Abstract

Title of Dissertation: ROLE OF THE PHOSPHOENOLPYRUVATE: CARBOHYDRATE PHOSPHOTRANSFERASE SYSTEM IN THE VIRULENCE OF THE GROUP A STREPTOCOCCUS

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Group A Streptococcus (GAS) is a fastidious microorganism that has adapted to a variety of niches in the human body by sensing its surroundings and modifying its metabolism to elicit a wide array of diseases. The Phosphoenolpyruvate Phosphotransferase System (PTS) is a primary mechanism by which many bacteria transport sugars and sense the carbon state of the cell. The PTS uses the non-sugar specific 'general' proteins EI and Hpr, and sugar-specific membrane-spanning proteins (EIIs) for the PTS-mediated uptake of each sugar. The role of PTS in

carbohydrate metabolism in GAS was investigated by generating a Δ*ptsI* mutant (EI deficient) in three different strains belonging to M1T1 and one of M4 GAS. All Δ*ptsI* mutant strains tested were unable to grow on multiple carbon sources (PTS and non-PTS). Complementation with *ptsI* expressed under its native promoter in single copy was able to rescue the growth defect of the mutant. Additional studies analyzing the role of PTS in pathogenesis of GAS, showed a 'hypervirulent' phenotype in the absence of Δ*ptsI* from M1T1 using soft tissue model of infection. The appearance of significantly larger and more severe ulcerative lesion observed in mice infected by Δ*ptsI* was correlated with increased transcript levels of *sagA* and early Streptolysin S (SLS) activity during exponential phase growth. The role of SLS in increased pathogenesis of Δ*ptsI* was investigated by the creation of a double mutant strain (Δ*ptsI sagB*) that lacks the ability to secrete SLS. The infection of mice with a Δ*ptsI sagB* double mutant resulted in a lesion comparable to either a wild type or a *sagB* mutant alone. In addition to SLS, it was found that PTS influences the secretion of cysteine protease SpeB and the ability of GAS to produce capsule. This regulatory effect was found to be dependent on the status of sensor kinase (CovS) of the CovR/S two-component system. Moreover, PTS was shown to phosphorylate PTS regulatory domains (PRD) of a global virulence regulator, Mga, resulting in alteration of its regulon in both M1T1 and M4 background, suggesting the ability of GAS to alter expression of Mga regulon in response to carbohydrate availability. Overall, our studies indicate that a functional PTS is important for utilizing PTS and non-PTS sugars and influences virulence during GAS infection.

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Dedication

This dissertation is dedicated to my parents for all their support and encouragement throughout my life.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADP	adenosine diphosphate
ARF	acute rheumatic fever
ATP	adenosine triphosphate
B. anthracis	*Bacillus anthracis*
bp	base pair
B. subtilis	*Bacillus subtilis*
BSA	bovine serum albumin
cAMP	cyclic
CCR	carbon catabolite repression
CDM	chemically defined medium
CFU	colony forming unit
CRP	cAMP receptor protein
CTD	C- terminal domain
CcpA	catabolite control protein A
Cov	control of virulence
cre	catabolite responsive element
dH2O	distilled water
DNase	deoxyribonuclease
E. faecalis	*Enterococcus faecalis*
E.coli	*Escherichia coli*
ECM	extra cellular matrix
EI	enzyme I

EII	enzyme II
EMP	Embden Meyerhof Parnas
Emm	M protein
GAS	group A streptococcus
GBS	group B streptococcus
GCS	group C streptococcus
gDNA	genomic DNA
HA	hyaluronic Acid
His	histidine
Hpr	histidine phosphocarrier
HprK/P	Hpr kinase/phosphatase
Kb	kilobase
LAB	lactic acid bacteria
LB	Luria-Bertani medium
MF	multiplication factor
Mga	multiple virulence gene regulator
NADH	nicotinamide adenine dinucleotide
NEB	New England Biolabs
ov	omnilog values
PEP	phosphoenol pyruvate
PPP	pentose phosphate pathway
PRD	phosphoenolpyruvate phosphotransferase regulatory domain

PSGN	post-streptococcal glomerulonephritis
PTS	phosphoenol phosphotransferase system
Pmf	proton motive force
RALPs	RofA-like proteins
sag	streptolysin associated gene
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
ser	serine
S. dysgalactiae	*Streptococcus dysgalactiae*
S. intermedius	*Streptococcus intermedius*
S. mutans	*Streptococcus mutans*
S. pneumoniae	*Streptococcus pneumonia*
S. pyogenes	*Streptococcus pyogenes*
S. suis	*Streptococcus suis*
S. typhimurium	*Salmonella typhimurium*
SLS	streptolysin S
SOF	serum opacity factor
Sic	streptococcal inhibitor of complement
Spe	streptococcal pyrogenic exotoxin
Srv	streptococcal regulator of virulence
TCA	tricarboxylic acid cycle
TCS	two-component system
THY	Todd Hewitt Yeast

WT	wild type

Chapter 1: Literature Review

In order to survive, bacteria (both pathogenic and non-pathogenic) need access to nutrients such as carbon sources. In the natural environment, the variety and the quality of available nutrients vary depending on the source or the niche. As a result, bacteria have evolved remarkably sophisticated strategies to allow them to take advantage of the wide range of essential nutrients, including carbon, nitrogen, sulphur and phosphorus. This enables them to feed the central carbon metabolic pathways-glycolysis, pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA), which integrate the transport and oxidation of main carbon source inside the cell.

Membrane transport systems play an important role in enabling the uptake of essential nutrients by bacteria. Since this dissertation focuses on Gram-positive bacteria, *Bacillus subtilis* will often be used as a model organism. There are four kinds of transporters in *B. subtilis* and other Gram-positive bacteria.

(a) Channel proteins that generally catalyze movement of solute in an energy independent manner.

(b) Secondary active transporters that use chemiosmotic energy in the form of electrochemical gradients to drive transport (e.g., proton motive force or pmf).

(c) Primary active transporters that use chemical, electrical or solar energy to drive transport (e.g., Adenoside Triphosphate (ATP) hydrolysis).

(d) Group translocating systems such as bacterial phosphoenolpyruvate phosphotransferase systems (PTS)

The overarching aim of this study is to characterize the PTS and explore its role in *Streptococcus pyogenes*. For many years, *B. subtilis* has been considered the model organism for Gram -positive bacteria for its long history of laboratory research, ease of cultivation and genetic manipulation. Although, it is well known that no single organism can be a perfect representative of all other species, for simplicity, we will focus on the knowledge of PTS available from *B. subtilis i*n the following sections.

1.1 The Phosphoenolpyruvate: Carbohydrate Phosphotransferase System (PTS)

PTS is a complex carbohydrate transport system found in both Gram-positive and Gram-negative bacteria that was first discovered in *Escherichia coli* in 1964 (1). It is ubiquitous in bacteria and is not found in plants or animals. It is a system that senses the presence of metabolites and is involved in regulation of a number of metabolic pathways (2). PTS is found mainly in obligate and facultative anaerobic bacteria, which synthesize ATP by substrate level phosphorylation under anaerobic conditions and thus make good use of ATP (3). It catalyzes the concomitant transfer and phosphorylation of the substrate using a phosphorylation cascade composed of the general cytoplasmic proteins enzyme I (EI) and histidine phosphocarrier (Hpr) along with carbohydrate specific translocation proteins called enzyme II or EIIs. Phosphoenol pyruvate (PEP) from glycolysis acts as a source of energy and a phosphate donor for the transport step and phosphorylation of carbohydrates via PTS pathway (4). The first protein of the PTS phosphorelay EI, receives the phosphate from the conversion of PEP to pyruvate in glycolysis of the incoming sugar by its PTS transporter. Upon receiving the phosphate from PEP, EI transfers it to histidine-

15 (his-15) of Hpr forming P~his-Hpr. The phosphate is then moved to the EIIAB proteins forming P~EIIBs, which then transfers the phosphoryl group to the substrate bound to its corresponding membrane spanning EIIC and the phosphorylated sugar is then released into the cell (4). Although the primary function of the PTS is to transfer phosphates to the carbohydrate, all the reactions up to and including the EII's are reversible (Fig. 1).

In Gram-positive bacteria, Hpr can also be phosphorylated at a second catalytic site serine-46 (ser-46). This reaction is carried out by an ATP-dependent Hpr kinase/phosphatase (HprK/P) that responds to the metabolite concentration in the cell. Phosphorylated ser-Hpr then participates in the activation of the carbon catabolite control (CCR) pathway (Fig. 1) of both Gram-negative and Gram-positive organisms, although the mechanism involved is quite different (2,5). More will be discussed on this later in the chapter.

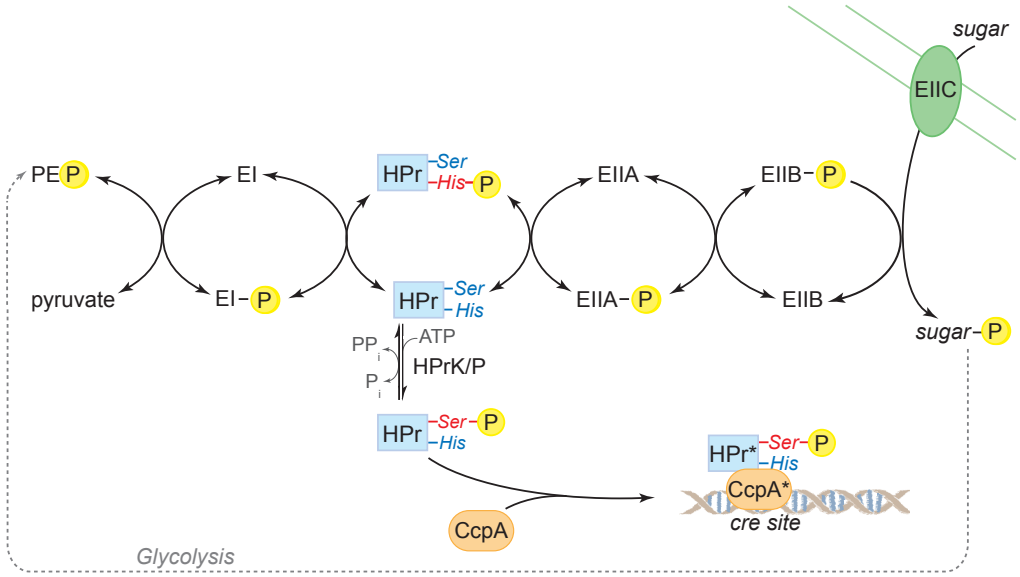

Figure 1: PTS in Gram-positive bacteria. The PTS couples carbohydrate transfer and phosphorylation. The general cytoplasmic proteins EI and Hpr and sugar-specific EII proteins form the phosphorelay transferring phosphate from the PEP of glycolysis to the incoming sugar. Carbon catabolite repression (CCR) results from HprK/P phosphorylation of serine 46 of Hpr which then complexes with CcpA to repress target promoters by binding to cre sites. Figure adapted from (6).

1.1.1 EI and Hpr

EI and Hpr are soluble, cytoplasmic proteins that are required to phosphorylate all EII proteins that participate in the phosphorylation of PTS carbohydrates in a given organism and thus have been called general PTS proteins (2,4). Unlike the sugar-specific EII components, EI and Hpr are common to each sugar transported by the PTS.

EI protein comprises of 577 amino acids that is encoded by the 1734 bp *ptsI* gene. The *ptsI* gene has been cloned and sequenced from *E. coli* (7), *Salmonella typhimurium* (8), *Staphylococcus aureus* (9), *Enterococcus faecalis* and *B. subtilis* (10). Sequence analysis has revealed that EI from various Gram-positive and Gram-negative bacteria exhibit significant identity (11). The structure of EI has been studied in a number of bacteria (12,13). EI is best described in *E.coli*. The functional form of EI is a ~128-kDa dimer of identical subunits (14) comprising two structurally and functionally distinct domains (15,16). The N-terminal phosphoryl-transfer domain contains the site of phosphorylation (his) and the binding site for HPr (17,18). In the phosphorelay, EI is autophosphorylated in the presence of PEP and Mg^{2+} on the histidine residue located in N terminus of the protein. The C-terminal domain is responsible for dimerization and contains the binding site for PEP (19-21). The EIN and EIC domains are connected to one another by a long helical linker that allows for the swivel mechanism to dimerize the protein.

Hpr protein comprises of 87 amino acids that is encoded by the 264 bp *ptsH* gene. Sequence analysis has revealed a high level of similarity around the active–site histidyl residue, his -15 in Hpr of most enteric bacteria and firmicutes (3). As mentioned before, in most low G+C Gram-positive bacteria, Hpr can also be phosphorylated by an ATP- dependent protein kinase (HprK) on a seryl residue at position 46 (ser- 46). The phosphorylation of this catalytic site has regulatory function and is not directly involved in phosphorylation of carbohydrates. In fact, the phosphorylation of the seryl residue slows the phosphoryl transfer from P~EI to Hpr by about 100 fold (2).

1.1.2 EII

The carbohydrate specificity of PTS occurs through resides in EII, and hence bacteria usually contain many different EIIs. Each EII complex consists of one or two hydrophobic integral membrane domains (domains C and D) and two hydrophilic domains (domains A and B), which together are responsible for the transport of carbohydrate across the bacterial membrane as well as the phosphorylation (4). In *E.coli* the EIIs have been classified into four families (22); (i) glucose- fructose-lactose family, (ii) the ascorbate-galactitol superfamily (23-26), (iii) mannose family, (iv) the dihydroxyacetone family (12). *E.coli* and *B. subtilis* contain about 15 different EII complexes.

1.2 Glycolysis (Embden, Meyerhof and Parnas Pathway)

In most bacteria, glucose is the most readily utilized carbohydrate and its metabolism starts with its uptake via PTS and proceeds with several interconnected pathways

mainly glycolysis, gluconeogenesis, pentose phosphate and tricarboxylic acid cycle (TCA). This represents the most common metabolic pathway in bacteria for sugar catabolism.

Glycolysis does not require oxygen and takes place in the cytosol of the bacterial cell. A series of enzymatic processes result in conversion of sugars into pyruvate, generating ATP (adenosine triphosphate) and NADH (nicotinamide adenine dinucleotide). The most common type of glycolysis is the Embden, Meyerhof, Parnas (EMP) pathway, which was first discovered by Gustav Embden, Otto Meyerhof and Jakub Karol Parnas. The overall reaction of glycolysis is represented as:

$$\text{Glucose} + 2NAD^+ + 2ADP + 2Pi \rightarrow 2 \text{ pyruvate} + 2NADH + 2ATP + 2H_2O$$

Thus, each molecule of glucose is converted into 2 molecules of pyruvate and a net of 2 ATP and 2 NADH. There are number of alternative fates for pyruvate; in the presence of oxygen (aerobic condition) pyruvate is converted to acetyl-CoA by the enzyme pyruvate dehydrogenase, which then enters the TCA or Krebs cycle. In the absence of oxygen (anaerobic conditions), fermentation occurs to regenerate NAD^+ for continued glycolysis. This results in the formation of lactate and as well as a non-oxidative decarboxylation of pyruvate, followed by reduction of acetaldehyde to ethanol.

1.3 TCA / Citric acid cycle

In aerobic bacteria, pyruvate from glycolysis, is combined with oxygen to produce Acetyl-CoA, which then goes through the citric acid or tricarboxylic acid (TCA) cycle first identified by Hans Adolf Krebs in 1937. The TCA cycle is a key metabolic pathway that is used by all aerobic organisms to generate energy. It is comprised of a series of chemical reactions by which glucose and other molecules are broken down in the presence of oxygen into carbon dioxide and water, resulting in the release of chemical energy in the form of ATP. In aerobic bacteria, a total of 36 ATP are produced from a single glucose molecule. In addition, the cycle provides precursors such as certain amino acids as well as the reducing agent NADH that is used in numerous biochemical reactions. The NADH generated by the TCA cycle is fed into the oxidative phosphorylation pathway. Eventually, through oxidative phosphorylation, NAD+ is regenerated from NADH and even more ATP is made from ADP.

1.4 Fermentation

Fermentation is a metabolic process that generates ATP by the process of substrate-level phosphorylation. Fermentation is important in anaerobic conditions where there is no oxidative phosphorylation to maintain the production of ATP. The energy for generating ATP comes from the oxidation of carbohydrates where pyruvate from glycolysis is used as an endogenous electron acceptor and metabolized to various compounds through several processes:

- Ethanol fermentation – pyruvate is converted into ethanol and carbon dioxide.
- Lactic acid fermentation – pyruvate produces lactic acid by two means

- Heterolactic fermentation is the production of lactic acid as well as other acids and alcohol
- Homolactic fermentation is the production of lactic acid from pyruvate.

As mentioned above, during the process of glycolysis NAD^+ is reduced to form NADH. For glycolysis to continue, NADH must be oxidized to form NAD^+ by an enzyme called lactate dehydrogenase. This occurs when NADH donates its electrons to pyruvate formed in glycolysis, resulting in the formation of lactic acid. In heterolactic acid fermentation, one molecule of pyruvate is converted to lactate and the other is converted to ethanol and carbon dioxide. In homolactic acid fermentation, both molecules of pyruvate are converted to lactate. Importantly, the fermentation reaction does not directly generate ATP molecules. Instead, the NAD^+ regenerated by lactate dehydrogenase is used in the glycolytic process to make ATP. Therefore, cells only get energy (2 ATP) from glycolysis and none from the TCA cycle.

1.5 Lactic acid bacteria (LAB)

LAB are Gram-positive, usually non-motile, non-spore-forming organisms. They lack the ability to synthesize cytochromes and porphyrins (components of respiratory chains) and therefore cannot generate ATP by creation of a proton gradient. Thus, LAB can only obtain ATP by fermentation, usually of sugars. LAB can be divided into two groups based upon the products produced from the fermentation of glucose. Homofermentative organisms ferment glucose to two moles of lactic acid, generating a net of 2 ATP per mole of glucose metabolized. Lactic acid is the major product of this process. Heterofermentative organisms ferment 1 mole of glucose to 1 mole of lactic acid, 1 mole of ethanol and 1 mole of CO_2. The LABs typically have limited

biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines and often a sugar as the carbon and energy source.

1.6 Regulation of carbon metabolism

Carbon catabolite repression (CCR) is a global regulatory mechanism of carbon source utilization that all bacteria employ for selectively utilizing carbon sources from the environment to conserve energy. Studies in model organisms show that the presence of glucose often prevents the use of other, secondary carbon sources (27). CCR enables the bacteria to sense the nutritional situation and adjust their catabolic capabilities through mechanisms such as inducer exclusion and global transcriptional control. The CCR mechanisms in Gram-negative and Gram-positive bacteria are quite different; however, the components of PTS play a major role in both (2,28). PTS– independent CCR mechanisms are also operative in some bacteria and will be briefly discussed later in the chapter.

1.6.1 Inducer exclusion in Enterobacteriaceae

Inducer exclusion is considered to be the major CCR mechanism in Enterobacteriaceae and serves as a paradigm for Gram-negative bacteria. In the presence of a rapidly metabolizable sugar, EII proteins of PTS become dephosphorylated. Dephosphorylated EII can interact with proteins of several non-PTS sugar transport systems (e.g., lactose- H^+ symport permease and glycerol kinase). Binding of the unphosphorylated EII to these target proteins results in the inhibition of their activities and thus, inhibits the uptake and utilization of lactose and

glycerol, respectively (29). Mechanisms of inducer exclusion have been most extensively studied in *E. coli* and *S. typhimurium* (4).

1.6.2 Inducer exclusion in Firmicutes

In Gram-positive of the phylum Firmicutes, Hpr plays the central role in CCR similar to EII in the Gram-negative Enterobactericiae. Inducer exclusion in Firmicutes is mediated via one of the phosphorylated forms of Hpr (P~ser-Hpr). The rapid metabolism of various sugars affects the activities of a bifunctional protein kinase/phosphorylase (HprK/P), which responds to changes of the ATP, Pi, PPi, and FBP concentrations, and of EI, which responds to alterations of the PTS phosphotransfer activity and the PEP-to pyruvate ratio (30). These two enzymes control the concentration of the various forms of HPr, which regulate carbon metabolism via protein-protein interactions (HPr and P~ser-HPr) or the phosphorylation of non-PTS proteins (P~his-HPr). In Gram-positive bacteria, HPr therefore functions as the "central processing unit" for carbon metabolism, as its phosphorylation state is determined by various signals, which in turn allow it to phosphorylate or to interact with numerous other proteins. P~ser-Hpr has been known to interact directly with specific ABC transporters and control their activity (29).

In addition, in the absence of glucose, P~his-Hpr phosphorylates the glycerol kinase GlpK and the PTS regulatory domain (PRD) containing transcriptional regulators that are necessary for its expression (31). P~his-Hpr phosphorylates and enhances the activity of transcriptional regulatory proteins, either antiterminators (e.g., SacT, SacY, LicT) or transcriptional activators (e.g., LevR, MtlR), resulting in enhanced

expression of operons important for utilization of less-preferred sugars. Therefore, this mechanism leads to CcpA-independent CCR (32).

1.6.3 CCR mediated by global transcription regulators

The Gram-negative Enterobactericiae contain several global transcriptional regulators playing a role in CCR, the best studied being cAMP receptor protein (Crp). When the preferred carbon source glucose is present, the $EIIA^{glc}$ remains unphosphorylated and inhibits the permeases transporting alternative carbon sources. However, in the absence of glucose, $EIIA^{glc}$ becomes phosphorylated and activates the conversion of ATP to cAMP via adenylate cyclase (111). The resulting cAMP can then bind Crp, which activates the expression of alternative catabolic operons.

All of the Gram-positive Firmicutes studied (except *Mycoplasma*) use the same pathway for CCR as *B. subtilis* (31). Since many Gram-positive bacteria, including *B. subtilis* and its close relatives, do not possess cAMP, a different mechanism operates in the cell (31). Catabolite control protein A (CcpA) is a global regulator and is a member of the LacI/GalR transcriptional regulator family that controls the expression of a wide variety of genes important for metabolism in Gram-positive bacteria (32). Numerous operons are subjected to catabolite control by both CcpA-dependent and CcpA-independent mechanisms. CcpA primarily represses expression of genes that might be involved in alternative sugar source utilization, but also activates transcription of genes that may function in glucose metabolism. CcpA regulates the expression of genes by binding to a catabolite response element (*cre*) sequence when complexed with P~ser-Hpr (27,33,34). Importantly, CcpA in lactic acid bacteria also

controls the general route of metabolism, such as glycolysis and lactic acid formation (35-37).

1.7 Carbon metabolism and Virulence

In many pathogenic bacteria, elements of CCR are crucial for the expression of virulence genes and for their pathogenicity. It is very interesting that many genes that encode virulence proteins, also influence in the uptake and use of alternate nutrients (38). Although the above statement is true for both Gram-negative and Gram-positive bacteria, my focus in this dissertation will be on the Gram-positive Firmicutes. Components of PTS and CcpA have been shown to be very important for the pathogenicity of the several Gram-positive organisms.

In *Bacillus anthracis,* the activity of the AtxA, virulence regulator of established virulence factors namely tripart toxin and capsule, is shown to be dependent on an intact PTS and the phosphorylation states of the components Hpr and EII (39). Toxin and capsule synthesis are both required for full virulence of *B. anthracis.* In addition, the induction of AtxA-mediated transcription was shown to be glucose-dependent and indirectly regulated by CcpA (40).

In *Listeria monocytogenes*, the transcriptional activator PrfA controls the expression of genes required for invading host cells for and release from phagosomes into the cytosol. The activity of PrfA is influenced by the presence of glucose or other PTS substrates (41). However, CcpA, HprK, P~ser-Hpr are not involved. Instead, the PTS sugar transporter EII^{Man} contributes to regulation of virulence gene expression by

glucose (42). Transcriptomic analysis suggested a possible role of the mannose-PTS in global CCR in *L. monocytogenes* and a link to bacteriocin resistance (43,44).

Enterococcus faecalis and *Enterococcus faecium* are the most prevalent enterococcal species cultured in humans. Interestingly, the PTS locus was found widespread in the isolates from hospital outbreaks of infection and clinical infection, but absent from human commensal isolates (45). In addition, CcpA modulates the expression of proteins involved in adhesion to host tissues. CcpA is important for the virulence of *E. faecalis*, where it regulates production of proteins involved in adhesion of the opportunistic pathogen to the host (46).

In the anaerobic *Clostridium perfringens*, the presence of glucose leads to repression of several virulence factor genes such as *pilT* and *pilD* that are involved in gliding motility and toxin production. Both of these functions are important for the pathogen to infect human tissues (47).

CcpA of *Staphylococcus aureus* appears to control a large regulon that comprises metabolic genes as well as genes encoding virulence determinants that are affected in a glucose-dependent as well as glucose-independent manner (48). Studies on CcpA and CCR in *S. aureus* revealed that CcpA mediates the expression of factors important for production of the superantigenic toxic shock syndrome toxin 1 (TSST-1) (49) and influences the enzymes involved in the proline reductase pathway (50). A *ccpA* mutant displayed reduced pathogen load in a murine model of staphylococcal

abscess formation (50). In addition, expression of an *agr*-regulated colony spreading protein is activated during *S. aureus* infection and is also regulated by glucose (51).

In pathogenic bacteria of the genus *Streptococcus,* CcpA and CCR are important for the expression of multiple virulence genes in several models of infection. In *S. pneumoniae*, CcpA is required for colonization of the nasopharynx as well as for survival and multiplication in the lung (52). Another study showed that the cellobiose PTS system is important for virulence in a murine pneumonia/sepsis model (53). *S. intermedius* secretes a human–specific cytolysin called intermedilysin (*ily*) that is required for infection and expression of *ily* is under CcpA control (54).

The oral pathogen *S. mutans* relies primarily on carbohydrates for energy production and their ability to generate acid for colonization of the oral cavity leading to dental caries. CcpA controls the expression of the virulence related functions such as expression of fructanase, acid formation and acid tolerance. Tight control of autolysis by *S. mutans* is critical for proper virulence gene expression and biofilm formation, and this process responds to environmental signals such as glucose (55). CcpA of another oral pathogen *S. suis* regulates the capsular polysaccharide synthesis important for virulence (56,57). CcpA and components of PTS such as Hpr, EI and some EIIs play a pivotal role in GAS pathogenesis and will be discussed in more detail later in this chapter.

1.8 Classification

1.8.1 General characteristics of *Streptococcus pyogenes*

S. pyogenes is a Gram-positive, non–motile bacterium that grows in chains of varying length. The typical size of a cell ranges from 0.5 μm - 1 μm in diameter. *S. pyogenes* is a non-spore forming facultative anaerobe and has an optimal growth temperature of 37°C. Like all streptococci, it is both catalase and oxidase negative. *S. pyogenes* typically produces large zones of β-hemolysis (the complete disruption of erythrocytes with the release of hemoglobin). Therefore, *S. pyogenes* is also called the Group A β-hemolytic Streptococcus (GABHS or GAS). GAS is a strict human pathogen.

1.8.2 Growth Requirement of *S. pyogenes*

S. pyogenes is a facultative anaerobe and is grown at 37°C in either ambient air or in 5-10% CO_2. GAS lacks the necessary enzymes for a functional TCA cycle and oxidative-cytochromes for electron transport; therefore, it relies completely on fermentation of sugars for growth and energy production. It is a member of the lactic acid bacteria and is homofermentative for lactic acid production from glucose fermentation. Specific components of a rich growth media for GAS include neopeptone extracts, glucose as a carbon source, and a complex mixture of nutrients from beef heart infusion as first described by Todd & Hewitt (58). GAS is considered a multiple amino acid auxotroph requiring nearly all amino acids to be present in its growth media. A Chemically Defined Medium (CDM) has been developed for GAS containing all of the necessary amino acids for GAS growth (59).

1.8.3 Lancefield grouping

The Lancefield typing assay was developed by Rebecca Lancefield in 1933 to serologically differentiate members of the genus *Streptococcus*. The protocol involves a precipitin assay that uses hot acid to extract the surface carbohydrate followed by incubation of the C-antigens to allow for the interaction (60). The strains were classified into five groups (A-E) and strains from human isolates were largely classified under the Group A.

1.8.4 M and T typing

In addition to differentiating streptococci by its surface carbohydrate, Lancefield also developed a method to characterize Group A Streptococcus based on the surface protein known as M protein. This assay involved acid extraction of the M protein from a given strain followed by a capillary precipitin reaction with standardized sera to determine the specific M type (60,61). Currently, there are more than 200 M-serotypes listed (62). To overcome the difficulty of antisera typing with great diversity of M types, a new method was developed by Beall and Facklam. In this method, a 5' hypervariable region of the *emm* gene encoding M protein is amplified, sequenced, and compared to a database of previously identified *emm* sequences (63). GAS strains are also characterized according to T antigen or pilin. A single T antigen may be found in strains belonging to different M types and sometimes the strains of the same M type may carry multiple T antigens.

1.8.5 Class determination

GAS serotypes based on M protein are further subdivided into two classes based on the presence or absence of an epitope detected by antibody against the C repeat region of the M protein and the presence of serum opacity factor (SOF) activity (64). Class I serotypes have an M protein that has a surface exposed C repeat region and lacks SOF. Class II serotypes lack the C repeat region, but contains the SOF.

1.8.6 Genetics

The complete sequence of *S. pyogenes* genome of the serotype M1 strain SF370 was first reported in 2001 (65). Soon after, many additional isolates were sequenced (66-71). Currently, the genome sequence of more than 13 strains, representing 10 serotypes, are publically available (72). The GAS genome ranges in size from 1.83 to 1.93 Mbp, and average GC content of 38.5% with the difference related to the number of bacteriophage present, which can vary between strains. Sequencing identified that the GAS contains a complete glycolytic pathway and 14 apparent PTS-sugar transport systems, but lacks the genes necessary for a TCA cycle. In addition, it was found that GAS only contains the genes for synthesis of a few amino acids, although there is a dedicated polyamine ABC transporter. M1T1 strain MGAS5005, a clinical isolate from the cerebrospinal fluid of an infected patient, was sequenced in 2005 (73) and contained the superantigen *speA2* allele. MGAS5005 was later found to be more virulent in animal models of GAS infection and has an invasive phenotype due to a mutation in *covS* (74-77).

1.9 GAS Clinical Presentation

GAS is capable of causing a wide range of diseases, ranging in severity from minor to life threatening. The minor and self-limiting infections include pharyngitis, impetigo and erysipelas. More severe and invasive infections include puerperal fever, streptococcal toxic shock syndrome (STSS), bacteremia and necrotizing fasciitis (flesh-eating disease). GAS is also associated with post immune sequelae such as acute rheumatic fever (ARF) and acute post streptococcal glomerulonephritis (APSGN). GAS is a cause of significant morbidity and mortality worldwide, where approximately seven hundred million people suffer from mild streptococcal infections every year (78). Globally, GAS causes an estimated 650,000 severe invasive infections at normally sterile sites.

1.9.1 Superficial Infections

1.9.1.1 Pharyngitis

The most common infection caused by GAS is streptococcal pharyngitis commonly known as “strep throat”. In 2005, a World Health Organization census found a minimum of 616 million cases of GAS pharyngitis worldwide. Strep throat is most prevalent in children between the ages of 5 and 12, although people of any age can be affected. The transmission of the bacteria primarily occurs by inhalation of aerosolized droplets or direct contact with respiratory secretions. The usual incubation period for streptococcal pharyngitis is 2 to 4 days. The common symptoms of pharyngitis include; fever, swollen cervical lymph nodes, headache, occurrence of petechiae on the soft palate, presence of erythema and exudates found on tonsils and pharynx (79). In acute cases of pharyngitis, nausea and abdominal pain can arise.

To confirm a pharyngeal infection, the "gold standard" practice involves swabbing the throat, including the pharynx and the tonsilar area, and culturing on a blood agar plate at 37°C with 5% CO_2. The plate is then checked for the presence of β-haemolytic colonies after 24-48 hours. Initial diagnosis is further confirmed by other methods including microscopy, resistance to bacitracin or latex agglutination (80). More recent systems use an optical immunoassay that detects the Lancefield group A carbohydrate (81). This method is called a "rapid strep test" and produces quicker results, although it lacks sensitivity and can sometimes lead to false negative results.

Pharyngitis is treatable by antibiotics, primarily penicillin and erythromycin. Even in the absence of antibiotics, a pharyngeal infection is self-limiting and all the symptoms disappear in a week. The predominant virulence factors associated with the throat infections are streptolysin O (*slo*), streptokinase (*ska*), pyogenic exotoxin A (*speA*), fibronectin-binding protein (*sfb*) and cysteine protease (*scp*) (82) (83).

1.9.1.2 Scarlet fever

Although scarlet fever was once a more prevalent and serious disease, the occurrence of this disease is now rare and mild in nature. It is described as pharyngitis with a rash or benign scarlet fever and is easily treatable (79). Benign cases can range from mild to moderate and usually presents with pharyngitis, a rash across the chest, fever, erythema of the cheeks, and strawberry tongue. More serious forms of scarlet fever include a high fever ranging from 107-108°F and painful swollen lymph nodes in addition to the above mentioned symptoms. These cases are typically mediated by a

toxin called streptococcal pyogenic exotoxins SpeA and SpeC, which have previously been associated with other outbreaks (84).

1.9.2 Streptococcal skin infections

1.9.2.1 Impetigo

Streptococcal impetigo, also known as pyoderma, is a skin infection of the dermis and epidermis. A small lesion on the skin of the extremities, usually the legs, characterizes typical streptococcal impetigo disease. These lesions can become a pus-filled, bacteria rich blister that with time, oozes and develops a thick golden crust. Impetigo is contagious, but is easily treated with penicillin (85,86). While impetigo, like pharyngitis, is a mild disease, both are linked to the streptococcal secondary sequelae, acute glomerulonephritis. Common virulence factors associated with skin infections are Mga (*mga*), M protein (*emm*), M-like proteins (*enn, mrp*), C5a peptidase (*scpA*), capsule (*has*), cysteine protease (*speB*), oligopeptide permease (*oppA – oppD),* streptokinase (*ska*), and regulator of protein F (*rof*) (87).

1.9.2.2 Cellulitis and erysipelas

Cellulitis is a streptococcal infection that results from a skin irritation or a puncture wound in the sub-cutaneous layer of the skin leading to inflammation. The typical symptoms include a pinkish skin color, along with swelling and pain. Cellulitis is frequently associated with individuals who have poor blood circulation or a weakened immune system. Cellulitis usually responds to penicillin treatment and is typically cleared in a week (79). Erysipelas is a more acute localized form of cellulitis, affecting the superficial layers of the skin. Typical symptoms are red, raised and well-demarcated skin lesions accompanied with pain and fever. Although it was a common

disease in the 19th century, it is rare today and is mostly seen in young children and elderly patients (88). As with other superficial GAS infections, penicillin is the treatment of choice and resolves the infection within a few weeks.

1.9.3 Invasive diseases

Invasive disease occurs when GAS leaves the primary sites (throat and skin) of infection and invades normally sterile tissues. These infections can be rapid and aggressive, resulting from a complex interaction between GAS and the human immune system.

1.9.3.1 Puerperal Fever

Puerperal sepsis (childbed fever) was one of the most deadly diseases of the 19th century. Puerperal fever can be contracted during pregnancy, miscarriage, or abortion and is marked by infection of the genital tract and endometrial lining. This acute endometritis is commonly masked by abdominal pain and is thus usually not recognized immediately following child delivery. Following entry into the women's endometrial lining, GAS can quickly spread to the surrounding structures and into the bloodstream (79). If left untreated, this infection can lead to death. However, with early diagnosis and antibiotic treatment, it is rarely fatal.

1.9.3.2 Bacteremia

Sometimes, GAS infection can spread from various sites to the bloodstream causing bacteremia. Pharyngitis-associated scarlet fever predisposes children to bacteremia, while in the elder, bacteremia is secondary to infection of the skin. Bloodstream infection is common in immune compromised patients, young children and intravenous (IV) drug users. Bacteremia will lead to death if not treated with

antibiotics intravenously (79).

1.9.3.3 Streptococcal toxic shock syndrome

Streptococcal toxic shock syndrome (STSS) consists of an invasive streptococcal infection followed by multiple organ failure. Skin infections are the most common portal of entry for streptococcal TSS (89) and it is often present alongside other deep-seated systemic infections such as bacteremia or necrotizing fasciitis. STSS is characterized by rapid onset of fever, high blood pressure, chills, nausea, confusion and pain that can lead to shock and overall tissue damage. Most of these symptoms are caused by GAS pyogenic exotoxins (SPEs) (79,90). Penicillin is the first-line antibiotic of choice for invasive disease (91).

1.9.3.4 Necrotizing fasciitis

Due to the severe tissue damage associated with this disease, this syndrome is also known as 'flesh-eating disease'. It is an infection of the subcutaneous tissue that results in progressive destruction of fascia and fat. Within a matter of days, the infection can progress from a benign skin lesion to a lethal disease. Unless appropriate immediate steps is taken, the infection may quickly become gangrenous, with inflammation spreading along the fascia and leading to organ failure (92).

1.9.4 Secondary Sequelae

In certain circumstances, otherwise uncomplicated benign infections with GAS can sometimes slowly progress to cause post-streptococcal glomerulonephritis (PSGN) and acute rheumatic fever (ARF) resulting in high mortality and morbidity. These diseases follow a few weeks or months after the primary GAS infection and after the bacteria have been cleared from the body. Symptoms are often due to an autoimmune

reaction where antibodies against GAS antigens instead attack healthy human tissue. More than 15 million people worldwide suffer from PSGN and ARF that claim over 350,000 human lives every year (78).

1.9.4.1 Acute Rheumatic fever

Acute rheumatic fever (ARF) is a major non-suppurative sequelae of GAS pharyngitis that occurs 2-3 weeks after the initial infection (85,89). The diagnostic criteria for ARF are referred to as the major Jones criteria and include arthritis, erythema, carditis and neurological dysfunctions (chorea) (93). Most patients with ARF are children, adolescents and young adults, and a third of the affected population suffers damaged heart valves that warrant replacement surgery. Several streptococcal factors such as superantigens (*spe*), group A carbohydrate, capsule (*has*), and M protein (*emm*) have been suspected to trigger the autoimmunity in ARF (93).

1.9.4.2 Poststreptococcal Glomerulonephritis (PSGN)

PSGN can occur from 1-3 weeks following GAS skin infection, pharyngitis, or scarlet fever (94). This severe disease results from immune complex formation in nephritic tissues that can lead to kidney failure. The diseased kidneys are seen to contain structures in the glomerular capillary basement membrane called sub-epithelial "humps" or "casts" (95). Streptococcal components, host complement factors and immunoglobulin are found localized in these structures (96).

1.9.5 Treatment and vaccines

GAS has been recognized as an important human pathogen since the early days of modern microbiology and remains among the top ten causes of mortality from an infectious disease globally. Although a historical perspective indicates major

reductions in overall GAS infection rates in the modern era, this pathogen still remains a leading health concern. Currently, there is no vaccine available for GAS. Existing treatments for GAS infection include the administration of penicillin, macrolides, or intravenous immunoglobulin (Ig). Penicillin remains the most common drug used for treating GAS infection for both superficial infections and prophylactically to prevent recurring GAS infection and immune sequelae (97).

Despite such treatment measures, GAS autoimmune sequelae persist at endemic levels in developing countries and in indigenous populations (78). Furthermore, some studies have measured a 20-40% failure rate of penicillin in the treatment of GAS pharyngitis, warranting the need for vaccine development (98). Possible factors contributing to the failure of penicillin treatment include co-colonization of other penicillin-resistant bacterial species, eradication of normal throat flora, invading the tissue sites, and patient compliance (98). Macrolides are utilized as an alternative to penicillin, particularly in cases of penicillin allergy, although there is concern that macrolide-resistant isolates may evolve and spread (99). In cases of invasive GAS disease, intravenous Ig is recommended as treatment. The timing of administration is critical, if administered too long after the initial diagnosis, intravenous Ig will not provide any benefits greater than if antibiotics alone were used for treatment. Furthermore, intravenous Ig only offers short-term protection, as no immunological memory is generated. While in most cases penicillin or alternate antibiotics remain effective in the treatment of superficial GAS infection, the costs of medical care and time off from work or school create a significant economic burden (99).

An effective vaccine should contain a conserved GAS epitope, be highly immunogenic, induce both IgG and IgA, and not provoke cross reactions with human tissues (100). Vaccine development has focused on the N-terminal of the M protein since Lancefield had shown that antibodies against this region are both protective and bactericidal (101). However for this strategy, even the newest 26-valent vaccine would be limited in the serotypes it can protect against, and some antibodies against M protein are cross-reactive with human tissue and could lead to ARF. Alternative vaccine strategies have focused on other antigens such as C5a peptidase, the group specific carbohydrate, and the pyrogenic exotoxins (100).

1.10 Virulence factors

GAS expresses a wide variety of virulence factors that have a known or predicted role in helping the GAS to colonize the host, evade the host immune system, or spread to sterile sites. Many of these molecules remain associated with the streptococcal cell, while others are released into the extracellular environment. Important cell associate factors include: M and M-like proteins, lipoteichoic acid, MSCRAMMs, streptococcal collagen like protein, C5a protease, capsule, serum opacity factor, streptolysin S, protein G-related **α**2-macroglobulin-binding protein (GRAB), and *S. pyogenes* cell envelope protease (SpyCEP). Important secreted factors include: streptolysin O, streptococcal pyrogenic exotoxin B (SpeB), streptococcal inhibitor of complement, streptokinase, *S. pyogenes* NAD-glycohydrolase, IgG-degrading enzyme, the superantigens (pyrogenic exotoxins) and DNases. Listed below are the virulence factors relevant to this study.

1.10.1 M and M-like Proteins

M protein is the major surface protein of GAS encoded by the gene *emm.* The genes encoding the structurally similar M-like proteins, including *mrp, arp, emmL, fcrA, enn, and sph*, have been categorized by their domain organization (85). M protein and M–like proteins are important for adhesion to various host tissues and extracellular matrix (ECM) components, immune evasion of GAS, and triggering internalization into non-phagocytic cells (102). Most strains of the GAS typically produce 1 to 3 M or M-like proteins, which in some cases leads to correlation with disease type (103). One of the major functions of the M protein is to protect the GAS cell from phagocytosis by the host. It does this by binding to the complement regulatory protein H to inhibit and interfere with opsonization of the cell.

1.10.2 Hyaluronic acid capsule

The hyaluronic acid (HA) capsule of the GAS, encoded by the *has* operon (*hasABC*), is composed of repeating units of glucuronic acid and N-acetylglucosamine identical to the HA produced in the human host. The presence of capsule leads to the mucoidy or glossy colony phenotype observed by Todd in 1928 (104). The *has* operon is negatively regulated by the CovR/S two-component system (105,106). The capsule of GAS is important in resistance to phagocytosis as acapsular mutant strains lose their ability to resist phagocytic killing and have 100-fold decreased virulence in mice (107). In a skin infection model, acapsular mutants produced no lesions or minor inflammation compared to the necrotic lesions with purulent inflammation seen in the encapsulated strain (108).

1.10.3 Streptococcal Opacity Factor (SOF)

SOF is an important adhesin of GAS usually found in Class II GAS strains. It helps to bind streptococcal cells to high-density lipoproteins through a fibronectin-mediated process (109,110). SOF appears to have two distinct activities, having roles in both serum opacification and fibronectin/fibrinogen binding, as mentioned previously (109). Although its direct role in virulence has been difficult to discern because of its bi-functional nature, studies have shown that inactivation of *sof* resulted in a reduction of virulence in an i.p. mouse model of infection (53).

1.10.4 Streptolysin S (SLS)

SLS is an oxygen-stable cytolysin/hemolysin responsible for the ß-hemolytic phenotype seen surrounding colonies on a blood agar plate. The structural toxin SLS is encoded by *sagA*, a streptolysin-associated gene (*sag*), and is the first gene of a 9-gene operon. This cytolysin is a potent virulence factor *in vivo* that lyses a variety of cell types including erythrocytes, lymphocytes (111), neutrophils, platelets, and even intracellular organelles such as mitochondria and lysozomes (112). Studies in mice using the subcutaneous skin infection model show a significant reduction in virulence with SLS mutant strains (106,113,114). Additionally, SLS has been shown to impair phagocytic clearance, promote epithelial cell cytotoxicity (115) and mediate the cytocidal effect of GAS on neutrophils (116), demonstrating SLS as a potent virulence factor used by GAS for host immune evasion.

1.10.5 Streptolysin O

Another secreted cytolysin/hemolysin is the oxygen-labile streptolysin O (SLO),

which is a member of the cholesterol-dependent cytolysin (CDC) family of pore forming toxins (117). Despite having pore-forming ability similar to SLS, SLO does not contribute to the ß-hemolytic phenotype due to its sensitivity to oxygen. SLO has been shown to be important for invasive disease in a mouse skin infection model. However, the exact role of SLO is still unknown, as it does not appear to be involved in the formation of necrotic lesion or bacterial dissemination for GAS (118).

1.10.6 Streptococcal pyrogenic exotoxin B (SpeB)

SpeB is a cysteine protease encoded on a chromosomal gene *speB* (119), therefore most streptococcal strains are capable of producing it. SpeB is secreted as an inactive 40-kDa zymogen that is converted into a mature 28-kDa active protease either by autocatalytic cleavage or aided by host or streptococcal proteases (120). Regulation of *speB* transcription and SpeB expression is complex and involves environmental factors, transcriptional and posttranscriptional regulation, and posttranslational regulation by an endogenous inhibitor encoded on the streptococcal chromosome (121). SpeB targets a large variety of both bacterial and human proteins (121). Although the role of SpeB in GAS infection is controversial, recent evidence has conclusively demonstrated that SpeB is critical for the pathogenesis of severe invasive disease caused by GAS (121).

1.10.7 Streptococcal inhibitor of complement (Sic)

Sic, encoded by *sic*, incorporates into the membrane attack complex of complement to inhibit complement-mediated lysis (122). Sic also aids in bacterial survival at mucosal surfaces and has been shown to bind and alter the activities of lysozyme and

the secretory leukocyte proteinase inhibitor (122). Sic is a multifunctional virulence factor, able to interact with various host cell proteins and members of the immune system.

1.11 Regulators of virulence

GAS expresses a large number of virulence factors in a coordinated manner to successfully cause infection in the human host (Fig. 2). To accomplish this, GAS employs a number of transcriptional regulators. GAS is able to sense changes in the host environment and adjust the expression of virulence determinants to its advantage. These virulence regulators include classical two-component regulatory systems (TCS) and stand-alone regulators.

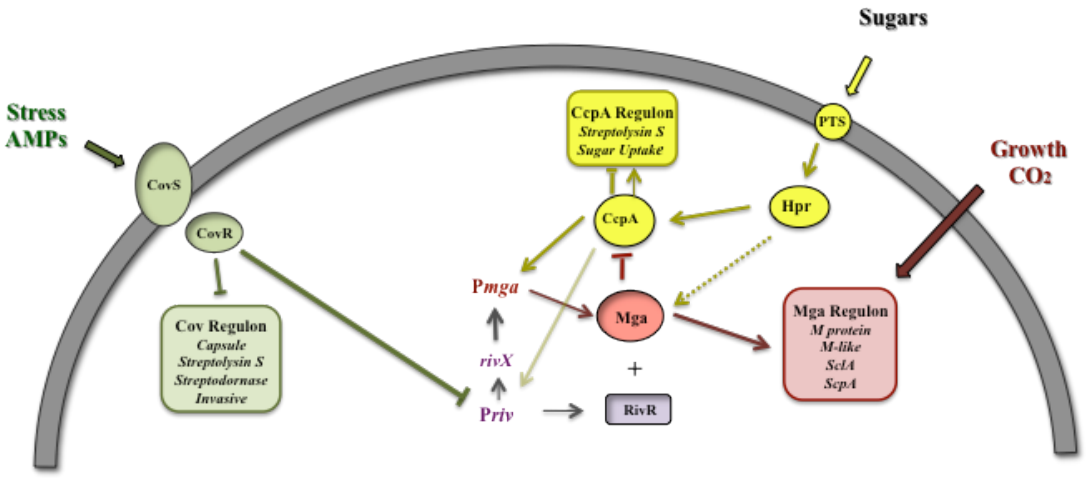

Figure 2: Interaction between virulence regulators of GAS. Schematic shows the two-component system CovR/S, stand-alone regulators Mga, RivR/RivX along with PTS and CCR regulator CcpA. Arrowheads indicate activation and flat ends indicate repression.

1.11.1 CovR/S

The most studied TCS of GAS is the Control Of Virulence (CovR/S) system, that responds to environmental stresses such as pH, temperature, and osmolarity (75). The predicted extracellular domain of the membrane bound sensor component of CovS is expected to bind an extracellular ligand or to sense the environmental conditions mentioned above. This interaction is predicted to trigger autophosphorylation of the cytoplasmic domain of the CovS protein. The phosphate group is then transferred on to the receiver domain of the cognate regulator CovR. The regulator component CovR, when phosphorylated, shows increased binding affinity for the promoters of several genes proposed to be regulated by the system. CovR/S has been shown to directly or indirectly influence expression of approximately 15% of the GAS genome (76), with the majority of the genes being repressed. Numerous studies have demonstrated the significance of CovR to expression of virulence factors within a host and they suggest that spontaneous mutations in CovRS allow GAS to become more invasive (75,123,124). These *cov* mutant GAS strains appear to be most prevalent in causing infections worldwide. Microarray analysis comparing wild type and a *covS* mutant show significant differences in the transcription of many genes that are associated with virulence *in vivo* (125). The data from this study and others suggest that CovR/S has an extremely significant role in modulating gene expression during GAS infection. The regulation of virulence within GAS is complex, and as the master regulator, CovR/S directly or indirectly interacts with multiple regulators such as Mga, CcpA, the two-component system TrxRS, and RivR.

1.11.2 Mga

Multiple virulence gene regulator of GAS (Mga) is a key stand-alone transcriptional regulator. The gene encoding the multiple gene regulator of GAS or Mga (*mga*) has been found in all sequenced GAS genomes and strains tested, exhibiting two divergent alleles (*mga-1*, *mga-2*) for M1 Mga or M4 Mga that correlate with different tissues sites of infection (126). Mga regulates expression of approximately 10% of the genome by binding to the core set of virulence gene promoters. The core regulon is composed of key virulence factors that Mga activates during the carbohydrate rich exponential phase, including genes encoding M protein (*emm*), M-like proteins (*arp* and *mrp*), C5a peptidase (*scpA*), and the streptococcal inhibitor of complement (*sic*) (127,128). Mga has an indirect effect on operons involved in carbohydrate metabolism and other metabolic processes (126). Mga is critical for multiple pathogenic phenotypes, including biofilm formation, growth in whole blood, resistance to phagocytosis and optimal virulence (126,129,130). Mga (62kDa) possesses two helix turn helix domains and one conserved Mga domain (CMD-1) at N terminus that have been shown to be involved in DNA binding (127,131). A C-terminal PTS Enzyme II-B (EIIB) domain has also been predicted. A recent study from our lab has shown that the catalytic histidine residues in the PRDs are important for the expression of Mga regulon (6). Based on sequence alignment, two regions within Mga were predicted to have strong homology to the dual system PRD in antiterminators (e.g., LicT) and activators (e.g., MtlR and LicR) involved in the regulation of sugar metabolism (126). Thus, Mga appears to represent the paradigm for a newly appreciated family of PRD-containing virulence regulators (PCVR) that

includes the important RofA-like protein (RALP) family of virulence regulators in GAS (RofA, Nra, Ralp3, and RivR).

1.11.3 RALPs

The RofA-like protein (RALP) family of transcriptional regulators was initially identified in GAS and now has orthologous members in several pathogenic streptococci, including *S. dysgalactiae* (GCS, GGS), *S. agalactiae* (GBS), and *S. pneumonia*. RALP's regulon comprises genes important for GAS-host cell interactions such as SLS and SpeB (132). GAS strains possess at least one copy of either RofA or Nra.

***RofA (RALP1)*:** RofA has been shown to negatively influence the expression of virulence factors such as *sagA*, *speB* and *mga* (132). RofA responds to changing atmospheric conditions and a *rofA* mutant leads to decrease in host cell viability along with reduced attachment and internalization to epithelial cells (133).

Nra (RALP2): Similar to RofA, Nra repressed the expression of several virulence genes, including *speB*, *speA, sagA,* and *mga, slo.* In addition, Nra represses the expression of other stand-alone regulators such as Rgg, RALP3, and RivR (RALP4). On the other hand, Nra positively influenced the expression of metabolic genes involved in transport and utilization of carbohydrates again, demonstrating a link between virulence and metabolism for RALPs (134).

***RALP3*:** Serotype specific distribution of *ralp3* has been observed. In an invasive M1T1 strain of GAS, mutations in *ralp3* influenced host-cell interactions and antimicrobial peptide sensitivity leading to inability to survive in whole blood and attenuation of systemic infection in mice (134). M1 Ralp3 was found to strongly

repress capsule synthesis (*has*) and protease (*speB*) expression (132).

***RALP4*:** Studies have shown that the expression of RivR is directly controlled by CovRS. DNA microarray analysis in an M1 strain determined that RivR activates Mga and its virulence regulon. RivR is associated with RivX and a *ΔrivRX* mutant was attenuated for invasive skin infection of mice (135,136).

1.12 Carbon metabolism and virulence in GAS

Regulation of virulence factor expression is critical for pathogenic microorganisms that must sense and adapt to a dynamic host environment. The global regulator, CcpA contributes to GAS virulence in various infection sites (137) and is involved in modulating the regulation of virulence factors. A study by Watson et al, demonstrated that in the absence of CcpA, lactate oxidase (*lctO*) levels are elevated, resulting in excessive generation of peroxide to self-lethal levels in a vaginal colonization model (138). In another study, Δ*ccpA* and Δ*covR*Δ*ccpA* isogenic mutant strains resulted in significant reduction in virulence in a mouse myositis model compared to the wild type parental strain (139). Another LacI/GalR family member, MalR also plays a role in GAS global gene expression. Inactivation of the maltose regulator, MalR, resulted in reduced GAS colonization of the mouse oropharynx by repressing a cell surface carbohydrate binding protein, but did not detrimentally affect invasive infection (140). To further the importance of the connection between carbohydrate metabolism and virulence in GAS, Almengor *et al.* suggested that CcpA regulates the expression of the virulence regulator Mga by binding to the *cre* site in the promoter region of Mga (141). In addition, carbohydrate utilization and metabolism genes are differentially regulated in the biofilm and tissue communities, suggesting that nutrient

acquisition is important at many steps in the disease formation (129). The presence of nutrients in the environment primarily sugars modulates the expression of factors crucial for pathogenesis (142).

1.13 Significance of Dissertation

The group A Streptococcus can cause a wide-range of infections. A hallmark of GAS is the ability to successfully colonize and adapt to many different tissue sites in the human host. Several studies have established that GAS exhibits transcriptomic changes during infection (123,129,130,143). Genes required for carbohydrate uptake (both PTS and ABC) along with metabolic operons and genes involved in CCR are induced *in vivo* and are required for full virulence in models of GAS infection (144-146). Therefore, GAS appears to depend upon carbohydrate uptake systems such as the PTS for their ability to survive in the host and elicit disease (6).

This study addresses the role of the uncharacterized PTS in both carbon metabolism and virulence of *Streptococcus pyogenes*. This work begins with a general characterization of the PTS by constructing a *ptsI* mutant in serotype M1T1 and M4 strains of GAS. The growth assays in Chemically Defined Media (CDM) and carbon metabolic assays using Biolog phenotype microarrays indicated that the PTS influences the uptake and metabolism of several carbohydrates.

The second focus of my research assessed the role of EI (PTS) in the pathogenesis of GAS. Interestingly, the Δ*ptsI* mutant strain resulted in a hypervirulent phenotype in a subcutaneous model of GAS infection, which is attributed to the temporal expression

of the hemolysin SLS. It was also found that the PTS influences the activity of additional virulence factors, SpeB and Capsule.

The third focus of thesis study was to investigate the regulatory role of PTS in influencing the activity of the PRD-containing virulence regulator Mga. Transcriptional studies in M1T1 and M4 suggested that PTS influences Mga regulon in strain specific manner.

Overall, this study demonstrates that PTS in GAS is required for uptake of PTS and non-PTS associated carbohydrates. In addition, PTS also influences the virulence of Group A Streptococcus by modifying the virulence factors and global regulators.

Chapter 2: Materials and Methods

2.1 Bacterial Strains

2.1.1 *E.coli* strains, medium, and growth conditions

E. coli strains DH5α and C41 [DE3] were used as the host for plasmid constructions (Table 1). All *E. coli* strains were grown in Luria-Bertani LB broth (EMD Chemicals). *E. coli* was grown at 37ºC with shaking at 250 rpm under normal aerobic conditions. Growth was assayed by a spectrophotometer (Ultraspec 10, Amersham Biosciences) at $OD_{600.}$ Antibiotics were used at the following concentrations: spectinomycin at 100 μg/ml, kanamycin at 50 μg/ml, and erythromycin at 500 μg/ml.

2.1.2 GAS strains, media, and growth conditions

Group A streptococcus (GAS) strains are listed in Table 1. Strain 5448 is a clonal M1T1 strain isolated from an invasive infection and 5448AP is an isogenic animal passaged *covS* variant (124). MGAS5005 (*covS*) is also an invasive M1T1 strain with an available genome sequence (73). GA40634 is a clinical isolate of the GAS M4 serotype. GAS was cultured in complete Todd-Hewitt medium supplemented with 0.2% yeast extract (THY; Alpha Biosciences). A 2X Chemically Defined Medium (CDM) was prepared by MP Biomedical as previously described (59,147). Prior to use, freshly prepared sodium bicarbonate (59.51 μM) and L-cysteine (11.68 μM) were added along with a carbohydrate source at a final concentration of either 0.5% (D-glucose) or 1% (all other sources). C medium was prepared as mentioned in (148). Growth of GAS was assayed by measuring absorbance in Klett tubes using a

Klett-Summerson colorimeter (A filter). Alternatively, overnight cultures of GAS (10 ml) in CDM were adjusted to OD_{600} of 0.2 in saline, appropriate carbohydrate was added, and 50 µl aliquots were added to each well of a 24-well microplate (Corning) followed by sealing with plate tape. Growth was followed for 12 hours at 37ºC using a FLUOstar Omega microplate spectrophotometer (BMG), with measurements taken at 30 minutes intervals after 15 minutes shaking. Antibiotics were used at the following concentrations: erythromycin at 1.0 µg/ml for GAS, spectinomycin at 100 µg/ml and kanamycin at 300 µg/ml for GAS.

2.2 DNA manipulations

2.2.1 Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* using the Wizard® Plus SV Miniprep system (Promega) following manufacturer's instructions. DNA fragments were gel purified from agarose using the Qiaquick Gel Extraction kit (Qiagen) or the Wizard® SV Gel and PCR Clean-Up system (Promega).

2.2.2 Genomic DNA extraction

GAS genomic DNA (gDNA) was isolated as previously described (149). Briefly, 15 ml cultures were grown overnight at 37ºC with 20mM glycine. Cells were pelleted and washed with 10mM Tris by centrifugation at 8000x g for 10 minutes. The washed pellet was resuspended in Solution I (1M Tris pH 8.0, 0.25 M EDTA pH 8 and 50% Sucrose) supplemented with fresh lysozyme (130 mg/ml) and incubated for 90 minutes with rotation at 37ºC. Cells were pelleted and resuspended in Solution II (1M Tris pH 8.0, 0.25 M EDTA, 20% SDS) and incubated at 37ºC for 15 minutes. To this,

RNAse A (10mg/ml) and Proteinase K (20 mg/ml) were added and incubated at 55ºC for 30 minutes with frequent mixing. The cell lysate was extracted once with an equal volume of TE-saturated phenol, with 1:1 phenol-chloroform until the interface is cleared, and once with 24:1 chloroform-isoamyl alcohol. A 2:1 volume of ethanol: lysate was added and DNA was precipitated overnight. The DNA was pelleted after overnight incubation and the pellet was resuspended in water. DNA concentrations were measured by the A_{260}.

2.2.3 Polymerase Chain Reaction (PCR)

PCR was performed using Phusion high-fidelity polymerase for cloning and Taq DNA polymerase for diagnostic assays (New England Biolabs, NEB). To use Phusion polymerase, annealing temperatures for primers were determined by using Tm calculator (https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator). Amplification was carried out by an initial denaturation step at 98ºC for 4 minutes, followed by 35 cycles of denaturation at 98ºC for 10 seconds, a 30 seconds annealing step at a pre-determined temperature, an extension step of 72ºC for approximately 15-30 seconds per kb of DNA. A final extension step for 4 minutes was added for the completion of the reaction. PCR reactions were purified according to manufacturer's instructions using Wizard SV PCR clean up kit. To use Taq polymerase, the PCR protocol was modified from Phusion: briefly, 30 cycles were used, denaturation was performed at 95ºC, and extension time used was ~1kb/min. Annealing temperatures for use with Taq polymerase were determined through Vector NTI software (version 11.0). Genewiz, Inc performed DNA sequencing analysis.

2.2.4 Enzymatic DNA modifications

Enzymatic DNA modifications were performed using enzymes with conditions suggested by the manufacturer (NEB). Restriction digests were performed in the buffers supplied with the enzymes by the manufacturer for 2 hours. Ligation reactions using T4 DNA ligase were set up using 1:16 vector to insert ratio with overnight incubations at 16ºC. Antarctic shrimp alkaline phosphatase was used to dephosphorylate the vector ends for cloning with incubation at 37ºC for 1 hour.

2.3 Bacterial transformation

2.3.1 *E. coli* competent cells

To prepare DH5α or C41 [DE3] competent cells, an overnight starter culture was started from a single colony streaked out on a LB agar plate. The next morning, 500 ml of LB broth was inoculated with 5 ml of an overnight culture and was grown to an OD_{600} of 0.5-0.7 nm. Cells were then placed on ice for 30 minutes to cool cells and stop growth prior to centrifugation at 7000x g for 30 minutes at 4ºC. After pelleting, cells were washed and resuspended in ice-cold sterile 10% glycerol (EP solution) and washed twice more. After the final wash, cells were resuspended in 800 µl of EP solution and split into 50 µl aliquots, which were stored at -80ºC for 6 months to one year.

2.3.2 GAS competent cells

To prepare competent GAS cells for transformation, 150 ml of THY broth with 20 mM glycine was inoculated with 7.5 ml of an overnight starter culture and incubated

static at 37ºC until OD_{600} was between 0.2 and 0.4 nm. Cells were kept on ice prior to centrifugation at 7000 x g for 30 minutes at 4ºC. The pelleted cells were washed and resuspended in 20 ml of EP solution, and centrifuged again twice more. Upon completion of the washes, the pelleted cells were resuspended in 1 ml EP solution and split into 200 µl aliquots, which were stored at -80ºC for 6 months.

2.3.3 Electroporation

To remove excess salts prior to electroporation of DNA into either *E. coli* or GAS, the DNA was drop dialyzed against H_2O using 0.025 µm membrane filters (Millipore) for 30 minutes for *E. coli* and 1 hour for GAS. Electroporation of both *E. coli* and GAS was performed using a GenePulser Xcell (Bio-Rad). The 50 µl *E. coli* competent cell aliquot was mixed with drop dialyzed DNA in a pre-chilled 2 mm cuvette and transformed using the electroporator settings as follows: 2.5 kV, 200 Ω, and 25 µF. Cells were then added to 1 ml LB broth and outgrown for 1 hour at 37ºC with shaking, prior to centrifugation at 6,000 x g. Pelleted cells were resuspended in 200 µl saline and 100 µl were plated with the appropriate antibiotic for selection. Electroporation of GAS was carried out using the electroporator settings as follows: 1.75 kV, 400 Ω, and 25 µF. After electroporation, GAS cells were added to 10 ml THY broth and outgrown for 2-4 hours, static at 37ºC. Cells were pelleted at 8,000 x g and resuspended in 200 µl saline and plated with the appropriate antibiotic for selection.

2.3.4 Temperature-sensitive allelic exchange

Transformation of GAS with the temperature-sensitive plasmid pCRK (Table1)

containing kanamycin (Kan) was used for selection for allelic exchange. Briefly, after electroporation, cells are outgrown at 30ºC prior to plating with Kan selection at 30ºC overnight to allow for plasmid replication. Isolated colonies are then inoculated into liquid cultures with Kan and any other applicable antibiotics for selection and passaged overnight at 30ºC. Cells are passaged one more time in fresh media, without Kan selection to allow for possible integration.

2.4 Genetic Constructs

2.4.1 Construction of a Δ*ptsI* mutant in GAS

The plasmid to generate the Δ*ptsI* allele was created as previously described (6). Briefly, the primers ptsI-1a and ptsI-1b (Table 2) were used to amplify from MGAS5005 genomic DNA (gDNA) an 819 bp upstream region containing 578 bp of *ptsI*, and a BglII restriction site. Primers ptsI-2a and ptsI-2b (Table 2) were used to amplify an 812 bp region containing the 3′of *ptsI* with BglII ends and a 10 bp overlap with the first fragment at the 5′end. These fragments were combined as template DNA for PCR SOEing (150) with ptsI-3a and ptsI-3b (Table 2) to generate the *ptsI* deletion. The resulting product was blunt end ligated into pBluescript II KS- to create pBlueΔptsI (Table 1). The non-polar *aad9* spectinomycin resistance cassette was amplified from pSL60-1 using primers aad9L2-bglII and aad9R2-bglII (Table 1), digested with BglII, and ligated into BglII-digested pBlueΔptsI. The resulting XbaI/XhoI*ΔptsI*::*aad9* fragment from pBlueΔptsI was ligated into XbaI/XhoI-digested pCRK to yield pKSM645, which was used to construct the Δ*ptsI* mutant MGAS5005.Δ*ptsI* (Table 1). Additional mutants were constructed in 5448

(5448.Δ*ptsI*) and 5448AP (5448AP.Δ*ptsI*) using the same protocol (Table 1). GAS Δ*ptsI* mutants were screened for sensitivity to kanamycin and verified by PCR on genomic DNA for specific junction regions.

2.4.2 Single-copy complementation of MGAS5005.Δ*ptsI*

A thermo sensitive integration plasmid for single-copy complementation was constructed using primers ptsIcomplementL and ptsIcomplementR (Table 2) to amplify a 1919 bp fragment containing a promoterless wild type copy of *ptsI* and BamHI restriction site on both ends. The resulting PCR fragment was digested with BamHI, ligated into BamHI digested pCRK to yield pKSM456 and pKSM455 (Table 1), and confirmed by sequence analysis. pKSM456 was electroporated into the mutant MGAS5005*ΔptsI* at the permissive temperature (30°C) and integrants were isolated following growth at the non-permissive temperature (37°C) to allow for complementation of the *ΔptsI* allele in single copy. The resulting strain MGAS5005.Δ*ptsI*c was verified by PCR and sequence analysis. Similarly, GA40634. .Δ*ptsI*c was constructed using pKSM455.

2.4.3 Construction of insertional- inactivation of *sagB* in MGAS5005.Δ*ptsI*

A *sagB* insertional inactivation vector, pKSM732 (145) was transformed in MGAS5005.Δ*ptsI* using temperature sensitive inactivation strategy as described previously. Strains were verified by loss of hemolysis on blood agar plate and PCR.

2.5 Murine infections

2.5.1 Subcutaneous route

An overnight culture (10 ml) was used to inoculate 75 ml of THY and incubated static at 37°C until late logarithmic phase. Approximately 3 x 10^7 CFU/ml, as determined by microscope counts and verified by plating for viable colonies, was used to infect 5 to 6-week-old female CD-1 mice (Charles River Laboratories). The mice were anaesthetized with ketamine and depilated for ~ 3 cm^2 area of the haunch with Nair (Carter Products) and 100 µl of a cell suspension (3 x 10^8 CFU/mouse) was injected subcutaneously. Mice were monitored twice daily for 7 days and were euthanized by CO_2 asphyxiation upon signs of morbidity. Lesion sizes (L x W) were measured at 38 hours post infection with length (L) determined at the longest point of the lesion and width (W) as the widest point. Lesion size data was analyzed using GraphPad Prism (GraphPad Software) and tested for significance using an unpaired two-tailed t-test with 99% confidence.

2.6 RNA analysis

2.6.1 RNA isolation

GAS from an overnight culture were inoculated 1:20 into 10 ml THY supplemented with appropriate antibiotics and grown to the appropriate Klett unit. Cells were then pelleted by centrifugation at 8,000 x g for 20 minutes at 4°C. Cells were resuspended in 1 ml of TE buffer (10 mM Tris pH 7.4 and 1 mM EDTA) with 0.2% (v/v) Triton X-100 and boiled for 10 minutes. The lysate was extracted three times using

chloroform-isoamyl alcohol, EtOH precipitated for 20 minutes at -80°C, pelleted at 13,000 x g for 15 minutes at 4°C, and the RNA pellet was resuspended in DEPC-treated H_2O. RNA quality was assessed on a formaldehyde gel (18% (v/v) formaldehyde, 1% (w/v) agarose, 72% (v/v) DEPC-treated H_2O, and 10% (v/v) 10x MOPS buffer (0.4 M 3-[N-Morpholino] propanesulfonic acid pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA). To quantify RNA, absorbance at 260/280 was determined using a spectrophotometer (Amersham BioSpec). RNA (5 ug) was treated with DNase using the Turbo DNA Free kit (Ambion) per manufacturer's protocol. Treated RNA was assessed for quality as mentioned above and for gDNA contamination by PCR analysis.

2.6.2 Real-time RT-PCR

25 ng of DNase-treated total RNA was added to SYBR Green Master mix (Applied Biosystems) along with 5 μg of each gene specific real-time primer (Table 2) using the 1-step protocol. The real-time RT-PCR experiments were completed using a Light Cycler 480 (Roche) and levels presented representing ratios of wild type/experimental relative to the level of *gyrA* transcript as the internal control. The real-time primers were designed using Primer 3: WWW Primer tool (biotools.umassmed.edu/bioapps/primer3_www.cgi).

2.7 Protein Analysis

2.7.1 TCA precipitation of secreted proteins

GAS cultures were grown to stationary phase as described elsewhere and culture supernatants were prepared by centrifugation at 8,000 g for 15 minutes at 4ºC. Bovine

Serum Albumin (BSA) was added to a final concentration of 50 µg/ml and trichloroacetic acid (TCA) to a final concentration of 15%. The resulting solution was incubated on ice for 30 minutes and centrifuged for 15 minutes at 12,000 g. The pellet was washed twice with 500 µl of ice-cold acetone, centrifuged for 15 minutes at 12,000 g, and the resulting pellet was resuspended in 50 µl of 1X β-mercaptoethanol.

2.7.2 Western Blot

GAS proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with α-SpeB antiserum at a 1:500 dilution for 2 hours at room temperature. After three 10 minutes washes with PBS-Tween, blots were incubated with goat α-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma) at a 1:12500 dilution for 1 hour. The blots were then washed three times with PBS-Tween for 20 minutes. Blots were developed using the Western Lightning chemiluminescence system (Femto) and visualized using a Fuji LAS3000 imager (GE Healthcare).

2.7.3 Biofilm formation

Overnight cultures of GAS grown at 37ºC (5% CO_2) in THY were harvested and used to inoculate fresh THY. Cultures were then grown to an OD_{600} of 0.5 nm. Tissue culture treated polystyrene six-well cell culture plates (Corning) were seeded with 3 ml of culture per well. Plates were incubated for 24 hours at 37°C, 5% CO_2. Medium was removed without disturbing the biofilm, wells were washed three times with distilled H_2O, and 1 ml aliquots of 0.1 % crystal violet (CV) (Sigma-Aldrich)

dissolved in dH_2O were dispensed to each well. Surface-attached bacteria were allowed to stain for 15 minutes at room temperature and then washed three times with dH_2O, after which 1 ml ethanol was added to each well to solubilize the CV. Absorbance was measured at 600 nm for each sample.

2.7.4 Hyaluronic acid capsule production

The amount of hyaluronic acid (HA) capsule produced by each GAS strain was determined as follows: cells from a 10 ml exponential phase culture were washed twice with 10 mM Tris pH 8.0, suspended in 0.5 ml 10 mM Tris pH 8.0, and capsule was released by extraction with 1 ml chloroform. After clarifying the sample by centrifugation, the HA content of the aqueous phase was determined by measuring absorbance at 640 nm after adding 2 ml of a solution containing 20 mg of 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naptho-[1,2-d]thiazolium bromide (Stains-all; Sigma Chemical Co., St. Louis, MO) and 60 µl of glacial acetic acid in 100 ml of 50% formamide.

2.7.5 Hemolysis assay

GAS were grown in THY supplemented with 10% heat-inactivated horse serum (Sigma). Samples were taken every hour for a total of 8 or 14 hours and immediately frozen at −80°C, bacterial cells were pelleted, and a 1:10 dilution was made of the supernatant. 500 µl of this dilution was added to an equal volume of 2.5% (v/v) difibrinated sheep red blood cells (RBC, Sigma) and washed three times with sterile PBS, pH 7.5. This mixture was incubated at 37°C for 1 hour and cleared by centrifugation at 3,000 x *g*. Absorbance of the supernatants were measured at 541 nm

by spectrophotometer (Molecular Dynamics) to determine release of hemoglobin by lysed RBCs. Percent hemolysis was defined as follows: [(sample A − blank A)/(100% lysis A)] × 100. To assay for streptolysin O-specific hemolytic activity, the streptolysin S inhibitor trypan blue (151) (13 μg/μl) was added to samples prior to incubation.

2.8 BIOLOG phenotype microarrays

Carbohydrate metabolic profiles were determined using the BIOLOG Omnilog system (Biolog Inc.) per the manufacturers protocol. Tests were performed utilizing the Carbon panel 1 (PM1) and Carbon panel 2 (PM2) carbon panels, each as standard 96-well microplates containing 95 different carbon sources and one negative control. Each well contained a redox dye (tetrazolium violet) for colorimetric determination of cells metabolizing the carbon source. Strains were first cultured on TSA 5% blood agar plates for 24 hours at 37°C. Cells were swabbed off the plate and resuspended in Inoculating Fluid (BIOLOG) to adjust to an absorbance at 600 nm of 0.14. An aliquot of 100 μl of the suspension was immediately dispensed into each well of the PM microplate with a multichannel pipette. The plate was incubated at 37°C for 48 hours and the data was analyzed using the Omnilog software (BIOLOG).

Table 1: Bacterial strains and plasmids

Name	Description	Reference
***E. coli* strains**		
DH5α	*hsdR17 recA1 gyrA endA1 relA1*	(22)
C41[DE3]	F^- *ompT gal dcm hsdS*$_B$(r_B^- m_B^-)(DE3)	(23)
***S. pyogenes* strains**		
MGAS5005	M1T1	(73)
5448	M1T1	(124)
5448AP	M1T1	(124)
GA40634	M4	(128)
MGAS5005.Δ*ptsI*	*ΔptsI* mutant in MGAS5005	(152)
MGAS5005.Δ*ptsI*c	*ΔptsI* complemented with pKSM456 insertion	(152)
MGAS5005.Δ*ptsIsagB*	*ΔptsI* mutant with insertional inactivation of *sagB*	(152)
5448.Δ*ptsI*	*ΔptsI* mutant in 5448	(152)
5448AP.Δ*ptsI*	*ΔptsI* mutant in 5448AP	(152)
GA40634.Δ*ptsI*	*ΔptsI* mutant in GA40634	(6)
GA40634.Δ*ptsIc*	*ΔptsI* complemented with pKSM455 insertion	This study
Plasmids		
pCRK	Temp. sensitive conditional vector, Km^R	(153)
pBluescript II KS-	ColE1 ori Amp^r *lacZa*	Stratagene
pSL60-1	Vector containing non-polar *aad9* gene	(154)
pKSM645	Δ*ptsI* mutagenic plasmid; non-polar *aad9*	(6)
pBlue.Δ*ptsI*	Δ*ptsI* in pBluescriptII KS-; bla (Ap^r)	(6)

pKSM455	promoterless ptsI intergration plasmid; Km^R	This study
pKSM456	promoterless *ptsI* integration plasmid; Km^R	(152)
pKSM732	*sagB* insertional inactivation vector	(145)

Table 2: Primers used in this study

Target	PCR Primers	Sequence (5'-3')	Reference
aad9	aad9L2-bglII	gcgcagatctGGGTGACTAAATAGTGAGGAG	(145)
	aad9R2-bglII	gcgcagatctGGCATGTGATTTTCC	(145)
ptsI	ptsI-1a	agatctGCTGAGTGACTTGTACGA	(152)
	ptsI-1b	GGTATTCATGCGCGTCCA	(152)
	ptsI-2a	gcgGCCTTGTGTTGGTGGTTTAAGAGCAAC	(152)
	ptsI-2b	*GTCACTCAGCa*gatctCGTGCGCTTACAGAATGT	(152)
	ptsI-3a	tctagaGCGGCCTTGTGTTGGTGGTTT	(152)
	ptsI-3b	ctcgagGGTATTCATGCGCGTCCA	(152)
	ptsl-internal sense	CAAATTGGTCTGCAAGC	(152)
	ptsl-internal antisense	CAGATACTGCTCAACTTAACA	(152)
	ptsl-external sense	GCTAGCAAAAAAGAGCTGGTTTA	(152)
	ptsl-ext antisense	CTCTTGACTACAAAGGTAAAGCAGTAAA	(152)
	5005.645jxn-sense	GCGGGTTATTTTTTAAATGTTTCCGAAG	(152)
	5005.645jxn-	GGGCCATCTGCAAAATACAAAGCAT	(152)
	ptsIcomplementL	cccggatccCATCACTCTTGACTACAA	(152)
	ptsIcomplementR	cccggatccTTAATCTTCAGAAACGTA	(152)
	Real time primers		
arp	arp M4 RT L	TAGCTGTTTCGCCTGTTGAC	(155)
	arp M4 RT R	GCTAAAGTAGCCCCACAAGC	(155)
emm	emm M1 RT L	ACTCCAGCTGTTGCCATAACAG	(128)
	emm M1 RT R	GAGACAGTTACCATCAACAGCTGAA	(128)
gyrA	gyrA M1 RT L	CGACTTGTCTGAACGCCAAAGT	(128)
	gyrA M1 RT R	ATCACGTTCCAAACCAGTCAAAC	(128)

Target	PCR Primers	Sequence (5'-3')	Reference
hasA	*hasA* M1 RT L	CGACTTGTCTGAACGCCAAAGT	(128)
	hasA M1 RT R	ATCACGTTCCAAACCAGTCAAAC	(128)
ptsI	*ptsI* M1 RT L	CGGAAACCAAGGAATGGAT	(152)
	ptsI M1 RT R	TGGCAAACCTGTTGTGGTT	(152)
sagA	sagA M1 RT L	GCTACTAGTGTAGCTGAAACAACTCAA	(145)
	sagA M1 RT R	AGCAACAAGTAGTACAGCAGCAA	(145)
sic	sic M1 RT L	AAGCCAGCTGAAAACCCTCTATC	(156)
	sic M1 RT R	CCTCGTGTGCCAGAAAAACC	(156)
sof	sof M28 RT L2	CTCATCACTTGCCTGCATCTG	(128)
	sof M28 RT R2	AACCTGCAGCTCCAATAATTGTTAG	(128)
speB	speB M1 RT L	GGTAAAGTAGGCGGACATGCC	(156)
	speB M1 RT R	CACCCCAACCCCAGTTAACA	(156)

Chapter 3: PTS represses SLS activity and lesion severity during soft tissue infection

3.1 Introduction

The ability to obtain essential nutrients during an infection is critical for bacterial pathogens to successfully colonize and proliferate within host tissues. One such key process involves the ability to import and catabolize optimal carbon sources such as carbohydrates. Bacteria have evolved elegant regulatory pathways to regulate their metabolism based on the presence of preferred carbohydrates (4,31). In fact, many pathogens tightly control the genes involved in carbohydrate utilization and regulation in response to *in vivo* growth and these same genes have been shown to be important to the disease process (6,129,157-160). Therefore, it is apparent that bacterial pathogens have closely linked their sugar metabolic sensing networks to virulence gene expression during infection.

Many Gram-positive pathogens including Streptococci, transport several sugars by the PTS which concomitantly catalyzes the phosphorylation and translocation of mono- and disaccharides via a chain of enzymatic reactions that transfer a phosphate group from PEP to the incoming sugar. The process was explained in details in Chapter 1 section 1.1. PTS-mediated signaling can also influence virulence gene expression in Gram-positive pathogens including the species of Streptococcus, as already mentioned in Chapter 1, section 1.7. The PTS is also involved in CCR via Hpr kinase (HprK)-mediated phosphorylation at ser-46 of Hpr in Gram-positive

bacteria. In the presence of glucose, P~ser-Hpr interacts with the catabolite control protein CcpA to mediate CCR (4,32). In addition to its central role in CCR, CcpA has been shown to be important for the virulence of a number of important Gram-positive pathogens (137,145,146,161-163). CcpA-independent pathways for CCR also exist in *S. mutans*, which is exerted through a network of PTS permeases (164). Thus, the sugar status of the bacterial cell can have a profound impact on virulence and expression of important virulence phenotypes in Gram-positive pathogens.

As already stated in Chapter 1, section 1.9, GAS is an important human pathogen that is responsible for numerous diseases with diverse clinical outcomes. The clinical impact of GAS is based largely on its ability to produce a large array of surface exposed (e.g., M protein, capsule) and secreted (e.g., Streptolysins SLS and SLO, and SpeB protease) virulence factors that contribute significantly to its pathogenesis. Global regulatory networks such as CcpA and the two-component system CovR/S coordinate virulence genes regulation in response to changing host environments. Genes involved in carbohydrate uptake and utilization are up regulated *in vivo* and contribute to virulence in GAS models of infection (144-146,165). CcpA-mediated CCR regulates over 6% of the genome, including indirectly repressing transcription of the *sag* operon encoding SLS (137,145,146).

Despite the increasing evidence that carbohydrate utilization influences GAS pathogenesis, the role of PTS transport and signaling in disease progression has not been investigated. In this study, we characterize the PTS pathway in multiple strains

of GAS by creating a deletion mutant of *ptsI* (EI), the first protein of the phosphorelay. We show that a functional PTS is not necessary for eliciting a GAS subcutaneous soft tissue infection. However, it does limit lesion severity at the site of subcutaneous infection in mice by regulating the temporal expression level of SLS. Thus, PTS transport and signaling contributes to GAS pathogenesis.

3.2 Results

3.2.1 GAS EI mutant (Δ*ptsI*) is defective for growth in several carbohydrates.

To assess the role of the PTS in GAS, an EI mutant (Δ*ptsI*) was constructed in the M1T1 strain MGAS5005 (Table 1), a representative strain of the most common GAS serotype associated with severe invasive disease worldwide (73). The genetic composition of the PTS in GAS is comparable to that found in many bacterial species (4). The *ptsHI* genes are typically found in an operon and are highly conserved among low G+C Gram-positive bacteria, including multiple species of *Streptococcus* (Fig. 3A). We replaced wild type *ptsI* with an in-frame deletion (Δ*ptsI*) containing a non-polar aad9 spectinomycin resistance cassette (166) in the MGAS5005 genome (Fig. 3A). The resulting MGAS5005.Δ*ptsI* mutant (Table 1) was verified by PCR (data not shown). Although MGAS5005.Δ*ptsI* had a slightly increased lag phase compared to wild type in rich THY media, the growth kinetics were comparable (data not shown).

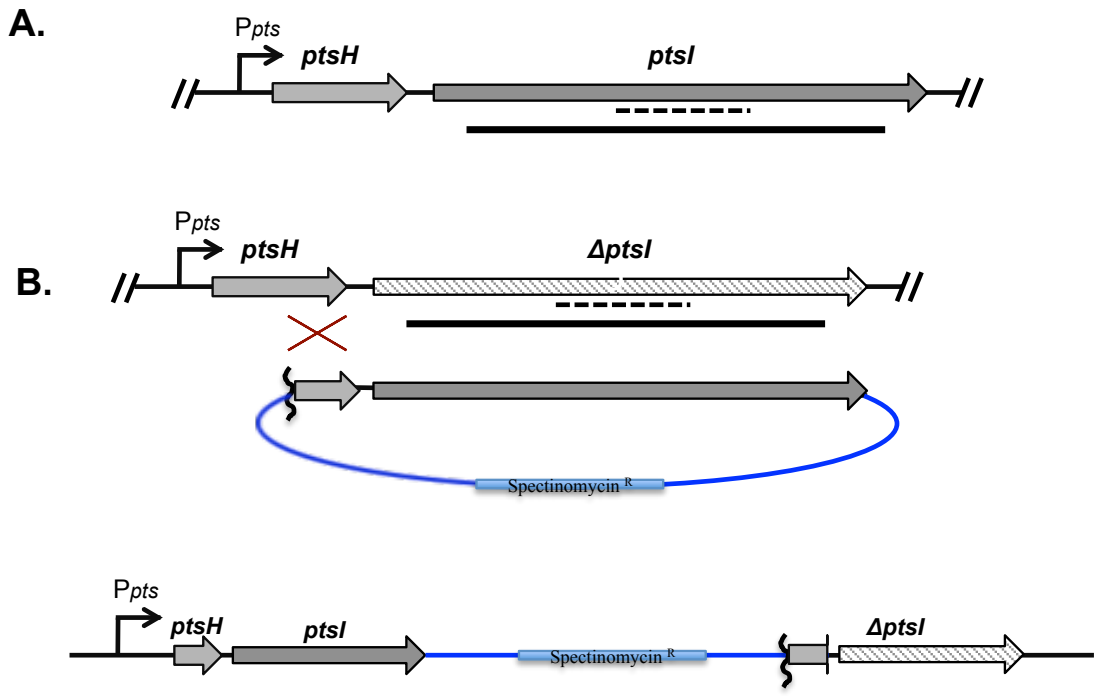

Figure 3: Construction of Δ*ptsI* mutant and Δ*ptsIc* of MGAS5005. (A) Schematic of *ptsHI* genomic region from GAS MGAS5005 with putative P*pts* promoter (arrow) driving transcription of *ptsH* (Hpr) and *ptsI* (EI). The Δ*ptsI* region (black bar) was replaced with a non-polar *aad9* cassette (spectinomycin-resistance). The region amplified for qRT-PCR analysis is shown (dashed line). (B) Single crossover integration of plasmid carrying 3'*ptsH* and full-length *ptsI* to complement Δ*ptsI* (striped) in MGAS5005. The homology region consists of ~300bp of 3' region of *ptsH*. The integration yields MGAS5005.Δ*ptsIc*

Growth of the parental MGAS5005 and the MGAS5005.Δ*ptsI* mutant was further analyzed by phenotypic microarray (BIOLOG) using the carbon panels PM1 and PM2, which contain a total of 95 different carbon sources. Each well was inoculated with either strain and the plates were incubated at 37ºC in an Omnilog plate incubator for 48 hours to allow generation of independent metabolic curves (Fig. 4). Each curve is plotted over time on a y-axis that represents random numbers called as omilog values (ov). For this assay, we devised a scoring scheme, where '+' represents utilization (ov ~ 200 and over), +/- represents a weak utilization (ov ~125-200) and a '-' for no utilization (ov ~<125). It was found that 45 carbon sources were able to support MGAS5005 metabolism (Table 3). Of these 45 carbon sources, the Δ*ptsI* mutant showed poor or no utilization of 19 (Table 3), including both PTS-transported (bold) and non-PTS-transported sugars.

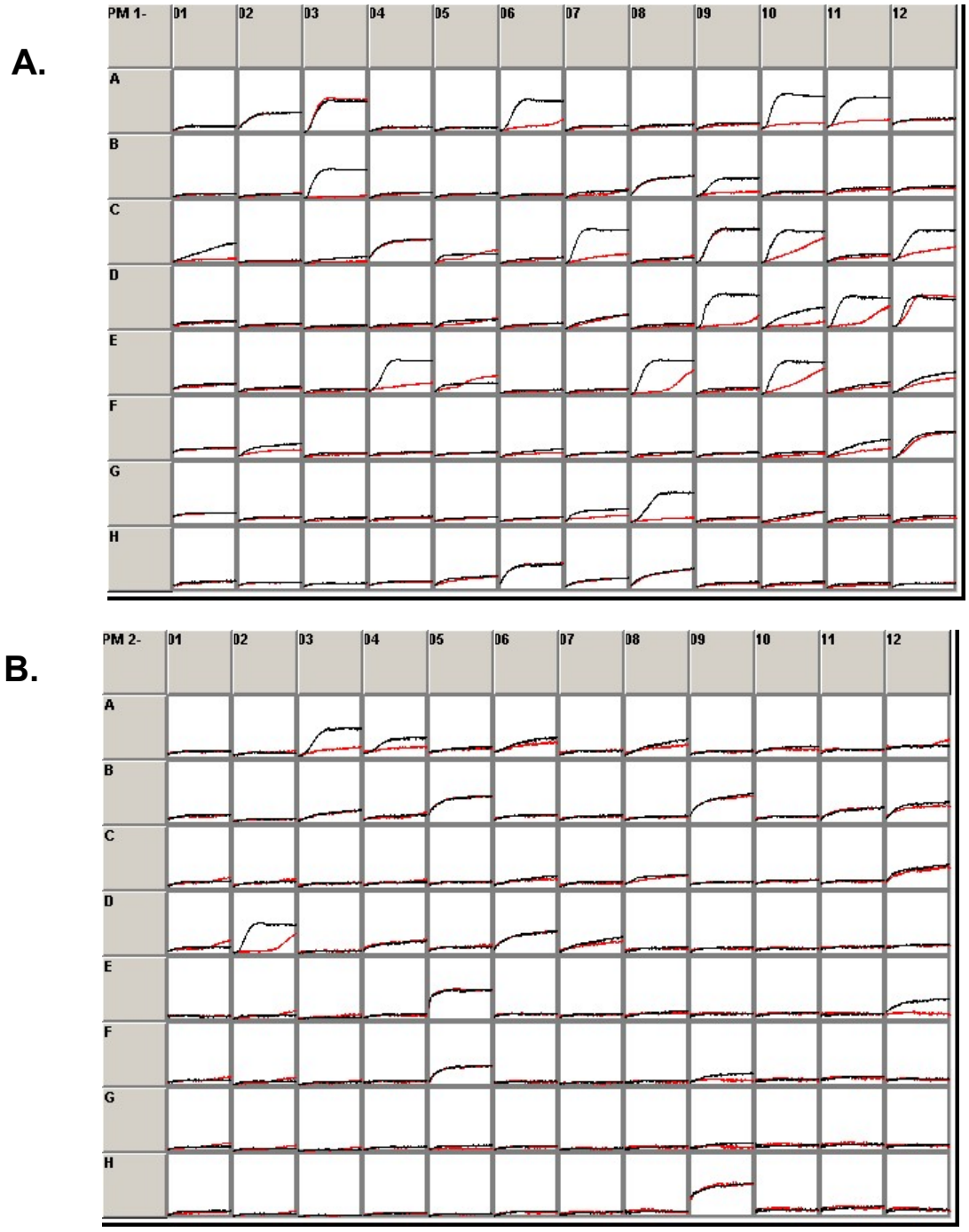

Figure 4: Carbon phenotype microarray panels using BIOLOG. Metabolic curves generated using carbon panels 1 (top) and 2 (bottom) by BIOLOG at the end of 48 hours. Black curves represent wild type MGAS5005 and red curves represent Δ*ptsI* mutant.

Table 3: Metabolism of MGAS5005.Δ*ptsI* in various carbon sources by BIOLOG

Carbon Source	Panel and well	MGAS5005	MGAS5005.Δ*ptsI*
D-galactose	**PM1-A6**	+	-
D-trehalose	**PM1-A10**	+	-
D-fructose	**PM1-C7**	+	-
D-mannose	**PM1-A11**	+	-
α-D-lactose	**PM1-D9**	+	-
sucrose	**PM1-D11**	+	-
maltotriose	**PM1-E10**	+	-
salicin	**PM2-D2**	+	-
β-methyl-D-glucoside	**PM1-E8**	+	-
L-lactic acid	**PM1-B9**	+/-	-
D-glucose-6-phosphate	**PM1-C1**	+/-	-
thymidine	**PM1-C12**	+	-
lactulose	**PM1-D10**	+/-	-
D-fructose-6-phosphate	**PM1-E4**	+	-
N-acetyl-β-D-mannosamine	**PM1-G8**	+	-
α-cyclodextrin	**PM2-A3**	+	-
β-cyclodextrin	**PM2-A4**	+/-	-
5-keto-D-gluconic acid	**PM2-E12**	+	-
glycerol	**PM1-B3**	+	-
L-arabinose	**PM1-A2**	+	+
N-actetyl-D-glucosamine	**PM1-A3**	+	+
D-xylose	**PM1-B8**	+	+
D-ribose	**PM1-C4**	+	+
α-D-glucose	**PM1-C9**	+	+
maltose	**PM1-C10**	+	+
uridine	**PM1-D12**	+	+
adenosine	**PM1-E12**	+	+
inosine	**PM1-F12**	+	+
L-lyxose	**PM1-H6**	+	+
pyruvic acid	**PM1-H8**	+	+
dextrin	**PM2-A6**	+	+
glycogen	**PM2-A8**	+	+
β-D-allose	**PM2-B3**	+	+
D-arabinose	**PM2-B5**	+	+
2-deoxy-D-ribose	**PM2-B9**	+	+
D-fucose	**PM2-B11**	+	+
3-O-β-D-galactopyranosyl-arabinose	**PM2-B12**	+	+
3-methyl-glucose	**PM2-C8**	+	+
palatinose	**PM2-C12**	+	+
L-sorbose	**PM2-D4**	+	+
D-tagatose	**PM2-D6**	+	+
turanose	**PM2-D7**	+	+
D-glucosamine	**PM2-E5**	+	+
oxalomalic acid	**PM2-F5**	+	+
dihydroxyacetone	**PM2-H9**	+	+

Carbohydrate-specific phenotypes observed by phenotype microarray were confirmed by growth assays in chemically defined medium (CDM) supplemented with 1% w/v of certain carbohydrate sources as the sole carbon source. MGAS5005.Δ*ptsI* showed comparable growth to MGAS5005 (wild type) when grown in CDM supplemented with 0.5% glucose (Fig. 5A). However, MGAS5005.Δ*ptsI* was unable to grow when the PTS-transported sugars sucrose and fructose (Fig. 5BC, Table 3) or lactose, galactose, trehalose, and mannose were tested using CDM (data not shown, Table 3). These results strongly support the conclusion that MGAS5005.Δ*ptsI* lacks a functional PTS.

The inability of the Δ*ptsI* mutant to metabolize some non-PTS sugars (Table 3) indicates the importance of a functional PTS for uptake and utilization of non-PTS sugars. In addition to the defined medium, C medium was also used for growth analysis. C medium consists of 0.5% (wt/vol) proteose peptone no. 3 (Difco), 1.5% (wt/vol) yeast extract (Difco), 10 mM K_2HPO_4, 0.4 mM $MgSO_4$, and 17 mM NaCl (pH 7.5). Unlike THY medium, C medium does not contain glucose and is rich in peptides and poor in carbohydrates (148). The growth kinetics observed with MGAS5005 and MGAS5005.**Δ***ptsI* were similar, however, the **Δ***ptsI* strain did not reach the final absorbance value as high as the wild type (Fig. 5D). To confirm that the growth defect phenotype was specific to the **Δ***ptsI* allele, we generated a complemented **Δ***ptsI* strain by introducing a wild type *ptsI* allele into the chromosome of MGAS5005.**Δ***ptsI* (MGAS5005.**Δ***ptsI*c, Table 1) (Fig. 3B). The MGAS5005.**Δ***ptsI*c

complemented strain showed a growth profile in all PTS specific sugars comparable to wild type MGAS5005 (Fig. 5BC, data not shown).

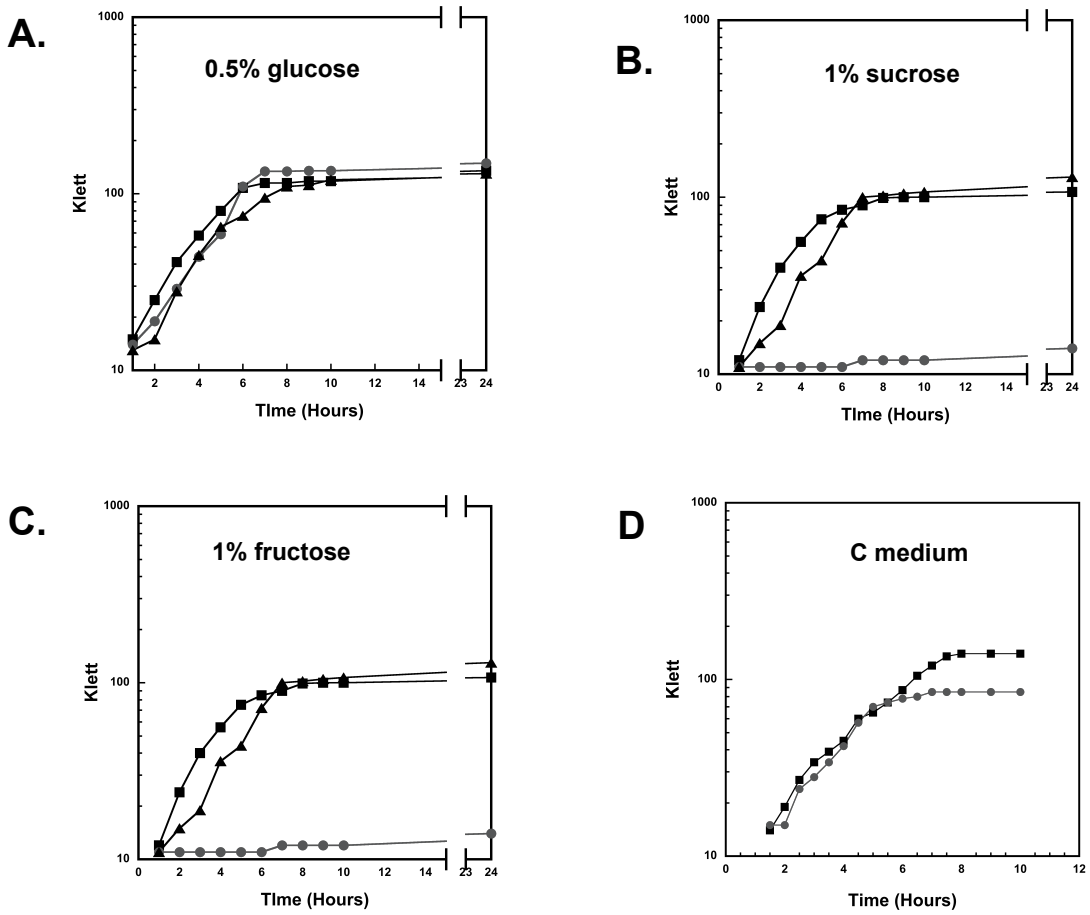

Figure 5: Growth analysis of MGAS5005, Δ*ptsI* in CDM. Growth curve of wild type MGAS5005 (squares), ΔptsI mutant (grey circles) and complemented ΔptsI (triangles) in CDM supplemented with (A) 0.5% (w/v) glucose (B) 1% (w/v) sucrose or (C) 1% (w/v) fructose is shown. (D) Growth curve of wild type MGAS5005 (squares) and ΔptsI mutant (grey circles) in C medium. Data is representative of three independent experiments.

3.2.2 Δ*ptsI* mutant shows increased soft tissue damage in a mouse model of GAS soft tissue infection.

The role of EI (*ptsI*) and the PTS in GAS virulence was assessed using a murine model of streptococcal soft tissue infection. MGAS5005 and MGAS5005.Δ*ptsI* were grown to late exponential phase and then injected subcutaneously (~3 x 10^8 CFU) into the haunches of 5-6 week old, female CD1 mice. For 7 days post infection, the progression of disease was monitored for both lesion size (at 38 hours post infection) and survival. Mice infected with wild type MGAS5005 developed purulent abscessed lesions with minimal ulceration after 38 hours (Fig. 6A). In contrast, mice infected with a comparable dose of MGAS5005.Δ*ptsI* developed lesions at 16 hours equivalent in size to wild type at 38 hours, and these lesions became fully ulcerative by 24 hours post infection. The visual difference in both the size and severity of lesions between the wild type and the Δ*ptsI* mutant at 38 hours post infection was quite striking (Fig. 6A). This coincided with a significant increase in the mean lesion size for mice infected with MGAS5005.Δ*ptsI* compared to MGAS5005 measured at the same time post infection (Fig. 6B). Interestingly, a corresponding increase in lethality over 7 days was not observed in mice infected with MGAS5005.Δ*ptsI*, suggesting there was no enhancement of dissemination from the skin or in systemic lethality (Fig. 6C). Thus, the Δ*ptsI* mutant exhibits a hypervirulent phenotype at the site of infection compared to wild type; however, systemic progression and lethality are not altered.

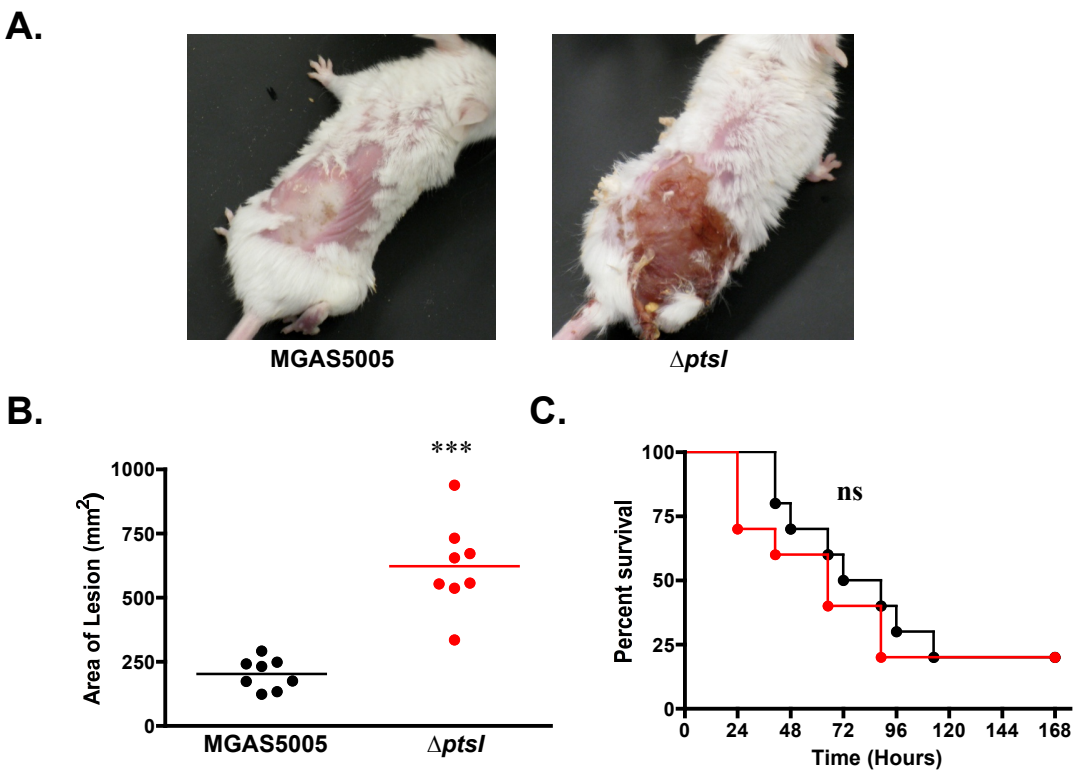

Figure 6: Effect of M1T1 MGAS5005 Δ*ptsI* mutant in mouse model of GAS infection. Mice were inoculated *s.c.* with ~3 x 10^8 CFU and monitored over 7 days (A) Representative images of mice infected with wild type MGAS5005 (left) or the Δ*ptsI* mutant (right) at 38 hours post infection. (B) Lesion sizes measured in mice infected with MGAS5005 (black) and Δ*ptsI* mutant (red) at 38 hours post infection. One of two independent experiments is shown (total n = 40). Each point represents a single animal and bars indicate the statistical mean. Significance was determined by unpaired two-tailed t test. (C) Survival plot of mice infected with MGAS5005 or its Δ*ptsI* mutant over the course of 7 days. Significance was determined by Kaplan-Meier survival analysis and log rank test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq .001$; NS, not significant).

3.2.3 Δ*ptsI* mutant exhibits comparable phenotype in multiple M1T1 GAS strains.

To explore the role of EI in different genetic backgrounds, we constructed independent Δ*ptsI* mutants in two additional M1T1 strains, 5448 and 5448AP (*covS*), with the same mutagenic plasmid used for MGAS5005.Δ*ptsI* (Table 1). Furthermore, these Δ*ptsI* mutants exhibited growth defects when grown in CDM supplemented with PTS sugars identical to that seen for MGAS5005.Δ*ptsI* (Fig. 7). Finally, similar growth phenotypes were observed in at least two independently isolated MGAS5005.Δ*ptsI*, 5448.Δ*ptsI* and 5448AP.Δ*ptsI* mutants. Thus, biologically independent Δ*ptsI* mutants exhibit comparable phenotypes in multiple M1T1 backgrounds. We also tested the 5448.Δ*ptsI* and 5448AP.Δ*ptsI* mutants and their respective parental strains in the murine model of streptococcal soft tissue infection (Fig. 8). As seen with MGAS5005.Δ*ptsI*, mice infected by either Δ*ptsI* mutant exhibited a more severe (Fig. 8A) and significantly larger (Fig. 8B) ulcerative lesion at 38 hours post infection compared to infection with its parental strain. In contrast to MGAS5005.Δ*ptsI* and 5448AP.Δ*ptsI* (*covS* mutants), only mice infected with 5448.Δ*ptsI* (intact *covS*) showed a significant increase in systemic lethality compared to wild type (Fig. 8C). These data suggest that EI is important in controlling virulence at the site of localized skin infections, as well as serving a CovS-dependent role in limiting dissemination to sterile sites of the body.

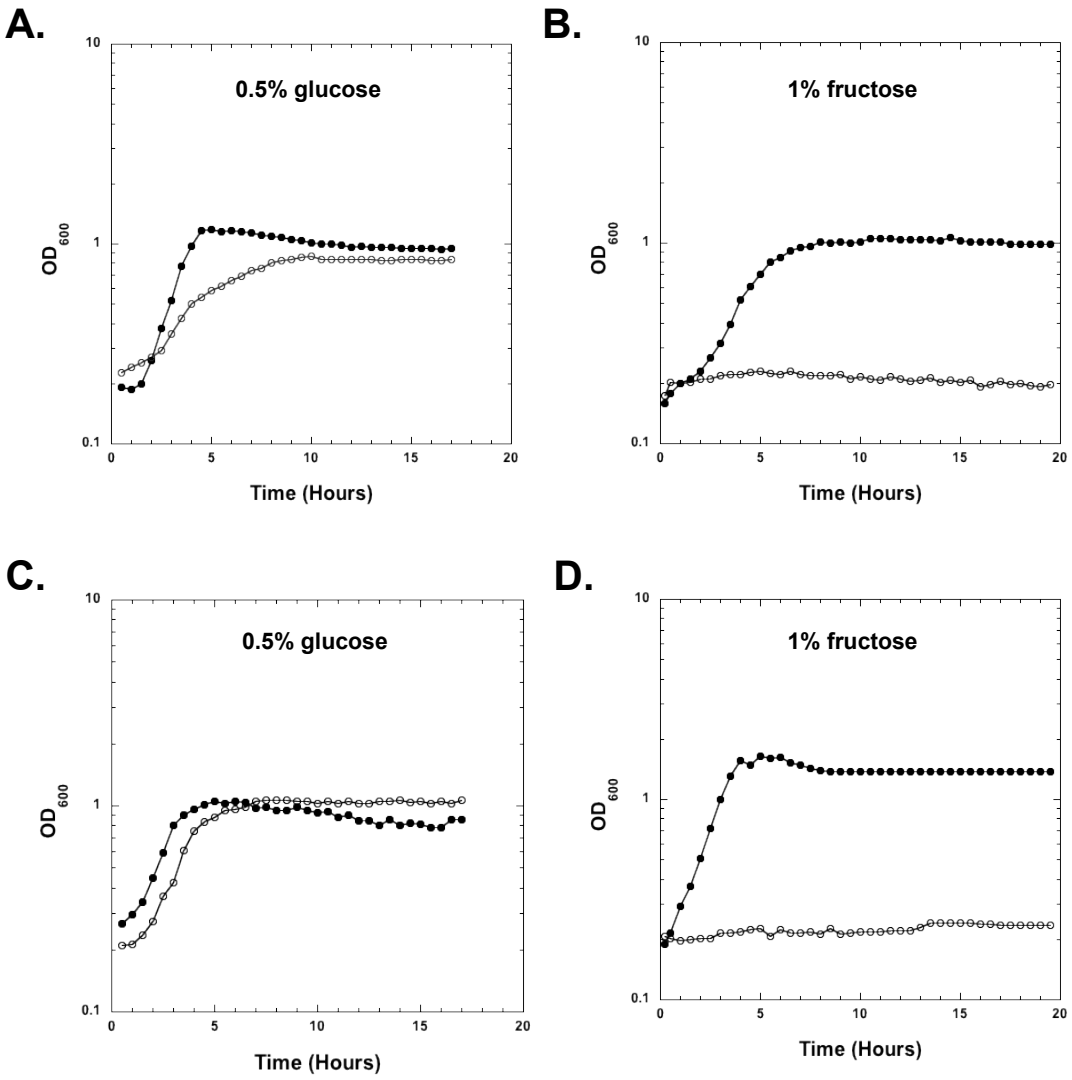

Figure 7: Growth curve of additional M1T1 strains in CDM. Wild type GAS (black circles) and their corresponding isogenic Δ*ptsI* mutant (grey circles) in M1T1 5448 (A, B) and 5448AP (C, D). GAS cells were grown in CDM supplemented with either 0.5% (w/v) glucose (left) or 1% (w/v) fructose (right). Data is representative of three independent experiments.

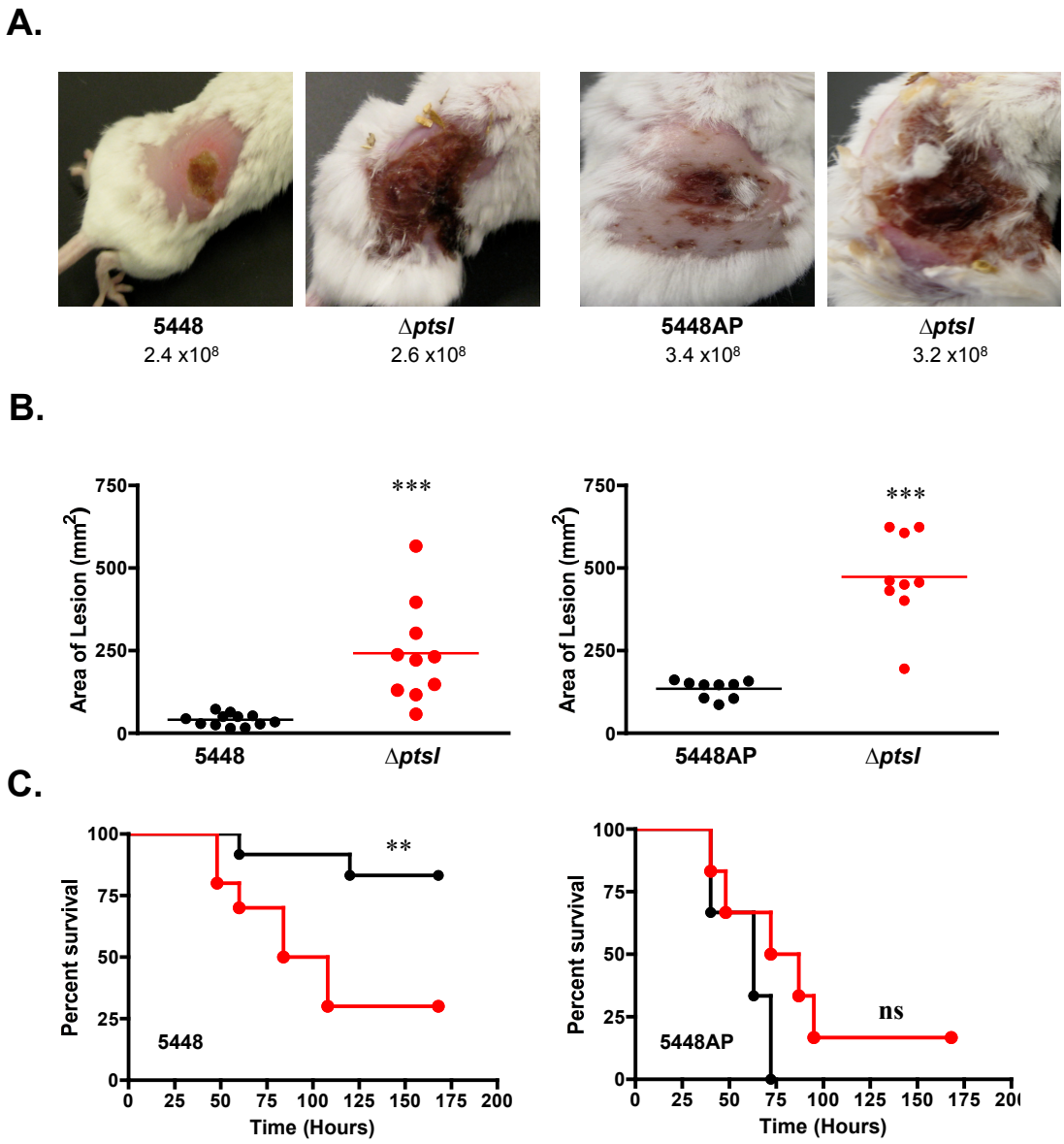

Figure 8: Δ*ptsI* mutants of additional M1T1 GAS strains. (A) Representative images of mice infected *s.c.* with 5448 (CovS$^-$) and its Δ*ptsI* mutant (left) or 5448AP (CovS+) and its Δ*ptsI* mutant (right) at 38 hours post infection. CFU used in infection are indicated. (B) Lesion sizes of same strains measured at 38 hours post infection from a representative experiment (total n = 60). Each point represents a single animal, with bars indicating statistical mean. Significance was determined by unpaired two-tailed t test. (C) Survival plot of mice infected with either wild type 5448 (left) and 5448AP (right) in black and their corresponding Δ*ptsI* mutants in red. Significance was determined by Kaplan-Meier survival analysis and log rank test. (* p≤0.05, ** p≤0.01, *** p≤.001, and NS, not significant).

3.2.4 Δ*ptsI* mutants exhibit expression of SLS during log phase growth.

Several virulence factors have been associated with increased lesion size and severity in soft tissue mouse model of GAS infection. Streptolysin S (SLS) is a secreted virulence factor that contributes to the formation of lesions in soft tissue models of mouse infection and GAS virulence in general (106,113,114). Biosynthesis and secretion of SLS in GAS requires the 9-gene *sag* operon, with *sagA* encoding the toxin precursor and the first position in the locus (167). To determine whether the Δ*ptsI* mutation affects *sag* operon expression, qRT-PCR was performed on the isolated mRNA of MGAS5005, MGAS5005.Δ*ptsI*, and the complemented MGAS5005.Δ*ptsI*c to quantify *sagA* transcript levels. The cells were collected at late exponential phase as the *sag* operon is maximally expressed at this point in growth. Changes in transcript levels greater than two-fold were considered significant. A modest yet significant increase in *sagA* transcript levels was observed in the Δ*ptsI* mutants compared to their corresponding wild type strains when grown in THY (Fig. 9A-C).

SLS-specific hemolytic activity was assayed using 2.5% defibrinated sheep RBCs incubated with culture supernatants from the wild type MGAS5005 and mutant MGAS5005.Δ*ptsI* GAS taken at 1 hour intervals across growth in THY. Addition of the SLS inhibitor, trypan blue, blocked all RBC lysis in each experiment, indicating that the observed hemolytic activity was due to SLS (data not shown). SLS hemolytic activity in the MGAS5005.Δ*ptsI* mutant supernatants showed a dramatic increase early in logarithmic phase and remained elevated during stationary phase (Fig 9D). In

comparison, SLS hemolytic activity from the supernatants of wild type and the complemented **Δ***ptsI* showed little activity during logarithmic phase and increased to maximum levels at the transition to stationary phase (Fig. 9D), as previously observed for SLS (145). Similarly, an early onset of SLS hemolytic activity was observed for the in 5448.**Δ***ptsI* and 5448AP.**Δ***ptsI* mutants in comparison to their respective wild type strains (Fig. 9EF). These data suggest that the early onset of SLS activity during exponential phase growth in all three M1T1 GAS strains lacking a functional PTS could lead to the increased severity of localized lesions observed in mice (Fig. 6 and 8).

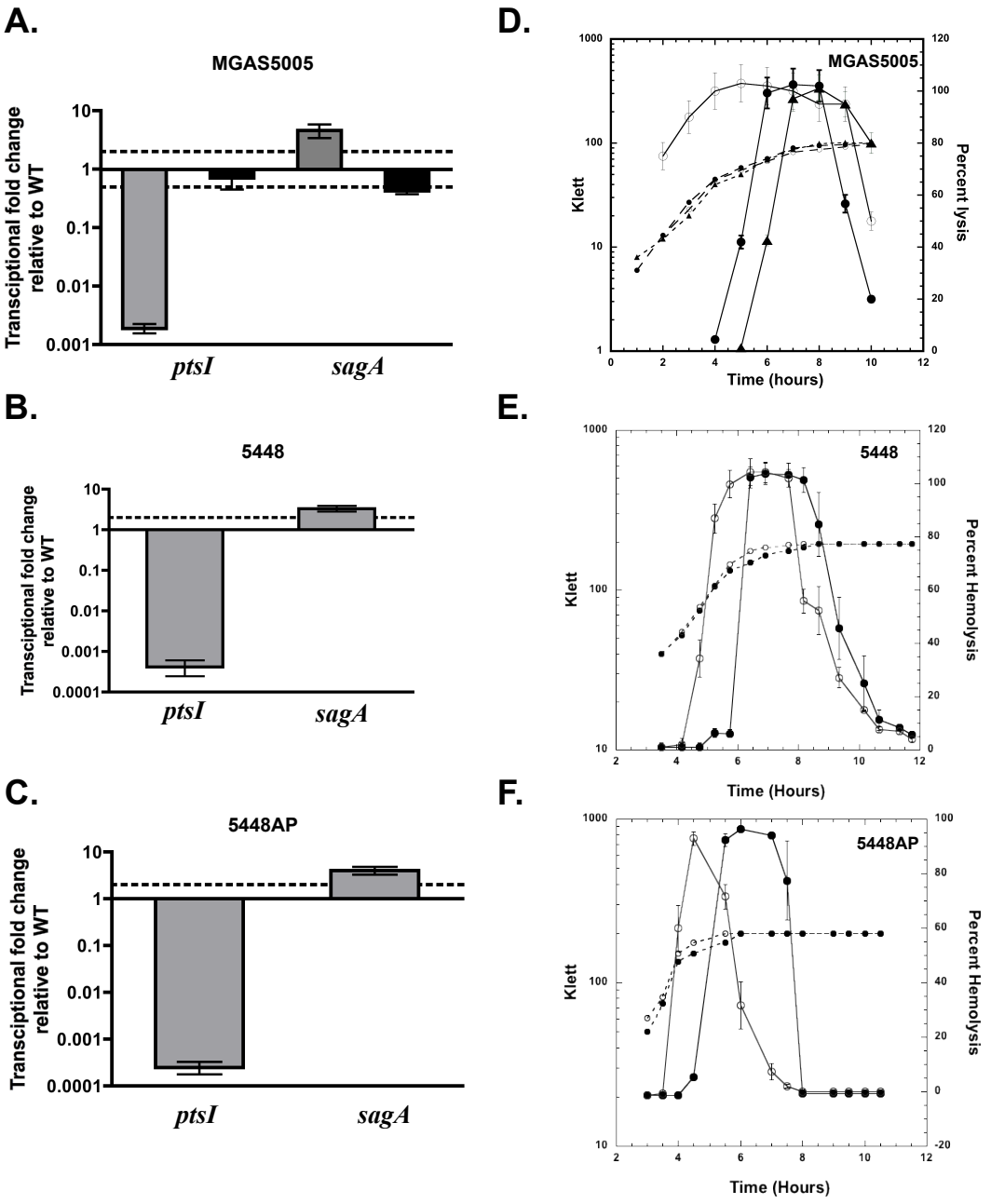

Figure 9: Influence of Δ*ptsI* on Streptolysin S (SLS) production. Transcript levels of *sagA* were measured by qRT-PCR at late-log phase in THY for wild type and Δ*ptsI* of (A) MGAS5005 (B) 5448 and (C) 5448AP. 2-fold differences in expression for mutant compared to wild type (dashed line) were considered significant. SLS hemolytic activity was measured in culture supernatants throughout growth for wild type (closed circles), Δ*ptsI* (open circles), and complement (closed triangles) of (D) MGAS5005 (E) 5448 and (F) 5448AP. Strains were grown in THY supplemented with 10% heat-inactivated horse serum. Data presented as percent hemolysis (solid lines) for 3 biological replicates. Representative growth is shown as dashed lines.

3.2.5 SLS is required for the lesion severity observed in M1T1 Δ*ptsI* mutants.

The role of SLS in the hypervirulence of lesions of mice infected with Δ*ptsI* mutants was investigated by creating a Δ*ptsI sagB* double mutant in MGAS5005. Inactivation of *sagB* has been shown to block SLS production in M1T1 GAS (145,167). As expected, a Δ*ptsI sagB* double mutant resulted in the absence of hemolytic activity both on blood agar plates (Fig. 10A) and also in RBC hemolysis assays (data not shown) comparable to an established MGAS5005.*sagB* mutant (Fig. 10) (145). To investigate the role of SLS in vivo, MGAS5005, MGAS5005.Δ*ptsI*, and the MGAS5005.Δ*ptsIsagB* double mutant were tested in the murine model of streptococcal soft tissue infection (Fig. 10BC). Despite the absence of a functional PTS, the Δ*ptsI sagB* double mutant lacking expression of SLS produced purulent lesions of the size and severity comparable to the wild type strain and not the Δ*ptsI* mutant (Fig. 10BC). These results strongly suggest that early expression of SLS during growth is the primary factor responsible for the increased lesion size and severity observed upon subcutaneous infection of mice with the Δ*ptsI* mutant.

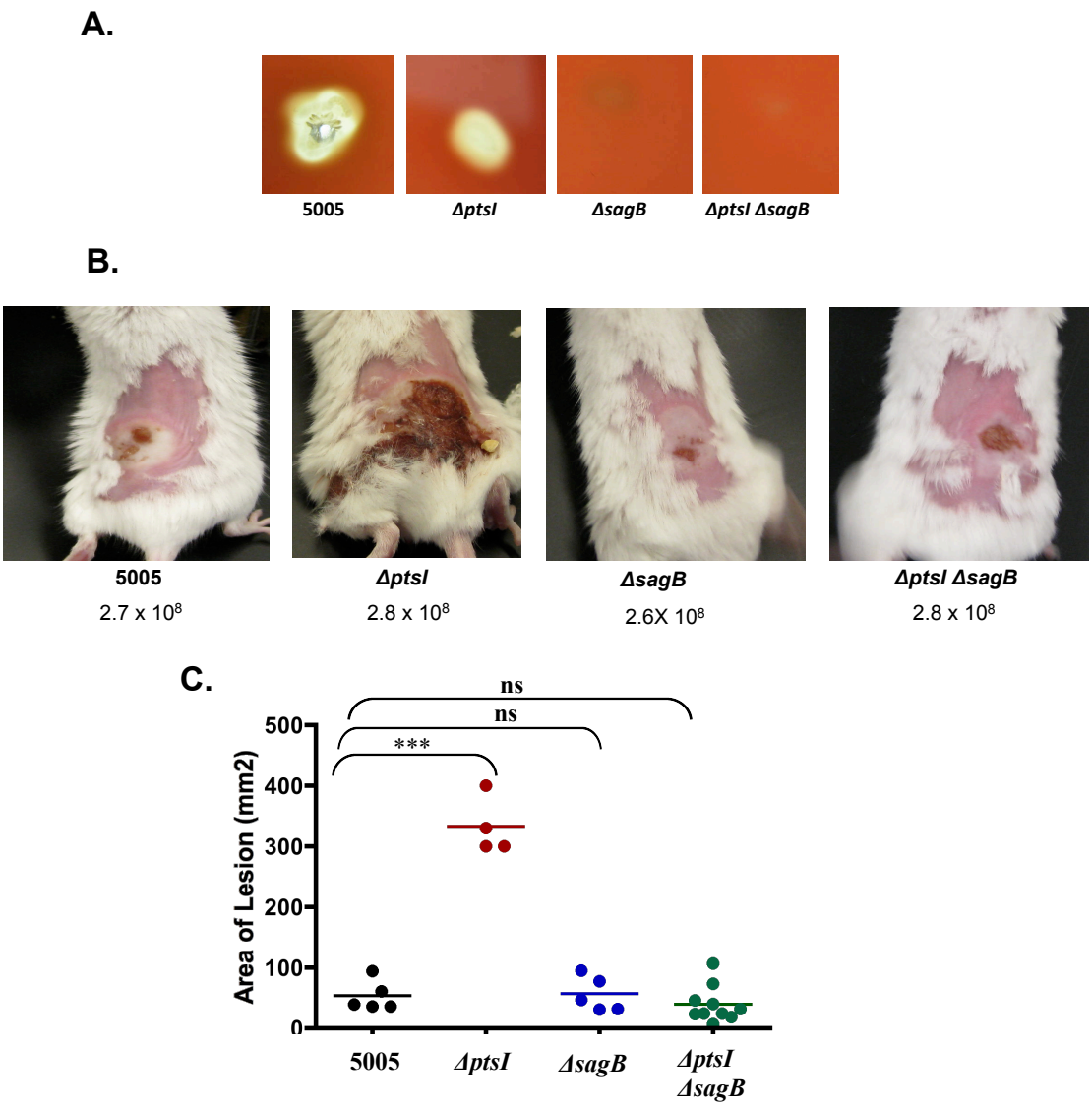

Figure 10: Role of Streptolysin S in Δ*ptsI* increased lesion formation. (A) Zones of hemolysis for WT MGAS5005, Δ*ptsI* single mutant, Δ*sagB* single mutant, and Δ*sagB* Δ*ptsI* double mutant strains on 5% sheep blood agar plates after growth at 37°C. (B) Representative images of mice infected *s.c.* with WT MGAS5005, MGAS5005.Δ*ptsI* mutant, Δ*sagB* single mutant and Δ*ptsI sagB* double mutant strains. CFU used in infection are indicated. (C) Lesion size of same experiment measured at 38 hours post *s.c.* infection of MGAS5005 (black), Δ*ptsI* mutant (red), Δ*sagB* mutant (blue), Δ*ptsI sagB* double mutant (green). Data represent two independent experiments and significance was determined using the unpaired two-tailed t-test (*** $p \leq .001$; NS, not significant).

3.3 Discussion

Although the connections between carbon metabolism and pathogenesis in GAS have been recognized for many years, the contribution of the PTS pathway to GAS virulence had not been investigated. In this study, we generated PTS mutants in multiple strains of GAS by deleting *ptsI* (EI) and characterized their growth profiles to identify the PTS-dependent carbon sources that support the growth of M1T1 GAS *in vitro*. Importantly, a PTS mutant was still able to colonize and elicit disease in a murine model of disseminating soft tissue infection. However, the PTS mutant resulted in a significant increase in localized lesion severity and size due to an increase in *sag* operon expression and an altered temporal expression of Streptolysin S (SLS). Thus, a functional PTS appears to limit the pathogenesis of GAS during invasive skin infection.

3.3.1 PTS pathway and carbon utilization in GAS

Typically, the genes encoding general proteins, Hpr (*ptsH)* and EI (*ptsI*) are organized as a tightly regulated *ptsHI* operon (168) and this appears to be the case for GAS as well. Several attempts to construct either a non-polar deletion or a polar insertion mutation of *ptsH* were unsuccessful (data not shown), suggesting that Hpr may be critical for GAS fitness under the conditions used here. This observation has been further supported by Tn-Seq essentiality studies ongoing in our lab (Le Breton and McIver, unpublished results). In contrast to *ptsH*, we were able to generate a non-polar *ptsI* (EI) deletion mutant in 3 different strains of M1T1 GAS and in an M4 GAS strain (6), indicating that the PTS itself is not required and that Hpr appears to possess a separate essential function in GAS. Despite being able to make *ptsI* null mutations

in GAS, attempts to clone *ptsI* on a multi-copy plasmid for complementation proved to be difficult and we were only successful when the construct lacked a functional promoter. This suggests that overexpression of *ptsI* can be deleterious in *E.coli* and that the *ptsHI* operon is likely under tight regulation.

By screening with the BIOLOG phenotypic microarray platform, we established that 45 of 190 carbon sources tested were able to support the metabolism (defined as 2-fold over background) of the M1T1 GAS invasive throat strain MGAS5005 (Table 3). In contrast, Kreikemeyer *et. al.* identified only 21 carbon sources with BIOLOG that supported the metabolism (defined as >= 10% of metabolism on glucose) of the M49 GAS "generalist" strain 591, a strain that is able to colonize both the throat and skin (169). Out of these 21 carbon sources, all but 5 (Tween40, Tween80, pectin, mannan, and gelatin) were in common with our studies in M1T1 (Table 3). An older study using CDM found that an M3 strain of GAS did not grow on lactose and glycerol, although both were found to support growth using BIOLOG (12). The different metrics used to determine functional growth between the two studies could explain some of the disparity in carbon sources. However, it could also indicate that metabolic requirements differ between strains of GAS, likely due to their varied tissue sites of infection.

The M1T1 MGAS5005 GAS genome possesses 14 putative EII loci (170) predicted for the transport of specific PTS sugars, although the exact substrates transported by these systems has not been experimentally determined. The isogenic Δ*ptsI* M1T1

MGAS5005 mutant was still able to utilize 26 of the 45 carbon sources required for wild type metabolism (Table 3), indicating that GAS does not need a functional PTS to utilize these carbon sources. However, the Δ*ptsI* mutant did have a metabolic defect for 19 carbon sources (Table 3), including 8 predicted PTS-specific carbohydrates (galactose, trehalose, fructose, mannose, lactose, sucrose, salicin, and maltotriose).

Growth defects in maltose and glucose were not found in our study likely due to the presence of non-PTS uptake systems. Transport of glucose in GAS has been shown to occur through both the MalE-dependent ABC transport system as well as the MalT-specific PTS pathway (13,170). In addition to the six carbon compounds, Δ*ptsI* blocked the utilization of α-glucosides (trehalose, sucrose, maltotriose), β-galactosides (lactose, lactulose), β-glucosides (β-methyl-D-glucoside, salicin), cyclodextrins, lactic acid and the three-carbon compound (Table 3). The defect in the use of non-PTS glycerol can be explained by the fact that glycerol kinase of Firmicutes is phosphorylated by PEP, EI and HPr, and that this modification is necessary for the activation of the enzyme (171). The results suggest that the PTS plays a role in regulating PTS and non-PTS transport, as well as the subsequent utilization of these substrates in central carbon metabolism.

3.3.2 PTS limits lesion severity during invasive skin infection in mice.

Despite the inability to metabolize 19 PTS and non-PTS sugars (Table 3), all three independent M1T1 Δ*ptsI* mutants were able to colonize mice following subcutaneous inoculation and elicit both localized and systemic disease to at least the levels of their

parental wild type strains. This provides strong evidence that GAS does not require a functional PTS or these specific sugars to infect at this tissue site and likely utilizes a carbon source that is not altered in the Δ*ptsI* mutant (Table 3). In fact, lesion formation was significantly more rapid with increased necrosis and size in mice infected with mutant strains (Fig. 6, 8 and 10). Furthermore, mice infected by *ptsI* mutants exhibited highly ulcerative and spreading lesions that resulted in severe hemorrhaging and tissue damage. This is in sharp contrast to the phenotype of a Δ*ptsI* mutant in *S. aureus*, where virulence was attenuated compared to wild type in an intraperitoneal (i.p.) model of systemic infection in BALB/c mice (172). The difference may merely reflect the different site of infections. Regardless, a functional PTS acts to limit localized tissue damage in GAS during invasive skin infection and this appears to be linked to the uptake and metabolism of sugars that require PTS transport (Table 3).

Dissemination of GAS from the subcutaneous site of infection to the bloodstream and organs leading to lethality was not significantly altered in MGAS5005.Δ*ptsI* and 5448AP.Δ*ptsI* compared to their parental strains (Fig. 6C and 8C). Both of these M1T1 strains possess a mutation in the histidine kinase gene *covS* that is associated with a molecular switch to a highly invasive phenotype for GAS. In contrast, deletion of *ptsI* in the M1T1 strain 5448 harboring a wild type *covS* gene exhibited a significant increase in systemic lethality due to dissemination (Fig. 8C) that correlated with the increase in lesion severity observed in all of the mutants. Thus, there appears to be a potential Cov-dependent influence on the ability of a functional PTS

to limit systemic spread as well as localized lesion formation. At this point, we do not know which Cov-regulated factor(s) are involved in this dissemination phenotype.

3.3.3 PTS represses *sag* expression and early SLS production

As mentioned above, the cytolysin SLS has been shown to contribute to the severity of skin lesions during GAS infection in mice (106,113,114). A modest up regulation of *sagA* and increased SLS activity in the supernatants of the M1T1 Δ*ptsI* mutants early in growth correlates nicely with the rapid onset of ulcerative lesions observed in the mice infected with the mutants. Importantly, inactivating *sagB* (an SLS-defective mutant) in the MGAS5005.Δ*ptsI* background reversed this phenotype and resulted in localized lesions comparable to the wild type parental strain alone (Fig. 10). These data strongly suggest that early expression of SLS during growth is likely the primary factor responsible for the increased lesion size and severity observed upon subcutaneous infection of mice with the MGAS5005.Δ*ptsI* and other M1T1 strains. Despite the absence of SLS hemolytic activity in the Δ*ptsI sagB* double mutant, lesion formation comparable to wild type was still observed (Fig. 10). These results suggest that other virulence factors besides SLS are contributing to the lesion development seen in the parental M1T1 MGAS5005.

A similar hypervirulent lesion phenotype, albeit not as significant, was previously observed by our lab when a Δ*ccpA* mutant in MGAS5005 was tested in the murine skin infection model. Importantly, this phenotype was attributed to CcpA-dependent overproduction of *sagA* and SLS (145). Other studies have actually found attenuation in virulence upon infection with a Δ*ccpA* mutant in M1T1 MGAS5005 and other

GAS strains (137,146). However, all studies observed an up regulation in *sag* operon transcription leading to increased SLS production in the mutants *in vitro*. Kietzman et al. showed that CcpA regulation of the *sag* operon in the M14 GAS strain HSC5 was indirect and that the repression did not appear to occur in infected skin tissue (137). The mechanism of indirect regulation of *sag* by CcpA has not been determined. Nevertheless, our results indicate that *sag* expression and SLS production are influenced based on carbohydrate availability. In addition to CcpA, the expression of *sagA* is under the transcriptional control of GAS global regulators such as CovRS, Mga, RofA, FasBCA and Nra (173). More will be discussed about this topic in later chapters.

In conclusion, we have used PTS-defective strains of M1T1 GAS to identify the PTS and non-PTS carbon sources that allow these invasive throat isolates to grow *in vitro*. The Δ*ptsI* mutants exhibited more severe and larger ulcerative lesions at the site of infection in a subcutaneous model of mouse infection. This phenotype was linked to up regulation of *sagA* and early onset of Streptolysin S (SLS) activity during exponential phase growth in the mutant. Infection of mice with a Δ*ptsI sagB* double mutant returned lesions to wild type levels, implicating SLS in the observed phenotype. Therefore, a functional PTS is not required for subcutaneous skin infection in mice; however, it does limit early expression of SLS and thus the overall severity of lesions *in vivo*.

Chapter 4: PTS influences other virulence factors associated with skin lesions in subcutaneous model of infection

4.1 Introduction

As already stated in chapter 1 section 1.9, group A streptococcal infections cause significant morbidity and mortality worldwide, making it a pathogen of global healthcare concern. The overall mortality rate of GAS infections is less than 0.1%, but the mortality rate of invasive GAS infections, which have resurged in the past 30 years, has now increased to 25% (78). This is largely attributable to the global dissemination and persistence of clonal lineage of M1T1 serotype (174-177). M1T1 clonal strains remain the most commonly isolated serotype from both invasive and non-invasive infections (177). One of the many unique genomic features contributing to the fitness of M1T1 is the ability to switch to a hyper virulent phenotype that is associated with the invasive diseases. This genetic switch occurs by a spontaneous mutation in the *covRS* locus due to selection pressure by the host immune system (123,124). This mutation results in a non-functional CovS, however, the activity of the response regulator CovR is not affected (75). The *covS* mutation in invasive M1T1 shuts off cysteine protease SpeB production, while upregulating the expression of several known virulence factors such as hyaluronic acid (178). CovR has been shown to bind to a region in the promoter region of the *has* operon (105,179). GAS strains with a functional CovR/S system produce small non-mucoid colonies, while CovS mutant strains produce large mucoid colonies (180). The capsule is encoded by the highly conserved *has* synthase operon consisting of *hasABC*, where two genes

(*hasA* and *hasB*) are required for biosynthesis of capsule (181). Hyaluronic acid capsule made by group A streptococcus is a major virulence factor and genetic inactivation of *has* genes significantly reduces virulence in multiple animal models of GAS infection (181-184). An important mechanism through which the capsule contributes to virulence is its capacity to confer resistance to complement–mediated phagocytic killing (185,186). Acapsular mutant strains lose their ability to resist phagocytic killing and have 100-fold decreased virulence in mice. In a skin infection model, acapsular mutants produced no lesions or minor inflammation compared to the necrotic lesions with purulent inflammation seen in the encapsulated strain (107,108).

SpeB is a broad-spectrum cysteine protease with proteolytic activity towards ECM proteins (such as Kininogen, plasminogen, fibrinogen etc.), modulators of host immunity (such as immunoglobulins, chemokines, complement C3 etc.), and GAS surface and secreted proteins (such as M protein, protein H, C5a peptidase, streptokinase etc.) (121). Discrepancies have been reported concerning the importance of cysteine proteinase for GAS disease in studies with *speB* mutants (181,187-191). Even though the role of SpeB is debated, recent evidence has conclusively demonstrated that SpeB is critical for the pathogenesis of severe invasive disease caused by GAS. Regulation of *speB* expression in GAS is extremely complicated and is governed by many factors, including environmental factors, growth phase, pH and salt concentration (121). SpeB expression involves multiple transcription factors and post-transcriptional regulation. Following translation, secretion and processing, the secreted protein folds into an inactive zymogen (40

kDa). This zymogen is enzymatically inactive and must be proteolytically cleaved to form an active mature form of protease (28 kDa). There has been no direct relationship between the presence of carbohydrates and SpeB expression, but the global carbon catabolite regulator CcpA positively regulates SpeB expression (137,139).

In addition to SLS, SpeB and capsule have been reported to contribute to lesion severity and spread of infection in mouse models of GAS skin infection. In this study, we investigated the influence of the PTS on the production of the virulence factors SpeB and capsule during GAS infection. We show that EI alters the expression of both the virulence factors, but not in all M1T1 strains tested, although we do not yet understand that mechanism involved. Overall, our results suggest that EI, and thus the PTS, plays a pleiotropic role in GAS virulence.

4.2 Results:

4.2.1 *ΔptsI* reduces *hasA* transcription and capsule production in a CovS-dependent manner.

Increased expression of capsule has been associated with enhanced lesions in GAS skin infection models (181-184). The small colony phenotype presented by all of the Δ*ptsI* mutants on blood agar plate suggests a reduction of capsule production. The difference in colony size was most pronounced in the MGAS5005 background (Fig. 11).

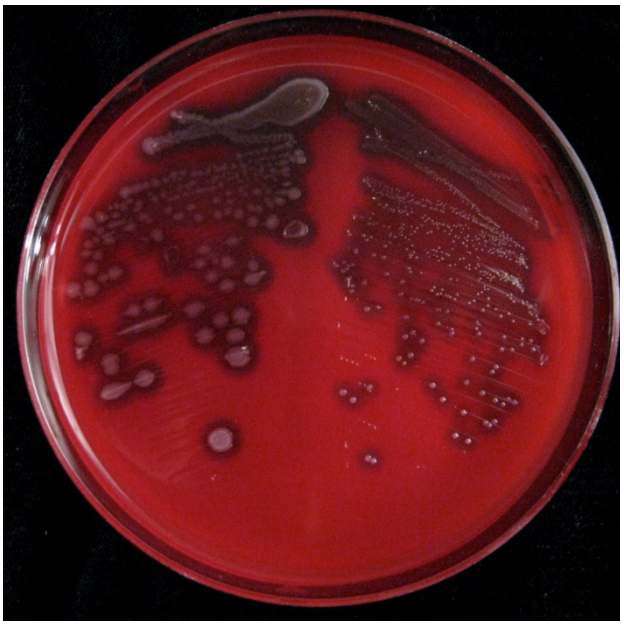

Figure 11: Streak of MGAS5005 and MGAS5005 *ΔptsI*. Streak plates showing the small colony phenotype of MGAS5005*.ptsI* (right) compared to WT (left) on TSA blood agar plate.

To investigate if PTS influences *hasA*, the transcript levels of *hasA* was quantified using qRT-PCR. The mRNA was extracted from wild type and the corresponding isogenic *ΔptsI* mutant from all three M1T1 grown in THY and collected at mid-log phase of growth as *hasA* gene transcripts are reported to be maximal during mid-log phase of growth (192). As shown in Fig. 12A, there was a significant reduction in *hasA* transcript levels in *ΔptsI* mutant compared to wild type MGAS5005. The same experiment was then repeated on RNA extracted with the cells grown in CDM + 0.5% glucose and C media to investigate if varying the carbohydrate concentration has any effect on *hasA* transcript levels. As observed for cells grown in THY, the transcript levels of *hasA* in MGAS5005.Δ*ptsI* were significantly reduced in both CDM and C media ranging from 2.5 to 4 fold (data not shown). Similarly, a significant reduction in the *hasA* transcript levels (3-4 fold) was observed in the Δ*ptsI* mutant compared to the wild type 5448AP (Fig 12C). In contrast, a 4-fold increase in *hasA* transcript levels was observed in 5448.Δ*ptsI* compared to 5448.

In addition to the three strains of M1T1 serotype, a Δ*ptsI* mutant was also constructed in a divergent serotype M4 (GA40634). A modest but a significant reduction (2.5 fold) was observed in *hasA* transcript levels (Fig. 12D). These data suggest that a functional PTS is important for optimal regulation of capsule production. However, the phenotype appears to depend on the *covS* status of the cell. Capsule formation correlated with significant reduction in *hasA* transcript levels only in the Δ*ptsI* mutants of M1T1 harboring a *covS* mutation and M4 GAS (Fig. 12ACD). Thus, reduction of capsule expression in the Δ*ptsI* mutants likely explains their small colony

size on blood agar.

Hyaluronic acid capsule of GAS is a major virulence factor, contributing to bloodstream survival through resistance to neutrophil killing and other immune effectors (193). Strains deficient in capsule production are readily phagocytosed. To test the fitness of the *ptsI* mutant in human blood, Lancefield bactericidal assays were performed for MGAS5005, 5448, 5448AP strains. The wild type and the *ptsI* mutant strains of each were grown in freshly drawn human blood for 3 hours and measured for survival by calculating the multiplication factor (MF). The multiplication factor was calculated by dividing the CFU obtained after blood challenge by the initial CFU inoculated. Data are presented (Fig. 13) as percent growth in blood corresponding to the MF of the mutant divided by the MF of the wild type (WT) *100. The *ΔptsI* mutant strains also carrying a mutation in *covS* showed a ca. 30% reduction in survival compared with the corresponding wild type strain (Fig. 13), correlating with a defect in capsule formation.

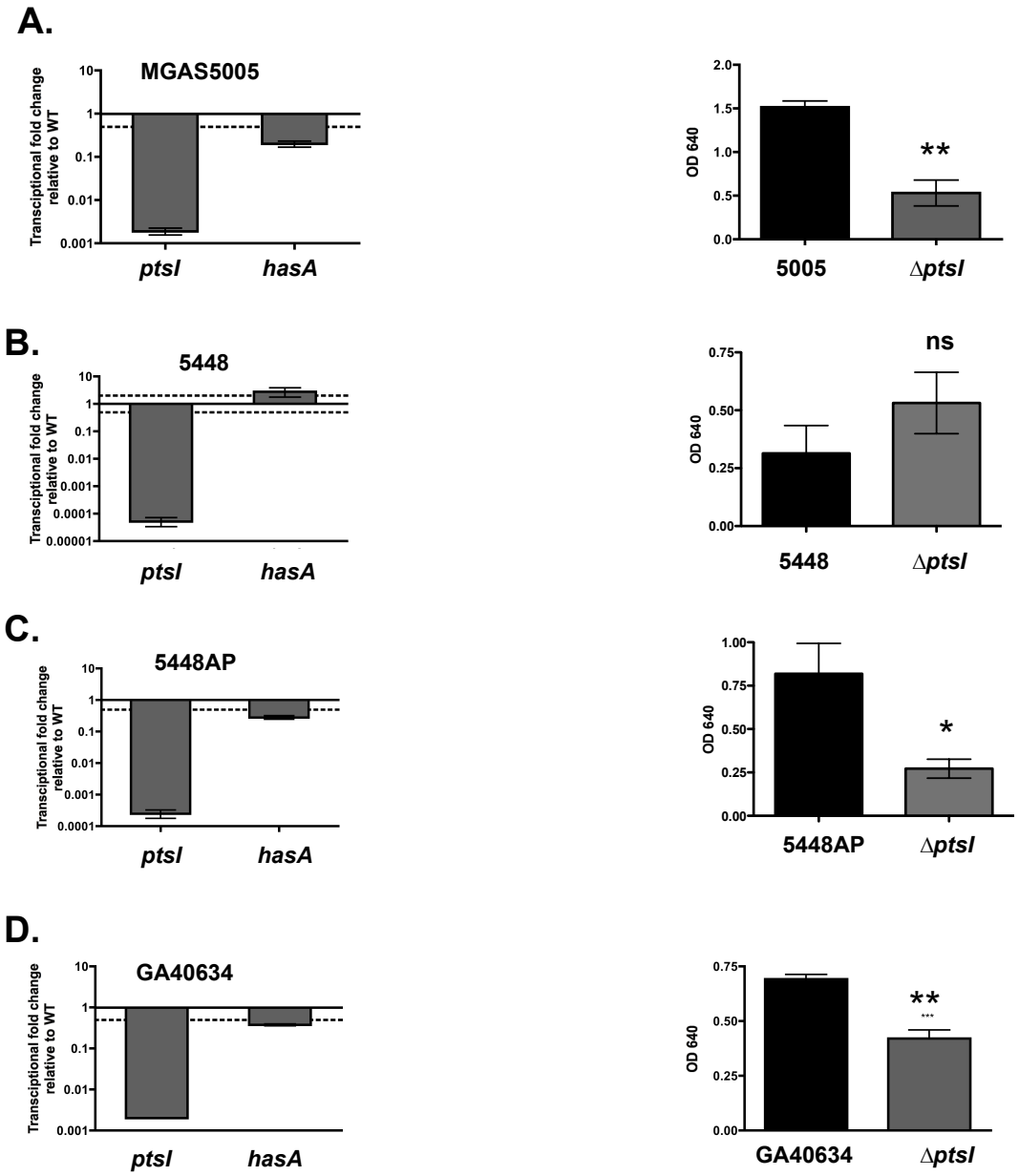

Figure 12: Effect of *ptsI* mutant on hyaluronic acid capsule production. Transcript levels and capsule production of (A) MGAS5005, (B) 5448, (C) 5448AP, and (D) GA40634 compared to each respective wild type. The transcript levels (left column) of *ptsI* and *hasA* measured by qRT-PCR at late-logarithmic growth in THY. Two-fold differences in expression (dashed line) were considered significant. Standard error was determined from three biological replicates. Hyaluronic acid production (right column) was measured using Stains-All from mid-log cells grown to OD_{600} of 0.5 and comparable cfu in THY. P values were determined using an unpaired two-tailed t test. **p≤0.01, ***p≤. 001; NS, not significant.

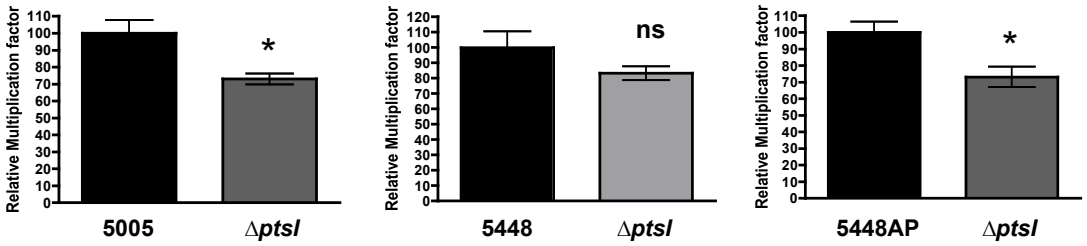

Figure 13: Lancefield bactericidal assay. Lancefield bactericidal assay of wild type and isogenic Δ*ptsI* mutant following growth in whole human blood for 3 hours Data are represented as relative multiplication factor. Significance was determined using an unpaired two-tailed t test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq .001$; NS, not significant).

4.2.2 Δ*ptsI* mutant increases secretion of SpeB in a strain-specific manner.

The cysteine protease SpeB is a well-characterized secreted virulence factor that has been shown to be a key contributor in various models of GAS skin infection, including ulcerative lesion formation (113,194,195). To investigate whether the M1T1 *ΔptsI* mutants resulted in altered *speB* transcript levels, qRT-PCR was performed on mRNA isolated from wild type and the corresponding isogenic *ΔptsI* mutant from all three M1T1 strains at the transition phase of growth (maximal expression of *speB*). No significant differences (greater than 2-fold) in *speB* transcript levels were observed for any of the three *ΔptsI* mutants compared to their respective parental strain (data not shown), suggesting that transcription of *speB* is not altered in any of the mutants.

Increased secretion of active mature SpeB by GAS has been shown to negatively effect the formation of biofilms (195-197) and can be used as an indirect reporter for SpeB activity. Thus, a biofilm formation assay was performed on each M1T1 wild type and its respective Δ*ptsI* mutant. Cells were grown in a 6-well plate and allowed to grow for 24 hours at 37°C and biofilm formation was quantified by crystal violet staining. MGAS5005.Δ*ptsI* and 5448AP.Δ*ptsI*, both *covS* mutant backgrounds, showed significantly less biofilm than their wild type parental strains (Fig. 14, left). Importantly, the reduction seen in these Δ*ptsI* mutants could be reversed with the addition of the cysteine protease inhibitor E64 (Fig. 14, left). In contrast, 5448.Δ*ptsI* showed no significant difference compared to the wild type 5448. It should be noted

that 5448 is reduced in its ability to form biofilm compared to MGAS5005 and 5448AP, and an MGAS5005.Δ*speB* control exhibited no significant difference in biofilm formation when compared to 5448 (Fig 14A).

Secretion of SpeB into the supernatant of all three *ΔptsI* mutants and their wild type strains was assayed by western blot using a polyclonal SpeB antibody. No differences were observed in the amount of cell-bound SpeB of *ΔptsI* mutants compared with their corresponding wild type strain (data not shown). However, dramatically increased levels of secreted SpeB (both 40-kDa zymogen and 28-kDa mature forms) were detected in the culture supernatant of MGAS5005.Δ*ptsI* and 5448AP.Δ*ptsI* compared to their isogenic *covS* parental strains (Fig. 14A-C, right). Interestingly, 5448.Δ*ptsI* had decreased levels of secreted SpeB compared to wild type 5448, which correlates with the biofilm forming ability. Although this strongly suggests that EI (PTS) may reduce SpeB secretion in a CovS-dependent manner (MGAS5005.Δ*ptsI* and 5448AP.Δ*ptsI),* this does not provide a mechanism for how increased lesion severity occurs in 5448.Δ*ptsI*.

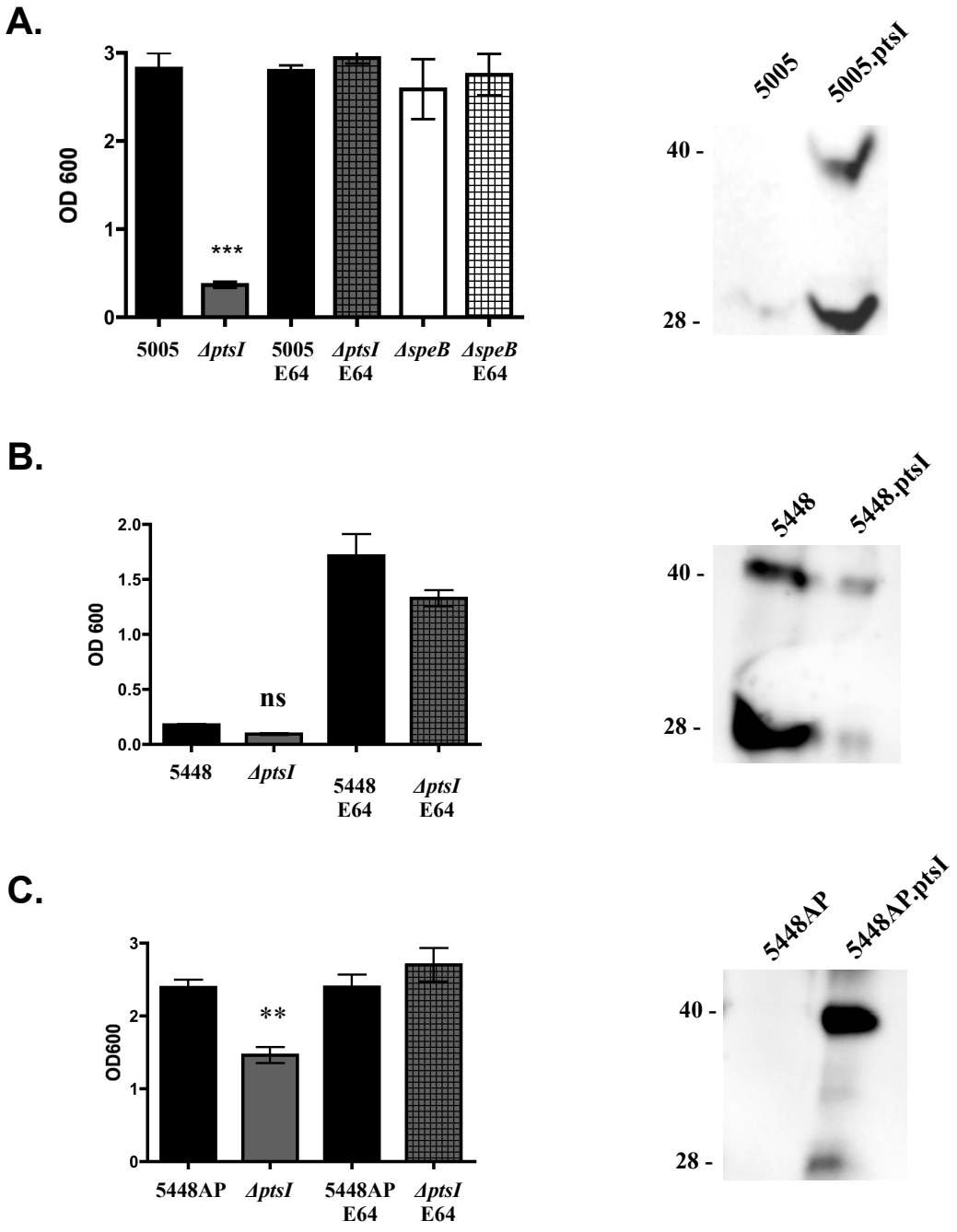

Figure 14: Effect of *ptsI* mutant on SpeB. Biofilm formation and SpeB secretion for MGAS5005 (A), 5448 (B), and 5448AP (C). Quantification of biofilm formation (left column) was determined by crystal violet staining with and without the cysteine protease inhibitor E64 (333µm). P values were determined using an unpaired two-tailed t test. **p≤0.01, ***p≤.001; NS, not significant. SpeB secretion (right column) was assayed Western blot for SpeB in cell-free culture supernatants collected at early stationary phase. Blot was probed with anti-SpeB. The 40 kDa band is the inactive zymogen and the 28 kDa band is the cleaved active mature protein.

4.3 Discussion

In this chapter we explored the influence of the *ptsI* mutation on capsule synthesizing operon and the protease SpeB in multiple strains of GAS. It was found that *ptsI* mutation plays a role in the regulation of both potential Cov-dependent and strain-specific manner. Furthermore, this work lays the groundwork for future experiments analyzing the influence of PTS on virulence factor regulation in GAS through possible interplay with the CovR/S two component system.

4.3.1 Role of Δ*ptsI* on capsule production:

The appearance of a small colony phenotype on blood agar plates (Fig. 11) suggested a possible metabolic defect. Although, this phenotype has not been linked directly with a *ptsI* mutant so far, the appearance of small colonies on blood agar has previously been connected to metabolic regulators. In *S. pneumoniae*, for example, a small colony phenotype on blood agar has been previously associated with a carbohydrate metabolic regulator CcpA that was linked to a metabolic defect (161). In another study, in *S. pneumoniae* a small colony phenotype on blood agar plate was attributed to reduced transcription from the capsule locus by RegM, causing closer packing of bacterial cells within colonies (198). In addition, Giammarinaro et al, reported attenuation in mice in Δ*regM* background. Our results show that *ptsI covS* double mutants of MGAS5005 and 5448AP show significantly reduced transcript levels of *hasA* leading to reduced capsule production (Fig. 12AC). In addition, significantly reduced capsule levels were also observed in a class II M4 serotype and the phenotype was rescued with the *ptsI* complement. Taken together, this suggests that EI positively influences capsule production. In contrast, a modest increase in

hasA transcript levels and comparable capsule production are observed in the 5448 background (Fig. 12B). However, it should be noted that due to the presence of intact CovS, less detectable levels of hyaluronic acid are produced (Fig. 12, right).

The reduced fitness observed in blood with the *ptsI* mutant in MGAS5005 and 5448AP (*covS*$^-$) nicely correlates with the reduction in capsule production. Capsule has been previously connected to GAS resistance to killing by blood leukocytes and opsonization (185,199). These data suggest that a functional PTS is required for an optimal fitness of GAS in human blood by altering the regulation of hyaluronic acid production. However, this regulation seems to be dependent on the CovS status of the cell (Fig. 13). Aziz et al. report that the primary difference between the strains lies in the CovRS two- component system (200). So far, CovR is the only regulator known to bind directly to the promoter region of *hasA*. The 'signal' that CovS senses is still not known, although Mg^2+ and antimicrobial peptides can induce the system (201,202). The CovS mutation in the invasive strains results from the selection pressure driven by the host immune response. Under stress conditions, CovS acts as a phosphatase has been reported to dephosphorylate CovR, reversing the gene regulon of CovR (75,203). The absence of a functional PTS results in inability to utilize a number of carbohydrate sources and likely acts as a stress to the cell, resulting in alteration of the regulation of the genes controlled by CovS.

4.3.2 Role of PTS in SpeB secretion

The cysteine protease SpeB is Cov-regulated and has been directly associated with the formation of severe lesions in skin models of GAS infection (113,194,195). Our

study shows that the transcription of *speB* is not altered in any of the *ptsI* mutants tested, but these mutations influence the secretion of SpeB in a *covS*-dependent manner. Since we did not observe a PTS-dependent effect on SpeB common to all three M1T1 backgrounds, does not explain the hypervirulent phenotype observed by the mice infected with *ptsI* mutant (Fig. 6,8,10). SpeB is a protease that cleaves surface proteins. Increased secreted SpeB levels also likely are involved in reduced capsule production observed. In addition to CovR/S, an array of GAS factors have been shown to influence *speB* transcription such as Srv, Rgg, RofA, and Mga (121). Although there is no known relation between carbon uptake and *speB* expression, expression of *speB* is growth-phase dependent and influenced by environmental factors. In vitro, expression of *speB* is induced during late exponential/stationary phase of growth and induction corresponds with nutrient depletion (204). In our study, we found no alteration in the transcript levels of these regulators in the Δ*ptsI* mutant of MGAS5005 when compared to the parental wild type strain. Only RopB, CovR and CcpA have been shown to directly bind the *speB* promoter. Therefore a detailed study of the interplay of proteins in this region will be crucial to understand clearly the events that lead to *speB* transcription.

Chapter 5: Effect of PTS on the Mga virulence regulon

5.1 Introduction

In addition to classical two-component systems (TCS), GAS controls global regulation of virulence factors by utilizing 'stand-alone' regulators (132,205), representing transcription factors that control virulence regulons in response to the growth phase. Mga was the first such stand-alone regulatory network described in GAS and allows the pathogen to adapt and flourish in host environments favourable for growth (126). The gene encoding Mga (*mga*) has been found in all sequenced GAS genomes and strains tested, exhibiting two divergent alleles (*mga-1*, *mga-2*) that correlate with different tissues sites of infection (206). Mga strongly activates transcription of a number of established virulence genes important for early colonization and adhesion during the exponential phase of growth (carbohydrate-rich conditions) and is critical for multiple pathogenic phenotypes, including biofilm formation, growth in whole blood, resistance to phagocytosis, and optimal virulence (207-210).

Genes regulated directly by Mga encode mostly cell-wall attached surface molecules important for adherence to host tissues, internalization into non-phagocytic cells and evasion of the host immune responses, including M protein (*emm*), M-like proteins (*arp*), C5a peptidase (*scpA*), collagen-like proteins (*scl1*, *sclA*), fibronectin-binding proteins (*fba*, *sof*), and the secreted inhibitor of complement (*sic*) (126). Numerous studies using a variety of animal models of GAS disease, including mice, non-human

primates and zebrafish, have established that the core Mga regulon plays a critical role in GAS pathogenesis *in vivo* (85). Over 100 different serotypes of GAS have been identified based upon variability in the cell-surface M protein (*emm* gene product). Strains expressing class II M protein also produce serum opacity factor (SOF+), whereas class I GAS strain do not (SOF^-) (132). Transcriptomic analyses in several GAS serotypes found that Mga regulates over 10% of the genome, including apparent indirect repression of operons encoding proteins involved in the transport and utilization of sugar sources (128). Thus, Mga is able to regulate genes important not only for virulence, but also for sugar metabolism and the catabolite control protein A or *ccpA* (211). In addition, synthesis of the Mga-regulated M protein can be influenced by specific sugars such as glucose (142,211). Furthermore, Mga regulon expression was shown to peak during the acute phase of infection in a primate model of GAS pharyngitis, directly correlating with carbohydrate utilization genes (210). Taken together, these findings suggest that Mga activity is linked to sugar metabolism; however, it is not clear how Mga is able to monitor the carbohydrate status.

A recent study by Hondorp et al. demonstrated that the conserved carboxy-terminal region of Mga, containing a PTS EIIB-like domain, is important for oligomerization of Mga as well as activation of its regulon *in vivo* (212). In addition, an *in silico* analysis comparing Mga to proteins of known structure in the Protein Database (PDB) revealed two potential PTS regulatory domains (PRDs) in the central region of Mga with three conserved histidines; two in PRD-1 and one in PRD-2, which might

serve as a site of phosphorylation (Hondorp et al. 2013) (Fig.15). The resulting domain prediction of Mga most closely resembles that of the mannose operon activator MtlR from *Geobacillus stearothermophilus*, which is modulated via phosphorylation of conserved histidine residues within its PRD domains (213). Proteins composed of dual PRD domains include both antiterminators (e.g., LicT) and activators (e.g., MtlR and LicR) involved in the regulation of sugar metabolism (4). The predicted structure of Mga also closely resembles the *B. anthracis* virulence regulator AtxA. Activity of these PRD-containing regulators is modulated by phosphorylation of conserved histidine residues within their PRD domain(s) via the PTS phosphorelay in response to the utilization of different carbohydrate sources (39).

In GAS and other Gram-positive pathogenic bacteria, when a favorable carbon source is present, carbon catabolite repression (CCR) is mediated by CcpA to prevent metabolism of an inferior sugar (213-215). However, in the absence of a rapidly metabolizable sugar, P~his-Hpr may begin to accumulate. Both P~his-Hpr and P~EIIB are also capable of phosphorylating conserved histidines within PTS regulatory domains (PRDs) of transcriptional antiterminators (e.g., LicT) and activators, (e.g., MtlR, LevR) thereby modulating their ability to regulate the expression of alternative sugar operons (213). Thus, the PTS provides a sophisticated sensory pathway for monitoring the metabolism of carbohydrates in Gram-positive bacterial pathogens.

Studies performed in our lab investigated whether the PRDs of Mga were involved in influencing Mga activity and, as a result, altering the Mga regulon in a the GA40634 (M4) serotype of GAS containing the *mga-2* allele. Hondorp et al. demonstrated that PTS phosphorylates Mga when the pathway (EI + Hpr) is reconstituted *in vitro* in the presence of radiolabelled [^{32} P]-PEP (Fig. 16A) (6). In addition, they showed that this *in vitro* phosphorylation by the general proteins of the GAS PTS (EI + Hpr) is specific to the region of Mga containing predicted PRD domains. Moreover, EI/Hpr mediated phosphorylation of Mga appears specific to the PRD histidines in the folded protein, since mutants lacking the conserved histidines or heat denatured wild type were not phosphorylated (6). This phosphorylation results in inactivation of Mga as shown by *in vitro* transcription of Mga-regulated *emm* in the presence or absence of PEP (Fig. 16B).

In this study, we propose that PTS-mediated phosphorylation of Mga serves to modulate its activity to regulate the Mga regulon in response to the metabolic state of the cell. This would provide a link between the metabolic state of the cell and Mga regulation of virulence gene expression.

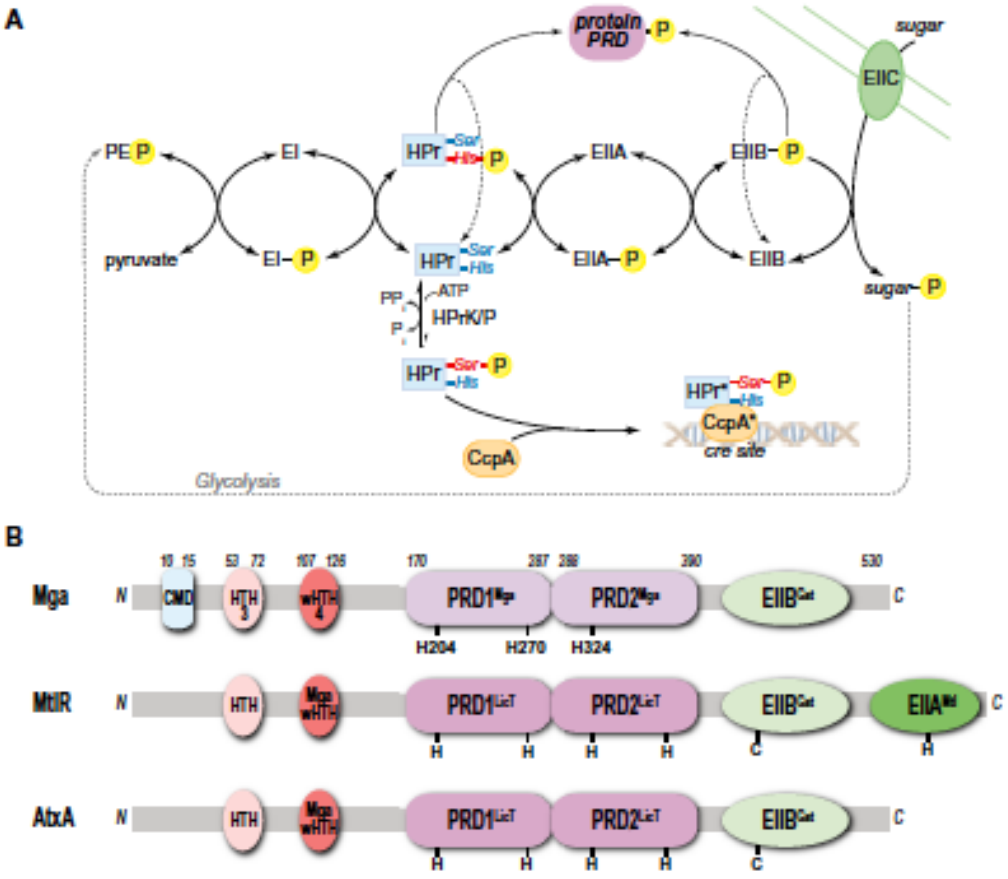

Figure 15: PTS pathway and alignment of Mga with PRD-containing regulators. (A) The phosphotransferase system (PTS) in Gram-positive bacteria couples the phosphorylation and import of sugars. The general cytoplasmic enzymes (EI and HPr) and sugar-specific EII components form a phosphorelay to transfer phosphate from phosphoenol pyruvate (PEP) produced by glycolysis to the incoming sugar. Carbon catabolite repression (CCR) results from HPrK/P phosphorylation of serine 46 of HPr, which then complexes with CcpA to repress target promoters via *cre* sites. HPr-His~P and EIIB~P can also phosphorylate PTS regulatory domains (PRDs) of sugar regulators, thereby modulating their activity. (B) Domain alignment of GAS Mga with *B. subtilis* MtlR and *B. anthracis* AtxA. EII (green), PRD (purple), DNA-binding (red) and conserved (blue) domains are indicated with conserved histidines (H) and cysteines

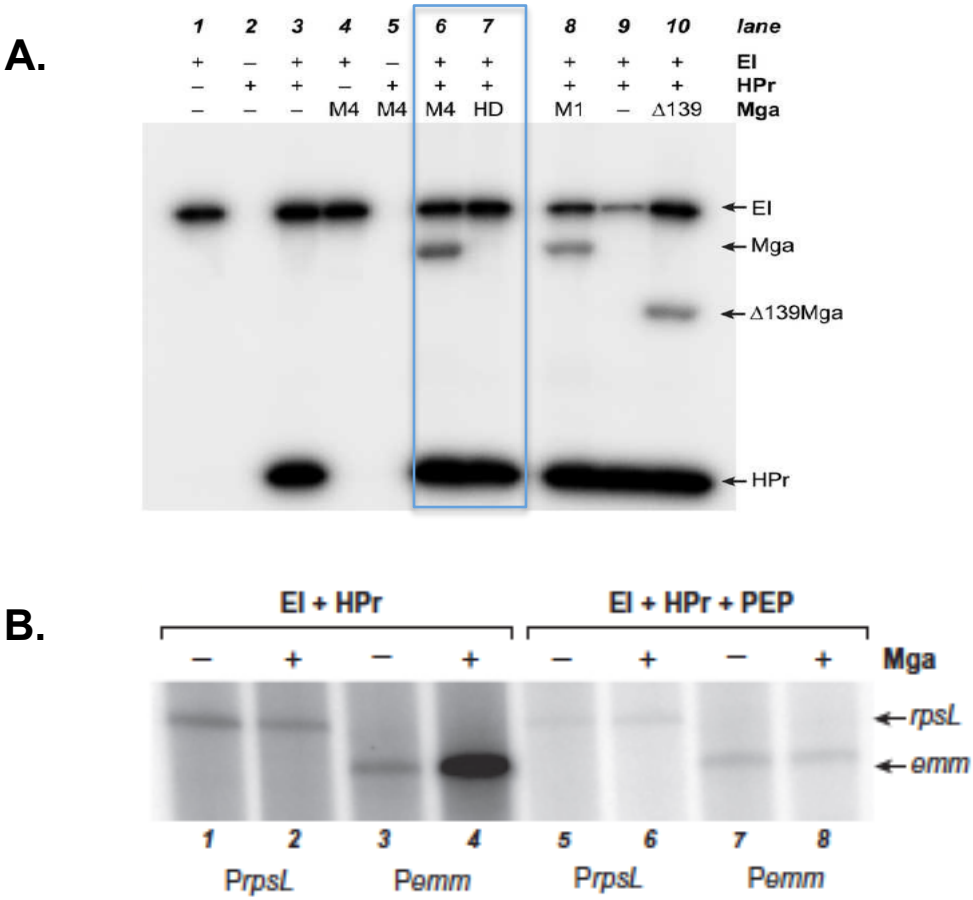

Figure 16: *In vitro* phosphorylation and inactivation of Mga by the PTS. Phosphorylation was assessed by incubation of [^{32}P]-PEP with purified His$_6$-EI, His$_6$-HPr, and Mga4-His$_6$. Reactions were then subjected to SDS-PAGE and phosphorimager analysis. (A) Proteins included in each reaction are indicated; HD refers to heat-denatured Mga4-His$_6$ (B) The ability of Mga to activate transcription of the constitutive P*rpsL* and Mga-regulated P*emm* promoters was assessed *in vitro* immediately following phosphorylation reactions (containing or lacking PEP). Products of the *in vitro* transcription assays were then separated on a 6% sequencing acrylamide gel and subjected to phosphorimager analysis. Figure adapted from (6).

To specifically explore the role of PRD histidine residues in the PTS-mediated phosphorylation, a series of Mga mutants were constructed in which each histidine was replaced by an alanine (H204A, H270A, and H324A) to prevent phosphorylation or an aspartate (H204D, H270D, and H324D) to mimic the phosphorylated residue (Fig. 17). Double PRD1 H204/H270 mutants (A/A and D/D) and triple H204/H270/H324 mutants of both PRD1 and PRD2 (A/A/A and D/D/D) were also prepared. Plasmids expressing wild type and mutant *mga4* alleles from the native promoter (P*mga4*) were transformed into an M4 Δ*mga* GAS background and Mga-regulated expression of *arp* and *sof* was assayed by qRT-PCR. None of the single alanine (non- phosphorylated) or aspartate (phosphomimetic) substitutions of the conserved histidine residues significantly altered Mga dependent *arp* and *sof* expression (Fig. 17) (6). The PRD1 double A/A Mga mutant exhibited a modest reduction in *sof* and *arp* expression; in contrast, the phosphomimetic double D/D Mga mutant was almost completely inactive compared to wild type (Fig. 17) (6). The PRD1/PRD2 triple A/A/A mutation also showed a ca. 9-fold reduction in Mga activity, perhaps due to structural perturbations, but not to the level of the PRD1/PRD2 D/D/D protein, which was similar to PRD1 D/D Mga4 (Fig. 17) (6). Thus, Hondorp et al. established that doubly phosphorylated PRD1 phosphomimetic (D/DMga4) is completely inactive *in vivo*, shutting down expression of the Mga regulon (6).

Mga controls its regulon by binding to the promoters of the genes. Thus, the inactive D/DMga4 was thought to be unable to bind to the DNA. Interestingly, Hondorp et al.,

also report that D/DMga4 was still able to bind DNA *in vitro*, but the homomultimerization of Mga was found to be disrupted and the protein was unable to activate transcription (6). This also suggested that while all three PRD histidines of Mga appear to be phosphorylated, phosphoryation of PRD1 leads to inactivation of Mga and seems to have the most significant functional consequence for GAS as seen by qRT-PCR at late exponential phase of growth (Fig 17). These data suggested that the mutations result in changed Mga structure, thus, interfering with its activity as transcription factor. Hence, it became important to understand the *in vivo* relevance of these results with a wild type Mga by PTS.

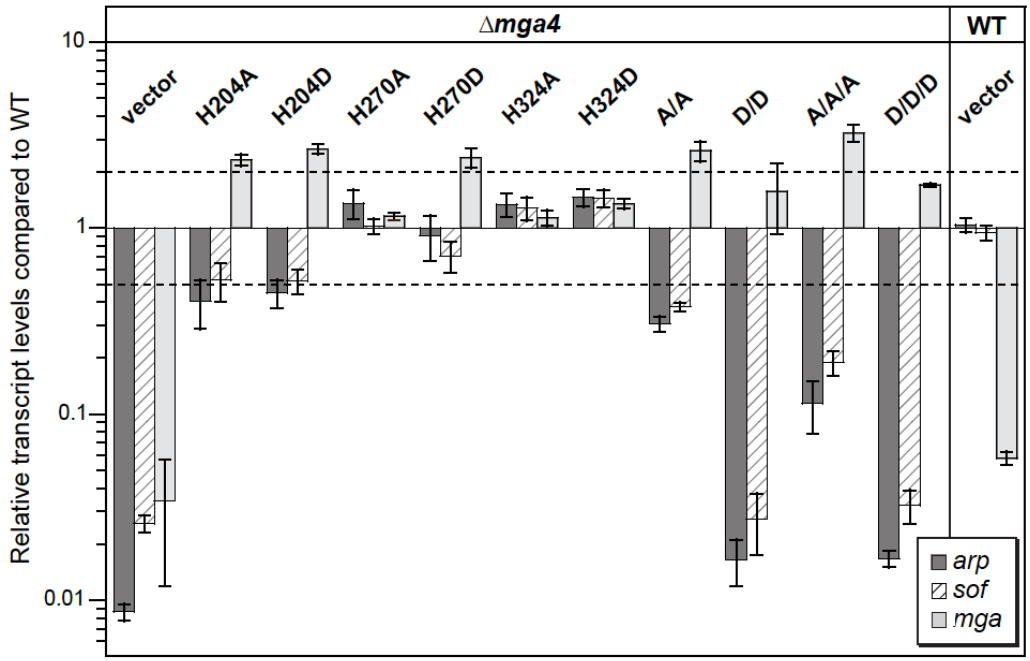

Figure 17: The conserved PRD1 histidines are important for M4 Mga activity *in vivo*. A) In vivo Mga activity was assessed by real-time RT-PCR analysis of arp (dark grey), *sof* (striped) and *mga* (light grey) mRNA. Transcript levels that were greater than 2-fold different compared to wild type (dotted lines) were considered significant. Single mutations in Mga are indicated, along with H204A/H270A (A/A), H204D/H270D (D/D), H204A/H270A/H324A (A/A/A), H204D/H270D/H324D (D/D/D), and an empty vector (vector) in the mga4-inactivated GAS strain KSM547.4. An isogenic wild type M4 strain GA40634 (WT) with vector was also assayed to show endogenous Mga4 (end WT) activity.

5.2 Results

5.2.1 GAS EI mutant (Δ*ptsI*) is defective for growth in several carbohydrates:

To assess the role of the PTS in Mga-dependent regulation in M4, an EI mutant (*ΔptsI*) was constructed in the strain GA40634 following the same strategy as Δ*ptsI* mutants in M1T1 background mentioned in chapters 3 and 4. Wild-type *ptsI* was replaced with an in-frame deletion (*ΔptsI*) containing a non-polar *aad9* spectinomycin resistance cassette (216) in the GA40634 genome. Although wild-type GA40634 had a slightly increased lag phase compared to the mutant in rich THY media, growth kinetics of the wild type, *ptsI* mutant and the complemented *ΔptsI* strain were comparable (Fig. 18A). Similar to the M1T1 Δ*ptsI* strains, the growth rate of GA40634.Δ*ptsI* was comparable to parental GA40634 in low glucose C medium, except the mutant strain reached a slightly lower overall yield (data not shown). Carbohydrate-specific phenotypes of the wild-type GA40634 and the GA40634.Δ*ptsI* mutant were analyzed by growth assays in chemically defined media (CDM) supplemented with various carbohydrates serving as the sole carbon source. In CDM containing 0.5% glucose, GA40634.*ΔptsI* showed a comparable growth rate to GA40634, except with higher yields and no significant lag phase (Fig. 18B) The complemented Δ*ptsI* strain, exhibited similar growth rate to wild type but reached higher final OD (Fig 18B). In contrast, GA40634.Δ*ptsI* was unable to grow when the PTS sugars fructose, sucrose (Fig. 18CD), lactose, sucrose, galactose, trehalose, and mannose (data not shown) were tested. This growth defect phenotype was rescued by the strain containing the complemented *ptsI* allele as indicated by the growth profile

exhibited a growth profile comparable to wild type (Fig 18). This was identical to that found for independent Δ*ptsI* mutants generated in three different M1T1 GAS strains MGAS5005, 5448, and 5448AP (Fig 5, 7) and confirms that GA40634.Δ*ptsI* lacks a functional PTS.

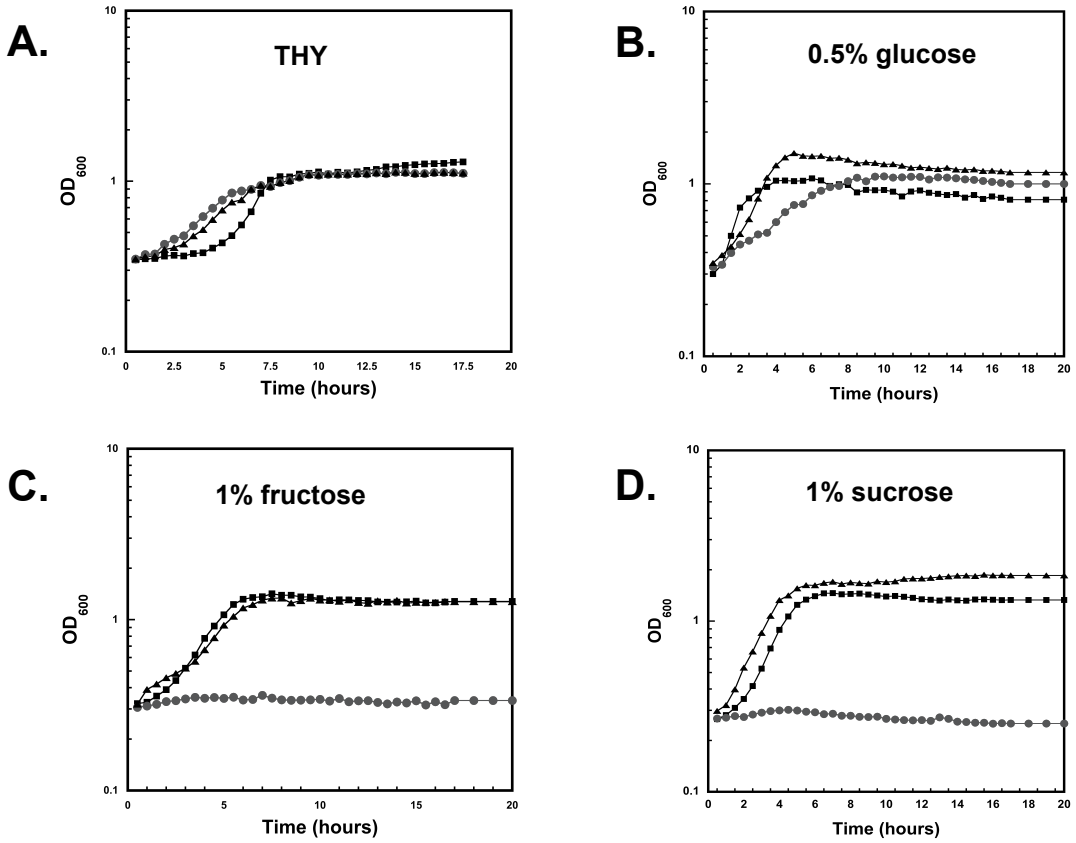

Figure 18: *A* Δ*ptsI* mutant of GAS is altered in PTS-dependent growth. (A) Growth curves of wild type GA40634 (squares), GA40634Δ*ptsI* (grey circles), complemented GA40634Δ*ptsI (*triangles*)* in (A) THY (B) CDM supplemented with either 0.5% (v/v) glucose or (C) 1% (v/v) fructose or (D) 1% (v/v) sucrose. Data are representative of the average of three independent experiments

5.2.2 Δ*ptsI* alters the Mga dependent virulence gene regulation in M4 GAS:

Given our hypothesis that PTS phosphorylates Mga to alter its activity, we sought to determine if a Δ*ptsI* mutant in GA40634 would exhibit Mga activity similar to the AAA (non-phosphorylatable) mutant strain. For this, qRT-PCR was performed on mRNA isolated from wild type and the *Δptsl* mutant at late logarithmic phase of growth in both THY (rich), C media (low glucose) and CDM with 0.5% glucose, probing for the Mga-regulated genes *arp* and *sof*. Relative transcript levels of GA40634.Δ*ptsI* compared to wild-type GA40634 were determined, such that full activity gives a ratio of 1.0 and a difference of greater than 2-fold was considered significant (Fig. 19, dotted lines). As expected, *ptsI* transcript levels were reduced 2 to 3 logs in the mutant (Fig. 19). In addition, both *arp* and *sof* transcript levels were significantly reduced (3- to 10-fold) in the Δ*ptsI* mutant compared to wild type.

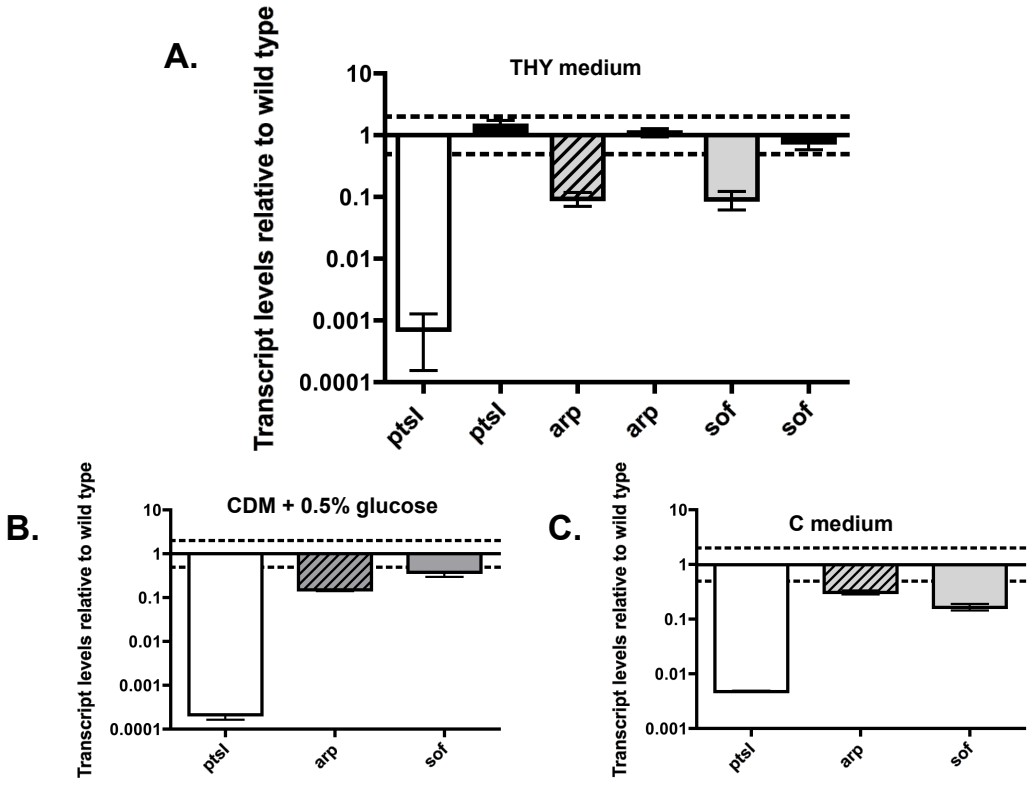

Figure 19: ***A*** **Δ*ptsI* mutant of GAS alters Mga regulon in M4.** Transcript levels of *ptsI* (white) *ptsI*c (black), *arp* (striped), and *sof* (grey) were measured by qRT-PCR from late logartithmic phase cultures grown in (A) THY and (B) CDM +0.5% (v/v) glucose (C) C medium for GA40634 Δ*ptsI* compared to GA40634. Two-fold differences in expression (dashed line) were considered significant. Standard error was determined from three biological replicates.

5.2.3 Δ*ptsI* influences the Mga virulence regulon differently in M1T1 GAS:

We investigated the effect of a Δ*ptsI* mutation on Mga regulated genes by qRT-PCR in the mutant strains MGAS5005.Δ*ptsI*, 5448.Δ*ptsI* and 5448AP.Δ*ptsI* compared to their respective M1T1 wild type parents. Since the strains expressing class I M protein do not express *sof*, we probed for the Mga-regulated secreted inhibitor of complement (*sic*) along with *emm*. Interestingly, the effect of loss of *ptsI* on Mga-regulated gene expression was more variable in the different M1T1 backgrounds. In MGAS5005.Δ*ptsI*, a significant reduction (2-3 fold) was observed for *sic* transcript levels, but not for *emm* transcript levels (Fig. 20A). However, no significant difference was seen in the transcript levels of *emm* or *sic* in 5448AP.Δ*ptsI* when compared to wild type 5448AP in THY, C media and CDM (Fig 20B, data not shown).

In contrast, 5448.Δ*ptsI* showed a significant up regulation of *emm* and *sic* (3-60 fold) observed in THY along with CDM + 0.5% glucose and C media (Fig 20CDE). Together, these data indicate that a functional PTS influences Mga-dependent virulence gene expression during logarithmic phase growth in M1T1 GAS.
However, that influence can vary considerably in different genetic backgrounds.

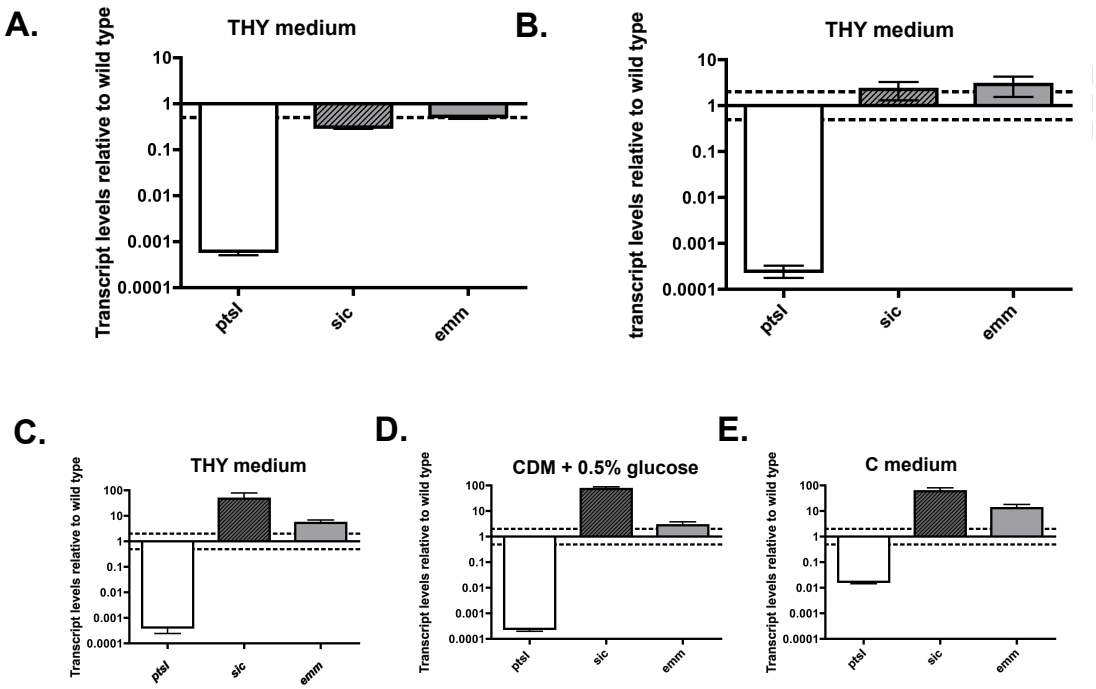

Figure 20: A Δ*ptsI* mutant of GAS alters Mga regulon in M1T1. Transcript levels of *ptsI* (white) , *sic* (striped), and *emm* (grey) were measured by qRT-PCR from late logartithmic phase cultures grown in THY of (A)MGAS5005Δ*ptsI* compared to MGAS5005 and (B) 5448APΔ*ptsI* compared to 5448AP. Transcript levels of 5448Δ*ptsI* grown in (C) THY (D) CDM +0.5% (v/v) glucose and (E) C media compared to 5448. Two-fold differences in expression (dashed line) were considered significant. Standard error was determined from three biological replicates.

5.3 Discussion

There is an increasingly appreciated connection between bacterial pathogenesis and sugar availability during infection; however, the molecular mechanisms that might allow this interplay are not well understood. The finding in our lab by Hondorp et al. that the Mga stand-alone regulator possesses homology to PRD domains found in sugar-specific regulators suggested a mechanism by which Mga might link the PTS to global virulence regulation in GAS (209,217). Together with the Hondorp study, findings in this dissertation validate the hypothesis that PTS influences the Mga activity resulting in altered expression of its regulon. Therefore, this provides a mechanism whereby the pathogen could modulate expression of Mga-regulated virulence genes in response to environmental sugar status.

While Mga regulation of target genes has been well studied, the means by which Mga is controlled was not understood. The predicted presence of PRD and conserved histidines within the domain in Mga led to the hypothesis that Mga activity could be regulated via PTS. For PRD-containing sugar regulators, the functional impact of PTS phosphorylation can be quite variable, with phosphorylation often showing antagonistic effects depending on which domain is targeted (213). According to Hondorp et al, while all three PRD histidines of Mga appear to be phosphorylated *in vitro* (data not shown), phosphorylation of PRD1 seems to have the most significant functional consequences for GAS *in vivo*. Real-time RT-PCR analysis of a doubly phosphorylated PRD1 phosphomimetic protein (D/D Mga4) showed complete loss of

activity regardless of the phosphorylation status of PRD2 (Fig. 17), indicating that PRD1-mediated inactivation of Mga is dominant (6).

Yet interestingly, the data also suggest that phosphorylation of PRD2 might enhance Mga activity. When phosphorylation of PRD1 is prevented (in the A/A PRD1 mutant), the detrimental effect of not being able to phosphorylate His324 in PRD2 is evidenced by a 2- to 3-fold loss in activity for the A/A/A mutant compared to the A/A background (Fig. 17) (6). Although qRT-PCR analyses indicate that single mutations in PRD2 do not affect Mga activity, it is quite likely that this is an artifact of the multi-copy plasmid-based system employed for these experiments. Even though Mga levels are significantly higher than when endogenously expressed, equivalent activity is observed (Fig. 17), which suggests that another factor may be limiting and maximal activity is capped under these conditions. Therefore, even if preventing PRD2 phosphorylation in the H324A mutant decreases Mga activity, protein concentrations may be high enough to compensate such that expression of the Mga regulon by the H324A mutant appears similar to that of the wild-type protein. Furthermore, because Mga activity is already saturated in this system, the H324D phosphomimetic would not be expected to exhibit increased activity. Thus enhancement of Mga activity by PRD2 phosphorylation may only be observed in the compromised A/A background (Fig. 17) or in the Δ*ptsI* mutant where *mga* is in single copy (Fig. 20).

Moreover, the results from this work show that the lack of a functional PTS in the GA40634.Δ*ptsI* mutant result in decreased Mga activity (Fig. 19), suggesting that the

PTS can also serve a stimulatory role. In contrast to GA40634.Δ*ptsI*, an increase in transcription of Mga-regulated genes was observed in 5448.Δ*ptsI* compared to its parental strain 5448 (Fig. 20CDE). In addition, absence of a functional PTS did not seem to influence transcript levels of *emm* at late exponential phase in MGAS5005 and 5448AP background. However, a modest decrease was observed in transcript levels of *sic* in MGAS5005. Δ*ptsI*. This may suggest that Mga binds to the promoters of *emm* and *sic* with different affinities.

In general, phosphorylation of PRD-containing sugar regulators by an inducer-specific $EIIB^{sugar}$ protein inactivates the regulator while phosphorylation by EI/HPr enhances activity (213). Hondorp et al. found that EI/HPr phosphorylates and inactivates Mga *in vitro* (Fig. 16). Yet, because EI/HPr is known to be able to phosphorylate PRDs *in vitro* when an $EIIB^{sugar}$ is the *in vivo* phosphodonor (213), the specific roles of the PTS protein(s) modulating Mga activity *in vivo* are uncertain. However, the results of the GA40634.Δ*ptsI* from this work, combined with the known physiology of GAS allows for speculation. Assuming that non-phosphorylated Mga is active, the PRD1-phosphorylated protein is inactive (and dominant) and the PRD2-phosphorylated species has enhanced activity, the impacts on Mga function can be proposed under various growth scenarios (Fig. 21). Based on this model, the observed decrease in Mga activity in the Δ*ptsI* mutant compared to the isogenic wild type (Fig. 19) would only be expected to occur in the presence of glucose and absence of inducer if EI/HPr inactivates Mga and an $EIIB^{sugar}$ enhances activity.

In both THYB and C media, cells are growing in the presence of glucose, which argues that EI/HPr inactivates Mga via phosphorylation of PRD1 and $EIIB^{sugar}$ phosphorylation of PRD2 enhances activity (Fig. 21). Hence, EI/HPr and $EIIB^{sugar}$ may modulate Mga activity in an opposite manner to that which is common for PRD-containing sugar regulators. However, this mechanism of control would make sense given the biological role of Mga in pathogenesis. Niches where preferred PTS sugars are available appear to present a desirable setting for GAS colonization such that expression of the Mga regulon would be highly beneficial. Under these favorable growth conditions, ready phosphorylation of incoming sugars would limit the availability of P~his-Hpr to phosphorylate and inactivate Mga; furthermore, buildup of serine-phosphorylated HPr may act in concert with CcpA to increase *mga* expression (218). In contrast, carbohydrate-poor conditions may provide a signal for GAS dissemination in host tissues mediated by down regulation of Mga-regulated adherence factors. A lack of preferred PTS sugars would lead to the accumulation of P~his-Hpr, which could phosphorylate PRD1 of Mga, thereby precluding the formation of active multimers and shutting down expression of the Mga regulon (Fig. 21). Thus while these results allow us to speculate on the nuances of the mechanism in M4 GAS, the mechanism seems to be different in M1T1. Furthermore, the influence of phosphorylation by PTS on modulation of Mga activity in the strain with the presence of an intact CovS (5448), appears to be opposite. Nevertheless, this study clearly shows that the PTS plays direct role in regulation of Mga activity and virulence of the pathogen.

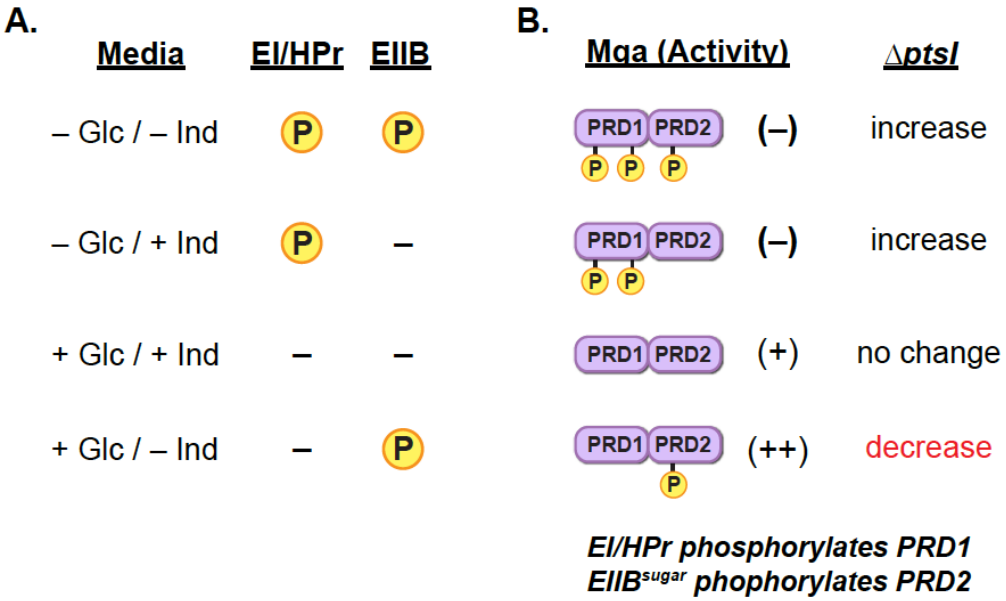

Figure 21: Model for PTS/Mga interactions in M4 *in vivo*. (A) Predicted availability of phosphate for transfer to Mga by either EI/Hpr or a sugar-specific EIIB component when growing in the presence or absence of a preferred sugar source (i.e., glucose) and/or an EIIB-specific inducer sugar. In the absence of glucose, EI/Hpr can phosphorylate PRD domains, whereas it cannot in its presence. Phosphorylation of PRD domains by a cognate EIIB protein would only occur in the absence of inducer. (B) Proposed role of PTS-mediated Mga phosphorylation on activity based upon sugar source in (A). In the absence of glucose, Mga would be inactivated through phosphorylation of PRD1. Inactivation of PTS (Δ*ptsI*) would be expected to increase Mga-regulated expression. With both glucose and inducer present, Mga is not phosphorylated at either PRD and is active. Loss of PTS (Δ*ptsI*) would have no effect. In the presence of glucose only (THY, C media, or CDM + glucose only), phosphorylation of Mga PRD2 by inducer-specific EIIB leads to enhancement of activity. In this case, loss of PTS (Δ*ptsI*) would result in a decrease in Mga regulon expression. Figure adapted from (21).

Chapter 6: Conclusions and Recommendations

The Group A Streptococcus (GAS) is a fastidious microorganism that has developed the remarkable ability to colonize a variety of niches in the human body, including the respiratory tract, the skin, and the bloodstream to cause a wide array of diseases. GAS relies heavily on carbohydrates as its energy source and to successfully elicit disease. While there is increasing evidence that carbohydrate utilization influences GAS pathogenesis, the role of the PTS pathway had not been investigated. The PTS represents a carbohydrate transport system that is also important for monitoring the metabolic status of the cell. Evidence presented in this dissertation shows that PTS signaling is not only important for carbon utilization, but also contributes to the pathogenesis of GAS by influencing virulence factor expression and global transcriptional regulators. Importantly, a functional PTS appears to limit the pathogenesis of GAS during invasive skin infection.

6.1 Carbon utilization by the PTS of GAS

The PTS of Gram-positive bacteria constitute a major carbohydrate transport pathway as evidenced by the presence of approximately 20 different PTS-associated proteins in the cell. The PTS has been studied in great detail in the *Bacillus* species and oral streptococci, but prior to this study, not much was known about the PTS in GAS. The *S. pyogenes* genome encodes 14 putative EII proteins whereas the cytoplasmic proteins Hpr and EI are common to all the carbohydrates imported via PTS EII transporters. EI from different bacteria are highly homologous, and most bacteria have only single *ptsI* gene encoding for EI (2) (4).

Deletion of EI is thought to result in a non-functional PTS pathway by disrupting the phosphate transfer to the histidine residue of the central regulator of carbon metabolism, Hpr. In this dissertation, we utilized a total of 4 GAS strains, of which three belong to the serotype M1T1 that is most associated with both invasive and non-invasive diseases in North America. Of these three strains, MGAS5005 and 5448 are clinical isolates whereas 5448AP is an animal-passaged variant of 5448. In addition, a strain, belonging to a divergent serotype M4 (GA40634) was also utilized. In all the strains tested, it was found that the PTS is required for utilization of not only predicted PTS-associated carbohydrate sources, but also non-PTS carbon sources; a phenotype that was rescued in the complemented *ptsI* strain. Using the carbon panels by BIOLOG, it was found that, in addition to PTS-associated carbon sources, MGAS5005.Δ*ptsI* also exhibited a metabolic defect that prevented utilization of 11 non-PTS-associated carbon sources, including glycerol. Beside PTS-associated EII proteins, P~his-Hpr phosphorylates glycerol kinase that is necessary for the activation of this enzyme (171). There are additional examples of P~his-Hpr influencing the non-PTS associated EII's such as the activity of non-PTS lactose permease of *S. thermophiles* is also influenced by the phosphorylation reaction of P~his-Hpr (219).

Interestingly, amongst the 26 sugars utilized by the MGAS5005.Δ*ptsI* mutant is glucose. This suggests the presence of a non-PTS transporter for glucose. In the related species *S. mutans* and *S. salivarus,* the utilization of glucose and other PTS sugars such as fructose and sucrose, have been reported to be PTS-independent and utilize an ABC- type active transport system called Msm for multiple sugar

metabolism (220,221). The presence of such a glucose transporter system still remains unknown in GAS. In *L. lactis*, a non-PTS glucose uptake system (GlcU) has been reported; however, genes encoding a homologous system are not found in the genomes of *S. pyogenes* or *S. pneumoniae* (222). This leads to question what mechanism is involved in uptake of glucose in GAS. To investigate this, further experimentation is required. There is a putative glucokinase gene present in the MGAS5005 genome that could act to phosphorylate glucose after uptake via a non-PTS transport system. For this, growth assays in the presence of glucose as a sole carbon source utilizing a strain carrying mutation in glucokinase encoding gene would be an important future experiment. Inability to take up glucose in this strain would not only confirm the presence of a non-PTS transporter of glucose in GAS, but also indicate that PTS is not required for glucose uptake. However, if we observe growth on glucose in the above experiment, constructing a glucokinase and a *pts* double mutant would be crucial to investigate the presence of additional transporters for glucose. Furthermore, a *ptsI* mutant from *Lactobacillus casei* has been reported to be able to metabolize glucose (223). Therefore, the uptake of glucose in the absence of a PTS is not uncommon; however, the identity of the non-PTS glucose transporter in GAS remains to be determined.

The balance between different phosphorylated forms of Hpr during growth contributes to the regulation of carbon metabolism in the bacterial cell. PEP-dependent phosphorylation of Hpr by EI yields P~his-Hpr, which is required for PTS-mediated transport of carbon sources. When PTS substrates are metabolized, the

level of glycolytic intermediates such as fructose-1,6-bisphosphate rises and stimulates HprK/P to generate P~ser-Hpr, which is required for CCR. Concomitantly, the rate of sugar consumption by PTS is reduced, because P~ser-Hpr is not a substrate for EI and does not participate in uptake of carbon sources. When the level of glycolytic intermediates drops and the level of inorganic phosphate rises, the bifunctional HprK/P catalyzes dephosphorylation of P~ser-Hpr yielding Hpr, which then leads to increased PTS dependent transport. In addition, carbohydrate uptake into Gram-positive pathogens can be regulated by several distinct mechanisms, including: 1) competition for a common permease, 2) inhibition by intracellular sugar-P, 3) inhibition by proton- electrochemical gradient, 4) PTS-mediated regulation, 5) competition for phosphor-Hpr, 6) regulation by ATP-dependent phosphorylation of a regulatory seryl residue in Hpr and 7) expulsion of intracellular sugars (28). It still remains a question, which of these mechanisms participates in the regulation of non-PTS sugars in the absence of functional PTS in GAS.

6.2 PTS and its role in virulence of GAS

Although EI is not essential for cell growth on a rich medium in the laboratory, a requirement for EI in a less friendly environment, such as during infection, cannot be excluded. However, little is known about the role of the PTS in bacterial virulence. Prior to this study, there was just one study done by Kok et al. that investigated the role of *ptsI* in a mouse model of staphylococcal disease (172). Kok et al. found that a *ptsI* mutant in the Gram-positive *S. aureus* was attenuated in virulence via the i.p. route of infection in C57BL/6 mice (172). This is in sharp contrast with our data (Fig. 6, 8, and 10). However, the differences could be attributed to the different pathogens,

the route of infection chosen, or the susceptibility of different inbred mouse strains. For GAS, the host genetic background largely determines the susceptibility of mice to this pathogen (224). For this dissertation, we infected the mouse via the subcutaneous (s.c.) route of infection in outbred CD1 mice that represented the diversity in the population, thus mimicking the natural infection by GAS in the human population. This route also allowed us to monitor progressing of infection from localized to systemic. All three independent M1T1 Δ*ptsI* mutants were able to colonize mice following subcutaneous inoculation and elicit both localized and systemic disease to at least the levels of their parental wild type strains (Fig 6, 8, and 10). This provides strong evidence that GAS does not require a functional PTS or the specific sugars transported by the PTS to infect at this tissue site. The mutants likely utilize a carbon source that is not altered in the Δ*ptsI* mutant (Table 3).

In fact, lesion formation was significantly more rapid with increased necrosis and size in mice infected with mutant strains (Fig. 6, 8, and 10). While this result was initially unexpected, the subsequent analysis identified that there was early production of SLS activity in all the Δ*ptsI* mutant strains tested. This potent cytolysin plays a significant role in pathogenesis of GAS and this hypervirulent phenotype has previously been observed in a *ccpA* mutant in the M1T1 background (145). Interestingly, Kinkel et al. found the entire operon encoding for SLS to be highly repressed by CcpA (145). Since Hpr closely interacts with CcpA to carry out CCR, it would be important to determine if CCR is playing a role in the phenotype observed in our study (Fig. 6, 8, and 10). To investigate this, *sagA* regulation and SLS activity in *ptsI ccpA* double

mutant will be important. However, since the CCR pathway remains intact in our Δ*ptsI* mutants (Hpr Kinase, Hpr, CcpA), our findings may reflect a novel pathway for influencing *sag* expression and SLS production based on carbohydrate availability. Also, we cannot rule out that both CcpA and the PTS are impinging indirectly on the same regulatory pathway and leading to repression of SLS production.

Additionally, the *sag* operon is also under tight regulation of the two-component response regulator CovR (173). Our study suggests the effect of *ptsI* on SLS activity is CovS-independent; yet, there is published evidence that CovR regulates the *sag* operon by direct binding to the P*sagA* promoter (225). Thus, although we think that CovS is not involved, we cannot rule out a role for CovR. Other known regulators of the *sag* operon include the PTS regulatory domain (PRD)-containing virulence regulators (PCVR) Mga and RofA (6). We do not understand the mechanistic details of how PTS influences the expression of SLS and further studies would be required to investigate this.

In addition to the *sag* operon, CovR/S regulates the transcription of virulence factors such as capsule synthesis operon (*hasABC*) and cysteine protease SpeB (*speB*). In response to the stress signal sensed, CovS modulates the activity of CovR by phosphorylating it on an aspartate residue D53, leading to a conformational change and allowing binding to DNA (75). Interestingly, it has been reported that CovR retains some activity even in the absence of wild type CovS. This suggests a possibility that CovR may be modulated by kinases or phosphatases other than CovS.

This could involve other phosphorelay systems that interact with CovR, cross talk with a non-cognate histidine kinase, or, in the absence of CovS, low molecular weight compounds such as acetyl phosphate or carbomyl phosphate might serve as phosphodonors (226) (75). However, it has also shown that in order to be active, CovR needs to be phosphorylated. Moreover, under the general stress conditions (*in vivo*), CovS can reverse the regulation of CovR leading to derepression of virulence genes such as *hasA* and activation of *speB* (75).

Our results indicate the influence of EI on SpeB and HasA depends on the CovS status of the cell (Fig. 12, and 14). In MGAS5005 and 5448AP (strains with a spontaneous mutation in CovS) results in diminished SpeB secretion and derepression of *hasA*, forming a mucoid colony. Interestingly, we observe that deletion of *ptsI* in 5005 and 5448AP background leads to reversing the regulation of *speB* and *hasA* by CovR. This may potentially suggest that in the absence of CovS, EI can possibly act as a kinase for CovR. In broth, CovS responds to recognize Mg^{2+} as a stimulus that can cause a CovS- dependent repression of *has* transcript but only at high non-physiological concentration (203). EI autophosphorylates in the presence of Mg^{2+} and as evidenced by Gryllos et al., $Mg^{2}+$ is a specific stimulus for CovS/R mediated regulation of *hasABC* and *sag* operon (227).

In 5448, with an intact *covS*, no significant change in regulation was observed. This may suggest that EI can donate the phosphate to CovR only in the absence of CovS. While these are just speculations based on the evidence presented in this dissertation,

further studies need to be carried out to understand the interplay between PTS and the two component system CovR/S. Since we know that CovR needs to be phosphorylated to alter its regulon, it would be important to perform an *in vitro* phosphorylation assay that determines if CovR gets phosphorylated by EI in the absence of CovS. Overall, the data presented in this dissertation indicate that signaling through PTS might influence the expression of global regulatory networks affecting the expression of SLS and other virulence factors in GAS.

6.3 PTS influences Mga regulon

PRD regulators can be phosphorylated by the PTS components, and these phosphorylation events modulate their activity. All known PRD-regulators possess two PRDs that are phosphorylated by different PTS proteins, thereby allowing an input of multiple signals. In all the cases studied so far, a sugar-specific permease of the PTS acts as a negative effector of the PRD-containing regulators: in the absence of the substrate, these permeases transfer a phosphate group to the PRDs. Thus, phosphorylation by a specific PTS permease links the activity of PRD-containing regulator to the presence of a specific sugar. Many but not all regulators are controlled by second phosphorylation event that stimulates the activity. The second phosphorylation is performed by P~his-Hpr, which will be available only in the case of a poor carbon supply. Thus, PRD regulators are active if glucose is absent and the inducer is present in the medium.

Even though we have proposed a model of how PTS interacts with Mga in M4 (Fig. 21), there are a lot of details that need to be explored. For example, what is the

inducer sugar for GAS, is it glucose? Which EII protein participates in the transport of the inducer carbohydrate? Moreover, the results obtained with M1T1 strains (Fig. 19, and 20) do not fit this model and suggest a more sophisticated mode of control of Mga. It is also possible that an EIIB is playing a role in modulating Mga activity in one strain but not in the other. The difference may possibly depend on the varied site of infection. M1T1 strains belong to Class I Mga and are best known to be responsible for causing throat infections. In contrast, M4 belongs to Class II Mga that comes under the category of 'generalists' strains and can infect both throat and skin. In this study, by mutating the first protein of the PTS phosphorelay, we blocked the uptake of several carbohydrates. It would be important to find what carbohydrate(s) source acts as a signal for Mga regulation. Furthermore, it is important to ask if specific sugars have a unique effect on Mga through their respective EII systems. To answer this, it would be important to create defined mutations in the EIIs and explore their effect on Mga regulon along with the available PRD mutants. This will certainly help us define the metabolic signals that regulate Mga activity and guide us to understand how this relates to the *in vivo* environment. According to Hondorp et al., PRD1 appears to inhibit Mga activity; however, the role of PRD2 phosphorylation needs to be explored further. Orthologs of Mga have been identified in several Gram-positive pathogens (*S. dysgalactiae*, *S. pneumoniae*, *S. equi*, *S. gordonii*, *S. mitis*, *S. sanguinis*, *S. uberis, E. faecalis, L. monocytogenes, and B. anthracis*), indicating that it represents an important class of regulators and PTS mediated phosphorylation might help understand the regulation of this class of regulators.

Phyre analyses also indicate that GAS RofA-like proteins (RALPs) (Chapter 1, section 1.11.3) have predicted domain structures that are similar to Mga and therefore may also function as PRD-containing virulence regulators (PCVRs). The RALPs represent another important family of stand-alone virulence regulators in GAS and other pathogenic streptococci that affect host cell attachment and avoidance of host cell damage as the pathogen transitions from exponential to stationary phase growth (132). Several RALPs are known to directly impact the expression of *mga* in the GAS cell; RofA and Nra repress the transcription of *mga* at this point in growth, while Ralp-3 and Ralp-4 (RivR) appear to enhance Mga expression and Mga-dependent transcriptional activation, respectively. Therefore, it is distinctly possible that direct interaction with the PTS also impacts the activity of the RALPs.

Finally, to explore the role of PTS in carbon utilization and virulence, additional studies in other serotypes of GAS would be informative. Evidence presented in previous chapters indicates that EI (PTS) has a pleiotropic effect on GAS. Although this study was limited to exploring the influence of EI on three virulence factors and one transcriptional regulator, performing a transcriptome profiling such as RNA-seq would be interesting to explore the effect of PTS on a global level.

In conclusion, the results of this work have identified that PTS of GAS is required for utilization of both PTS associated and non-PTS carbohydrates. It was also found that a functional PTS influences virulence factors and transcriptional regulators important for pathogenesis of GAS. Thus PTS plays a pleiotropic role in GAS by impacting

both metabolic state and ability to cause infection, helping to adapt GAS to the changing environment for better survival within the host.

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