and that this defective operon architecture is conserved in all *S. pyogenes* whole genome sequences that are currently deposited in the Genbank database.

Interestingly, LacD.1 retains the enzymatic activity characteristic of an aldolase, and has the ability to cleave TBP, and its isomer fructose 1,6 bis-phosphate (FBP) into the glycolytic intermediates di-hydroxy acetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P) (26). However, its ability to participate in transcriptional regulation is

independent of enzymatic activity, since mutation of a catalytic lysine residue (K204) abolishes the ability to cleave substrate, but has no effect on its ability to regulate virulence factor expression. In contrast, mutation of key residues involved in binding substrate does lead to an abrogation of regulatory activity, suggesting that an ability to bind substrate is an essential component of LacD.1's adapted regulatory function (26).

Further support for the specific adaptation of LacD.1 to a regulatory role comes from the observation that LacD.2 can not substitute for LacD.1 in regulation despite the fact the two aldolases are 82% similar. Comparison of all aldolases has revealed similarity in sequence, although they are distributed between 2 general classes that are distinguished by their mechanism of catalysis (class I and class II) (30). Unlike the metal-requiring class II aldolases found in most prokaryotes, LacD.1 and LacD.2 are class I enzymes that utilize a lysine to form a Schiff base intermediate. The crystal structures of LacD.2 in both its apo form and bound to DHAP and the apo form of LacD.1 have recently been determined, revealing strong similarity to the well-studied structure of rabbit muscle aldolase (RMA) (23, 28). All three enzymes share a conserved $(\alpha/\beta)_8$ fold and the structures show that RMA and LacD.2 undergo a similar conformational response upon binding DHAP. In addition, the structures show that the LacD.1 and LacD.2 active sites share an identical sequence. Taken together, these data suggest that LacD.1's adaptation to a regulatory function relied upon its original function as an aldolase and that adaptation involved alterations to residues outside of the active site.

As a first step in understanding how these two similar proteins have been adapted to diverse function, we sought to compare and contrast various properties of LacD.1 and LacD.2 in order to identify characteristics unique to each enzyme. We found that

LacD.1's adaptation is associated with a measurable divergence in catalytic function and that this altered enzymatic activity is necessary for its adapted regulatory function. However, an altered enzymatic activity is not sufficient to account for LacD.1's divergence, as its regulatory function requires additional variant residues. LacD.1, but not LacD.2, was found to confer a significant competitive advantage under growth conditions that mimic a deep tissue environment. Furthermore, this gain in fitness did not depend on its divergent catalytic properties, but was specifically associated with its regulatory activity. Thus, these data provide insight into the molecular basis of the evolution of LacD.1's novel regulatory function.

RESULTS

LacD.1 and LacD.2 are structurally similar. Recently, crystal structures of LacD.1 and LacD.2 from *S. pyogenes* (PDB codes 3MYO and 3MHF, respectively) have both been solved (23, 28), but have not been directly compared. To compare the tertiary structures of the aldolases, the algorithm FATCAT was utilized to allow for an alignment that takes flexibility into consideration (44). This comparison indicated that the two proteins were significantly similar ($P < 0.001$) with an r.m.s.d of 1.0 (Cα atoms) (Fig. 1A) and that the positioning of active site residues between the apo forms of the two enzymes are essentially identical (Fig. 1B).

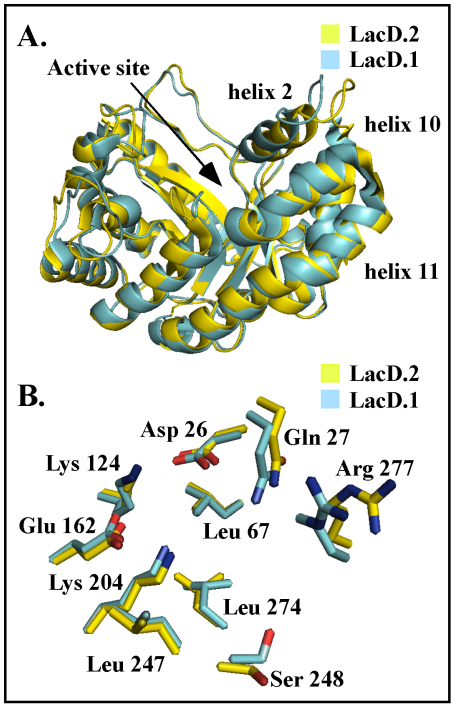


FIGURE 1. Structural comparison of LacD.1 and LacD.2. The crystal structure of LacD.1 (3MYO) and LacD.2 (3MHF) were compared: **A.** Superposition of LacD.1 and LacD.2 crystal structures. **B.** Superposition of LacD.1's active site (cyan) and LacD.2's active site (yellow), active site residues from LacD.1 D_{26}, Q_{27}, L_{67}, K_{124}, E_{162}, K_{204}, L_{247}, S_{248}, L_{274}, and R_{277} are labeled.

LacD.1 has a higher K_m for substrate than LacD.2. Given that LacD.1 and LacD.2 share identical active site residues and nearly identical three-dimensional structures, aldolase activity was directly compared to determine if LacD.1's divergent function was associated with any variation in enzyme efficiency. Activity was assessed using a standard enzyme-coupled assay linking cleavage of the phosphorylated sugar to oxidation of β-NADH by glycerol-3-phosphate dehydrogenase, which was measured spectroscopically. This analysis revealed that LacD.2 readily cleaved both TBP and FBP (Table 1) at rates similar to those reported previously (28) and similar to the class II TBP aldolase found in *E. coli* (43). When the *S. pyogenes* enzymes were compared, the k_{cat} of LacD.1 was similar to that of LacD.2, although slightly lower for both substrates (approximately 1.5-2-fold, Table 1). In contrast, K_m for LacD.1 was approximately four-times higher than for LacD.2, indicating a considerable decrease in affinity for both TBP and FBP (Table 1). Considering that the two enzymes share identical active sites, these data demonstrate that LacD.1's adaptation to a regulatory role is associated with a roughly 6-8 fold decrease in enzyme efficiency as calculated by k_{cat}/K_m (Table 1). These data also suggest that variant non-active site residues influence LacD.1's divergent properties.

Table 1: Steady state kinetic parameters of LacD chimeric enzymes for the cleavage reaction of TBP and FBP

	FBP			TBP		
Enzyme[a,b]	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
LacD.2	6.35±0.19	498±57	12.8±0.1	24.3±1.1	199±28	122±18
LacD.1	4.41±0.19	1993±144	2.1±0.8	14.62±1.3	920±193	16±3.6
$LacD.1_{2A}$	12.6±0.6	293±34	42.9±5.4	18.3±0.7	183±19	100±11
$LacD.1_{2B}$	5.20±0.12	404±39	12.8±1.3	12.9±0.38	298±55	39±7.6
$LacD.1_{2C}$	5.46±0.11	684±46	7.9±0.8	15.1±1.5	446±112	33±8.8
$LacD.1_{2(L1)}$	5.48±0.18	864±98	5.8±0.4	10±0.58	497±88	20±3.7
$LacD.1_{2(T2)}$	4.76±0.19	907±121	4.9±0.3	12.6±0.48	477±55	26±3.2
$LacD.1_{2(L1+T2)}$	5.56±0.19	667±84	8.4±0.7	12.3±0.39	343±37	36±4.0
$LacD.2_{1G}$	4.88±0.11	538±46	9.1±0.8	18.5±1.0	216±31	86±13

[a]Kinetic parameters determined using enzyme coupled assay described in methods.
[b]Enzyme assay solution conditions: 50 mM HEPES pH 6.8 100 mM NaCl at 25°C

Reaction pH has a similar influence on both LacD.1 and LacD.2. The observation that pH is one signal that modulates LacD.1-dependent regulation (26) suggested that adaptation may have involved a modification to a pH-sensitive step associated with LacD.1's differential catalytic activity. To test this, steady-state kinetic parameters for FBP cleavage were determined over a range of pH values that reflect those encountered by *S. pyogenes* during infection. Comparison of enzyme efficiency revealed a decrease for LacD.1 at every pH value tested (Fig. 2). However, the profiles for LacD.1 and LacD.2 resembled similar bell-shaped curves, with optimum enzyme efficiency occurring at pH 6.8 and efficiency decreasing at both more acidic and basic solution conditions (Fig. 2). Although two different buffer systems were required for the full range of pH values analyzed, measurement of activity of each buffer at their overlap (pH 6.8) produced similar results for both enzymes, indicating that the buffer composition had no measurable influence on catalysis (data not shown). Comparison of the apparent pKa and pKb of both enzymes revealed essentially identical values (Fig. 2). Thus, pH has a similar

influence on catalysis for both LacD.1 and LacD.2, suggesting adaptation did not require a change in LacD.1's catalytic mode.

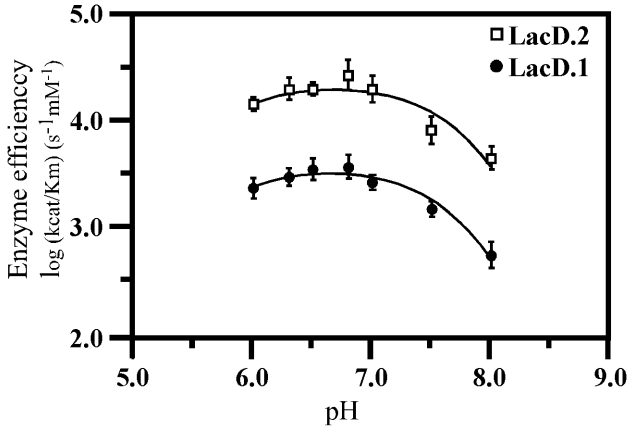


FIGURE 2. The influence of pH on the enzyme efficiency of LacD.1 and LacD.2. The influence of pH on the enzyme efficiency of both LacD.1 and LacD.2 was determined by measuring the Michaelis-Menten constants for cleavage of FBP at several different pHs between the range of 6.0 – 8.0. LacD.2 (pK_a – 4.71±0.82, pK_b - 8.63±0.23) LacD.1 (pK_a – 4.82±0.45, pK_b - 8.46±0.12) See (Materials and Methods) for details.

Catalysis is not influenced by changes in enzyme concentration. Class I aldolases in higher eukaryotes typically function as homotetramers with an extremely low oligomerization constant (42) with quaternary structure linked to enzyme thermostability (3) and non-catalytic aldolase function (39). However, to determine if a change to oligomeric state is associated with altered enzymatic activity, LacD.1 and LacD.2 quaternary structure was compared. Analysis using size-exclusion chromatography revealed that both proteins eluted as a single peak with a nearly-identical elution volume (Fig. 3). The calculated apparent molecular weight for LacD.1 and LacD.2 was 67±3 and 71±3 respectively, close to the expected molecular weight for a dimer (75 kDa, LacD.1; 76 kDa, LacD.2) and consistent with the crystallization of each as homodimers (23, 28).

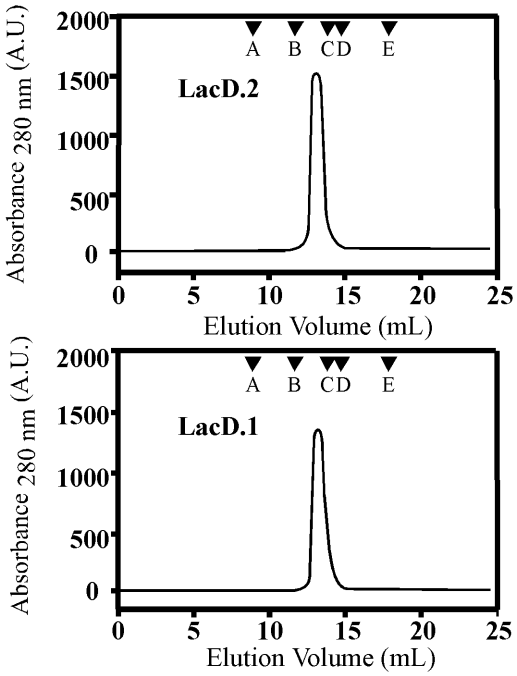


FIGURE 3. Gel filtration chromatography of LacD.1 and LacD.2. Purified LacD.1 and LacD.2 were subjected to gel filtration chromatography over Superdex 200 10/200 GL with their elution profiles illustrated above. Based on the elution of the standards (A-E are: Thyroglobin-669 kDa, Aldolase-158 kDa, Conalbumin-75kDa, Ovalbumin-43 kDa, and RnaseA-13.7 kDa, respectively) the molecular weight of LacD.1 and LacD.2 was calculated to be 67±3 kDa and 71±3 respectively.

To investigate if enzyme concentration influences catalysis, the FBP aldolase activity of both LacD.1 and LacD.2 was measured at six different concentrations ranging between 100 nM to 5 mM. Plots of enzyme activity as a function of enzyme concentration revealed a linear relationship for both LacD.1 and LacD.2 (Fig. 4), suggesting that the differential activity of LacD.1 is not the result of a concentration dependent change in activity. Taken together, these data suggest that adaptation did not involve major changes to the quaternary structure of LacD.1.

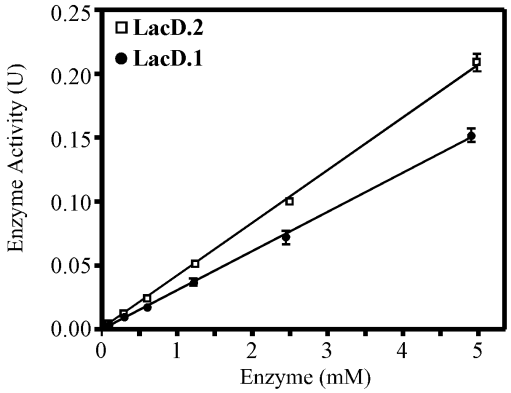


FIGURE 4. Influence of enzyme concentration on enzyme activity. The FBP aldolase activity of LacD.1 and LacD.2 was measured at the enzyme concentrations 0.1, 0.325, 0.625, 1.25, 2.5, and 5 mM. Plotting of activity versus enzyme concentration revealed a linear relationship with R^2 = 0.99 for both LacD.1 and LacD.2. See (Materials and Methods) for details.

The C-terminus of LacD.1 contributes to its differential enzymatic activity. To determine the amino acid residues involved in LacD.1's differential catalytic activity, the divergent LacD.1 residues were mapped onto the structure of LacD.2. This comparison revealed that these residues were located predominantly on the surface of the enzyme (Fig. 5). However, they were not noticeably clustered in any specific region, but rather were dispersed throughout the protein (Fig. 6). Thus, in the absence of any obvious candidate residues involved in the differential activity, several chimeric proteins were created that swapped extended contiguous regions of LacD.1 with the corresponding LacD.2 region (Fig. 6) followed by analysis of their steady state kinetic properties (Table 1). Initially LacD.1 was bisected, replacing its C-terminal half with 162 amino acid residues from the C-terminus of LacD.2 (LacD.1_{2A}, Fig. 6). This enzyme behaved very similar to LacD.2 in the cleavage of TBP, but was over 3 times more efficient than LacD.2 at cleaving FBP, as measured by the quotient (k_{cat}/K_m) (Table 1).

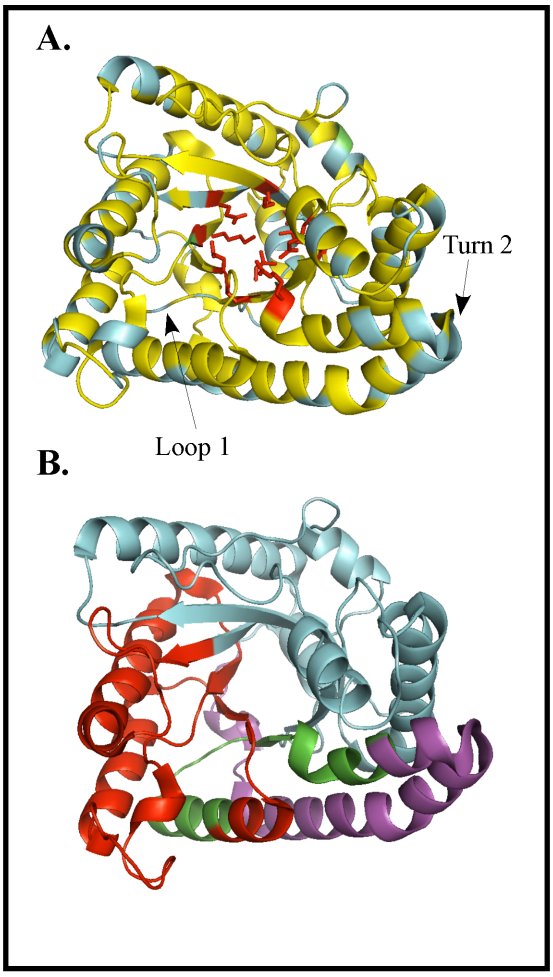


FIGURE 5. Identification of polymorphic amino acid residues in LacD.1. A. The polymorphic residues of LacD.1 (cyan) are mapped on the crystal structure of LacD.2. Catalytic residues identified above are highlighted in red. The location of the Loop 1 and Turn 2 region are labeled with arrows. B. Identification of LacD.1 regions swapped with LacD.2, regions swapped are highlighted: LacD.1_{2A} include residues red, green, and purple, LacD.1_{2B} include residues green and purple, and LacD.1_{2C} include the residues that are purple.

The increase in enzyme activity by LacD.1_{2A} as compared to LacD.1 suggested the residues in the C-terminus contributed to the decreased activity displayed by LacD.1. To help further identify the specific residues involved in modulating LacD.1's enzymatic activity two smaller swaps were made, LacD.1_{2B} and LacD.1_{2C} (Fig. 6), resulting in the exchange of either 19 or 13 polymorphic residues, respectively. Comparison of LacD.1_{2B}

to LacD.2 revealed the enzyme was similar in activity in cleaving FBP, but less efficient at cleaving TBP due to a lower k_{cat} (Table 1). LacD.1_{2C} exhibited a decrease in the K_m for both TBP and FBP compared to LacD.1 (Table 1). However, compared to LacD.1_{2B} or LacD.2, LacD.1_{2C} was less efficient at cleaving both FBP and TBP due to a relative increase in K_m, suggesting that the 13 polymorphic residues mutated in LacD.1_{2C}, as well as the 6 additional polymorphic residues mutated in LacD.1_{2B}, contribute to the enzymatic efficiency of LacD.1. Taken together, comparison of the chimeric enzymes illustrate that polymorphic residues found throughout the C-terminus half of LacD.1 contribute to its decrease in enzymatic activity, primarily by influencing LacD.1's K_m for both TBP and FBP, and to a lesser extent the turnover number or k_{cat} for both substrates.

Examination of the polymorphic residues swapped on the crystal structures of LacD.1 and LacD.2 revealed that the identified residues primarily clustered into two structural regions. The first region, Loop 1, connects the ninth alpha helix to the eighth beta sheet and contains 4 polymorphic residues. The second region, Turn 2, connects the tenth helix to the eleventh helix (Fig 5) and contains 6 polymorphic residues including a proline that changes position and likely influences the flexibility of the protein (Fig. 6).

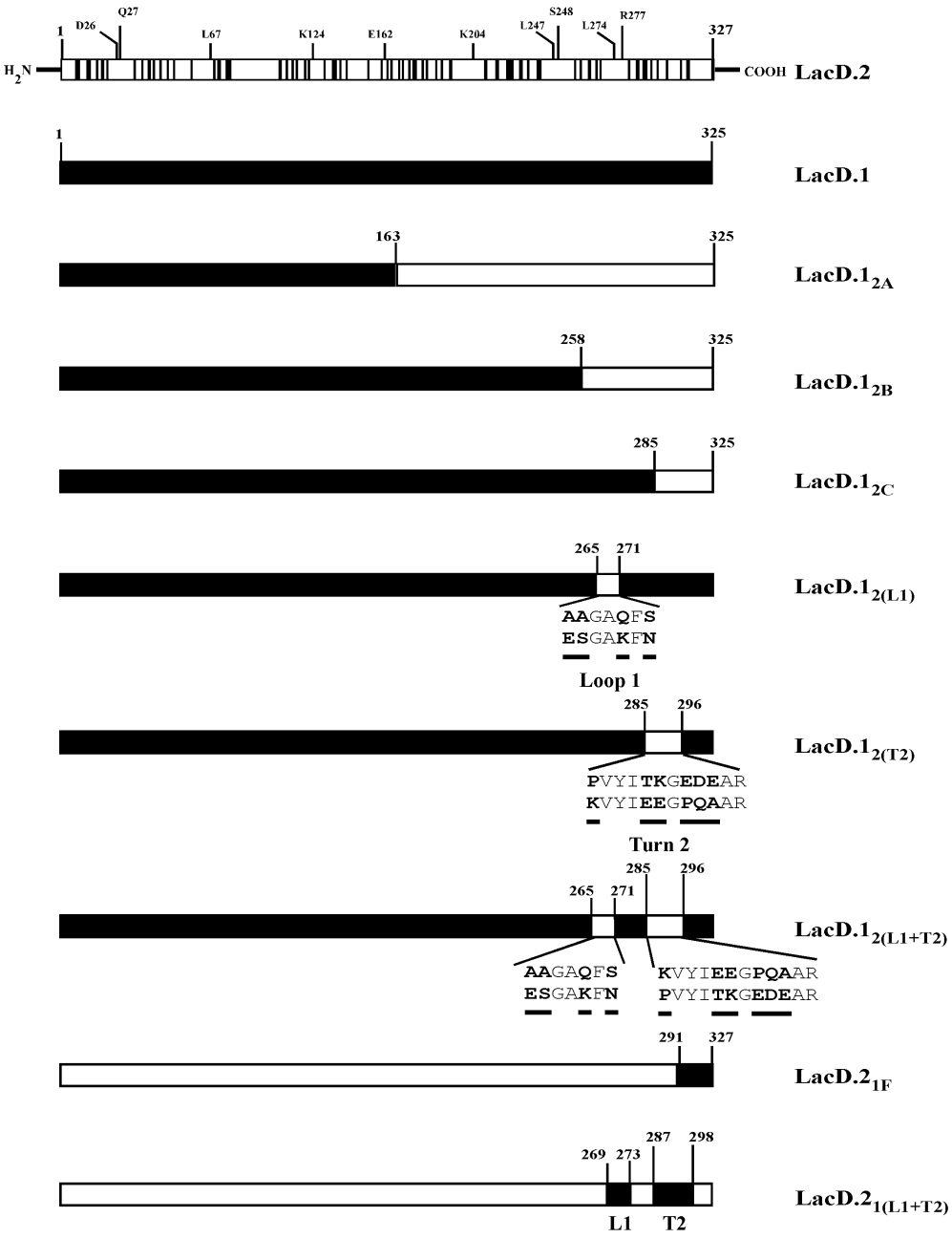


FIGURE 6. Construction of LacD.1 chimeras. The amino acid sequence of LacD.2 is illustrated at the top of the Figure with black boxes representing the polymorphic residues between LacD.2 and LacD.1. The catalytic residues from LacD.1 are labeled. The LacD.1 sequence is indicated by the white box and for each chimera, the black and white boxes represent the sequences derived from LacD.2 and LacD.1, respectively. The Loop 1 and Turn 2 sequences are highlighted below the boxes of selected chimeras, with the specific mutations introduced into the chimera underlined as represented by the sequence shown on the lower line. The name of each chimera is shown at the right of the Figure.

Loop 1 and Turn 2 influence the differential enzymatic activity of LacD.1. To determine the role of these two structural regions in the enzymatic activity of LacD.1, the amino acid sequence of the LacD.1 Loop 1 or the Turn 2 region were swapped with the corresponding LacD.2 sequence. The two enzyme's catalytic activities were then characterized and compared to LacD.1 and LacD.2. Mutation of Loop 1 to create LacD.$1_{2(L1)}$ (Fig. 6) resulted in an enzyme with a considerably lower K_m for both FBP and TBP compared to LacD.1 along with an increase in k_{cat} for FBP (Table 1). However, the Loop 1 chimera remained less efficient than LacD.2 in cleavage of both phosphorylated sugars. The Turn 2 chimera, LacD.$1_{2(T2)}$ (Fig. 6), also resulted in an enzyme that was more efficient for cleavage of both substrates than LacD.1, although not to the same extent as LacD.2 (Table 1). To examine the contribution of these two structural regions together an additional chimera was constructed to introduce both the LacD.2 Loop 1 and Turn 2 into LacD.1 (LacD.$1_{2(L1+T2)}$, Fig. 6). The swap of both regions generated an enzyme with activity comparable to LacD.2 for FBP (Table 1), but was less efficient at cleaving TBP due to a lower k_{cat} and a slightly higher K_m. (Table 4). Chimeras with the reciprocal swaps to exchange the LacD.2 Loop 1 and Turn 2 into LacD.1 were constructed, however, expression of these in *E. coli* resulted in low yields and proteins that were insoluble (LacD.$2_{1(L1+T2)}$, Fig. 6; Supplementary Fig. 1). This protein instability was further seen with replacement of LacD.2's 36 C-terminal residues to LacD.1 (LacD.2_{1F}, Fig. 6; Supplementary Fig. 1). Additionally, a LacD.2 chimera was created, that replaced the LacD.2 residues 165 through 260 with the LacD.1 sequence leaving intact both LacD.2's Loop 1 and Turn 2 sequences (LacD.2_{1G} , see Fig. 8 below). Kinetic analysis of this aldolase revealed that for both FBP and TBP this enzyme behaved similar

to LacD.2 (Table 1). Taken together these data indicate: 1. That the residues responsible for divergent activity reside in the C-terminal half of LacD.1; 2. That the polymorphisms in Loop 1 and Turn 2 make a major contribution to differential catalysis of TBP by LacD.1 due to an increase in the enzyme's K_m and a decrease in the k_{cat}; and 3. That the LacD.2 Loop 1 and Turn 2 do not completely restore LacD.1's catalysis of TBP, with enzyme efficiency remaining approximately four-fold lower than for LacD.2.

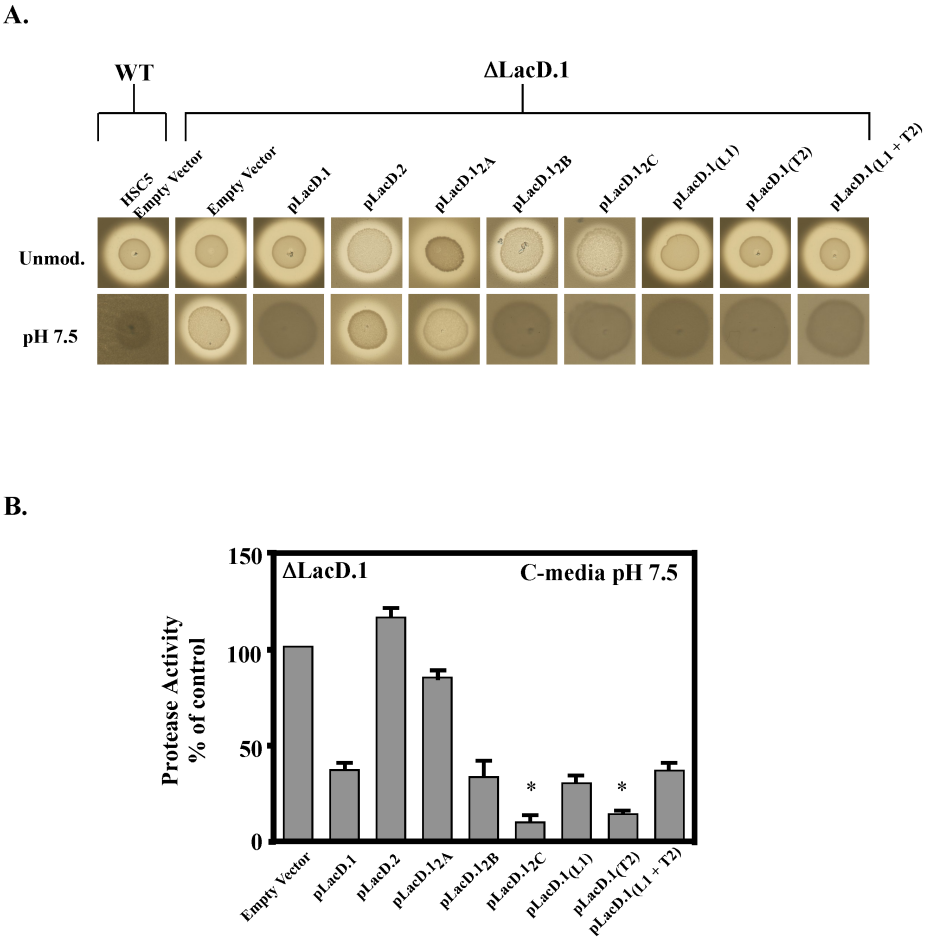


FIGURE 7. Regulation of *speB* by LacD chimeras. A. Spotting of overnight cultures on protease indicator media. Protease activity is apparent as a zone of clearing around the colony. The media is unmodified or buffered to a pH of 7.5 with the addition of 100 mM HEPES pH 7.5. The HSC5 and LacD.1$^-$ strains contain empty vectors, while the other strains in a LacD.1$^-$ background contain either wild type LacD.1, LacD.2, or the indicated chimeras described in Figure 6. B. Secreted protease assay from supernatant fluid of indicated strains described in Figure 6. Buffered media prepared as described in Methods. Activity is reported as a percentage of LacD.1$^-$. Data represents the mean of at least three independent experiments, with samples analyzed in triplicate. An asterisk denotes a significant difference from LacD.1$^-$ pLacD.1 / LacD.1$^-$ complemented with LacD chimeric enzymes in the same medium (* = $P < 0.05$).

The polymorphic residues responsible for differential enzyme activity contribute to regulatory function. The adaptation of LacD.1 to a regulator resulted in an aldolase with decreased enzyme efficiency. However, it is unclear if this decrease in aldolase activity was necessary for adaptation or coincidental. To begin to answer this question the ability of the chimeric enzymes to regulate a virulence factor, the secreted cysteine protease, *speB* was examined. Expression of SpeB is routinely monitored on protease indicator plates, where the diameter of zone of clearing around bacterial growth in milk-containing media results from the proteolytic cleavage of casein reflecting the amount of SpeB produced (WT, Umod., Fig. 7A). However, buffering the medium to pH 7.5 results in repression of *speB* transcription ((25)) and a loss of the halo (HSC5 Empty Vector, pH 7.5, Fig. 7). In contrast, *speB* expression is derepressed in a LacD.1 null mutant under these conditions (ΔLacD.1 Empty Vector, pH 7.5, Fig. 7A) and the wild type phenotype can be complemented by introduction of a plasmid expressing LacD.1, but not LacD.2 (compare pLacD.1 to pLacD.2, pH 7.5, Fig. 7A). To determine the influence of polymorphic residues on this regulatory activity, plasmids encoding various chimeric enzymes were tested for the ability to complement a LacD.1-deficient mutant. Expression of the bisected chimera (LacD.1_{2A}), which has LacD.2-like enzymatic properties, revealed a similar regulatory phenotype to LacD.2, as it was unable to repress expression of SpeB (Fig. 7A, 7B). In contrast, the chimera with only the 67 C-terminal residues of LacD.2 (LacD.1_{2B}) and reduced enzyme efficiency was fully functional in regulation with a phenotype similar to LacD.1 (Fig. 7A, 7B). Swaps of the 40 C-terminal residues (LacD.1_{2C}) or the individual regions of Loop 1, Turn 2, or Loop 1 & Turn 2 did not alter LacD.1's ability to regulate SpeB expression, as similar to WT or pLacD.1, no

halo formed around growth at pH 7.5 on the indicator plates (Fig. 7A). Measurement utilizing a more quantitative FITC-Casein cleavage assay to measure SpeB activity following growth in liquid media revealed that chimeras containing the LacD.2 Turn 2 (LacD.1_{2C} and LacD.$1_{2(T2)}$) were even more effective repressors than LacD.1, as they exhibited a super-repressor phenotype by producing significantly less SpeB activity (Fig. 7B). Interestingly, super-repression was abrogated when the LacD.2 Turn 2 was combined with the cognate LacD.2 Loop 1 residues as chimeras with matched domains had regulatory activity identical to LacD.1 (LacD.$1_{2(L1+T2)}$, Fig. 7). This context dependent super-repressor phenotype along with the influence of these domains on aldolase activity (see above) suggests that the polymorphic residues in Loop 1 and Turn 2 are functionally coupled. Taken together, these data indicate that chimeras with LacD.2 levels of catalytic efficiency lacked the ability to function as regulators. This latter property was only associated with those chimeras that have a four-fold or greater decrease in efficiency for cleavage of TBP.

Extensive regions of LacD.1 are required for regulatory activity. The ability of LacD.1_{2B} to function as a regulator and the inability of LacD.1_{2A} to complement a LacD.1 mutant suggested that variable residues within the 94 amino acid sequence located in the center of LacD.1 are necessary for its regulatory function. To determine if these residues are sufficient an additional chimera was constructed to swap the LacD.1 region into LacD.2 (LacD.2_{1G}, Fig. 8A). However, when examined on indicator plates and in liquid culture, this chimera was unable to repress expression of SpeB (Fig. 8B, 8C), suggesting additional variable residues within the N-terminus are required. Three additional

chimeric enzymes were then constructed to swap larger regions of LacD.1 into the LacD.2 sequence (Fig. 8A).

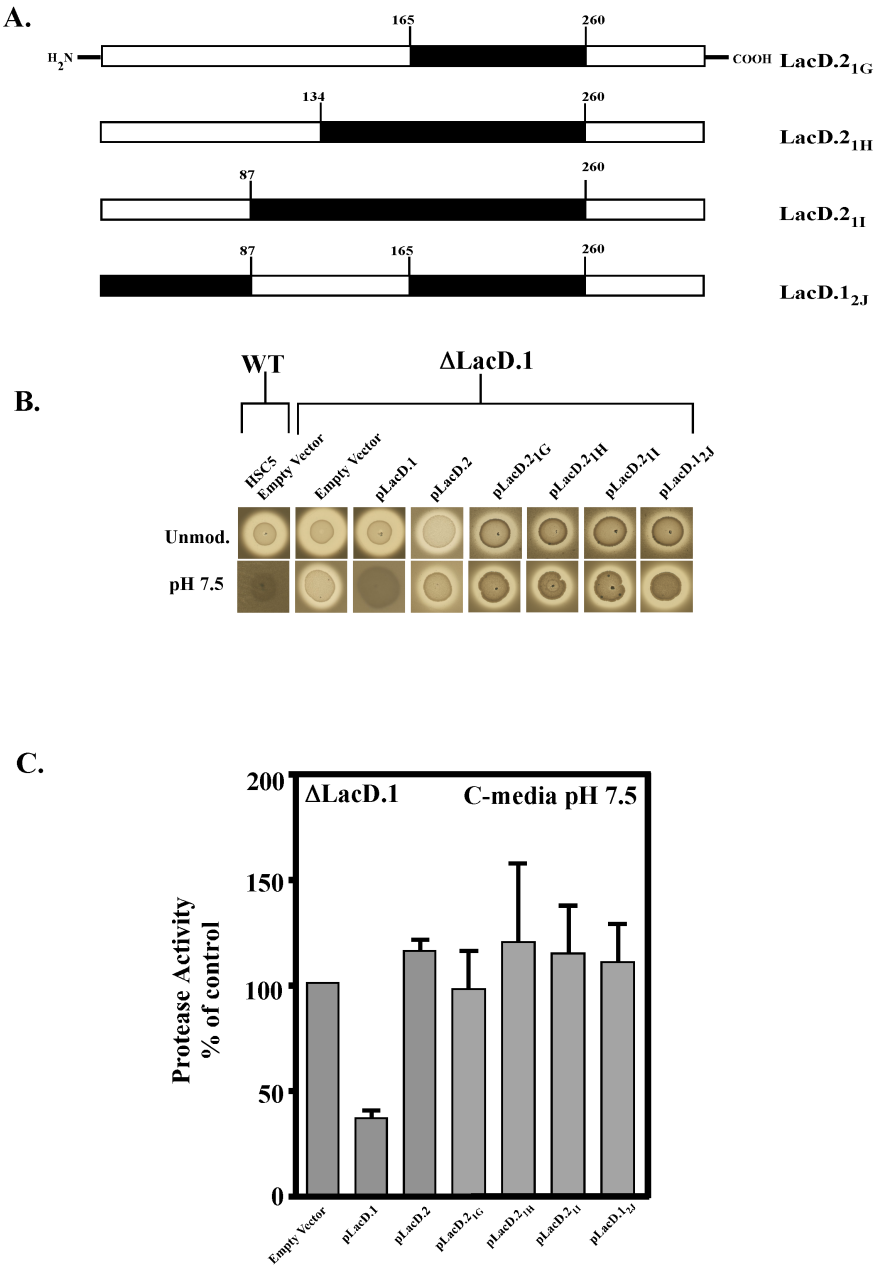


FIGURE 8. Identification of regulatory regions of LacD.1. A. Several additional LacD.1 and LacD.2 chimeras are shown, and are represented as described for Fig. 6. B. Protease activity of the indicated strains determined by a spotting assay on protease indicator media. The clear zone around the bacterial growth indicates production of proteolytic activity. **C.** Quantitative determination of proteolytic activity in cell-free supernant fluid of the indicated strains. Data represents the mean of at least three independent experiments, with samples analyzed in triplicate.

Neither the introduction of 126 or 173 LacD.1 residues was able to generate a chimera with regulatory activity (LacD.2_{1H} , LacD.2_{1I} ; Fig. 8b, 8C). Furthermore, a final chimera which swapped the 87 N-terminal residues of LacD.1 along with the central 94 amino acid region into LacD.2 also did not gain regulatory activity (LacD.2_{1J}, Fig. 8B, 8C). These data suggest that multiple polymorphic residues dispersed through out LacD.1 are required for its function as a regulator.

The adaptation of LacD.1 results in increased fitness. In order to determine if LacD.1's adaptation is associated with any selective advantage, competition analyses were performed using culture conditions formulated to mimic growth in a deep tissue environment (25). This analysis was conducted by complementing ΔLacD.1 with plasmids that expressed LacD.2 or selected enzyme chimeras. These plasmids were identical in all other respects, including the ectopic promoter driving expression of the chimeric gene and the kanamycin and chloramphenicol resistance determinants. An exception was the plasmid for the competitor strain, which expressed LacD.1 as above, but lacked the kanamycin-resistance determinant (pLacD.1^{-kan}). A co-culture was then initiated between the competitor strain (ΔLacD.1 with pLacD^{-kan}) and a test strain expressing one of the chimeric enzymes. The number of colony forming units for each strain was then enumerated following serial passage of the culture over a period of several days. As measured by the competitive index, the LacD.1 test strain had fitness equivalent to that of the competitor (pLacD.1^{-kan}) over a 4-day period (pLacD.1, Fig. 9). In contrast, the LacD.2 test strain had a loss of fitness that was apparent by Day 2 that continued to decline precipitously, achieving a net decrease of 4 logs by Day 4 (pLacD.2, Fig. 9). Test strains expressing LacD.1 chimeras with increased catalytic properties, but

intact regulatory activity, demonstrated no loss of fitness over the period of observation (pLacD.1_{2B}, pLacD.1_{2C}; Fig. 9). However, when the chimera tested also lacked regulatory activity, it also lost fitness in a pattern identical to the LacD.2 test strain (pLacD.1_{2A}, Fig. 9). These data demonstrate that LacD.1 makes an important contribution to fitness, and that mutations in the C-terminus linked to increased enzymatic activity did not influence this fitness under the conditions tested. Taken together, these results implicate its regulatory activity in LacD.1's contribution to fitness.

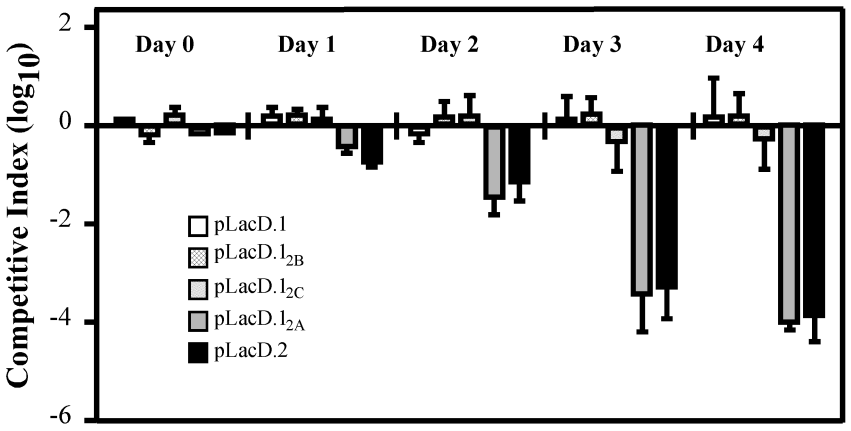


FIGURE 9. The regulatory activity of LacD.1 confers increased fitness. Competition during growth *in vitro* was determined in co-culture with a competitor strain (DLacD.1 complemented with pLacD.1^{-kan}) and a test strain (DLacD.1 complemented with one of the various plasmids indicated in the Figure). Cultures were back-diluted into fresh medium following 24 hrs of growth and CFUs enumerated by plating on media with and without kanamycin for calculation of the competitive index. Data shown are the mean of at least two independent experiments.

DISCUSSION

Comparison of protein paralogs with divergent functions can provide detailed information regarding the structure/function relationship of each protein as well as insight into the evolutionary mechanism that resulted in novel protein function. In this study, comparison of the paralogs LacD.1 and LacD.2 has revealed that while these enzymes share extensive sequence identity and structures, they are divergent with regards to their enzymatic properties and overall functions. The polymorphic residues associated with divergent catalytic activity were primarily confined to two discrete segments. However, the residues responsible for LacD.1's novel regulatory function were more widely dispersed. Furthermore, LacD.1's less efficient catalytic activity was associated with its ability to function as a regulator. Together these data provide insight into the specific functions of LacD.1 and LacD.2 and into the evolutionary process that fostered their divergence.

One interesting insight came from the observation that the Loop 1 and Turn 2 features were functionally coupled. This is consistent with studies showing that protein evolution can be driven by networks of statistically coupled amino acids, termed sectors, that co-evolve throughout protein families (11, 41). Sectors may be linked to conservation of specific function, including enzymatic reactions or substrate-binding motifs, and thus, constitute fundamental units of evolution for repurposing to novel function. Examples of specific sectors include conserved networks for allosteric regulation of enzymes that link residues on the surface of the protein to the active site (37). While the residues within the sector can continue to change, their coevolution allows allosteric regulation to remain

intact. Similar to what was observed for LacD.1 and LacD.2, cognate sectors can be adapted in order to modulate an enzymes activity (37).

This mechanism of variation has also been reported for other families of aldolase enzymes, suggesting a common mechanism underlays the evolution of the *S. pyogenes* enzymes. Similar to the streptococcal enzymes, the aldolases found in higher eukaryotes are 70% identical and have identical active sites, yet they can have divergent activity with respects to their substrates. Mutational studies have shown that the residues responsible for differential activity are clustered in two specific patches, the terminal surface patch (TSP) and the distal surface patch (DSP), that are distal to their active sites (2, 20, 21, 31). Examination of cysteine reactivity and the analysis of crystal structure *B*-factors suggest that these two patches influence flexibility to coordinate motion of the enzyme (14, 33, 34). Comparison of these aldolase isozymes to the *S. pyogenes* enzymes reveals that the polymorphic residues of Turn 2 adjacent to helix 10 and helix 11 and across from helix 2 correspond to a region within the TSP. Thus, Turn 2 could have a similar influence on movement. An increase in motion could explain the increase in the K_m as a result of the higher entropic penalty accompanying binding. Additionally, a disruption in the coordination of the protein motions that are required for substrate binding could also be a contributing factor. Taken together, these similarities suggest that Loop 1 and Turn 2 are sectors that are constituents of an allosteric network in type 1 aldolases.

Conservation of an allosteric network in type I aldolases further supports the hypothesis that these enzymes evolved from a common ancestor. Structural comparison of LacD.2 and rabbit muscle aldolase revealed a strong conservation of active site and catalytic residues (28), with the absence of the flexible C-terminal region found on class I

FBP aldolases being the largest deviation in structure. This C-terminus region aids in product release and likely contributes to LacD.2's decrease in FBP cleavage rate compared to other FBP aldolases. Interestingly the LacD.1_{2A} chimera showed an increase in cleavage of FBP compared to LacD.2 suggesting that polymorphic residues found between 163 and 258 have an influence on catalysis. However swapping this region in LacD.2 to create LacD.2_{1G} did not drastically influence this enzyme's activity toward TBP or FBP. The epistatic nature of these residues suggests that additional amino acids found in the N-terminus of LacD.1 are functionally linked to this region and the enzymatic activity of the aldolase. Furthermore, given that the residues in this region are required for the regulatory function of LacD.1, this further suggests that the adaptation of LacD.1 into a metabolic sensor required the co-evolution of multiple amino acid residues.

Often, the best option for informing the cell of its metabolic state are enzymes that have evolved to be sensitive to the concentration of a specific metabolite (6, 13, 40). Thus, an important question is whether the manipulation of allostery to repurpose enzymes for alternate enzymatic activity can also be used for the adaption to more diverse functions, including roles as transcriptional regulators and/or metabolic sensors. Investigation into a number of different bacteria and regulatory pathways has found a variety of examples by which metabolic enzymes have evolved to function in a regulatory fashion. In the case of PutA, a proline degrading enzyme found in enterobacteria, the acquisition of a DNA binding domain has allowed this protein to function both as a metabolic enzyme and transcriptional regulator through direct interaction with DNA. This regulation is controlled by the presence of proline that leads to a distinct conformational change in the protein that changes its localization in the cell

and the ability to bind DNA (32). Alternatively, in *E. coli* the regulation of the maltose pathway has evolved through a number of protein-protein interactions of enzymes in the pathway that sequester the transcriptional activator MalT in the absence of substrate (17). Additionally the human aldolase isozymes that are expressed in a specific tissue manner have also acquired secondary interactions with cytoskeleton proteins expressed in these tissues. These interactions occur in and near the active site of the aldolase and thus are also regulated by the presence of substrate. Interestingly the tissue specific aldolases have increased specificity for the cytoskeleton proteins expressed in the tissue in which they are expressed, suggesting an additional role of the isozyme specific residues (19, 20). Together, these examples suggest that evolution has routinely drawn upon an enzyme's ability to recognize a metabolic substrate to link the structural changes that occur with binding to a regulatory output. Comparison of these examples to LacD.1 highlights some key similarities and differences. Previous mutagenesis studies examining LacD.1 have shown that catalysis does not contribute to its regulatory function, but that disruption of the ability to bind substrate does result in loss of regulatory activity (26), suggesting LacD.1's evolution likely required retention of the ability to accurately sense a phosphorylated sugar substrate. However, noticeably different from the previous examples is the division of the metabolic and regulatory function between two proteins. Occasionally, the acquired secondary regulatory function can coexist with the enzyme's primary metabolic role, however in instances when these two functions are mutually exclusive, gene duplication can serve to resolve this conflict.

Adaptation that acts to harness an enzyme's ability to sense a metabolite to a transcriptional regulatory output often occurs subsequent to a gene duplication event.

However, the most common fate of the initially redundant gene is that it accumulates deleterious mutations leading to gene death (7). Thus, retention of the duplicate requires that it is adapted to provide a selective advantage for the organism. In this regard, LacD.1's adaption may share some features in common with the well-characterized GAL network in the yeast *Saccharomyces cerevisiae*, in which an adapted galactose kinase functions as the sensory component of a transcriptional regulatory complex. This adapted enzyme is apparently the product of a gene duplication event and comparisons to the ancestral enzyme found in related yeasts have suggested that duplication was necessary in order resolve an adaptive conflict that arose when positive selection for regulatory activity proved deleterious to enzymatic function. The ancestral enzyme has dual functionality, with a limited ability to act both as a transcriptional regulator and as a galactose kinase. However, in *S. cerevisiae*, duplication promoted a process of sub-functionalization that partitioned regulatory and enzymatic activity between the two genes. This "escape from conflict" then allowed positive selection to optimize each of these individual functions. In the streptococci, it is not yet known whether regulatory function is unique to LacD.1 of *S. pyogenes* or whether it was derived from an ancestor with dual function. However, since one of LacD.1's regulatory targets, the SpeB protease, is unique to *S. pyogenes*, it is possible that LacD.1's regulatory activity is also novel and thus, its adaptation may be distinct from that of the yeast enzyme. In this scenario, an escape from conflict would have promoted neofunctionalization, where instead of partitioning and perfecting existing functions, the gene would gain a novel function.

Another distinction is that while the subfunctionalization of the yeast regulatory paralog resulted in the loss of its galactose kinase activity (35), LacD.1 retains an aldolase activity. Interestingly, an increase in aldolase efficiency to levels comparable to LacD.2 resulted in a loss of activity. Currently it is unclear whether these residues have a direct role in regulation or if an increase in activity indirectly influences LacD.1's regulatory function; it is possible that the reduced efficiency of LacD.1's catalytic activity represents sub-functionalization of catabolic activity between LacD.1 and LacD.2. For the yeast regulatory paralog, it has been shown that both the partitioning of regulatory activity and the loss of enzymatic activity each contributes to significant gains in fitness (15, 24). However, the gain of fitness provided by LacD.1 appears to be associated with a gain in regulatory activity acquired concomitantly with a decrease in enzymatic activity. Thus, while LacD.1's decrease in enzymatic activity toward TBP represents subfunctionalization, the polymorphisms that occur in Loop 1 and Turn 2 region of LacD.1, and their associated role in enzymatic activity is not clear. In the present study, we examined fitness under conditions that mimicked growth in soft tissue, which is relatively poor in its content of carbohydrates that can be metabolized by *S. pyogenes*. This is likely not the case at other sites that can support streptococcal colonization. Thus, an alternative is that rather than being required for LacD.1's function during growth in deeper tissue, polymorphisms in Loop 1 and Turn 2 may be required to maintain fitness under carbohydrate-rich conditions where the Lac.2 operon is highly expressed. An inappropriate aggregate level of aldolase activity could alter the flow of carbon through the glycolytic pathway or altered the fine balance of regulation of the Lac.2 operon relative to other carbohydrate processing pathways.

LacD.1's regulatory function has been demonstrated to include genes expressed both in exponential and early stationary phase(1). This study represents the initial stage of an investigation into the complex evolutionary process that resulted in LacD.1's novel protein function. Taken together, the data suggest that LacD.1's adaptation represents a form of neofunctionalization where duplication facilitated the gain of regulatory function important to growth in tissue and pathogenesis. However, confirmation of this idea will require further investigation into a number of different areas, including an understanding of the molecular details of how LacD.1 acts to control transcription and its partners in the regulatory pathway. This information will prove invaluable for identification of residues that are specifically required for LacD.1's regulatory function and the role of selection in divergence at these positions relative to LacD.2. The rapid accumulation of sequence from additional *S. pyogenes* strains and from other streptococcal species will allow a higher resolution analysis of selective pressure on individual residues and should provide insight into the relative contributions of subfunctionalization vs neofunctionaliztion in the evolution of virulence in *S. pyogenes*.

Supplementary Table I. Strains used in this study[a]

Strains	Relevant genotype	Plasmid	Description	Reference
E. coli				
JL266	BL21(DE3)	pJL132	Expression of LacD.1-His	Loughman *et al*. (2006)
JL268	BL21(DE3)	pJL134	Expression of LacD.2-His	Loughman *et al*. (2006)
ZC81	BL21(DE3)	pZC15	Expression of LacD.1_{2A}-His	This work
ZC209	BL21(DE3)	pZC53	Expression of LacD.1_{2B}-His	This work
ZC227	BL21(DE3)	pZC54	Expression of LacD.1_{2C}-His	This work
ZC282	BL21(DE3)	pZC79	Expression of LacD.$1_{2(L1)}$-His	This work
ZC283	BL21(DE3)	pZC80	Expression of LacD.$1_{2(T2)}$-His	This work
ZC318	BL21(DE3)	pZC99	Expression of LacD.$1_{2(L1+T2)}$-His	This work
ZC240	BL21(DE3)	pZC64	Expression of LacD.2_{1F}-His	This work
ZC364	BL21(DE3)	pZC116	Expression of LacD.$2_{1(L1+T2)}$-His	This work
ZC214	BL21(DE3)	pZC55	Expression of LacD.$2_{1(L1+T2)}$-His	This work
S. pyogenes				
HSC5	wild-type	none	wild-type	Hanski *et al*. (1992)
ZC369	wild-type	pLZ12	HSC5 (vector)	This work
JL151	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	none	In-frame deletion of *lacD.1*	Loughman *et al*. (2006)
ZC374	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pLZ12	JL151 (vector)	This work
JL340	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pJL154	JL151 (pLacD.1-HA)	Loughman *et al*. (2006)
JL342	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pJL156	JL151 (pLacD.2-HA)	Loughman *et al*. (2006)
JL347	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pJL162	JL151 (pLacD.1_{2A}-HA)	This work
ZC719	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC206	JL151 (pLacD.1_{2B}-HA)	This work
ZC714	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC207	JL151 (pLacD.1_{2C}-HA)	This work
ZC451	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC127	JL151 (pLacD.$1_{2(L1)}$-HA)	This work
ZC452	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC128	JL151 (pLacD.$1_{2(T2)}$-HA)	This work
ZC453	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC129	JL151 (pLacD.$1_{2(L1+T2)}$-HA)	This work
ZC715	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC208	JL151 (pLacD.2_{1G}-HA)	This work
ZC716	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC209	JL151 (pLacD.2_{1H}-HA)	This work
ZC717	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC210	JL151 (pLacD.2_{1I}-HA)	This work
ZC718	HSC5 *lacD.1*$_{\Delta 21\text{-}321}$	pZC211	JL151 (pLacD.2_{1J}-HA)	This work

a. Gene names are based on current annotation of the genome of *S. pyogenes* SF370 in the NCBI database (http://www.ncbi.nlm.gov).

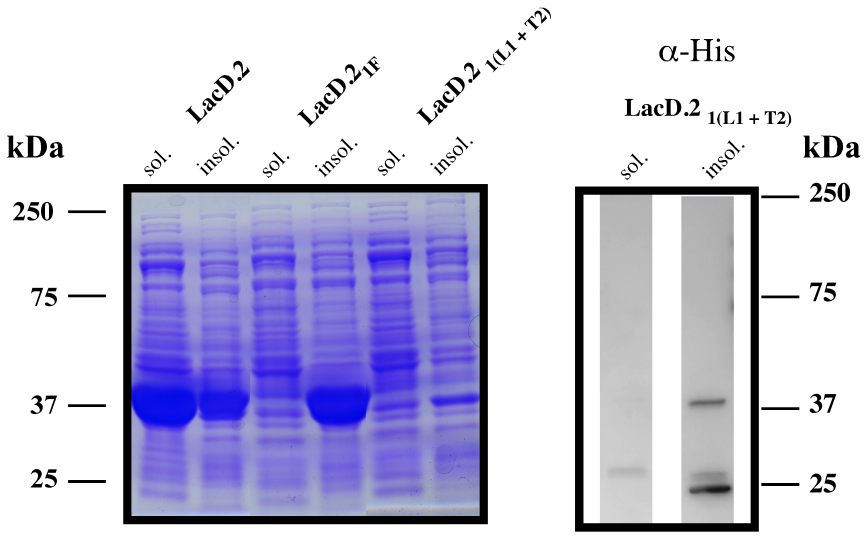
Supplementary Table II. Primers used in this study

Name	Sequence[a]	Template	Plasmid	Description
JLP104	TAAAGAATTCTATCAGCAACTTCAAGTAAG	HSC5	pJL155	Primers for expression of LacD.1
JLP173	ATCACACTGCAGTTAAGCATAATCTGGAACATCATATGGATAGATTTTTTCCGTCCAAGG			
JLP130	CAATGAATTCAAGTCAAAGAGGTATAAAAAATG	HSC5	pJL156	Primers for expression of LacD.2
JLP174	TTTCTTCTGCAGTTAAGCATATCTGGAACATCATATGGATACATTTTTTCAGTCCAAGG			
JLP165	GAGACCATGGACATGACAAATCACAGT	HSC5	pJL132	Primers for pET24d/LacD.1
JLP166	GGTTCTCGAGGATTTTTTCCGTCCAAGG			
JLP167	GAGGCCATGGAAATGACAATTACTTTAAC	HSC5	pJL134	Primers for pET24d/LacD.2
JLP168	AATACTCGAGCATTTTTTCAGTCCAAGG			
OJOY3S	GGGGTCCCTTTCCTTGATAG	pJL75	pJL162	Primers for first half of LacD.1_{2A} for expression
JLP160	AAAGCTCGAGAAAAAAAGGGATGTCTTC			
OJOY37	CTGCACTTTGTTATTTACCAATACC	pJL126	pJL162	Primers for second half of LacD.1_{2A} for expression
JLP161	CTACCTCGAGATTCTAACTTATGATG			
ZC34	GAGACCATGGACATGACAATAACAGC	pJL162	pZC15	Primers for pET24d/LacD.1_{2A}
ZC33	AATAGTCGACCATTTTTTCAGTCCAAGG			
JLP165	GAGACCATGGACATGACAAATCACAGT	HSC5	pZC53	Primers for first half of pET24d/LacD.1_{2B}
ZC99	/5PHOS/GCCTGCACTCAAATAGATAT			
ZC100	/5PHOS/GTTTCTGCCAAATTGTTCCA	HSC5	pZC53	Primers for second half of pET24d/LacD.1_{2B}
JLP168	AATACTCGAGCATTTTTTCAGTCCAAGG			
JLP165	GAGACCATGGACATGACAAATCACAGT	HSC5	pZC54	First half of pET24d/LacD.1_{2C}
ZC104	/5PHOS/GGATCCTGCCCACGTGGCACGCC			
ZC103	/5PHOS/GTAAAAGTCTATATCGAAGAAGG	HSC5	pZC54	Second half of pET24d/LacD.1_{2C}
JLP168	AATACTCGAGCATTTTTTCAGTCCAAGG			
ZC117	CAAGAGACATTATACTTTGCAGCAGAATCAGGTGCTCAATTTAGTGG	pJL132	pZC43	Mutagenesis primers for LacD.1 (A265E A266S)
ZC118	CCACTAAATTGAGCACCTGATTCTGCTGCAAAGTATAATGTCTCTTG			
ZC169	CTTTGCAGCAGAATCAGGTGCTAAATTTAACGGTGTTTTGTGTGG	pZC43	pZC79	Mutagenesis primers for LacD.1_{L1} (A265E A266S Q269K S271N)
ZC170	CCACACAAAACACCGTTAAATTTAGCACCTGATTCTGCTGCAAAG			
ZC85	GCCACGTGGGCAGGATCCGTTAAAGTTTATATTACTAAAGG	pJL132	pZC41	Mutagenesis primers for LacD.1 (P285K)
ZC86	CCTTTAGTAATATAAACTTTAACGGATCCTGCCCACGTGGC			
ZC157	CAGGATCCGTTCCAGTTTATATTACTAAAGGACCGCAGGCAGCAAGAAAGTGGCTATGCAC	pZC41	pZC76	Mutagenesis primers for LacD.1 (P285K E292P D293Q E294A)
ZC158	GTGCATAGCCACTTTCTTGCTGCCTGCGGTCCTTTAGTAATATAAACTGGAACGGATCCTG			
ZC171	GCCACGTGGGCAGGATCCGTTAAAGTTTATATTGAGGAGGGACCGCAGGCAGCAAG	pZC76	pZC80	Mutagenesis primers for LacD.1_{T2} (P285K E292P D293Q E294A T289E K290E)
ZC172	CTTGCTGCCTGCGGTCCCTCCTCAATATAAACTTTAACGGATCCTGCCCACGTGGC			
ZC157	CAGGATCCGTTCCAGTTTATATTACTAAAGGACCGCAGGCAGCAAGAAAGTGGCTATGCAC	pZC79	pZC88	Mutagenesis primers for LacD.1_{L1+T2} (A265E A266S Q269K S271N, E292P, D293Q, E294A)
ZC158	GTGCATAGCCACTTTCTTGCTGCCTGCGGTCCTTTAGTAATATAAACTGGAACGGATCCTG			
ZC85	GCCACGTGGGCAGGATCCGTTAAAGTTTATATTACTAAAGG	pZC88	pZC91	Mutagenesis primers for LacD.1_{L1+T2} (A265E A266S Q269K S271N, E292P, D293Q, E294A, P285K)
ZC86	CCTTTAGTAATATAAACTTTAACGGATCCTGCCCACGTGGC			
ZC171	GCCACGTGGGCAGGATCCGTTAAAGTTTATATTGAGGAGGGACCGCAGGCAGCAAG	pZC91	pZC99	Mutagenesis primers for LacD.1_{L1+T2} (A265E A266S Q269K S271N, E292P, D293Q, E294A, P285K, T289E, K290E)
ZC172	CTTGCTGCCTGCGGTCCCTCCTCAATATAAACTTTAACGGATCCTGCCCACGTGGC			
JLP130	CAATGAATTCAAGTCAAAGAGGTATAAAAAATG	HSC5	pZC64	First half of pET24d/LacD.2_{1F}
ZC128	/5PHOS/TCCTTCTTCGATATAGACTTTTAC			
ZC130	/5PHOS/GAGGACGAAGCAAGAAAGTGGCTATG	HSC5	pZC64	Second half of pET24d/LacD.2_{1F}
JLP166	GGTTCTCGAGGATTTTTTCCGTCCAAGG			
ZC173	ATTTGCAGCATCAGGTGCTCAGTTCAGCGGTGTCTTATGTGGACG	pJL134	pZC81	Mutagenesis primers for LacD..2_{L1+T2} (K271Q N273S)
ZC174	CGTCCACATAAGACACCGCTGAACTGAGCACCTGATTCTGCTGCAAAT			
ZC175	GGGCAGGATCTGTAAAAGTCTATATCGAAGAAGGAGAGGATGAAGCTCGTGAATGGTTGCG	pZC81	pZC87	Mutagenesis primers for LacD..2_{L1+T2} (K271Q, N273S, P294E, Q295D,A296E)
ZC176	CGCAACCATTCACGAGCTTCATCCTCTCCTTCTTCGATATAGACTTTTACAGATCCTGCCC			
ZC177	CTACCTGGGCAGGATCTGTACCAGTCTATATCGAAGAAGG	pZC87	pZC97	Mutagenesis primers for LacD..2_{L1+T2} (K271Q, N273S, P294E, Q295D,A296E, K287P)
ZC178	CCTTCTTCGATATAGACTGGTACAGATCCTGCCCAGGTAG			
ZC182	GGGCAGGATCTGTAAAAGTCTATATCACGAAAGGACCACAAGCAGCTCGT	pZC97	pZC116	Mutagenesis primers for LacD..2_{L1+T2} (K271Q, N273S, P294E, Q295D,A296E, K287P, E291T, E292K)
ZC183	ACGAGCTGCTTGTGGTCCTTTCGTGATATAGACTTTTACAGATCCTGCCC			
JLP162	GAATCTCGAGGTAGAATGGAATATCTTC	pJL126	pJL164	Primers for expression of LacD.2_{1K}
OJOY3S	GGGGTCCCTTTCCTTGATAG			
OJOY37	CTGCACTTTGTTATTTACCAATACC	pJL75	pJL164	Primers for expression of LacD.2_{1K}
JLP159	TTTTTCTCGAGCTTTTGAGTTATGATGAG			

a. Engineered restriction sites are underlined. Sequence for Influenza hemagglutanin (HA) epitope tag is in italics

Name	Sequence[a]	Template	Plasmid	Description
JLP167	GAGGCCATGGAAATGACAATTACTTTAAC	pJL164	pZC55	Primers for first half of pET24d/LacD.2_{1G} for expression
ZC99	/5PHOS/GCCTGCACTCAAATAGATAT			
ZC100	/5PHOS/GTTTCTGCCAAATTGTTCCA	pJL164	pZC55	Primers for second half of pET24d/LacD.2_{1G} for expression
JLP168	AATACTCGAGCATTTTTTCAGTCCAAGG			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	HSC5	PCR 1	Primers for construction of LacD.2_{1H}
ZC402	GTAAATTGATTTGTTCATCTCCATCAATATCATAGTAAAGAAGGAATTTAACTGCTTC			
ZC403	GATGGAGATGAACAATCAATTTAC	HSC5	PCR 2	Primers for construction of LacD.2_{1H}
ZC399	CTGCTGCAAATTCCAAAGTATCTTGAAAAAGTTTGGCAGATAC			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	PCR 1 + PCR 2	PCR 3	Primers for construction of LacD.2_{1H}
ZC399	CTGCTGCAAATTCCAAAGTATCTTGAAAAAGTTTGGCAGATAC			
ZC400	ACTTTCGAATTTGCAGCAG	HSC5	PCR 4	Primers for construction of LacD.2_{1H}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	PCR 3 + PCR 4	pZC209	Primers for expression of LacD.2_{1H}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	HSC5	PCR 5	Primers for construction of LacD.2_{1I}
ZC397	GTTTTTTCGTAAGCTAACAGTAAGCCTGCTTCTTCTGAACGAACACG			
ZC398	GGCTTACTGTTAGCTTACGAAAAAAC	HSC5	PCR 6	Primers for construction of LacD.2_{1I}
ZC399	CTGCTGCAAATTCCAAAGTATCTTGAAAAAGTTTGGCAGATAC			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	PCR 5 + PCR 6	PCR 7	Primers for construction of LacD.2_{1I}
ZC399	CTGCTGCAAATTCCAAAGTATCTTGAAAAAGTTTGGCAGATAC			
ZC473	TTGAATTCTATCAATCTATTAAAGTCAAAGAGGTATAAAAAATGACAATTACTTTAACTGAAAACAAACG	PCR 7 + PCR 4	pZC210	Primers for expression of LacD.2_{1I}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	HSC5	PCR 8	Primers for construction of LacD.1_{2J}
ZC423	CACGACTTGCTGGCAAGCCATATTCTGGATCTAATAAAATAGATGAAGC			
ZC424	GGCTTGCCAGCAAGTCGTG	pZC55	PCR 9	Primers for construction of LacD.1_{2J}
ZC401	TGATCTGCAGTTAAGCGTAATCTGGAACATCGTATGGGTACATTTTTTCAGTCCAAGGACTTGC			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	PCR 8 + PCR 9	pZC211	Primers for expression of LacD.1_{2J}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	pZC53	pZC206	Primers for expression of LacD.1_{2B}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	pZC54	pZC207	Primers for expression of LacD.1_{2C}
ZC401	TGATCTGCAGTTA*AGCGTAATCTGGAACATCGTATGGGTA*CATTTTTTCAGTCCAAGGACTTGC			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	pZC79	pZC127	Primers for expression of LacD.$1_{2(L1)}$
ZC260	ATCACACTGCAGTTA*AGCATAATCTGGAACATCATATGGATA*GATTTTTTCCGTCCAAGG			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	pZC80	pZC128	Primers for expression of LacD.$1_{2(T2)}$
ZC260	ATCACACTGCAGTTA*AGCATAATCTGGAACATCATATGGATA*GATTTTTTCCGTCCAAGG			
ZC259	TTTGAATTCGTAAGAGAGGTATCACATGACAATCACAGCAAATAAAAGACATTACCTAG	pZC99	pZC129	Primers for expression of LacD.$1_{2(L1+T2)}$
ZC260	ATCACACTGCAGTTA*AGCATAATCTGGAACATCATATGGATA*GATTTTTTCCGTCCAAGG			

a. Engineered restriction sites are underlined. Sequence for Influenza hemagglutanin (HA) epitope tag is in italics



Supplementary Figure 1

MATERIALS AND METHODS

Strains, media and growth conditions. Molecular cloning experiments utilized *Escherichia coli* TOP10 (Invitrogen) and BL21 (DE3) was used for expression of recombinant proteins. Experiments with *S. pyogenes* used strains HSC5 (12) and JL151 (26), a ΔLacD.1 derivative of HSC5 with an in-frame deletion in *lacD.1* (SPy_1704). Culture for all assays of function was at 37^{o}C in C medium (29). In selected experiments, C-medium was buffered by the addition of 1 M HEPES pH 7.5 to a final concentration of 0.1 M. Routine culture of *S. pyogenes* and *E. coli* utilized Todd Hewitt Yeast Extract medium under anaerobic conditions and Luria-Bertani medium aerobically, respectively. When appropriate, antibiotics were added to media at the following concentrations: chloramphenicol, 15 μg/mL for *E. coli* and 3 μg/mL for *S. pyogenes* and kanamycin, 50 μg/mL for *E .coli* and 500 μg/mL for *S. pyogenes*.

Purification of enzymes. Proteins were expressed in BL21 DE2 cells as described previously (26). Following expression, cells were collected by centrifugation and disrupted by sonication(26). The six-His-tagged proteins were purified by chromatography over a cobalt affinity resin, according to the recommendations of the manufacturer (Clontech). Eluted proteins were then dialyzed against a buffer consisting of 50 mM $NaPO_4$ (pH 7.5), 300 mM NaCl, and 25 mM βME and further purified by gel filtration chromatography using Superdex 200 10/300 GL (GE Healthcare). Fractions containing purified protein were identified by SDS PAGE, were pooled and then dialyzed against a buffer consisting of 50 mM $NaPO_4$ (pH 7.5), 300 mM NaCl, 1 mM DTT in a final volume of 25% glycerol and stored at -20°C. Protein concentrations were determined by absorbance at 280 nm of aliquots diluted into 6M Guanidine hydrochloride

and final values calculated from the average of three different dilutions. Calculation of protein extinction coefficients utilized ProtParam (http://web.expasy.org/ protparam/) (9).

DNA and computational techniques. Plasmid purification and transformation of *E. coli* and *S. pyogenes* were performed as described previously (5, 22). The genes encoding various LacD chimeric enzymes were constructed using the PCR products generated using 5' phophorylated primers for three-way ligations, or through a process of overlap extension PCR (16). Restriction enzymes and additional reagents were used according to the manufacturer's recommendations (New England Biolabs). Site-directed mutagenesis was performed by PCR followed by *DpnI* digestion of template using a commercial kit (QuikChange Site-Directed Mutagenesis kit, Stratagene) and the primers and templates described in Supplementary Table 1. The fidelity of all constructs was confirmed by determination of DNA sequences using the services of a commercial vendor (SeqWright, Houston, TX). Coordinates for protein structures were obtained from the Protein Data Bank (www.pdb.org) and were compared using the PyMOL Molecular Graphics System (Version 1.5.0.4, Schrödinger, LLC).

Aldolase cleavage assay and determination of steady state kinetic constants. Aldolase activity was measured using a standard enzyme coupled assay that monitored oxidation of β-NADH by following the decrease in absorbance at 340 nm in the presence of the coupling enzymes glycerol-3-phosphate dehydrogenase and triosephosphate isomerase (4). Enzyme was diluted to 100-170 nM into a reaction mixture consisting of 50 mM HEPES pH 7.5, 100 mM NaCl, 2.5 units glycerol-3-phosphate dehydrogenase, 20 units triosephosphate isomerase, and 0.25 mM β-NADH. The initial velocity (V_0) was plotted at varying concentrations of substrate ([S]) and fit to a standard equation (V_0 =

$V_{max}[S]/K_m + [S]$) to determine the Michaelis-Menten constant (K_m) and the V_{max}. The k_{cat} was calculated by dividing the V_{max} by the enzyme concentration. For cleavage of FBP at pH 7.5, a substrate range of 0.2-8 mM was used for LacD.2 and 0.2-40 mM used for LacD.1. For cleavage of TBP at pH 7.5 (supplied by Dr. Wolf-Dieter Fessner of Technische Universität, Darmstadt, Germany), a substrate range of 0.1-2 mM was used for LacD.2 and 0.5-16 mM used for LacD.1. For cleavage of FBP at pH 6.8, a substrate range of 0.2-8 mM was used for all enzymes. For cleavage of TBP at pH 6.8, a substrate range of 0.04-4 mM was used for all enzymes.

Influence of pH on aldolase activity. The aldolase cleavage assay was performed as described above in reaction mixture buffered by 50 mM BisTris (pH range 6.0 - 6.5) or by 50 mM HEPES (pH range 6.8 – 8.0). The k_{cat}/K_m was calculated and fit to the equation log Y = log(C/(1+[H]/Ka + Kb/[H] + [H]2 /KaKb)) using Graphpad Prism (Version 5, GraphPad Software, San Diego, CA).

Determination of molecular weight. Purified protein and molecular weight standards (GE Healthcare) were either dialyzed against or resuspended in a buffer of 50 mM $NaPO_4$, 300 mM NaCl, and 25 mM βME. Aliquots ranging from 100-200 μLs were then developed over a Superdex 200 HR 10/300 GL column with a flow rate of 0.5 mL/min. Elution of protein was monitored by absorbance at 280 nm. The value of K_{av} was calculated for each standard using the equation $K_{av} = (V_e – V_o)/(V_t – V_o)$, where V_e is the elution volume, V_o is the void volume (8.107 mL), and V_t is the bed volume (24 mL). For each standard, the K_{av} was plotted against the log Mr (molecular weight). The calculated molecular weight of LacD.1 and LacD.2 represents the average of at least three separate experiments.

Influence of aldolase concentration on activity. To determine the enzymatic activity of LacD.1 and LacD.2, an aliquot of 5 μL containing enzyme at concentrations ranging from 100 nM to 5 mM was added to 95 μLs of a mixture containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 15 units GDH, 600 units TIM, 22.5 mM FBP and 250 or 500 μM of β-NADH. The measured rate of decrease in absorbance at 340 nm was used to calculate the activity at each concentration of enzyme. The slope of enzyme activity plotted versus enzyme concentration is equal to the specific activity of each aldolase and was comparable to the specific activity calculated from the k_{cat} of each enzyme determined from steady state experiments.

SpeB Protease Activity. Protease activity was measured as described previously by spotting a 5 μL aliquot of an overnight bacterial culture from each strain under analysis onto the surface of solidified unmodified or modified C medium supplemented with 2% milk (29). Following overnight culture under anaerobic conditions, protease activity was apparent as a zone of clearing surrounding the bacterial growth. Quantitative analysis of protease activity following overnight culture in liquid medium was determined using a fluorescein isothio-cyanate casein substrate as described (26).

Determination of Fitness. Overnight cultures in C-medium supplemented with choloramphenicol of the strains indicated in the text were back-diluted 1:100 into fresh C-medium with chloramphenicaol and incubated for approximately 4 hrs. Culture densities were normalized by OD_{600} and co-cultures established by co-inoculation of 50 μL of both test and competitor strains into 10 ml of C-medium containing chloramphenicol. Following overnight incubation, each culture was back-diluted 1:1000 in fresh medium and this process was continued for 4 days. The competitor strain was

ΔLacD.1 complemented with pLacD.1^{-kan}, a plasmid identical to pLacD.1 except for removal of the kanamycin resistance cassette. The test strains were ΔLacD.1 complemented with the various plasmids indicated in the text. Cultures were grown in the presence of chloramphenicol, as both test and competitor plasmids carry an identical chloramphenicol resistance cassette. Growth rates were calculated for each individual strain in the absence of competition to confirm that these were identical. To enumerate CFUs, serial dilutions from cultures were plated on Todd Hewitt Yeast Extract media with and without kanamycin at the indicated time points. Data shown are the average of at least 2 independent experiments.

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Chapter III

Contribution of Arginine and Citrulline Catabolism to *Streptococcus pyogenes* pathogenesis

SUMMARY

The ability of a bacterium to acquire nutrients from a host during an infection is essential for colonization and thus pathogenesis. For the pathogen *Streptococcus pyogenes*, the catabolic, arginine deiminase (ADI) pathway has been shown to be up-regulated in a mouse model of a soft tissue infection, suggesting that this pathway contributes to *S. pyogenes'* metabolism during an infection. Typically arginine catabolism in bacteria aids in the supplementation of energy and protects against acid stress through the production of ATP and ammonia, respectively. To investigate the role of arginine catabolism in the pathogenesis of *S. pyogenes* we utilized a subcutaneous mouse model of infection. In-frame deletions of enzymes through out the pathway revealed the inability to utilize arginine resulted in a disruption in pathogenesis. Further analysis demonstrated mutants unable to utilize arginine or citrulline were hyper attenuated, suggesting an important role for citrulline utilization in *S. pyogenes* pathogenesis. Additionally, infection of iNOS knock out mice with a mutant in the ADI pathway produced wild type lesions, suggesting a role for arginine catabolism in the inhibition of the inducible nitric oxide synthase (iNOS) through substrate depletion. Together this work demonstrates the importance of arginine and citrulline catabolism in an infection, and suggests a novel mechanism by which *S. pyogenes* modulates the innate immune response by limiting the production of the antimicrobial, nitric oxide.

INTRODUCTION

Nutrient acquisition by a pathogen is necessary for the colonization of a host, and is therefore an important aspect of bacterial pathogenesis. Identification of bacterial metabolic pathways utilized during an infection can provide unique targets for novel therapeutics, as well as provide insight into the environmental cues being sensed by the pathogen. Additionally, the metabolism of a microbe, shuttling key metabolites away from or toward the host, can contribute to how the host responds to a pathogen. The difficulty then becomes identifying the role of metabolic pathways active during an infection and deciphering their specific contribution to pathogenesis.

Streptococcus pyogenes (group A streptococcus) is a common Gram positive pathogen responsible for a large number of diseases that range in severity and invasiveness (12). The more common, non-invasive infections include bacterial pharyngitis and impetigo, while less common infections include the invasive and often life threatening, necrotizing fasciitis (12). Research examining the nutrients available to *S. pyogenes* during an infection has revealed that the environment encountered is likely deficient in glucose with an abundance of amino acids and peptides (35). This nitrogen rich environment encountered by the bacteria results in the up-regulation of amino acid and peptide transporters, as well as peptidases that likely aid in providing the cellular energy necessary for replication (26, 27). One metabolic operon that was found to be up-regulated in a soft tissue infection in a mouse model and in human blood was the arginine deiminase pathway (26, 27).

The arginine deiminase (ADI) pathway, encoded by the Arc operon in *S. pyogenes*, is involved in a number of aspects of bacterial metabolism including the production of cellular energy and the protection against acid stress (1, 11). The ADI pathway is composed of three key enzymes: ArcA, ArcB, and ArcC, and an antiporter, ArcD(2, 36, 50) (Figure 1). The first enzyme in the pathway is ArcA, an arginine deiminase, involved in the liberation of an ammonia group from arginine to produce citrulline. Next, the ornithine carbamoyl transferase, ArcB, cleaves citrulline to produce ornithine and carbamoyl phosphate, the latter being utilized by ArcC to form a molecule of ATP, carbon dioxide, and ammonia (2, 3, 14, 17, 28, 29, 36, 48, 50). The byproduct of ArcB's reaction, ornithine, is then utilized by ArcD in an ATP independent manner to transport ornithine out, and concomitantly bring arginine into the cell (18). Interestingly, carbamoyl phosphate is also a precursor in pyridine biosynthesis, suggesting a possible contribution of arginine catabolism to the production of prymidines.

In addition to generating ATP, this pathway also aids in protection against acid stress (11, 38). The production of two ammonia molecules can be utilized to buffer

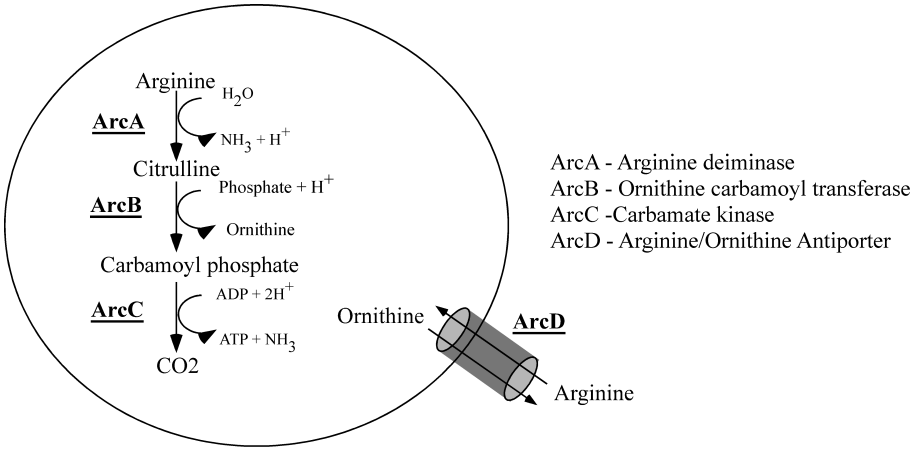

Figure 1: Arginine catabolism via the arginine deiminase (ADI) pathway. Through the multienzyme pathway arginine is catabolized to produce one molecule of carbon dioxide, one molecule of ATP and two molecules of ammonia.

against the decrease in pH, often associated with the production of lactic acid, a byproduct of fermentation (49). Disruption of this pathway in *S. pyogenes* results in an increase in susceptibility to acid stress (16). Given the ability of this pathway to contribute to both ATP production as well as protect against acid stress, it is not surprising that arginine catabolism has been found to play a key role in the pathogenesis of a number of bacterial species (31, 47). Indeed, in addition to contributing to the bacteria's metabolism, arginine utilization *in vivo* can also greatly influence how the host responds to a pathogen (15).

Upon detection of a pathogen, immune cells including neutrophils and macrophages are recruited to the site of infection in an attempt to contain the bacteria. To do so, they enlist a number of bactericidal mechanisms, including the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) (41). This enzyme is typically expressed by macrophages and utilizes arginine to produce NO and citrulline. Given the requirement for arginine, iNOS is often directly regulated by the availability of this extracellular substrate (40, 42). This link of NO production to arginine availability has resulted in a number of examples where microbes attempt to regulate the production of the toxic molecule, NO, by limiting arginine availability (6, 15). Identification of the role of macrophage recruitment to an *S. pyogenes* infection has established macrophages as an important mediator for clearance and controlling dissemination of bacteria, suggesting a likely role of iNOS and NO in this process (24, 39).

To investigate the role of arginine catabolism in *S. pyogenes* pathogenesis we began by making an in-frame deletion in the first enzyme in the pathway, ArcA, and monitoring the influence on pathogenesis in our subcutaneous mouse model of infection.

Our results demonstrate that arginine catabolism does contribute to pathogenesis as demonstrated by a decrease in lesion size, as well as a reduction in bacterial colony forming units. Further investigation into the contribution of this pathway revealed that deletion of ArcB or ArcC resulted in significantly smaller lesions compared to both wild type bacteria, as well as the ArcA mutant. This decrease in lesion size and bacterial burden was found to be associated with the ability to utilize both arginine and citrulline *in vitro*. Furthermore, infection of an iNOS knock out mouse with a ΔArcA mutant revealed an increase in lesion size comparable to a wild type infection, suggesting a unique contribution of arginine catabolism in *S. pyogenes* to the modulation of the host innate immune response.

RESULTS

An ArcA mutant is attenuated in a subcutaneous mouse model of infection.

To investigate the role of arginine catabolism in the pathogenesis of *S. pyogenes,* we began by making an in-frame deletion in the first enzyme in the pathway, ArcA. Following deletion of *arcA,* the generated mutant was assayed for the ability to catabolize arginine by measuring growth in C-medium supplemented with 10 mM arginine. For wild type cells, the addition of 10 mM arginine to C-medium results in an increase in cellular density, as measured by OD_{600} (Figure 2). However, with ΔArcA there was no increase in the OD_{600} in the presence of 10 mM arginine, confirming that the Arc operon encodes a functional ADI pathway capable of utilizing arginine as an energy source (Figure 2). Following this *in vitro* characterization, we next wished to investigate the role of arginine catabolism in our mouse subcutaneous infection model. Subcutaneous infection of *S. pyogenes* in a mouse typically results in the formation of a lesion after twenty-four hours with the lesion growing in size and peaking on day three. Monitoring the lesion size formed by both wild type and the *arcA* mutant bacteria

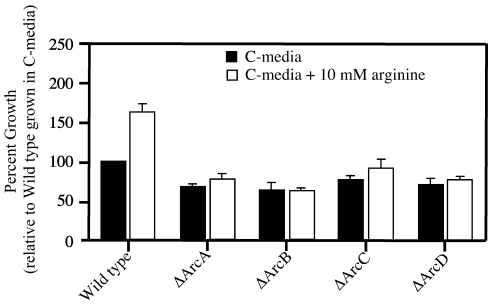

Figure 2. Disruption of arginine catabolism in *arc* mutants. Wild type (HSC5) bacteria and mutant strains were grown in C-media and C-media supplemented with 10 mM arginine for 16 hrs. Data is presented as percent growth relative to wild type grown in C-media, and is the mean and standard deviation from at least three independent experiments.

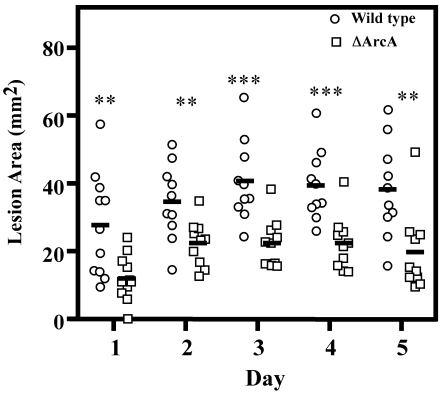

Figure 3. Deletion of *arcA* disrupts virulence in *S. pyognes*. Hairless SKH1 mice were infected subcutaneously with wild type (○) or ΔArcA (□) strain in order to determine relative virulence. At indicated times the resulting skin ulceration was imaged and the 2-dimensional lesion area measured using ImageJ. The data presented are plotted lesion area from mice from multiple expreiments. Differences in mean lesion area between mutant and wild type were tested for significance using the Mann-Whitney U test. (** P<0.01, *** P<0.001)

revealed that for the five-day period examined, the *arcA* mutant formed significantly smaller lesions compared to wild type on all days measured (Figure 3). This data demonstrates the importance of arginine catabolism *in vivo* and suggests that arginine is an important energy source encountered by *S. pyogenes* during an infection.

Deletion of ArcB and ArcC result in significantly smaller lesions compared to an ArcA mutant.

Arginine catabolism can contribute to bacterial metabolism and pathogenesis in a number of different ways, including the production of ATP, carbamoyl phosphate, and the inhibition of iNOS. To determine the specific contributions of arginine catabolism on *S. pyogenes* pathogenesis, we decided to make in-frame deletions in *arcB*, *arcC*, and *arcD*. By comparing each mutant to ΔArcA we hoped to be able to dissect arginine catabolism's contribution to an *S. pyogenes* infection. Initial *in vitro* characterization of all three *arc* mutants revealed a disruption in the ability to catabolize arginine comparable to an *arcA* mutant, as determined by growth in C-medium supplemented with 10 mM

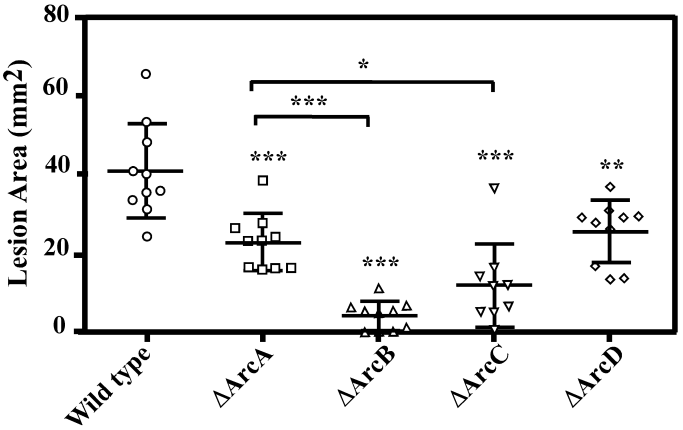

Figure 4. Deletion of *arc* genes influence virulence to varying degrees. Hairless SKH1 mice were innoculated subcutaneously with designated strain. The resulting skin ulceration was imaged on Day 3 and the 2-dimensional lesion area measured using ImageJ. The data presented are plotted lesion area from mice from multiple expreiments. Differences in mean lesion area between mutant and wild typewere tested for significance using the Mann-Whitney U test. (* P<0.05, ** P<0.01, *** P<0.001)

arginine (Figure 2). These results confirm the requirement of all four proteins for a fully functioning ADI pathway. Following *in vitro* characterization, the *in vivo* contribution of all three mutants was analyzed in our mouse model of infection. Comparison of lesion sizes on day three revealed two distinct phenotypes compared to wild type bacteria (Figure 4). The first phenotype, including the mutants ΔArcA and ΔArcD, consisted of lesions significantly smaller than the wild type strain (Figure 4). The second phenotype, included the mutants ΔArcB and ΔArcC, and was hyper attenuated, forming significantly smaller lesions compared to ΔArcA (Figure 4). Given that these enzymes function in a distinct metabolic pathway, we had hypothesized that deletion of genes downstream of ArcA would either phenocopy an *arcA* mutant or behave similar to our wild type strain, however this was not what was observed.

One proposed explanation for this phenotype is the build up of intermediates in ΔArcB and ΔArcC strains that are either detrimental to the bacteria or beneficial to the host. To test this hypothesis, we repeated our subcutaneous infection with wild type bacteria, the two single mutants ΔArcA and ΔArcB, and a double mutant ΔArcA ΔArcB. Deletion of *arcA* in a ΔArcB background should prevent production of any detrimental intermediates and alleviate their influence. In addition to measuring lesion size on day 3, we also harvested the lesions and plated them for CFUs. Bacterial CFUs recovered from a ΔArcA infection was significantly lower compared to wild type (Figure 5B), but remained significantly higher than the bacteria recovered from a ΔArcB infection, confirming the phenotype observed previously (Figure 5B). Analysis of the infection with the double mutant resulted in significantly smaller lesions on day three compared to ΔArcA as well as a reduction in recoverable bacterial CFUs (Figure 5). These results

suggest that the attenuation *in vivo* seen for ΔArcB and ΔArcC are due to the lack of a functional ArcB or ArcC enzyme, respectively, and not due to the build up of intermediates in the Arc pathway. Given that ArcB functions downstream of ArcA on the amino acid citrulline, the resulting decrease in lesion size associated with an *arcB* and *arcC* mutant can likely be attributed to the inability to utilize both arginine and citrulline.

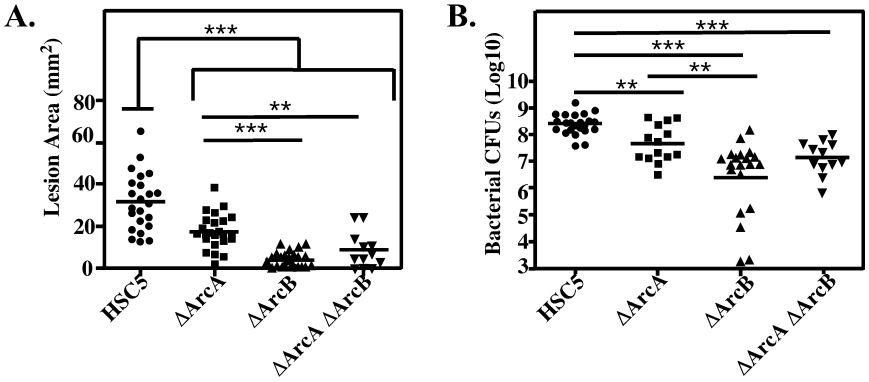

Figure 5. A double mutant ΔArcAΔArcB phenocopies a ΔArcB mutant. (A).Hairless SKH1 mice were infected subcutaneously with wild type or the designated strain andt at indicated times the resulting skin ulceration were imaged and the 2-dimensional lesion area measured using ImageJ. Additional the lesionarea was harvested. The data presented are plotted lesion area from mice from multiple expreiments. Differences in mean lesion area between mutant and wild type were tested for significance using the Mann-Whitney U-test. (** P<0.01, *** P<0.001) **(B.)** CFUs recovered from lesions. Each symbol represents the number of CFUs recovered from and individual mouse with the indicated strain. Data are presented from multipleexperiments. Differences between indicated strains were tested for significance using the Mann-Whitney U-test.

An *arcA* mutant can utilize citrulline *in vitro* but an *arcB* or *arcC* mutant cannot.

To test whether *S. pyogenes* could utilize citrulline as an energy source, the growth of bacteria in medium supplemented with 10 mM citrulline was monitored by OD_{600}. Growth of wild type bacteria or any *arc* mutant revealed no increase in OD over time compared to unmodified medium, suggesting cellular density could not be increased through the utilization of citrulline (data not shown). Previous research involving arginine catabolism in *S. pyogenes* has demonstrated the utilization of citrulline to protect against acid stress (16). To confirm these results and further investigate the influence of our

mutants on citrulline utilization, we monitored the ability of both arginine and citrulline catabolism to protect *S. pyogenes* against acid stress.

Protection from acid stress was measured by growing the bacteria in a rich medium, ThyB, containing a high concentration of glucose. As a consequence of fermentation the pH of the medium is rapidly acidified. This acidification has been determined to be lethal to *S. pyogenes* and ultimately results in a decrease in bacterial CFUs that can be monitored over time, with no detectable bacteria remaining by day six. Bacteria cultured in C-medium buffered to pH 7.5 remained viable throughout day six suggesting the loss of bacterial viability is associated with a decrease in the medium's pH (data not shown). For wild type bacteria, addition of 10 mM arginine or 10 mM citrulline both mitigated the decrease in viability with roughly 10^6 and 10^5 bacteria still being detected on day six, respectively. Following six days of growth, the medium in which the bacteria grew was collected, filter sterilized and the pH measured. Comparison of the medium pH for wild type bacteria containing arginine or citrulline revealed a significant increase in pH due to the production of ammonia, thus confirming utilization of both substrates (Figure 7). This result revealed that despite being unable to increase the cellular density of a culture, utilization of citrulline did protect the bacteria from acid stress. Comparison of an *arcA* mutant to wild type reveals a more rapid drop off in CFUs over time as well as no increase in viability in the presence of arginine (Figure 6). However, the addition of 10 mM citrulline was able to protect the mutant through the six-day time course (Figure 6), confirming the ability of this mutant to utilize citrulline.

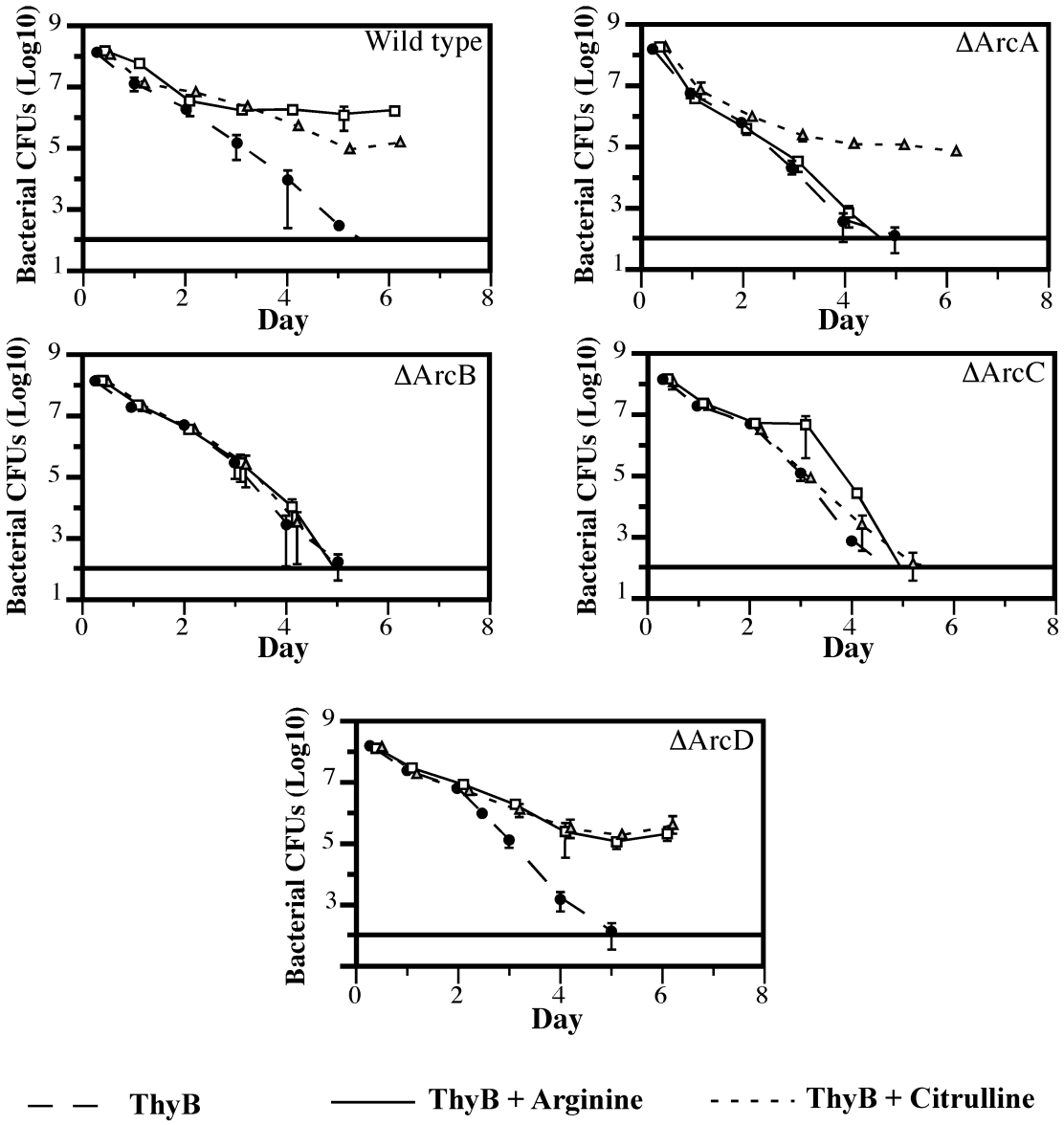

Figure 6. Deletion of *arc* genes disrupt ability to utilize arginine and citrulline for protection against acid stress. Wild type bacteria and the indicated strains were cultured in ThyB (long dash), ThyB + 10 mM arginine (solid line), or ThyB + 10 mM citrulline (short dash) medium for a six day period. At indicated times the cultures were re-suspended and a small aliquot removed and serially diluted in PBS, followed by plating on ThyB medium for enumeration of CFUs. Data displayed are the mean of recovered CFUs from multiple experiments.

Measurement of the final pH of ΔArcA medium containing citrulline did not reveal as large of an increase in pH as wild type (Figure 7), likely due to the inability to utilize additional arginine already present in the unmodified medium. Analysis of the *arcB* or *arcC* mutant's ability to withstand the acid stress in medium supplemented with arginine or citrulline revealed that they were both defective in protecting against the acid stress regardless of the supplemented amino acid (Figure 6). However surprisingly the *arcD* mutant was able to survive in both the presence of arginine and citrulline (Figure 6) suggesting a redundancy in the transport of arginine and perhaps citrulline. However, given that ΔArcD cannot utilize arginine for growth, this redundant transport of arginine is not sufficient for utilization as an energy source. This insufficiency could be due to a lower affinity for arginine directly or a reduced expression compared to ArcD.

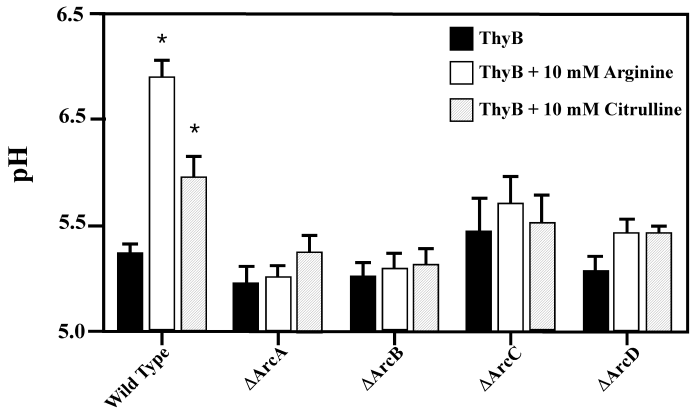

Figure 7. Influence of arginine and citrulline catabolism on medium pH. Wild type bacteria and the indicated strains were grown in ThyB supplemented with nothing, arginine, or citrulline. Following six days of growth the cultures were filter sterilized and the pH of the media measured using a pH probe. Data is presented as the mean and standard deviation from at least three independent experiments. Difference in the mean pH from unmodifed medium to the media supplemented with arginine or citrulline were tested for significance within the designated strains using the Mann-Whitney U-test. (* $P<0.05$)

Utilization of citrulline by *S. pyogenes* to protect against acid stress has been previously reported and is confirmed here. Disruption of the ability to utilize both arginine and citrulline correlates with the formation of significantly smaller lesions during an infection, suggesting utilization of both amino acids *in vivo* is crucial for pathogenesis. Furthermore, deletion of *arcD* did not disrupt the ability of *S. pyogenes* to utilize arginine or citrulline for protection against acid stress (Figure 6), suggesting

redundant mechanisms of transport for these amino acids. Additionally, analysis of the medium pH after six days revealed that survival by ΔArcA or ΔArcD in the presence of citrulline or arginine, respectively, did not correlate with a significant increase in extracellular pH (Figure 7), which may indicate a role of ATP production in protection against acid stress.

RAW264.7 Macrophages up-regulate iNOS during an infection with *S. pyogenes*

Identification of the role of arginine catabolism in *S. pyogenes* pathogenesis suggested that during infection arginine is a viable energy source and likely rapidly catabolized. This catabolism of arginine, while important for bacteria's energy production and protection from acid stress, can also greatly influence the host, specifically immune cells targeted to the site of an infection. Macrophages are often recruited to an active infection, and have been demonstrated to be essential for controlling dissemination of *S. pyogenes*. Upon arrival, macrophages can target bacteria through a number of different bactericidal effectors including NO. To begin to address the influence of bacterial arginine catabolism on the host's ability to produce NO, we first

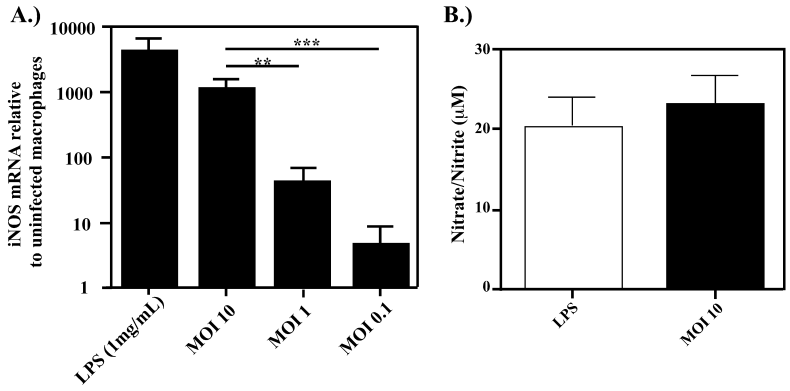

Figure 8. Activation of iNOS in RAW 264.7 macrophages by *S. pyogenes*. A.)RAW 264.7 Macrophages were infected with *S. pyogenes* at varying MOIs as described in the methods. Relative transcript abundance was determined by RT-PCR. Transcript levels are reported relative to that detected in uninfected macrophages. Data represent the mean and standard deviation of at least two independent experiments with samples analyzed in triplicate. Statistical significance was determined using Mann-Whitney U-test (** P<0.01, *** P<0.001).**B.)** Measurement of NO formed nitrates/nitrites using Griess reagent. A standard curve of nitrite in culture medium was used to determine the absolute concentration. Data is presented as the mean and standard deviation from at least two independent infections measured in triplicate.

looked to see if the enzyme iNOS was up-regulated in macrophages during an infection with *S. pyogenes*. To augment the production of nitric oxide, INFγ, which has been shown to be important for protection against *S. pyogenes* infections (43), was added to the macrophages 24 hours prior to the addition of bacteria. Following infection with *S. pyogenes* at varying MOIs, the RNA from macrophages was purified and the transcript levels of iNOS quantified using real-time RT-PCR. Analysis of transcripts levels revealed that with increasing MOI, the mRNA levels of iNOS increased proportionately in a dose dependent manner, with the highest MOI of 10 producing levels of transcript comparable to the positive control, LPS (1mg/mL) (Figure 8A). Furthermore, production of NO by macrophages can be indirectly measured using Griess reagent, and was found to be produced to levels similar to LPS, thus demonstrating the induction of iNOS transcription and production of NO by macrophages in the presence of *S. pyogenes* (Figure 8B).

An ArcA mutant forms wild type lesion in an iNOS knock out mouse.

Given the induction of iNOS and the production of NO by macrophages in the presence of *S. pyogenes,* we next examined if arginine utilization by the bacteria *in vivo* was actively inhibiting NO production through substrate depletion.

To test this hypothesis infection in C57BL/6 mice lacking a functional iNOS was compared to infection in a wild type mouse strain with the same genetic background. Both mouse strains were infected with either HSC5 (wild type), ΔArcA, or ΔArcB followed by measurement of lesions and enumeration of bacterial CFUs on Day 3. Comparison of infection in the wild type or iNOS knock out (KO) mice revealed that with either HSC5 (wild type) or ΔArcB, no significant difference was observed in lesion

area or bacterial CFUs. Conversely, infection of iNOS KO mice with ΔArcA resulted in significantly larger lesions compared to those produced by ΔArcA in wild type mice. In addition, the bacterial burden was significantly greater in iNOS KO mice compared to wild type. This data suggests that that during an infection ArcA functions to inhibit NO production through substrate depletion. In the instance when this activity is abolished by deletion of *arcA*, the host enzyme iNOS is unabated in its production of NO, resulting in the killing of *S. pyogenes*. Additionally this work demonstrates that attenuation of virulence seen with a ΔArcB mutant is not due to an increase in NO production, but rather due to the disruption of citrulline catabolism. Taken together this data confirms the role of arginine catabolism in modulation of host response and further supports the important contribution of citrulline catabolism in *S. pyogenes* pathogenesis.

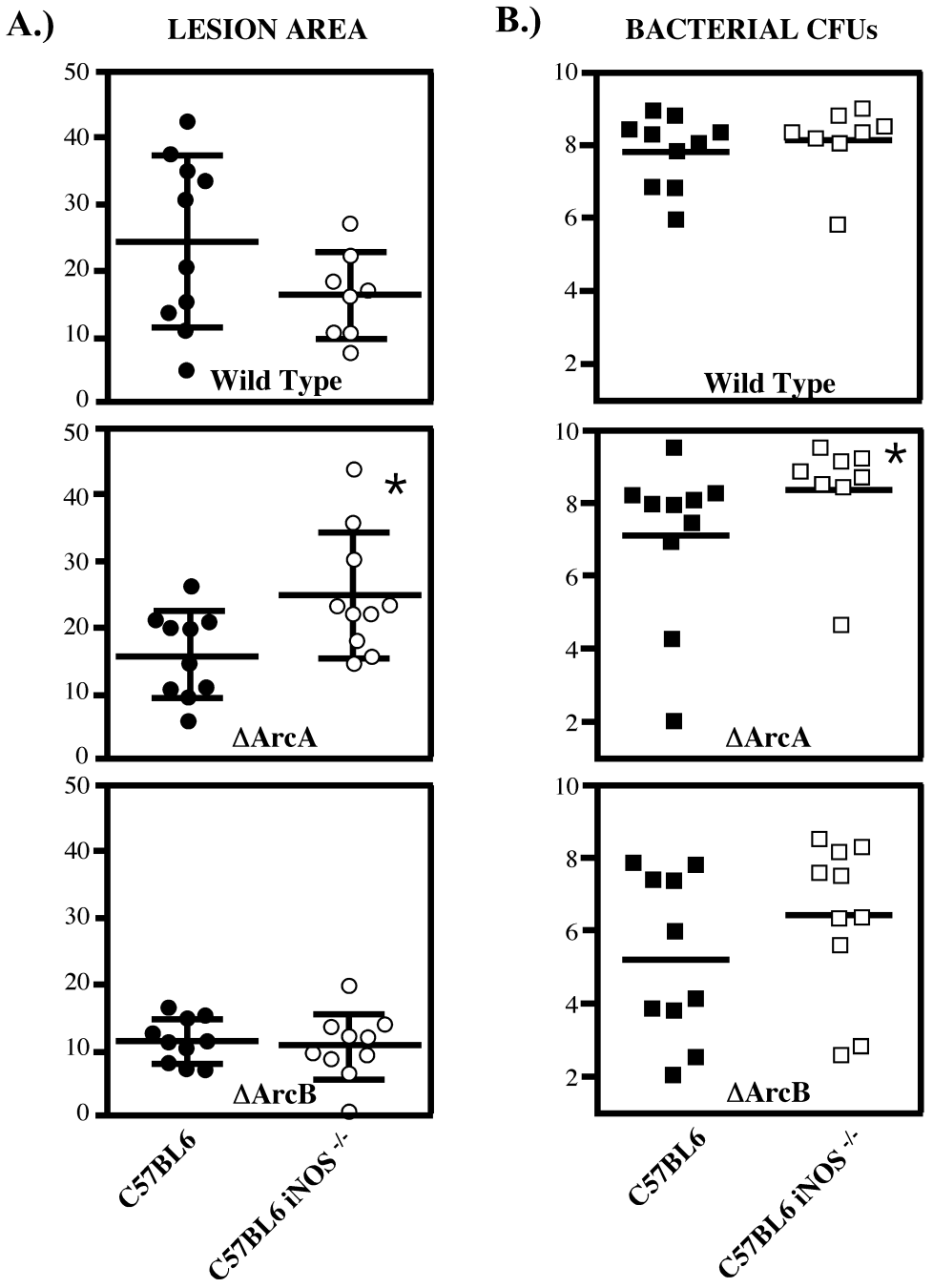

Figure 9. An *arcA* mutant forms wild type lesions in iNOS knock out mice. A.)C57BL/6 or iNOS $^{-/-}$ mice were infected with wild type, ΔArcA, or ΔArcB. The lesions formed on day 3 were imaged and the area measured using image analysis. The data presented are plotted lesion area from multiple mice from at least two independent expreiments. Differences in mean lesion area between C57BL/6 and iNOS $^{-/-}$ were tested for significance using the Mann-Whitney U test. (* $P<0.05$) B**.)** CFUs recovered from lesions. On day 3 the lesions were harvested and the recoverable bacteria enumerated. Each symbol represents bacteria recovered from an individual mouse. Differences between the two mouse strains were tested for significance using the Mann-Whitney U test. (* $P<0.05$)

Table 1. Strains used in this study[a]

Strains	Relevant genotype	Plasmid	Description	Reference
S. pyogenes				
HSC5	wild-type	none	wild-type	Hanski *et al*. (1992)
ZC156	HSC5 $arcA_{\Delta 10-409}$	none	In-frame deletion of *arcA*	This work
ZC512	HSC5 $arcB_{\Delta 13-326}$	none	In-frame deletion of *arcB*	This work
ZC498	HSC5 $arcC_{\Delta 9-312}$	none	In-frame deletion of *arcC*	This work
ZC500	HSC5 $arcD_{\Delta 7-490}$	none	In-frame deletion of *arcD*	This work
ZC604	HSC5 $arcA_{\Delta 10-409}$ $arcB_{\Delta 13-326}$	none	In-frame deletion of *arcA* and *arcB*	This work

a. Gene names based on annotation of the SF370 genome of *S. pyogenes*

Table 2. Primers used in this study[a]

Name	Sequence[a]	Template	Plasmid	Description
ZC78	TTTTGTCGACTTTGATTAATTATTTTTTGTGAGAT	HSC5	pZC32	Primers for In-fram deletion of *arcA*
ZC66	/5PHOS/GAAGATATTTAATAAGCTAT			
ZC67	/5PHOS/GGTGTTTGAGCAGTCAT	HSC5	pZC32	Primers for In-fram deletion of *arcA*
ZC65	TTTCTGCAGCTCGAGTTAC			
ZC317	CCCCCTCGAGGTCGACGGTAAAGGTGGTTGTAGGTCAG	HSC5	pZC141	Primers for In-fram deletion of *arcB*
ZC318	GGAATAAAGAGGTTACCAAGGGTTGCGCTAGGAAGCTACGTCCTTGAAATACTTGTGTCAT			
ZC319	GCAACCCTTGGTAACCTCTTTATTCCAAAAGTGTAATAAGGACTGGTACTCCTTAACTCATTTC	HSC5	pZC141	Primers for In-fram deletion of *arcB*
ZC320	GATCCCCCGGGCTGCAGTGTTTTTACGAACCACTGACGCAATCCC			
ZC279	AGGGTCGACCTTGCCAAGGCTTTAGTGGTCCTTG	pJL75	pZC137	Primers for In-fram deletion of *arcC*
ZC280	/5PHOS/ACTACGATTTTTTGTTTCGTCATAATTACTCCTC			
ZC281	/5PHOS/GGAACACAAATTATCGCAGGGTAATCAAGAGG	pJL126	pZC137	Primers for In-fram deletion of *arcC*
ZC282	TTTTCTGCAGCAAGACATTGACAGATACTGGATTTTC			
ZC324	CCCCCTCGAGGTCGACTATCCTTATCACAGAAGATGCTGACGAAGC	pJL162	pZC15	Primers for In-fram deletion of *arcD*
ZC325	CTATCTGTTAAAAGAATGTTGCAACTACAAGTCCGTTTTTTTTCTTCTGTCATAATGATTACCTC			
ZC326	ACTTGTAGTTGCAACATTCTTTTAACAGATAGGAGGTTCCTATGGAATCCTATATCACACCTAAAC	HSC5	pZC53	Primers for In-fram deletion of *arcD*
ZC327	GATCCCCCGGGCTGCAGGCAAACCCAAAGGTAGCCTGTTCTTCTAC			
iNOS Forward	GCAAACATCACATTCAGATCC			RT-PCR primers for iNOS
iNOS Reverse	TCAGCCTCATGGTAAACACG			
GAPDH Forward	CCACCCAGAAGACTCTGGAT			RT-PCR primers for GAPDH
GAPDH Reverse	CACATTGGGGGTAGGAACAC			

a. Engineered restriction sites are underlined.

DISCUSSION

In the present study we looked to examine the contribution of arginine catabolism to *S. pyogenes* pathogenesis. Through a series of in-frame deletions we were able to determine that the utilization of both arginine, as well as citrulline contribute to bacterial survival during an infection. Additionally, infection of iNOS KO mice with wild type and an *arcA* mutant revealed the novel contribution of arginine catabolism to the inhibition of NO production by iNOS, demonstrating a unique role of arginine catabolism in the modulation of the host innate immune response. Together this work highlights the influence a pathogen's metabolism can have on both the bacteria and host during an infection.

Utilization of arginine can aid in both the production of ATP, as well as through protection against acid stress. Given that these two contributions occur simultaneously it is difficult to determine, which is required during an infection. In an attempt to segregate the influence of arginine catabolism on pathogenesis, in-frame deletions in enzymes through out the ADI pathway were examined. Interestingly, *in vitro* analysis of these mutants revealed the requirement for both ArcB and ArcC in survival against acid stress. Indeed, analysis of the *arcC* mutant revealed that despite maintaining a medium pH equal to or higher than the *arcA* mutant in ThyB supplemented with citrulline, it was unable to survive to similar levels. Previous research has suggested production of ATP is necessary for protection against acid stress, which may explain this phenotype (10). To confirm this hypothesis, a closer examination of the intracellular pH of the bacteria under these conditions, as well as investigation into the *in vivo* environment encountered, will be required to determine the exact role of the ADI pathway in pathogenesis.

Another interesting insight from this work came from the discovery of citrulline utilization by *S. pyogenes* during an infection. Citrulline is a non-essential amino acid that is not incorporated into proteins, and until recently has only been thought of as a metabolic intermediate in the urea cycle (44). Citrulline production can occur by the enzyme iNOS, as described above, or through the conversion of ornithine by the host enzyme ornithine carbamoyl transferase (45). Recent investigation into arginine-citrulline conversion has revealed a possible role of citrulline in fine-tuning arginine homeostasis in response to diet (13). Given this flux of nitrogen between arginine and citrulline, it is not surprising that both amino acids present themselves as viable nutrient sources for *S. pyogenes* and other pathogens during an infection.

Recently, utilization of citrulline by *Francisella tularensis* has also been shown to contribute to pathogenesis (37). *F. tularensis* is a Gram negative pathogen, and the causative agent of tularemia. In *F. tularensis,* catabolism of citrulline occurs via the enzyme citrulline ureidase, a hydrolase enzyme involved in cleaving citrulline to produce ornithine and ammonia (20). Production of ammonia by *F. tularensis* in the endosomal compartment of macrophages is believed to inhibit phagosomal maturation, thus protecting the bacteria (34). Additionally, this enzyme has recently been proposed to inhibit NO production by macrophages through depletion of citrulline (37).

Citrulline conversion back into arginine occurs through the enzymes arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) (13). These enzymes function in macrophages to recycle citrulline into arginine, keeping a steady supply available for iNOS (7). Indeed, up-regulation of ASS gene expression by pro-inflammatory cytokines demonstrates the shift in arginine availability required to combat

an infection (4). Thus utilization of citrulline by a pathogen could possibly aid in an infection by limiting the supply of arginine, as believed to be the case with *F. tularensis* (37). In contrast, in the present study deletion of the enzyme ArcB resulted in a drastic reduction in virulence in both the wild type and iNOS KO mice, suggesting the inability to catabolize citrulline likely contributes to bacterial survival irrespective of NO production. However, given the severe phenotype displayed by the *arcB* mutant, it is possible that this mutant is refractory to changes in iNOS activity. Thus, the lack of a phenotype may be misleading to what is actually occurring during the infection. Alternatively, given that ΔArcB still possesses a functional ArcA it is also plausible that depletion of arginine by ΔArcB *in vivo* is sufficient to inhibit the production of NO. These conflicting theories raise many interesting questions regarding the utilization of citrulline *in vivo*, and will require further investigation to answer.

In contrast to ΔArcB, infection of iNOS KO mice with an *arcA* mutant resulted in lesion sizes comparable to wild type bacteria, along with increased recovery of bacterial CFUs. Demonstrating ArcA's major contribution to virulence is through the inhibition of iNOS. Inhibition of NO production has been demonstrated to occur in other pathogens including *Heliobacter pylori* that depletes arginine availability with an encoded arginase, RocF (22). The targeting of iNOS activity by such a range of pathogens highlights the importance of this antimicrobial in the host innate immunity (15). Previous research looking at macrophage activation during an *S. pyogenes* infection has suggested iNOS is not up regulated in the presence of this bacterium (25). These experiments involved intra-peritoneal infection of mice, followed by isolation of macrophages from the peritoneal cavity at one, four, and 16 hours post infection. Analysis of the macrophages

transcriptome revealed iNOS was not up regulated under these conditions. However, both the route of infection as well as the time at which the macrophages were examined can explain these contradicting results. Variation in the environment in which an infection occurs, as well as changes in the temporal expression of cytokines, will drastically influence macrophage transcription. Previous research examining an intravenous infection of *S. pyogenes* in mice demonstrated an increase in NO in serum during an infection (23). This work further revealed a host genetic component of NO production as illustrated by changes in the amount of NO produced in two different mouse strains (23). Furthermore, recent experiments looking at the recruitment of macrophages into soft tissue following infection have found that peak expression of the pro-inflammatory cytokine, TNFα, does not occur until 48 hrs post infection (39). Additionally, this research has also demonstrated an important contribution of macrophages to limiting the dissemination of *S. pyogenes*, suggesting an alternative function of macrophages is necessary for controlling an *S. pyogenes* infection. Continuing research into the role of macrophages in a soft tissue infection, and examination of their activation profile over time will be essential in elucidating their contribution to an *S. pyogenes* infection.

In addition to a reduction in NO production, inhibition of iNOS can have a large influence on a macrophage's response to a pathogen. Competition for arginine utilization by iNOS can occur by both a pathogen and the host enzyme arginase. Several pathogens including *Salmonella* have used this competition in their favor by inhibiting NO production by iNOS through the activation of the host arginase (33). This up-regulation of arginase results in a decrease in arginine availability as well as an increase in downstream metabolites including ornithine, which is converted into polyamines such as

spermidine and spermine. Spermine has been demonstrated to inhibit pro-inflammatory gene expression increasing survival of the pathogen (9). This balance between iNOS and arginase in the host raises interesting questions as to additional influences arginine catabolism by *S. pyogenes* may have on an infection. Indeed, can increased production of ornithine by *S. pyogenes* be funneled into the host to increase production of spermidine and spermine, and how do these polycations influence the infection. Further investigation into production of these metabolites as well as others involved in wound healing will be essential in understanding the influence *S. pyogenes'* metabolism has on host-pathogen interactions and pathogenesis.

MATERIALS AND METHODS

***E. coli* strains, media, and growth conditions**. The *E. coli* strain DH5α was cultured in Luria-Bertani medium at 37ºC and utilized for common molecular cloning techniques. When appropriate antibiotics were added to the medium at the following concentrations: erythromycin 750 μg/mL.

***Streptococcus pyogenes* strains, media, and growth conditions.** *Streptococcus pyogenes* HSC5(30) and mutant derivatives of this strain (Table 1) were utilized for these experiments. Strains were grown in Todd-Hewitt Broth (THYB) (DIFCO) supplemented with 0.2% Yeast Extract (DIFO). When indicated in text, strains were also grown in C-medium (0.5% Protease Peptone #3 (DIFCO), 1.5% Yeast Extract (DIFCO), 10 mM K_2HPO_4, 0.4 mM $MgSO_4$, 17 mM NaCl). Supplementation of medium with arginine or citrulline occurred after autoclaving medium and was prepared by adding from a 0.5 M filter sterilized stock to a final concentration of 10 mM. In all media conditions growth of indicated strains utilized sealed culture tubes at 37ºC under static conditions. Streptococci grown on THYB medium solidified by the addition of 1.4% Bacto Agar (DIFCO) were cultured anaerobically in sealed jars in the presence of commercially available gas-generating packets (GasPak cat. #70304, BBL). When appropriate antibiotics were added to the medium at the following concentrations: erythromycin 1 μg/mL.

Construction of deletion mutants. All references to genomic loci are based upon the genome of *S. pyogenes* SF370 (19). In-frame deletion mutations in genes encoding ArcA (SPy_1547), ArcB (SPy_1544), ArcC (SPy_1541), and ArcD (Spy_1543) were generated using allelic replacement as previously described(46). Construction of the individual allelic replacement vectors utilized the vector pJRS233 (21) and were generated using 5' phosphorylated primers for three-way ligations, or through a process of overlap extension PCR (32) with the indicated primers listed in Table 2. Verification of the allelic replacement was confirmed by PCR.

Acid stress assay. Indicated strains were grown in unmodified ThyB, ThyB supplemented with 10 mM arginine, or 10 mM citrulline. At indicated time points the culture was re-suspended and a small aliquot removed for serial dilution in PBS. The bacteria were then plated on ThyB medium. Following overnight culture, the number of CFUs on the plate were enumerated. At the end of six-day period the culture was centrifuged and five mL of the medium removed and filter sterilized. The pH of the medium was then measured using a pH probe (Accumet AB15 pH meter).

Subcutaneous infection of mice. As described previously infection of 6 to 8-week old female SKH1 (Charles Rive Labs), C57BL/6 (cat. #000664), or B6.129P2-*Nos2tm1Lau*/J (iNOS $^{-/-}$ cat. #002609) mice were infected subcutaneously with 10^7 bacterial CFUs of each indicated strain (5, 8). Following infection the resulting ulcers were imaged on the day noted in the text, and analyzed using the image software ImageJ (http://rsb.info.nih.gov/ij/index.html) to determine the area of the irregular border of each

lesion. The data presented are from at least two independent experiments with at least a total 10 mice in each experimental group. Differences in lesion area were tested for significance using the Mann-Whitney *U*-test. When noted in the text, recoverable CFUs were measured by excision of the lesion and placement into 1 mL of sterile phosphate-buffered saline (PBS). The tissue was immediately homogenized using sterile homogenizer probes until no detectable tissue fragments remained. Following homogenization the resulting suspensions were serially diluted in PBS and plated on THYB medium. Following overnight culture, the number of CFUs on the plate were enumerated. Data displayed represents the pooling of least two independent experiments with at least 10 mice per experimental group. The difference in CFUs between experimental groups was tested for significance using the Mann-Whitney *U*-test.

Preparation of *S. pyogenes* for Macrophage infection. *S. pyogenes* was grown overnight in THYB, diluted in fresh THYB and allowed to grow for approximately 3-4hrs to an OD600 of 0.2. The bacteria were then collected by centrifugation and washed with 10 mLs of 0.9 % (Endotoxin-free) Saline solution (Teknova). Next the bacteria were collected by centrifugation and suspended in 1 mL of 0.9 % Saline solution. The cells were then sonicated on ice to disrupt chains and the number of bacterial cells enumerated using a hemocytometer (Hausser Scientific Partnership cat # 3900). When necessary the bacterial suspension was diluted in 0.9 % saline solution to the appropriate density. Bacterial CFUs were determined by serially diluting the bacterial suspension in PBS and plating on THYB medium, followed by enumeration of CFUs after overnight growth.

***S. pyogenes* infection of RAW264.7 Macrophages.** The murine macrophage cell line RAW 264.7 was plated in a six-well tissue culture plate (TPP) and maintained in RPMI-1640 (Life Technologies) with 10% FBS in a humidified 5% CO_2 atmosphere. Prior to infection macrophages were washed with Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies) and fresh medium added. An aliquot of the bacterial suspension was added to each macrophage-containing well and incubated at 37ºC in 5 % CO_2 for 30 minutes As a positive control LPS from *E. coli* (Sigma cat # L4391) was added to a well at a final concentration of 1μg/mL. Following this incubation all the wells were washed with 1-2 mL of DPBS followed by addition of fresh cell culture medium containing 100 units/mL of penicillin and 100 μg/mL streptomycin and allowed to incubate for an additional four hours. Macrophages were harvested in 1 mL of DPBS using a cell scraper and collected by centrifugation. The macrophage cell pellet was placed at -80ºC for storage. Prior to collection of the cells by centrifugation a small aliquot was removed and the macrophages counted with a hemocytometer (Hausser Scientific Partnership cat # 3200) to determine the total number of macrophages/well. For measurement of NO, macrophages were prepared as above and plated in a twelve-well tissue culture plates (TPP). Following a 60-minute infection with *S. pyogenes* or LPS (1μg/mL) the wells were washed with DPBS and fresh medium containing 100 units/mL of penicillin and 100 μg/mL streptomycin was added. Macrophages were allowed to incubate for an additional twenty-four hours, after which a small aliquot was removed and centrifuged at high speed to remove any cells or cellular debris.

Isolation of RNA and real-time RT-PCR. Total RNA was isolated from macrophages using Qiagen QIAshredder and RNeasy Mini kit per the manufacturer's protocol. RNA was subjected to reverse transcription (RT) using iScript (Bio-Rad) per the manufacturer's instructions. RT-PCR analysis of cDNA samples was performed using iQ SYBR green Supermix (Bio-Rad) using the primers listed in Table 2. Relative transcript levels were determined using the $\Delta\Delta C_t$ method using GAPDH transcript as a standard followed by comparison to uninfected cells. Data presented are the means derived from triplicate determinations of two separate samples prepared from at least two independent experiments. Differences between the means of experimental groups were tested for significance using Mann-Whitney *U*-test.

Measurement of NO Concentration. The concentration of byproducts formed by the oxidation of NO, NO_3- and NO_2- were measured using the Griess reagent system (Promega) per manufacturer's instructions. Absolute concentrations were determined by preparation of a standard curve in cell culture medium.

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CHAPTER IV

Regulation of the Arc operon in *Streptococcus Pyogenes* by the Transcriptional Regulators ArcR and AhrC.2

SUMMARY

In *Streptococcus pyogenes* arginine utilization occurs via the arginine deiminase pathway (ADI) encoded by the Arc operon. The utilization of arginine by the pathogenic bacterium *S. pyogenes* has recently been determined to contribute to virulence during a soft tissue infection. The importance of this metabolic pathway on pathogenesis suggests investigation into the regulation of the Arc operon will provide valuable information regarding the mechanism by which activation of transcription occurs, as well as insight into the environmental signals being sensed by the bacteria during an infection. Here we report, that in-frame deletion of either of the two putative transcriptional regulators *arcR* or *ahrC.2* resulted in disruption in the ability to catabolize arginine. Transcriptional analysis of these mutants demonstrated a down regulation of the Arc operon in all phases of growth, revealing a requirement for both *arcR* and *ahrC.2* for expression of genes in this operon. Furthermore, utilization of a biotinylated-DNA pulldown assay demonstrated the specific binding of AhrC.2 to the promoter region of the Arc operon in an ArcR-dependent manner, further supporting a role for both regulators in the transcriptional control of this operon. Analysis of these mutants in our mouse model of infection revealed that deletion of *ahrC.2* resulted in significantly smaller lesions compared to wild type, while deletion of *arcR* did not produce any significant phenotypic change. Together this work demonstrates the importance of Arc operon regulation in *S. pyogenes*, and raises interesting questions regarding the regulation of the Arc operon *in vivo*, and the influence of these regulators on genes outside of the Arc operon.

INTRODUCTION

The ability of a pathogen to control the spatio-temporal expression of virulence genes required for pathogenesis requires the accurate sensing of the environment in which it is found. Often many pathogens will coordinate the expression of virulence genes with metabolism, by linking the regulation of these factors to known metabolic regulators (19, 30). Investigation into the regulation of metabolic pathways activated during an infection can thus provide insight into identification of the environmental and cellular signals being sensed by the bacteria. Additionally, examination of the transcriptional regulators involved in regulating the metabolic operon can reveal the novel coordination of virulence factor expression with metabolism. A challenge then becomes identifying the mechanism of regulation and delineating the contribution of multiple regulators on a gene's expression.

In the Gram positive pathogen *Streptococcus pyogenes* (group A streptococcus), adaptation to the environment often involves the coordination of virulence gene expression with the bacteria's metabolism (27, 29). *S. pyogenes* is the known causative agent of a wide variety of diseases including common superficial infection of the throat and skin (pharyngitis and impetigo) as well as more invasive infections of soft tissue and fascia (cellulitis and necrotizing fasciitis) (7). In *S. pyogenes*, linking the expression of virulence genes to the metabolic state of the cell allows for the bacterium to quickly adapt to changes in the environment. This adaptation requires not only changes in expression of virulence factors, but also changes in metabolic pathways necessary for the production of cellular energy. Recent identification of the role of the Arc operon and the utilization

of arginine during an infection suggests that arginine catabolism, and the regulation of this operon is necessary to efficiently colonize and infect a host.

Arginine catabolism in *S. pyogenes* occurs through the arginine deiminase pathway encoded by the Arc operon (Figure 1), and aids to bacterial survival through both the production of ATP and protection from acid stress (1, 9) (Figure 1). This pathway involves utilization of three key enzymes: ArcA, ArcB, and ArcC along with the antiporter, ArcD (2, 3, 8, 11, 14, 15, 24, 31, 32) (Figure 1). In addition to these proteins the Arc operon is also found to encode a putative dipeptidase, ArcT, and a small protein with homology to the GNAT family of acetyl transferases (17). Upstream of ArcA lies an FNR/CRP-like transcriptional regulator, ArcR, which has been found to be essential for activation of transcription in several Gram positive bacteria (25, 26, 33). Additionally upstream of ArcR, transcribed in the opposite direction is the gene, *ahrC.2*, which encodes a protein homologous to the arginine regulator ArgR found in *Lactococcus lactis* and ArcR found in *Streptococcus gordonii* (11, 21). Research conducted in *Streptococcus gordonii* has found activation of the Arc operon in response to arginine requires the transcriptional regulator ArcR, which is homologous to AhrC.2 in *S. pyogenes* (11), while activation of the Arc operon in response to oxygen availability requires the activator Flp homologous to ArcR (10). Despite demonstrating the separate function these regulators under these two conditions, analysis of both regulators influence on transcription in both conditions have not been examined. This complex regulation of the Arc operon is suggestive of its importance in bacterial growth and survival.

Gene SPy #	Description
1541	arcC, carbamate kinase
1542	arcT, putative Xaa-His dipeptidase
1543	arcD, arginine/ornithine antiporter
1544	arcB, ornithine carbamoyl transferase
1546	?, putative GNAT family acetyl transferase
1547	arcA, arginine deiminase
1548	arcR, FNR/CRP-like transcriptional regulator
1549	ahrC.2, transcription regulator

Figure 1. Organization of the Arc operon in *S. pyogenes*. A.)The structure of the genome loci encoding the enzymes of the Arginine Deiminase pathway are shown based on the annotation of the S. pyogenes SF370 genome. **B.)** A table containing the SPy number of all genes associated with the Arc operon along with a description of their putative function.

In addition to the putative regulation by both AhrC.2 and ArcR, regulation of the Arc operon in *S. pyogenes* has also been found to involve the two catabolite-sensing regulators CcpA and LacD.1 (19, 20). CcpA, the major carbon regulator in *S. pyogenes* is involved in prioritizing the utilization of metabolic substrates and has been found to repress expression of *arcA* in the presence of glucose (20). Conversely, the regulator LacD.1 has been found to activate the Arc operon during the exponential phase of growth (20). In addition to controlling expression of the Arc operon both of these metabolic regulators have also been found to regulate the expression of known virulence factors, including the secreted cysteine protease SpeB (19, 20, 23).

The recent discovery of the contribution of arginine and citrulline catabolism to *S. pyogenes* pathogenesis, suggests the requirement for the activation of the Arc operon during an infection. Thus, inquiry into the *in vitro* regulation of this operon will provide insight into the environmental signals being sensed by *S. pyogenes* that lead to the activation of this operon. In order to initiate this investigation, in-frame deletions of the two arginine-like regulators associated with the Arc operon were made. Analysis of these

regulators influence on arginine catabolism demonstrated that both were required for transcriptional activation of the Arc operon. Further experiments examining the ability of the each regulator to bind to the region upstream of *arcA* revealed the binding of AhrC.2 occurred in an ArcR-dependent manner. Additionally, despite being both necessary for the *in vitro* activation of the Arc operon, these two regulators produced varying phenotypes in our mouse model of infection. Suggesting a complex but important role for arginine regulation in the pathogenesis of *S. pyogenes.* Together this work will help elucidate the manner in which the Arc operon is regulated in *S. pyogenes*, and further help illuminate it's ability to coordinate the expression of both metabolic and virulence genes during an infection.

RESULTS

Expression of the Arc operon requires both AhrC.2 and ArgR

To initiate investigation into the regulation of the Arc operon in *S. pyogenes*, in-frame deletions in both *ahrC.2* and *arcR* were generated. Initial attempts to assay for arginine utilization involved growth of both mutants in the presence of 10 mM arginine. Comparison to wild type showed a noticeable difference in final optical density (OD) in the presence of arginine indicative of a disruption in the ADI pathway (Figure 2). To verify that this disruption was occurring on the transcriptional level, the transcripts of several genes through out the Arc operon were measured by real-time RT-PCR and compared to wild type. Transcript levels were measured in both the exponential and stationary phase of growth for bacteria grown in C-medium. C-medium is a complex medium rich in amino acids and peptides with a low concentration of glucose. Previous research has found that growth in this medium results in activation of the Arc operon, and thus would allow the function of both regulators to be assayed. Measurement of transcripts during exponential phase revealed that for both mutants nearly all genes in the operon were down regulated between 60-90 fold (Figure 3). While in stationary phase the two mutants showed a down regulation of Arc genes between 2-16 fold (Figure 3). Comparison of transcripts in wild type cells during

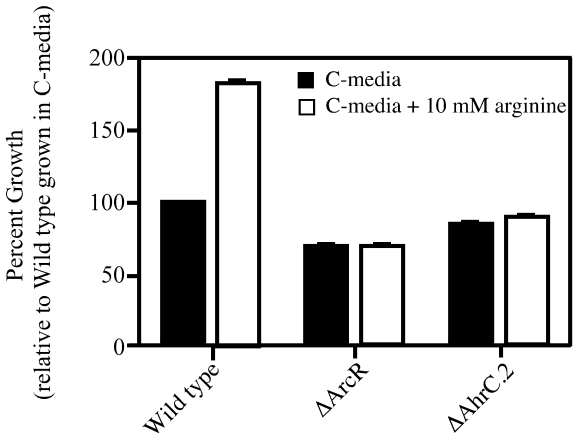

Figure 2. Disruption of arginine catabolism in ΔArcR and ΔAhrC.2. Wild type (HSC5) bacteria and mutant strains were grown in C-media and C-media supplemented with 10 mM arginine for 16 hrs. Data is presented as percent growth relative to wild type grown in C-media, and is the mean and standard deviation from at least two independent experiments.

exponential and stationary phase showed no significant shift in abundance during growth (Figure 4), suggesting that the change in down regulation seen in the mutants was not due to a shift in transcript abundance in wild type cells. Taken together this data suggests that both ArcR and AhrC.2 are required for transcriptional activation of the Arc operon in C-medium, and that additional growth phase dependent regulators may also influence the transcription of the Arc operon.

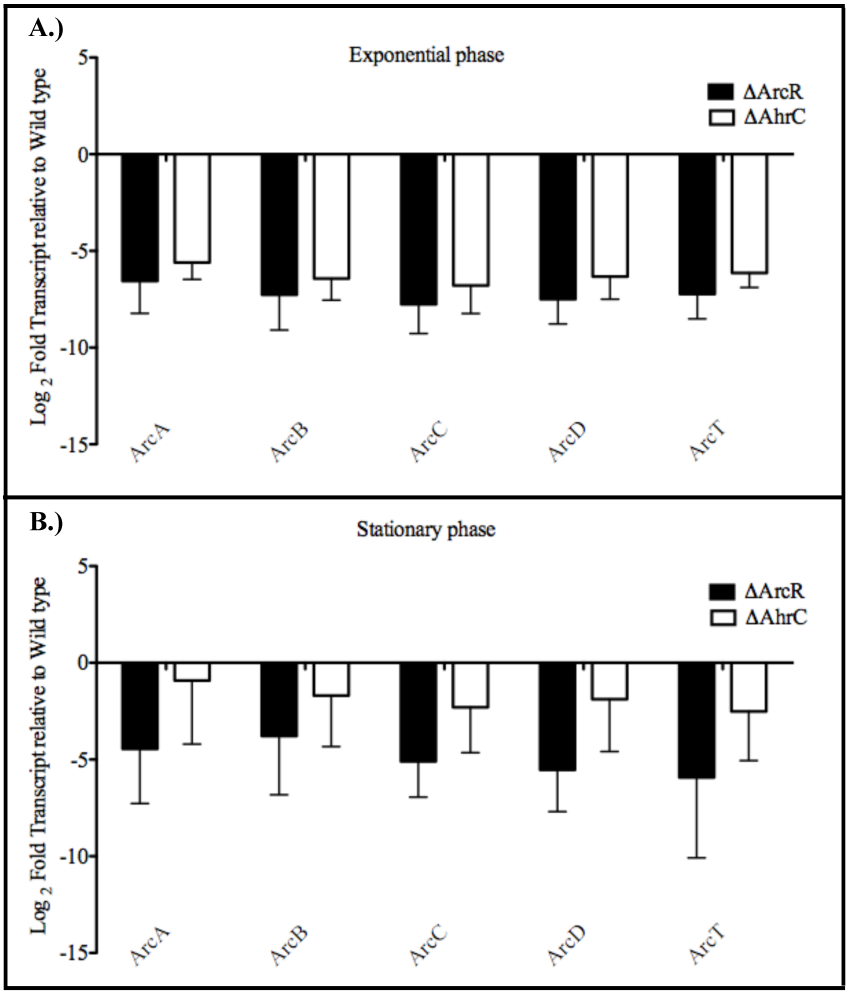

Figure 3. Mis-regulation of the Arc operon in both ΔArcR and ΔAhrC. Wild type, ΔArcR, and ΔAhrC were grown in C-medium until **A.)** exponential phase or **B.)** early stationary phase. Total RNA was isolated from these cultures and used for real-time RT-PCR analysis of the transcript of the indicated Arc gene. Data are presented as the ratio of transcript abundance in the mutant to that in the wild type and represent the mean and standard deviation from three independent experiments each analyzed in triplicate.

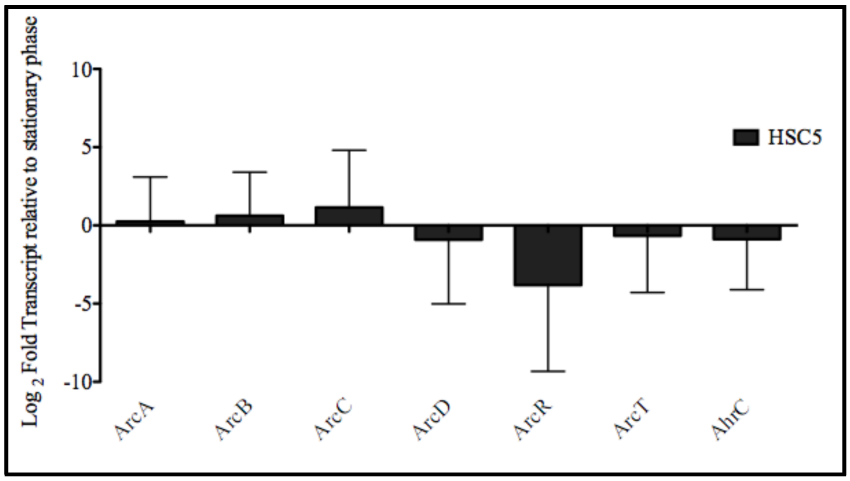

Figure 4. Growth phase dependent regulation of Arc operon in C-medium. Wild type were grown in C-medium until exponential phase or early stationary phase. Total RNA was isolated from these cultures and used for real-time RT-PCR analysis of the transcript of the indicated Arc gene. Data are presented as the ratio of transcript abundance in the exponential phase to that in stationary phase and represent the mean and standard deviation from three independent experiments each analyzed in triplicate.

Binding of AhrC.2 to the promoter region of *arcA* is ArcR dependent

Previous investigation of the Arc operon in *Enterococcus faecalis* has revealed that expression of the genes occur as a polycistronic mRNA with a single transcription initiation start point upstream of *arcA* (3). To further investigate the mechanism of regulation of the Arc operon, we next looked to observe the direct binding of ArcR and AhrC.2 to the putative promoter region upstream of *arcA* using a previously described biotinylated DNA pulldown assay (19). To monitor binding both ArcR and AhrC.2 were modified with a C-terminal hemagglutinin (HA) tag and expressed ectopically from a plasmid. To verify that both ArcR-HA and AhrC.2-HA were functional, the ability of these constructs to complement the single mutants ΔArcR and ΔAhrC.2, respectively, were monitored by growth in arginine (data not shown). The relative increase in growth of the complemented strains compared to the mutants in the presence of arginine, suggested that the addition of the C-terminal HA tag did not disrupt the function of either

protein. Initial analysis of binding to the *arcA* promoter revealed that under these conditions AhrC.2-HA but not ArcR-HA was able to bind (Figure 5B). Binding of the *arcA* promoter was found to be specific as demonstrated by the lack of binding to DNA amplified from the upstream region of the *arcR* gene (data not shown).

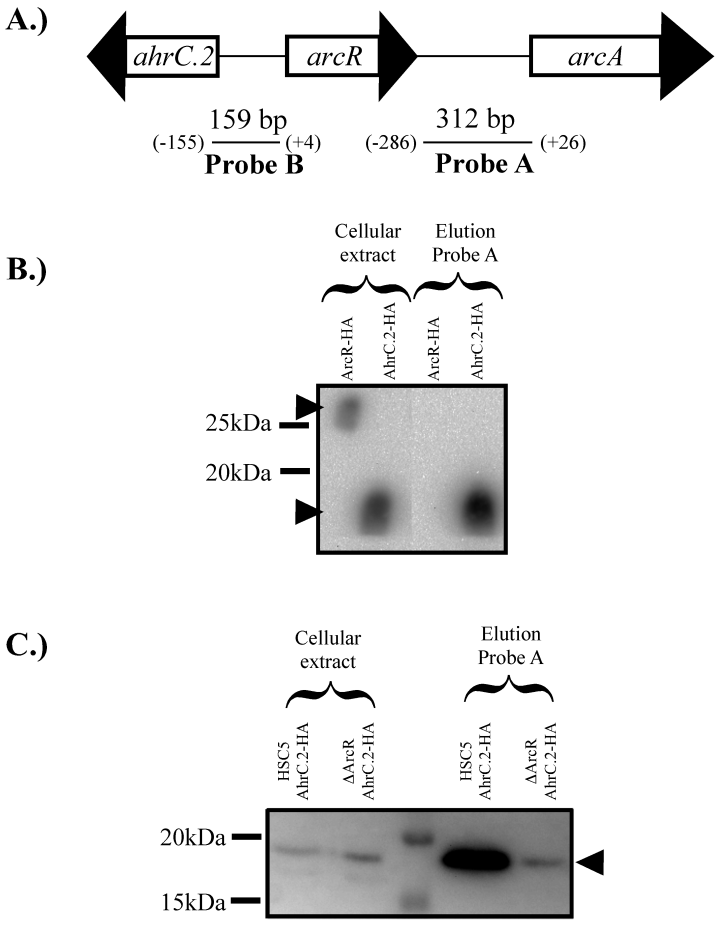

Figure 5. AhrC.2 binds to the *arcA* promoter in an ArcR dependent manner. A.)Diagram of the beginning of the Arc operon. The two probes described in the text are illustrated with positions of the DNA features described relative to the start of translation of the *arcA* and *arcR* genes, with the A of the ATG start codon considered +1. **B.)** Immuno blot developed with αHA antibody of cellular lysates or the elution of proteins precipitated by probe A as described in the methods from wild type bacteria expressing ArcR-HA or AhrC.2-HA. The positions of molecular weight standards are indicated on the left **C.)** Immuno blot developed with αHA antibody of cellular lysates or elution of proteins precipitated by probe A from wild type bacteria expressing AhrC.2-HA or ΔArcR bacteria expressing AhrC.2-HA. Arrows indicate the correct molecular weight for ArcR (28 kDa) or AhrC (19 kDa). Interactions with probe B were used as a negative control which showed no interaction with either ArcR-HA or AhrC.2-HA (data not shown).

Previous research in *Lactococcus lactis* had demonstrated that AhrC.2 and ArcR functioned as a complex (21). To investigate the influence of ArcR on the binding of AhrC.2 to the *arcA* promoter, cellular extract of AhrC.2-HA expressed ectopically in ΔArcR was used in the DNA binding experiments. Surprisingly in the absence of ArcR, the binding of AhrC.2 to the *arcA* promoter was nearly completely abolished. Together this data suggests that under conditions tested AhrC.2 is able to bind to the DNA region upstream of *arcA* in an ArcR dependent fashion.

ΔAhrC and ΔArcR display different phenotypes *in vivo*

Given the complex regulation of the Arc operon and the recent identification of the role of arginine catabolism in an *S. pyogenes* infection, we next wish to investigate the influence deletion of these two regulators may have in our subcutaneous infection model. Comparison of lesions formed in mice infected with our wild type strain, ΔArcR, or ΔAhrC.2 revealed that despite producing similar phenotypes *in vitro*, deletion of *ahrC.2* results in significantly smaller lesions compared to wild type, while ΔArcR does not (Figure 6). This surprising result raises many questions concerning the additional role of these regulators *in vivo* and the mechanism by which they regulate the Arc operon.

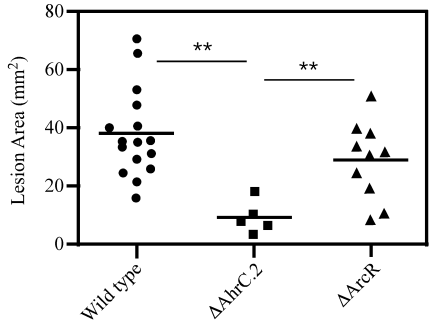

Figure 6. ΔArcR amd ΔAhrC.2 display different phenotypes *in vivo*. SKH1 Hairless mice were inoculated subcutaneously with the WT, DAhrC, or DArcR. The resulting ulcerations formed were imaged at day 3 postinjection and the area measured. Data shown are lesion area from individual mice. Differences between groups were tested for significance using the Mann-Whiteny U-test (** P<0.01).

Table 1. Strains used in this study[a]

Strains	Relevant genotype	Plasmid	Description	Reference
S. pyogenes				
HSC5	wild-type	none	wild-type	Hanski *et al.* (1992)
ZC161	HSC5 $arcR_{\Delta 7-219}$	none	In-frame deletion of *arcR*	This work
ZC606	HSC5 $ahrC.2_{\Delta 10-151}$	none	In-frame deletion of *ahrC.2*	Loughman *et al.* (2006)
ZC649	HSC5 $SPy1496_{\Delta 6-151}$	none	In-frame deletion of *SPy1496*	This work
ZC651	HSC5 $SPy2150_{\Delta 7-141}$	none	In-frame deletion of *SPy2150*	Loughman *et al.* (2006)
ZC698	HSC5	pZC200	HSC5 (pArcR-HA)	Loughman *et al.* (2006)
ZC699	HSC5	pZC201	HSC5 (pAhrC.2-HA)	This work
ZC700	HSC5 $arcR_{\Delta 7-219}$	pZC200	ZC161 (pArcR-HA)	This work
ZC721	HSC5 $ahrC.2_{\Delta 10-151}$	pZC201	ZC606 (pAhrC.2-HA)	This work
ZC701	HSC5 $arcR_{\Delta 7-219}$	pZC201	ZC161 (pAhrC.2-HA)	This work

a. Gene names based on annotation of the SF370 genome of *S. pyogenes*.

Table 2. Primers used in this study[a]

Name	Sequence[a]	Template	Plasmid	Description
ZC79	TTTGTCGACAAGAGATTCTGCAGCTAAGGTCTCA	HSC5	pZC32	Primers for in-frame deletion of *arcR*
ZC81	/5PHOS/TATTTTTTACAATACACAAAGTGAA			
ZC82	/5PHOS/ATCTTCTCTTCGAATCACACAAATC	HSC5	pZC32	Primers for in-frame deletion of *arcR*
ZC80	TTTCTGCAGACCGTTGCTACAATTTCTGGAATTTGCA			
ZC384	CCCCTCGAGGTCGACCGACTGGAAAGTAAAAATAAGTCACGTC	HSC5	pZC173	Primers for in-frame deletion of *ahrC.2*
ZC385	GGTTTTCTGTTAAGCCTTATTTGTTACTAAAGAAGGGTGACGCGTCTCTTTCTTATTCATTGTTATAATC			
ZC386	CTTCTTTAGTAACAAATAAGGCTTAACAGAAAACC	HSC5	pZC173	Primers for in-frame deletion of *ahrC.2*
ZC387	CCCCCGGGCTGCAGCAGATGGTGGTGACGACTTTTAC			
ZC437	CCCCCTCGAGGTCGACTTTTATAACGATCAAAAATAAGCTGATAGTTCTC	pJL75	pZC189	Primers for in-frame deletion of *SPy1496*
ZC438	GGAGTTTGAGAAACATGAAAAAGAGTGATGCAGGGTATAACCTAGAAAAAAATAGGGG			
ZC439	TCACTCTTTTTCATGTTTCTCAAACTCC	pJL126	pZC189	Primers for in-frame deletion of *SPy1496*
ZC440	GTGGATCCCCCGGGCTGCAGGGCGCTTCAACAGGTGGTTTTACC			
ZC441	CCCCCTCGAGGTCGACGGTCAAACCAATTTCTAAGAGTTGTGC	pJL162	pZC190	Primers for in-frame deletion of *SPy2150*
ZC442	GGGTGATATATCATGAATAAAATGGAGCGGGCTGCAGGCCTATGACTAGCTATC			
ZC443	CGCTCCATTTTATTCATGATATATCACCC	HSC5	pZC190	Primers for in-frame deletion of *SPy2150*
ZC444	GTGGATCCCCCGGGCTGCAGCTGTTCAGCTAACTCGCTAGCAATC			
ZC418	AACTCAATTGGTGATTCGAAGAGAAGATTATCAATACC	HSC5	pZC200	Primers for expression of ArcR-HA
ZC460	ATCTGCAGTTTAAGCGTAATCTGGAACATCGTATGGGTACTTTGTGTATTGTAAAAAATAGTCTTTATC			
ZC420	AACTGAATTCATGAATAAGAAAGAGACGCGTCACC	HSC5	pZC201	Primers for expression of AhrC.2-HA
ZC446	ATCTGCAGTTAAGCGTAATCTGGAACATCGTATGGGTATTTGTTACTAAAGAAGAAAGGTGGCGTG			
ZC479	/5BIOSG/ACATGAATTGGTGTTTGAGCAGTCATAAGAATTAC	HSC5		Primers for PCR of region upstream of *arcA*
ZC480	TACACAAAGTGAATTAAAGGTCAGAACAC			
ZC481	/5BIOSG/TCACACAAATCTCCTTTTTCTAAACATC	HSC5		Primers for PCR of region upstream of *arcR*
ZC482	TCTTTCTTATTCATTGTTATAATCTTCC			

a. Engineered restriction sites are underlined. Sequence for Influenza hemagglutanin (HA) epitope tag is in italics

Real-time RT-PCR Primers used in this study

RecA Forward	ATTGATTGATTCTGGTGCGG
RecA Reverse	ATTTACGCATGGCCTGACTC
ArcA Forward	TGACCGTAATGAAACCACTCG
ArcA Reverse	GAAATACCAACCGCAAGCAC
ArcB Forward	TGGGTCGTATGTTTGATGGG
ArcB Reverse	TGGCACACCTGAGAATTCTG
ArcC Forward	TGGCTTGGTCAGGTTTGTTGAA
ArcC Reverse	CAAGACCCTGACTGGTGTGGAA
ArcD Forward	ATGCCAAAATCTTCCCACGGAATC
ArcD Reverse	GAGCATAAGGAACTCTTCCAGTTGC
ArcT Forward	AGGCACTCAAGACGATAAAGG
ArcT Reverse	GATTCATACACCGCCACAAAG
ArcR Forward	GCTACATGAGTTGAGGGTTCG
ArcR Reverse	GTCTTTACCCATTTCAACCAG
AhrC.2 Forward	CTCATACGTTCCCTCATCTCTG
AhrC.2 Reverse	TCCAGAAGTTACTTTGACCAGG

DISCUSSION

In the present study we initiated investigation into the mechanism of regulation of the Arc operon in *S. pyogenes.* Previous research has identified the Arc operon and arginine catabolism is important for mediating bacterial survival during an infection, thus suggesting an important role for regulation of this operon *in vivo.* Analysis of regulation *in vitro* reveled that deletion of either *arcR* or *ahrC.2* abolished activation of the Arc operon, suggesting the requirement for both regulators for transcription. This requirement was further supported by additional experiments that demonstrated the ArcR-dependent interaction of AhrC.2 with the promoter region of *arcA*. However, analysis of these mutants *in vivo* produced varying phenotypes, suggesting an alternative form of regulation, or an additional function for these regulators during an *S. pyogenes* infection.

In bacteria, tight regulation of the ADI pathway is necessary to ensure arginine, required for protein synthesis is not degraded unnecessarily. Previous research in low G + C bacteria have revealed the conservation of two transcriptional regulators clustered near the Arc operon (34). Investigation in many of these low G + C bacteria have revealed that transcription of the operon is dependent on one of these regulators (10, 26, 33). However, they have often failed to examine the function of the other additional regulator associated with the Arc operon. In the present study the role of both regulators associated with the Arc operon, and their involvement in the transcriptional regulation of this operon were examined. In agreement with previously published research, deletion of either one of the regulators homologous to ArcR or AhrC.2 resulted in disruption of expression of the Arc operon (10, 26, 33). However, this work is the first to suggest the

requirement for both ArcR and AhrC.2 for the activation of the Arc operon, and suggests a more complex form of regulation previously envisioned.

While different, regulation of arginine metabolism in *Lactococcus lactis* reveals a unique form of regulation similar to *S. pyogenes*. In *L. lactis* transcription of genes involved in arginine catabolism and arginine biosynthesis are co-regulated to ensure efficiency in arginine metabolism (22). This coordination is believed to occur through a direct interaction between the homologs of AhrC.2 and ArcR, which is directly mediated by arginine (21). In low levels of arginine AhrC.2 binds to the promoter of the Arc operon preventing transcription. In the presence of increasing arginine, ArcR interacts with AhrC.2 resulting in a decrease in affinity for the Arc operon promoter and an increase in affinity for promoters regulating genes involved in arginine biosynthesis (21). This change in affinity for promoters leads to the de-repression of the Arc operon and repression of the arginine biosynthesis operon. Comparison of regulation of the Arc operon in *L. lactis* to *S. pyogenes* found in contrast to requiring both regulators, only the homolog of ArcR and not AhrC.2, is required for activation of this operon. This difference in regulation may be partially explained by the lack of an arginine biosynthesis operon in *S. pyogenes*, and thus the lack of requirement to coordinate expression with the Arc operon. Nevertheless, the requirement for both regulators to repress the arginine biosynthesis operon in *L. lactis* and their arginine-dependent interaction suggests these regulators may have evolved to function as a complex.

As an initial attempt to investigate the interaction of these regulators with the promoter of *arcA* we utilized a DNA pull down assay. This assay was efficient at detecting a specific interaction between AhrC.2-HA and the DNA region upstream of the

arcA promoter. Given the requirement for both regulators for the activation of transcription, we had hypothesized that these proteins may function in a complex, with both AhrC.2 and ArcR binding to the promoter simultaneously. However, despite multiple attempts, we were unable to detect an interaction between ArcR-HA and this DNA region. Despite this negative data, our previously stated hypothesis cannot be completely ruled out. Indeed, a putative interaction between ArcR and AhrC.2 may result in an increase in affinity for the *arcA* promoter, and that upon subsequent wash steps this interaction between ArcR and AhrC.2 is disrupted. The decrease in binding of AhrC.2-HA when expressed in a ΔArcR background supports this model, suggesting the requirement of both proteins for DNA binding. However, given that these interactions were performed from crude extract, deletion of *arcR* may result in changes to the concentration of another co-factor required for AhrC.2 binding. Further investigation into this model with purified protein will be essential in elucidating the direct mechanism of regulation, and the role that each regulator plays in activating transcription of the Arc operon. Additionally, analysis of the DNA region upstream of *arcA* has revealed a weak consensus with the previously identified binding sites of other arginine-like regulators. Determination of the transcriptional start site of the Arc operon will aid in discovering the exact DNA binding site of AhrC.2, as well as help identify additional genes regulated by AhrC.2 and ArcR through promoter analysis.

The recent discovery of the role of arginine catabolism in the pathogenesis of *S. pyogenes* suggested that regulation of this operon contributes to virulence. Given that deletion of either regulator disrupted activation of this operon, we had hypothesized that ΔArcR and ΔAhrC.2 would phenocopy each other in our mouse model of infection.

However, surprisingly deletion of *arcR* had no significant influence on virulence, while deletion of *ahrC.2* resulted in an attenuated strain with significantly smaller lesions compared to both wild type and ΔArcR. This variation in virulence raises many interesting questions regarding the activation of the Arc operon *in vivo* and the possible function of these regulators outside of the Arc operon. To further address these questions will require identification of additional genes mis-regulated by deletion of *arcR* and *ahrC.2*, as well as analysis of Arc transcription *in vivo.* Additionally, examination into the influence of other regulators, including CcpA and LacD.1, as well as additional environmental signals, including arginine, pH, and glucose, will allow for insight into the activation of this operon both *in vitro* and *in vivo*. Coupled with *in vivo* data, these experiments can provide insight into the environmental signals being sense by this bacterium and the influence of these regulators on virulence in *S. pyogenes*.

MATERIALS AND METHODS

***E. coli* strains, media, and growth conditions**. Common molecular cloning techniques utilized the *E. coli* strain DH5α, which was cultured in Luria-Bertani medium at 37ºC When appropriate antibiotics were added to the medium at the following concentrations: kanamycin 50 μg/mL and erythromycin 750 μg/mL.

***Streptococcus pyogenes* strains, media, and growth conditions.** *Streptococcus pyogenes* HSC5(16) and mutant derivatives of this strain (Table 1) were utilized for these experiments. Regular growth of strains was in Todd-Hewitt Broth (THYB) (DIFCO) supplemented with 0.2% Yeast Extract (DIFO) unless indicated in the text. Specific experiments utilized growth in C-medium (0.5% Protease Peptone #3 (DIFCO), 1.5% Yeast Extract (DIFCO), 10 mM K_2HPO_4, 0.4 mM $MgSO_4$, 17 mM NaCl). Supplementation of medium with arginine or citrulline occurred after autoclaving medium and was prepared by adding from a 0.5 M filter sterilized stock to a final concentration of 10 mM. Streptococci grown on THYB medium solidified by the addition of 1.4% Bacto Agar (DIFCO) were cultured anaerobically in sealed jars in the presence of commercially available gas-generating packets (GasPak cat. #70304, BBL). When appropriate antibiotics were added to the medium at the following concentrations: chloramphenicol 3 μg/mL and erythromycin 1 μg/mL.

Construction of deletion mutants. All references to genomic loci are based upon the genome of *S. pyogenes* SF370 (12). In-frame deletion mutations in genes encoding ArcR

(SPy_1548), AhrC (SPy_1549), (SPy_1496), and ArcD (Spy_2150) were generated using allelic replacement as previously described (28). Allelic replacement vectors were constructed with the vector pJRS233 (13) either through a process of overlap extension PCR (18) or by three-way ligation and 5' phosphorylated primers as listed in Table 2. All deletions were confirmed by PCR.

Isolation of RNA and real-time RT-PCR. As previously described(4) total RNA was isolated from strains grown in the media and at specific times in the growth cycle as indicated in the text. RNA was subjected to reverse transcription using iScript (Bio-Rad) per the manufacturer's instructions. Real-time RT-PCR analysis of cDNA samples was performed using iQ SYBR green Supermix along with primers listed in Table 2. Relative transcripts levels were determined using the $\Delta\Delta C_t$ method with the *recA* transcript as a standard as previously described (4).

Biotinylated DNA pulldowns. A biotinylated DNA pulldown assay was utilized as described previously (19). Briefly, DNA regions of interest were amplified from genomic DNA using 5' biotinylated primers listed in Table 2 followed by purification of the reaction using QIAquick PCR purification kit (Qiagen). Next 2 μg of the PCR reaction was bound to the strepavidin-coated magnetic beads and washed to remove unbound DNA. *S. pyogenes* cellular lysate of the specified strain was prepared and the total protein concentration measured using a bichinchoninic acid assay (Pierce/Thermo-Fisher). For the pulldown, 2 mg of total cellular lysate from the specified strain was

added to the DNA-coated beads, and incubated in 1 mL of binding buffer (10 mM Tris-HCl [pH 8], 100 mM KCl, 3 mM $MgCl_2$, 20 mM EDTA, 5 % glycerol, 40 μg/mL sheared salmon sperm DNA [Invitrogen], and 10 μg/mL bovine serum albumin [Sigma]) at room temperature. Following a 30-min incubation the beads were collected with a magnetic particle separator (Invitrogen cat # K1585) and washed four times with 500 μL of binding buffer. Protein was eluted by addition of 1 X protein sodium dodecyl sulfate (SDS) sample buffer (1X; 50 mM Tris-HCl[pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] SDS, 0.1 % [wt/vol] bromophenol blue, 10 % glycerol) followed by boiling of the sample for 5 mins. The magnetic beads were then removed with magnetic particle separator, and the resulting sample was subjected to electrophoresis using 12% SDS-polyacrylamide gel. The proteins were then transferred to a PVDF membrane for western blotting with a rabbit polycolonal αHA antibody (1:10,000 final dilution) and peroxidase conjugated goat αrabbit secondary antibody (1:10,000 final dilution; both from Sigma).

Subcutaneous infection of mice. As described previously infection of 6 to 8-week old female SKH1 (Charles Rive Labs) mice were infected subcutaneously with 10^7 bacterial CFUs of each indicated strain (5, 6). Following infection the resulting ulcers were imaged on the day noted in the text, and analyzed using the image software ImageJ (http://rsb.info.nih.gov/ij/index.html) to determine the area of the border of each lesion. Differences in lesion area were tested for significance using the Mann-Whitney *U*-test.

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CHAPTER V

Conclusions and Future Directions

CONCLUSIONS

For many pathogens, the acquisition of nutrients is the main driving force in virulence gene expression. Several virulence factors function through the delivery of important nutrients required for bacterial growth. Thus it is not surprising that for many pathogens changes in virulence gene expression and metabolism occur in a coordinated fashion. In *Streptococcus pyogenes*, this coordination is achieved by co-opting metabolic regulators for the expression of virulence genes. Previously, the catabolite-sensing regulator LacD.1 has been shown to function as both a regulator of virulence and metabolic genes, suggesting a unique confluence of metabolism and virulence gene expression. In this work, examination of LacD.1's function in *S. pyogenes* has provided insight into the adaptation of LacD.1 into a novel regulator, as well as revealed the importance of LacD.1 regulation in *S. pyogenes* fitness. Investigation of the LacD.1 regulon has identified the novel contribution of both arginine and citrulline metabolism to pathogenesis, and its modulation of the host innate immune response. The major conclusions of this work include: 1.) multiple residues dispersed throughout LacD.1 are required for its regulatory function, 2.) the adaptation of LacD.1 as a regulator results in a decrease in its ancestral enzymatic activity, 3.) the regulatory function of LacD.1 is required for *S. pyogenes* fitness, 4.) the arginine deiminase pathway contributes to *S. pyogenes* virulence through the catabolism of both arginine and citrulline, 5.) depletion of arginine from extracellular milieu by *S. pyogenes* results in an inhibition of iNOS activity, and thus NO production, 6.) Activation of the Arc operon requires both ArcR and AhrC.2, and 7.) AhrC.2 can directly interact with the DNA region upstream of *arcA*.

These studies reveal a unique coordination of virulence gene expression and metabolism by the regulator LacD.1 and demonstrate the direct influence of bacterial metabolism on pathogenesis. Upon infection, *S. pyogenes* encounters an environment void of easily metabolized carbohydrates. Through both de-repression via CcpA and activation via LacD.1, *S. pyogenes* is able to up-regulate alternative metabolic pathways involved in utilization of complex carbohydrates, as well as express catabolic pathways, such as the ADI pathway. The catabolism of arginine and citrulline by the bacteria results in the production of ATP and ammonia, which can buffer against the acidic, fermentation products created by the bacteria. In addition, depletion of arginine can serve to prevent the host from utilizing this amino acid crucial for the production of NO, a potent antimicrobial. During this shift in bacterial metabolism, LacD.1 regulation also results in the de-repression of *speB* resulting in expression and secretion of this potent protease. Upon secretion, SpeB can function to liberate peptides and arginine that can continue to supplement the ADI pathway. Ultimately, this co-regulation of both arginine catabolism and *speB* expression contributes to the fitness of *S. pyogenes* and demonstrates an example of how pathogens have evolved to coordinate the expression of virulence factors with metabolism.

FUTURE DIRECTIONS

The coordination of metabolism and virulence gene expression is becoming more and more evident in a number of pathogenic bacteria. Continuing to understand the influence bacterial metabolism has on both the regulation of virulence factors and the host response to a pathogen will be essential in furthering our understanding of host-pathogen interactions. The following discussion describes areas of research that will help to solidify our current understanding of *S. pyogenes* pathogenesis.

Examine the mechanism of LacD.1 regulation and its coordination of *speB* and the Arc operon.

Research examining the evolution of LacD.1 has revealed key residues necessary for LacD.1's regulatory function. Identification of these distinct regions suggests their possible role in protein-protein interactions. Previous attempts to detect these interactions have proven difficult, likely due to their short-lived lifetime along with fluctuations in important metabolites that mediate these interactions. To address these issues may involve the use of cross-linking agents to stabilize any putative interactions with protein or DNA. Given the recent characterization of several chimeric enzymes with altered ability to regulate, addition of these proteins in this analysis will allow for the identification of novel interactions relevant to LacD.1's regulatory function.

Previous studies have established LacD.1's role in the repression of *speB* along with the activation of the metabolic Arc operon (2, 6). However, examination of this regulation has occurred under different conditions and different phases of growth. A more in depth examination of LacD.1's role in regulating the Arc operon will be helpful

in understanding how environmental signals may influence both the expression of *speB* and the Arc operon. Given the role of arginine catabolism in protection against acid stress and the induction of *speB* transcription by an acidic pH (5), the coordination of expression of these two pathways may suggest a functional interaction. Indeed, it would be interesting to examine if the activation of the Arc operon via LacD.1 is increased by a decrease in medium pH. This increase in activation would suggest protection from acid stress via arginine catabolism might be supplemented by SpeB's protease activity. Further experimentation utilizing the established acid stress model would also allow us to determine if SpeB activity is able to protect *S. pyogenes* via the ADI pathway, revealing a unique contribution of SpeB activity to *S. pyogenes'* metabolism.

Examine the influence of arginine catabolism on virulence.

The recent identification of arginine catabolism's role in *S. pyogenes* pathogenesis raises many interesting questions regarding its exact contribution to virulence. Given that this pathway aids to bacterial metabolism with both the production of ATP and protection from acid stress occurring simultaneously, it is difficult to determine which is necessary *in vivo.* Preliminary evidence looking at the contribution of serine catabolism to *S. pyogenes* pathogenesis has revealed evidence suggesting that arginine and citrulline catabolism primarily contributes to protection against acid stress. Serine catabolism funnels through pyruvate metabolism with the conversion of serine to pyruvate. Due to this route of metabolism, supplementation of C-medium with serine results in a decrease in the medium's pH. Deletion of the two enzymes, SdhA and SdhB, responsible for this catabolism results in an inability to utilize serine, as well as attenuation in virulence (Figure 1). However, deletion of both *arcB* and *sdhA/B* does not result in further

attenuation compared to the single mutation (Figure 1). This phenotype may suggest that preventing the catabolism of serine diminishes fermentation, and thus the requirement for arginine catabolism to protect against acid stress. Further investigation into the pH of a lesion formed by both wild type *S. pyogenes* and a ΔArcB during an infection will be essential in understanding both the contribution of arginine/citrulline catabolism to acid protection, as well as the reveal the environmental pH encountered by *S. pyogenes*. Additionally, monitoring changes in pH through out an infection may reveal a novel correlation with the temporal expression of *S. pyogenes* virulence factors.

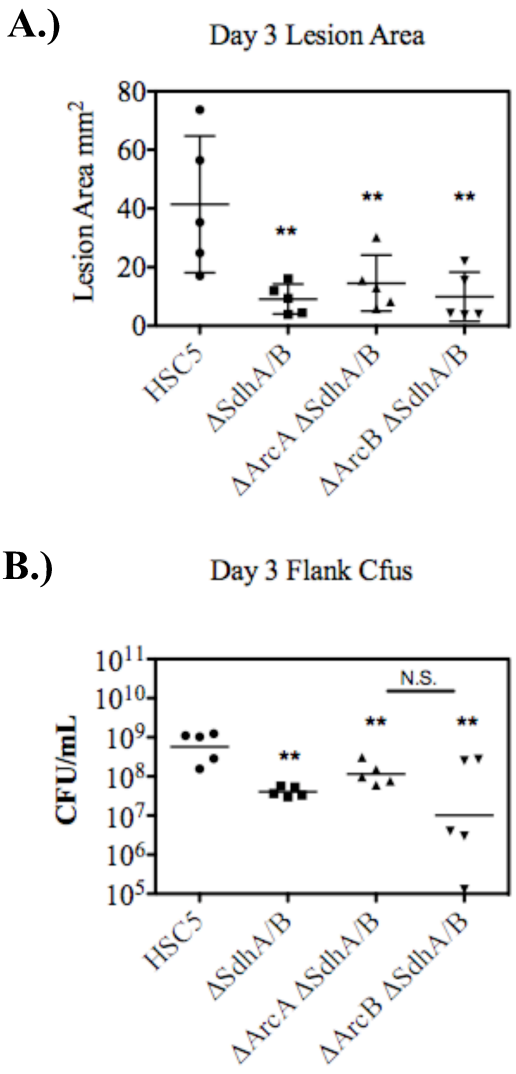

Figure 1. The influence of serine catabolism on S. pyogenes pathogenesis A.) Hairless SKH1 mice were infected with wild type(HSC5), ΔSdhA/B, ΔArcA ΔSdhA/B, or ΔArcB ΔSdhA/B. The lesions formed on day 3 were imaged and the area measured using image analysis. The data presented are plotted lesion area from multiple mice from at least two independent expreiments. Differences in mean lesion area compared to wild type were tested for significance using the Mann-Whitney U test. (** P<0.01) B**.)** CFUs recovered from lesions. On day 3 the lesions were harvested and the recoverable bacteria enumerated. Each symbol represents bacteria recovered from an individual mouse. Differences between wild type were tested for significance using the Mann-Whitney U test. (** P<0.01)

Identify the citrulline transporter in *S. pyogenes*, and the role of ATP production in protection against acid stress.

The recent discovery of the utilization of citrulline during an infection raises many interesting questions regarding its transport into the cell, and its influence on gene expression. Identification of the transporter involved in bringing citrulline into the cell will be essential in dissecting the exact contribution of citrulline catabolism in virulence. Additionally, examination of the role of arginine catabolism in protection against acid stress has raised many questions regarding the exact contribution of the production of ATP versus the production of ammonia in protecting bacteria. Further examination of the intracellular pH in the presence of arginine in both wild type and the *arc* mutants will provide insight into the exact mechanism of protection. While difficult, advances in pH-responsive flurophores may allow for monitoring of the intracellular pH in real time.

Influence of arginine sensing on the expression of the Arc operon and virulence genes in *S. pyogenes*

The recent discovery of the role of arginine catabolism in an *S. pyogenes* infection, suggests that activation of the Arc operon by AhrC.2 and perhaps ArcR is important for pathogenesis. Examination of the genome of *S. pyogenes* reveals the presence of two additional arginine-like regulators (SPy_1496 and SPy_2150). Preliminary studies have revealed that deletion of these additional regulators do not disrupt the utilization of arginine (data not shown), suggesting their regulatory function lies elsewhere. Given the utilization of arginine during an infection it will be interesting to examine if the presence of this amino acid triggers expression of additional metabolic

pathways or virulence genes via an arginine-responsive regulator. Indeed, in many pathogens including *Legionella pneumonphila, Staphylococcus aureus*, and *Streptococcus pneumoniae* regulators of arginine metabolism have been found to include regulation of several virulence factors (1, 4, 7). Investigation in to the genes mis-regulated in the four mutants (ΔArcR, ΔAhrC.2, ΔSPy_1496, and ΔSPy_2150) in the absence and presence of arginine will provide insight into the influence arginine sensing has on gene expression in *S. pyogenes.*

Examine influence of arginine/citrulline catabolism on host response.

The inhibition of iNOS activity during an infection through arginine depletion reveals a unique influence of *S. pyogenes'* metabolism on the host innate immune response similar to the influence of RocF in *Heliobacter pylori*. Further experiments looking at the effect of the arginase, RocF on gastric epithelial cells have shown a clear influence on cytokine expression and host signaling (3). Given this similar modulation of iNOS activity, it will be interesting to explore the influence of arginine catabolism on the host response further, by examining cytokine expression during an infection, and exploring possible changes to the activation profile of macrophages.

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ZACHARY CUSUMANO

5981 N. Cuba Ct, St Louis, MO, 63139
work: 314-362-7650 · cell: 920-809-1964 zac.cusumano@gmail.com

EDUCATION

Washington University — St. Louis, MO
Ph.D. Biochemistry — 2004 – 2012

Benedictine College — Atchison, KS
B.S. Biology — 1999 – 2003

EXPERIENCE

Washington University, Department of Molecular Microbiology, — St. Louis, MO
Dr. Michael Caparon, Ph.D. Laboratory — 2006 – 2012
Graduate Research Assistant

- Examined the evolution and mechanism of virulence gene regulation by the regulatory aldolase, LacD.1, in *Streptococcus pyogenes*
- Examined the contribution of arginine catabolism to *Streptococcus pyogenes* pathogenesis
- Performed protein purification using IMAC, ion-exchange chromatography, and size-exclusion chromatography
- Performed enzyme coupled assay to determine Michaelis-Menten kinetic constants
- Performed mutagenesis, cloning and sequencing to identify amino acid residues involved in enzymatic activity
- Generated in-frame deletions in *Streptococcus pyogenes*
- Examined pathogenesis of *Streptococcus pyogenes* in mouse model of infection
- Cultured macrophages and examined interaction with *S. pyogenes*
- Measured mRNA levels in bacteria, cell cultured macrophages, and in the mouse using qRT-PCR
- Mentored undergraduate and graduate students during their lab rotations.
- Radiation safety officer

Washington University, Department of Molecular Microbiology, — St. Louis, MO
Dr. George Gokel, Ph.D. Laboratory — 2005 – 2006

- Synthesized and purified synthetic ion channels
- Measured inhibition of bacterial growth and synergistic effect with antibiotics

Benedictine College, Department of Biology — Atchison, KS
Dr. Martin Simon, Ph.D. — 2001 – 2003
Undergraduate Research Assistant

- Examined the invertebrate biodiversity of the Benedictine Bottoms flood plain by collecting and identifying field samples
- Analyzed data and presented at the Missouri River Natural Resources Conference

TEACHING

Washington University — St. Louis, MO
Young Scientist Program Director and Teacher — 2004-2005

- Taught high school students about physics, forensic science, and microbiology
- Conducted Summer research boot camp to introduce common laboratory techniques
- Developed experiments and lesson plans

Washington University — St. Louis, MO
Teaching assistant for Introduction to the Nervous System — 2006

- Conducted discussion sections, graded tests and held office hours.

AWARDS

- Infectious Disease Pathway Scholar, Washington University in St. Louis — 2011
- Undergraduate Research Grant — 2003
- Benedictine College Discovery Scholar — 2003
- Heuer Scholarship — 2002
- Dean's List — 1999
- UNM Scholar Scholarship — 1999
- Presidential Education Award — 1999
- New Mexico State Department of Education Scholar — 1999

PUBLICATIONS

Cusumano, Zachary and M.G. Caparon. Contribution of arginine catabolism to the pathogenesis of *Streptococcus pyogenes.* In preparation.

Cusumano, Zachary and M.G. Caparon. Adaptive evolution of the *Streptococcus pyogenes* regulatory aldolase LacD.1. In Revision to *Journal of Bacteriology.*

Atkins, J.L., Patel, MB, **Cusumano, Z**. Gokel GW. 2010. *Enhancement of antimicrobial activity by synthetic ion channel synergy.* Chem Commun (Camb). **46**(43): p. 8166-7.

Cusumano, Zachary, Gokel, George. 2006. Synthetic Ion Channels as Novel Antimicrobial Agents. *Current Bioactive Compounds***.** 2:13-18.

Bowen, Daniel E., Martin P. Simon, John W. Davis, Tiffany M. Cope, **Zachary T. Cusumano**, Jill C. Hellmer, Virginia L. Winder, Sarah J. Soard, Allison M. Lidolph, Sarah E. Zielinski, Bethany James, Michelle Runchey, Trisha Hackmann. 2004. A list of plants observed along the lower Missouri River by the Lewis and Clark Expedition in 1804 and 1806*. Transactions of the Kansas Academy of Science*. 107:55-68.

POSTER AND ORAL PRESENTATIONS AT CONFERENCES AND SYMPOSIUMS

Inhibition of Nitric Oxide Synthase By Arginine Catabolism and the Novel Utilization of Citrulline by *Streptococcus pyogenes*. Oral presentation, International Conference on Gram-Positive Pathogens (2012)

Contribution of arginine catabolism to the regulation of the virulence factor SpeB. Poster presentation, Molecular Genetics of Bacteria and Phage Meeting (2011)

Comparative kinetic analysis of the regulatory tagatose bisphosphate aldolase, LacD.1, and its paralog in *Streptococcus pyogenes*, Poster presentation. Midwest Microbial Pathogensis Conference. (2010

Mechanism of virulence regulation by LacD.1 in *Streptococcus pyogenes*. Poster presentation American Society of Microbiology General Meeting. (2007)