

**GENERATION OF DIVERSITY DURING THE  
SURVIVAL OF *STREPTOCOCCUS PYOGENES***

---

A Dissertation  
Submitted  
to the Temple University Graduate Board

---

In Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

---

By  
Kathryn E. Weinstein  
August, 2010

Committee Members

Bettina Buttarò, Ph.D. - Advisor, Microbiology & Immunology

Patrick Piggot, Ph.D. - Microbiology & Immunology

Toby Eisenstein, Ph.D. - Microbiology & Immunology

Warren Masker, Ph.D. - Biochemistry

Richard Rest, Ph.D. - External Reader, Drexel University

©  
by  
Kathryn E. Weinstein  
2010  
All Rights Reserved

## ABSTRACT

*Streptococcus pyogenes* is a human-specific pathogen that can cause a wide variety of diseases. These diseases range from the relatively mild pharyngitis and impetigo to invasive diseases such as necrotizing fasciitis to post-streptococcal sequelae such as rheumatic heart disease. The bacteria are frequently carried asymptotically and may cause recurrent disease. Corresponding with their etiologic variation amongst diseases, clinical isolates demonstrate diverse virulence factor expression and random genetic mutations. In these studies, we examine the role of intracellular residence during survival as a niche for the diversification of *S. pyogenes*.

Survival was previously studied using two in vitro systems: long-term stationary phase survival in culture and survival within epithelial cells in the presence of extracellular antibiotics. The surviving populations diversified, giving rise to stable strains with alternate colony morphologies, distinct proteomes, and altered metabolic properties. Further analysis in these studies showed that alterations in colony morphology were not solely observed during survival, but could also be induced in models mimicking acute infection. However, diversification in certain metabolic pathways occurred only during survival, and this metabolic diversification was observed at the transcriptional level. Further, one of three clinical isolates from patients with recurrent pharyngitis was altered in its metabolic profile, suggesting metabolic diversification may be occurring in vivo.

The survivor strains had varied transcriptional changes in the genes encoding the virulence factors *emm*, *slo*, and *speB*. All of the stationary phase-derived survivor strains and two intracellular survival-derived strains had attenuated virulence in zebrafish. Most

of the attenuated strains disseminated to the spleen and were cleared within three days. A whole blood killing assay showed a strong correlation between bacterial killing and *emm* expression. While the diversification appeared random, these strains retained their multi-locus sequence type (MLST). These results suggest *S. pyogenes* strains with the same MLST, but diverse virulence properties, may arise during survival in the host.

## ACKNOWLEDGMENTS

I would first like to thank my advisor, Bettina Buttarò, for her many years of help and support in the development of this project as well as in my own development as a scientist. I would like to thank the members of my committee: Patrick Piggot, Toby Eisenstein, Warren Masker, and Richard Rest for their insight and advice. I would like to thank the members of my laboratory both past and present. In particular, Daniel Wood and Shannon Morgan were the pioneers of the *S. pyogenes* project. I also would particularly like to thank Bryan Utter for his great friendship, advice, and our ‘word of the day’ topics. Additionally, I would like to thank my family and friends. Their love and support during this process were endless and will never be forgotten.

# TABLE OF CONTENTS

	PAGE
ABSTRACT.....	iii
ACKNOWLEDGMENTS .....	v
LIST OF FIGURES .....	xi
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS.....	xiii
CHAPTER	
1. INTRODUCTION .....	1
Diseases Caused By <i>S. pyogenes</i> .....	2
Acute Diseases .....	3
Pharyngitis .....	3
Impetigo .....	4
Necrotizing Fasciitis .....	4
Streptococcal Toxic Shock Syndrome .....	5
Other Acute Diseases .....	6
Immune Response to <i>S. pyogenes</i> infection.....	7
Post-Streptococcal Sequelae .....	9
Rheumatic Fever / Rheumatic Heart Disease (RHD) .....	9
Post-Streptococcal Glomerulonephritis .....	10
Vaccine Development.....	10
<i>S. pyogenes</i> Carriage and Survival.....	11
Intracellular Invasion .....	12
Biofilm Formation .....	14
<i>Streptococcus pyogenes</i> Virulence Factors.....	16

General .....	16
M Protein .....	16
Capsule.....	17
Streptococcal Pyrogenic Exotoxin B (SpeB).....	18
Streptolysin O (SLO) .....	21
Fibronectin Binding Proteins .....	21
Pyrogenic Exotoxins .....	22
Pili.....	22
Other Virulence Factors .....	23
Summary of Virulence Factors .....	24
<i>Streptococcus pyogenes</i> Virulence Factor Regulation.....	24
General.....	24
Mga .....	25
CodY .....	25
Srv .....	26
RofA-Like Proteins (RALPs) .....	26
RopB/Rgg .....	27
CovR/S.....	27
Other Response Regulators.....	28
Survival and Persistence .....	29
<i>Mycobacterium tuberculosis</i> .....	29
<i>Pseudomonas aeruginosa</i> .....	30
<i>Staphylococcus aureus</i> .....	31
<i>Helicobacter pylori</i> .....	33
<i>Streptococcus pneumoniae</i> .....	33

The Distributed-Genome Hypothesis .....	35
Diversity of <i>Streptococcus pyogenes</i> .....	33
Diversification during survival .....	38
Project Aims.....	41
2. MATERIALS AND METHODS.....	42
Bacterial Strains .....	42
Bacterial Growth Media and Incubation Conditions .....	45
Survival Assays.....	45
Growth Curve.....	45
Generation of Northern Probes and Sequencing of Regulator Genes.....	46
Isolation of Genomic DNA from <i>S. pyogenes</i> .....	46
Polymerase Chain Reaction (PCR).....	47
<i>Taq</i> polymerase PCR reaction for production of Northern blot probes .....	47
<i>Pfu</i> polymerase PCR reaction for regulatory gene sequencing.....	51
Agarose Gel Electrophoresis.....	51
Set-up and Analysis for Sequencing Reactions .....	51
RNA Isolation, Detection, and Comparison .....	52
Isolation of Total RNA from <i>S. pyogenes</i> .....	52
Generation of Probes for Northern Blotting .....	54
Northern Blotting.....	54
Real Time PCR (RT-qPCR).....	56
Generation of cDNA from RNA.....	56
Real Time PCR .....	57
Metabolic Profiles for <i>S. pyogenes</i> .....	58

Zebrafish Virulence Assays .....	58
Mortality .....	58
Spleen Dissemination.....	59
Whole Blood Killing Assay .....	59
3. RESULTS .....	61
Survivor Strains Upregulate Transcription of the PA Pathway Genes.....	61
Changes in PA Pathway Expression were Observed Only After Survival and not in Strain Variants Isolated from Passage in Mice or Human Blood.....	63
One of Three Clinical Isolates Upregulated PA Pathway Expression.....	72
<i>S. pyogenes</i> Survivor Strains Diversify Virulence Factor Expression.....	77
<i>speB</i> Expression is Variable and Growth-Phase Dependent.....	80
Survivor Strains Show Diverse Virulence Characteristics .....	83
Avirulent Survivor Strains are Cleared.....	85
Survivor Strains are Killed Upon Incubation with Human Blood .....	85
The Generation of Diversity in Survivor Strains May be Due to Random Mutation .....	88
4. DISCUSSION.....	91
Metabolic Diversification is Selected During Survival .....	92
Survival in Stationary Phase and Eukaryotic Cells led to the Development of Polyclonal Populations with Respect to Virulence Factor Expression.....	97
Some but not all Survivor Strains were Attenuated in a Zebrafish Model and Attenuation was not Correlated with any Particular Virulence Factor Expression Pattern .....	102
Reduced Survival in Human Blood Correlated with Changes in <i>emm</i> Transcription .....	103

Changes in Survivor Strains are Likely Generated by Random Mutation.....	104
Survival Gives Rise to a Phenotypically Polyclonal Population with the Same MLST Type.....	112
General Conclusions .....	114
REFERENCES CITED.....	117
APPENDIX A.....	134

## LIST OF FIGURES

Figure	Page
1. The complex regulatory network of <i>S. pyogenes</i> .....	20
2. Metabolic pathways upregulated during long term survival .....	40
3. Survivor strains Alt. 1 and Alt. 2 upregulate transcription of the PA pathway genes during exponential phase .....	64
4. Colony morphologies of 64P and its passage-derived strains. ....	66
5. Transcription of genes for the PA pathway was not upregulated in 64P and its passage-derived strains.....	68
6. Survivor strains obtained from <i>S. pyogenes</i> strains CS101, 64P, and the 64P passaged strains demonstrate variable levels of formate production.....	69
7. Survivor strains that produce high levels of formate increase transcription of genes for the PA pathway .....	71
8. Transcription of genes for the PA pathway was upregulated in one of three clinical isolates.....	73
9. One of three clinical isolates produces increased levels of the metabolites involved in the PA pathway. ....	75
10. Parental <i>S. pyogenes</i> strains produce low levels of formate .....	76
11. Virulence factor transcription varies between survivor strains .....	78
12. <i>speB</i> expression is variable, and its expression increases earlier for survivor strain E47.8 than CS101 .....	81
13. Whole blood killing varies between survivor strains .....	87

## LIST OF TABLES

Table	Page
1. <i>Streptococcus pyogenes</i> strains used in this study.....	43
2. Oligonucleotides used in this study .....	48
3. Survivor strains showed variable virulence in zebrafish. ....	84
4. Attenuated survivor strains are cleared from zebrafish spleens. ....	86
5. Sequencing survivor strain regulators reveals random mutations .....	89
6. Summary of virulence properties in survivor strains.....	98

## LIST OF ABBREVIATIONS

AA	amino acid
APC	antigen presenting cell
ATP	adenosine triphosphate
bp	base pairs
C	Celsius
CF	cystic fibrosis
CFU	colony forming units
CDM	chemically defined medium
CNA	Colombia colistin-nalidixic agar
CLN	cervical lymph node
CovR/S	Control of virulence genes
CSPD	disodium-3-(4-methoxy Spiro[1,2-dioxetane-3'2'-(5-chloro)tricyclo(3.3.1.3 <sup>3,7</sup> )decan]-4-yl)phenylphosphate
DC(s)	dendritic cell
DEPC	Diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ETS	electron transport system

FBP	fibronectin binding protein
FNR	fumarate nitrate reductase
g	gram
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
HLA	human leukocyte antigen
HTH	helix-turn-helix
ICE	integrative conjugative element
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Indel	insertion/deletions
kb	kilobase
kDa	kilodalton
L	liter
LD	lethal dose
LTA	lipoteichoic acid
M	molar
Mb	megabase
mg	milligram
MHC	major histocompatibility complex
min	minutes

ml	milliliter
mM	millimolar
MMR	mismatch repair
MLST	multi-locus sequence typing
MSCRAMM	microbial surface components recognizing adhesive matrix materials
NADH	nicotinamide adenine dinucleotide hydrogen
NALT	nasal associated lymphoid tissue
NF	nuclear factor
nm	nanometer
NO	nitric oxide
OD	optical density
PA	pyruvate to acetate
PANDAS	pediatric autoimmune neuropsychiatric disorders associated with streptococci
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	pyruvate to ethanol
PFGE	pulse field gel electrophoresis
PMN	polymorphonuclear leukocyte
PRD	phosphotransferase system (PTS) regulatory domain
RALPs	RofA-like proteins
RAPD	random amplification of polymorphic DNA

RHD	rheumatic heart disease
RF	rheumatic fever
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RR	response regulator
RT-PCR	real time PCR
SCV	small colony variant
SNP	single nucleotide polymorphism
SDS	sodium dodecyl sulfate
SLO	Streptolysin O
SpeB	Streptococcal pyrogenic exotoxin B
SSC	sodium salts citrate
STSS	Streptococcal toxic shock syndrome
TAE	tris acetate EDTA
TCA	tri-carboxylic acid
TCS	two-component system
TCR	T cell receptor
TH	Todd-Hewitt
Th	T helper cell
THB	Todd-Hewitt Broth
TNF	Tumor Necrosis Factor

Tris	Tris(Hydroxymethyl)Aminomethane
μg	microgram
μl	microliter
μM	micromolar
UV	ultraviolet
URT	upper respiratory tract

## CHAPTER 1

### INTRODUCTION

*Streptococcus pyogenes* (Group A Streptococci) are Gram-positive cocci that cause a wide range of diseases in humans. They grow in chains, are non-motile, and are non-spore-forming. They are beta-hemolytic, meaning that they completely lyse red blood cells when grown on agar containing sheep's blood. They are fermentative and facultatively anaerobic. *S. pyogenes* is a well-recognized pathogen, with over 700 million cases of *S. pyogenes* infections world-wide, and with a mortality rate of 500,000 deaths/year (Carapetis *et al.*, 2005). Most commonly, especially among children, *S. pyogenes* causes pharyngitis and impetigo. It can also cause systemic disease such as toxic shock-like syndrome or necrotizing fasciitis. *S. pyogenes* infection can lead to post-streptococcal sequelae such as glomerulonephritis and rheumatic fever. In addition to causing acute disease, *S. pyogenes* can be asymptotically carried in children and, less frequently, in adults (Reviewed by Cunningham, 2000).

As predicted for a pathogen found exclusively in the human host and causing a wide variety of diseases, individual clinical isolates differ significantly in genetic content and have significant allelic diversity. The *S. pyogenes* genome is 1.9 Mb (Reviewed by Olsen and Musser, 2010; McShan *et al.*, 2008; Green *et al.*, 2005; Banks *et al.*, 2004; Nakagawa *et al.*, 2003; Beres *et al.*, 2002; Smoot *et al.*, 2002; Ferretti *et al.*, 2001) encoding ~1800 genes (Lefebure and Stanhope, 2007). Its genomic GC content is ~38% G + C (Banks *et al.*, 2004). Approximately 80% of the genome is conserved with another approximately 20% being variable in genic content (Beres and Musser, 2007; Lefebure

and Stanhope, 2007). The genome has multiple integrative conjugative elements (ICEs) as well as ~10% prophage content (Beres and Musser, 2007). In addition to differences in genic content, single nucleotide polymorphisms (SNPs) and insertion/deletions (Indels) are common and even can be seen in strains from multiple sequential outbreaks (Beres *et al.*, 2010). Clinically, *S. pyogenes* strains are primarily identified by their M-type, of which there are over 120 serotypes (Reviewed by Bisno *et al.*, 2003), with even more genomic variation present between the sub-strains. Multilocus sequence typing (MLST) is commonly used to characterize the relatedness of clinical isolates.

### **Diseases Caused by *S. pyogenes***

*S. pyogenes* causes a wide range of diseases, from relatively mild, acute infections to those that are life threatening. *S. pyogenes* is under-recognized in its global importance due to under-reporting of *S. pyogenes* infections in underdeveloped nations. However, even modest estimates place its mortality rate among pathogens only behind HIV, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, and *Streptococcus pneumoniae* (Carapetis *et al.*, 2005). *S. pyogenes* causes ~500,000 deaths/year worldwide. In the U.S alone, streptococcal pharyngitis causes \$1 to \$2 billion in direct health care costs (Musser and Shelburne, 2009). Antibiotic treatment greatly reduces the incidence of post-streptococcal sequelae; however, in underdeveloped countries physicians often do not treat with antibiotics or there is not full compliance with the antibiotic regime. This leads to elevated rates of complications, especially rheumatic fever and the subsequent rheumatic heart disease, and mortality due to *S. pyogenes* infection.

## Acute Diseases

### Pharyngitis

It is estimated that there are at least 600 million cases of pharyngitis due to *S. pyogenes* infection in those 4 years and older worldwide (Carapetis *et al.*, 2005). *S. pyogenes* pharyngitis presents itself with pharyngeal swelling, tonsillar exudate, swollen uvula, and swollen lymph nodes. Disease lasts 7 to 10 days if left untreated (Reviewed by Vincent *et al.*, 2004). Pharyngitis can be caused by viruses or bacteria such as *Staphylococcus aureus*, Group B Streptococci, and *S. pyogenes*. *S. pyogenes* is the predominant bacterial infectious agent associated with pharyngitis, and *S. pyogenes*-caused pharyngitis, commonly referred to as 'strep throat,' is of high concern since *S. pyogenes* can be carried within the oropharynx by patients which may lead to more serious streptococcal-related diseases (Reviewed by Vincent *et al.*, 2004). *S. pyogenes* accounts for 10 – 30% of pharyngitis cases in children.

The primary treatment for pharyngitis is penicillin V, or cephalosporin for patients who are allergic to penicillin. There is a high rate of treatment failure with antibiotic therapy, particularly for penicillin, but no penicillin resistant strains have been isolated to date. Therefore, penicillin treatment failure is attributed to multiple factors including poor compliance, antibiotic avoidance or tolerance, production of beta lactamases by flora, intracellular survival, and biofilm formation. 5-12% of children are carriers for *S. pyogenes*, and the treatment choice to eradicate carried bacteria is the lincosamide, clindamycin, which penetrates eukaryotic cells (Reviewed by Leung and Kellner, 2004). In patients allergic to penicillin, macrolides such as erythromycin or azithromycin are commonly prescribed. Macrolides, unlike penicillin, are able to enter into eukaryotic cells, and seem like a more promising treatment for *S. pyogenes* infection since the bacteria has been shown to invade eukaryotic cells (Reviewed by Passali *et al.*, 2007). However, there are *S. pyogenes* strains that are resistant to macrolides. Macrolide resistance is associated with high expression levels of serum opacity factor (SOF). In one

study, 126 of 127 isolated macrolide resistant strains were SOF positive (Dicuonzo *et al.*, 2002; Reviewed by Passali *et al.*, 2007). In addition, protein F/ SfbI has been associated with macrolide resistance. High expression of PrtF2 is linked to bacterial invasion of eukaryotic cells and to the carrier state; and hence, to penicillin failure. Macrolides are able to penetrate eukaryotic cells, but they are a poor choice for treatment due to bacterial resistance (Reviewed by Passali *et al.*, 2007).

### Impetigo

Impetigo, or pyoderma, is a superficial skin infection characterized by a localized, inflamed epidermis with a golden colored crust. The disease is most common in children ages 2 – 6 years and the lesions are highly contagious (Reviewed by Bisno and Stevens, 1996). It is estimated that there are currently >111 million cases of impetigo worldwide in children under 15 years old (Carapetis *et al.*, 2005). *S. pyogenes* and *S. aureus* are the main etiological agents for impetigo. There are two types of impetigo: Impetigo contagiosa, which is the more common, crusted form caused by Streptococci or Staphylococci, and bullus impetigo, which is characterized by blisters caused by *S. aureus* (Reviewed by Sladden and Johnston, 2005). Certain M types are associated with impetigo, such as M49. There is a strong association of impetigo infections leading to glomerulonephritis, but interestingly no association with rheumatic fever (Reviewed by Bisno and Stevens, 1996).

### Necrotizing Fasciitis

*S. pyogenes* infection can lead to more severe invasive diseases such as necrotizing fasciitis, also known as the flesh-eating disease. There are approximately 660,000 cases/year of invasive diseases a year worldwide, causing >160,000 deaths (Carapetis *et al.*, 2005). Necrotizing fasciitis can progress within a matter of hours from a minor-looking skin lesion to lethality. Initially the bacteria infect the fascial planes between muscles. The action of bacterial proteases and tissue-destructive enzymes from polymorphonuclear leukocytes (PMNs) that rapidly enter the location lead to localized

destruction of the surrounding tissues and facilitate systemic invasion of the bacteria into the surrounding muscles and vascular system. Invasive disease by *S. pyogenes* is believed to be caused by either progression of a mild infection, direct infection of an injury or by systemic infection after carriage (Reviewed by Olsen and Musser, 2010). Many investigators believe that the original *S. pyogenes* infection that leads to necrotizing fasciitis occurs long before the clinical disease. It may develop from deep tissue infection and/or carriage in the oropharynx (Reviewed by Olsen and Musser, 2010; Hoe *et al.*, 2003; Stevens *et al.*, 1989) Sera from infected patients have high levels of IL-1, TNF- $\beta$ , and IFN- $\gamma$ , suggesting possible involvement of streptococcal superantigens in creating a cytokine storm. The most effective treatment for necrotizing fasciitis is rapid debridement of the affected area and intravenous antibiotic therapy (Reviewed by Olsen and Musser, 2010). Necrotizing fasciitis caused by *S. pyogenes* is a relatively rare disease, with ~1 in 100,000 people developing the disease, but many cases from underdeveloped countries are not reported. It carries an extremely high mortality rate of up to 50%, even in the U.S.A. (Reviewed by Olsen and Musser, 2010).

#### Streptococcal Toxic Shock Syndrome (STSS)

Toxic shock syndrome can be caused by two toxin-producing bacteria, *S. aureus* and *S. pyogenes* (Reviewed by Lappin and Ferguson, 2009). Streptococcal toxic shock syndrome is an acute, multi-system toxin-mediated disease, and it can result in multi-organ failure. STSS is associated with a very high mortality rate of 66.7% (Huang *et al.*, 2001; Reviewed by Chiang-Ni and Wu, 2008). STSS is different from the mild, acute diseases pharyngitis and impetigo in that it is caused by a systemic infection (Reviewed by Lappin and Ferguson, 2009).

During infection, *S. pyogenes* secrete toxins which act as superantigens. Superantigens produce excessive T cell activation and cytokine/chemokine release, which causes multi-organ failure. The superantigens act differently than typical antigens in that they bind directly and unprocessed to both MHCII on the antigen presenting cell (APC)

and the T cell receptor (TCR), outside of the antigen binding pocket. Eleven superantigens have been identified in *S. pyogenes* including SpeA, SpeC, SpeG-M, SSA, and SmeZ (Reviewed by (Sriskandan *et al.*, 2007). Each superantigen interacts with a specific subset of V $\beta$  TCR causing expansion of those specific T cells. The T cells produce the cytokines lymphotoxin- $\alpha$ , interleukin-2 (IL-2), and interferon- $\gamma$  (IFN $\gamma$ ). APCs, such as monocytes, produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , and IL-6. These cytokines activate nuclear factor- $\kappa$ B (NF- $\kappa$ B), which controls expression of multiple genes needed to drive the inflammatory response. The massive inflammatory response subsequently leads to multi-organ failure. The T cell response leads to activation of more T cells and APCs which further exacerbates the cytokine storm. SpeA is the predominant toxin associated with STSS. STSS most often arises from a preexisting invasive infection, such as necrotizing fasciitis, but it can result from blunt trauma or joint effusion. STSS is confirmed when *S. pyogenes* is isolated from normally sterile body fluids (Reviewed by Lappin and Ferguson, 2009). Once it is confirmed that *S. pyogenes* is the infectious agent, the patient is treated with antibiotics similar to those used for pharyngitis treatment. Primarily penicillin or clindamycin are prescribed, but penicillin is not as effective at high bacterial loads. In the case of penicillin allergy, macrolides or fluoroquinolones with clindamycin are prescribed. A third treatment option is linezolid, daptomycin, or tigecycline (Reviewed by Lappin and Ferguson, 2009). Fluid replacement therapy and anti-chemokines can be used to control the capillary leakage induced by cytokines, thus preventing death due to shock before the infection can be controlled by the antibiotics.

#### Other Acute Diseases

*S. pyogenes* causes many diseases in addition to the ones discussed above. Here a few additional diseases are briefly described. Necrotizing pneumonia caused by *S. pyogenes* presents itself as pneumonia with substantial necrosis of lung cells (Lassalle *et al.*, 2005). Puerperal sepsis is a septicemia contracted after childbirth because of a

current infection spreading to the bloodstream due to breaks in epithelial layers (Byrne *et al.*, 2009). Myositis is an invasive disease that is characterized by inflammation of the muscles (Bharathi *et al.*, 2009). Pediatric autoimmune neuropsychiatric disorders associated with streptococci (PANDAS) link psychiatric disorders such as obsessive-compulsive disorder or Tourette's syndrome with *S. pyogenes* infections, but the correlations have not been fully established and the concept is still quite controversial (Shulman, 2009).

The large amount of diversity seen in clinical outcome of diseases due to *S. pyogenes* infection is mostly due to differences in allelic and genic content between strains. This affects expression of virulence factors, which determine the progression of disease. Many of these virulence factors exacerbate disease, but the immune response in the human host is able to control disease progression in many cases.

### **Immune Response to *S. pyogenes* Infection**

The innate immune response is the primary response for eliminating *S. pyogenes* upon infection. Neutrophils are the main cells responsible for phagocytosis of *S. pyogenes*, and they kill the bacteria using oxygen-dependent and independent mechanisms. Phagocytosis induces NADH oxidase that produces superoxide. Superoxide is converted to other reactive oxygen species (ROS) such as hydrogen peroxide and hypochlorous acid. Also, PMN granules fuse with phagosomes, in a process known as degranulation, which introduces antimicrobial peptides to the phagosome (Reviewed by Voyich *et al.*, 2004). *S. pyogenes* has evolved mechanisms to alter the action of neutrophils. M protein prevents degranulation and phagosomal fusion of azurophilic granules. Capsule, protein H and M protein allow for survival inside the neutrophil by mechanisms that have not been established (Stali *et al.*, 2003; Reviewed by Urban *et al.*, 2006). Although neutrophils are the primary cells responsible for clearing *S. pyogenes*, *S. pyogenes* may encounter dendritic cells (DCs), which are

abundant on the pharynx and skin, upon initial infection. Once DCs encounter *S. pyogenes*, they mature, and they produce pro-inflammatory cytokines and chemokines that induce the innate immune response. Also, as the DCs mature, they upregulate MHC and costimulatory molecules that will stimulate T cell responses. DCs therefore act to bridge the innate and adaptive immune response. Only a minority of *S. pyogenes* strains induces maturation of human-derived DCs in vitro, and the ability to inhibit activation varies between *S. pyogenes* isolates. The virulence factors capsule and streptolysin O (SLO) are associated with inhibition of DC maturation (Cortes and Wessels, 2009).

In mice, *S. pyogenes* invades nasal associated lymphoid tissue (NALT), which is the human tonsil homolog. M cells in NALT transport the bacteria across the epithelium where the host can mount an immune response, similar to the function of Peyer's patches in the intestinal tract (Park *et al.*, 2003). Once across the epithelial barrier, mature myeloid CD11c+ DCs as well as NALT macrophages present antigen to antigen-specific T helper cells. The T cells proliferate primarily in the NALT, but also in the cervical lymph node. By 21 days post-infection, memory T cells are recovered in the spleen that, upon restimulation, produce IL-2, TNF- $\alpha$ , and IFN- $\gamma$  which are typical of a Th1 response (Costalonga *et al.*, 2009).

Multiple proteins expressed by *S. pyogenes* are recognized as antigens to the immune system. The most studied is M protein, which is the basis for multiple vaccine candidates. The host response against specific M-serotypes protects against reinfection with the same serotype. Serological detection of current or prior *S. pyogenes* infection uses an antibody response against one or more of the following antigens: SLO, DNase B, hyaluronidase, NADase, or streptokinase. Other antigens include cell wall mucopeptide, group A streptococcal carbohydrate, and the cell wall proteins R and T (Reviewed by (Cunningham, 2000).

The immune response is effective, in many cases, at controlling streptococcal infection. However, sometimes it is the resulting immune response itself that can cause diseases of significant concern, as observed with post-streptococcal sequelae.

### Post-Streptococcal Sequelae

#### Rheumatic Fever/Rheumatic Heart Disease (RHD)

Since the 1940s there has been a significant decline in the occurrence of rheumatic fever. This is primarily due to the treatment of pharyngitis with penicillin or other antibiotics. However, in many undeveloped nations the frequency of rheumatic fever remains high since treatment for pharyngitis is not commonly available (Reviewed by Hahn *et al.*, 2005). Worldwide, there are approximately 471,000 cases of rheumatic fever, and approximately 15.6 to 19.6 million people currently alive suffer from rheumatic heart disease as a result of rheumatic fever. 1.5% of people with rheumatic heart disease die each year, which leads to the estimation that 233,000 people die from rheumatic heart disease each year. Of those who have rheumatic heart disease, 79% are from less developed countries (Carapetis *et al.*, 2005). Rheumatic fever presents itself as migratory arthritis in the joints, symptoms of carditis, erythematous rash, subcutaneous nodules, and jerking movements of Sydenham's chorea. The disease is due to an inflammatory process caused by the cross reactivity of T cells that recognize both M protein and cardiac myosin. The T cell which recognize self antigens on the heart produce cytokines to stimulate autoreactive B cells. Polarization of the immune response to T or B cells results in different symptoms of the disease. Mononuclear cells in RHD lesions produce TNF $\alpha$ , IFN $\gamma$ , and IL-10. The cross-reactive T cells also produce TNF $\alpha$  and IFN $\gamma$  (Reviewed by Guilherme and Kalil, 2007). Treatment of rheumatic fever consists of two types of therapy. First, anti-inflammatory drugs such as salicylates and steroids are prescribed. Second, antibiotics such as penicillin are prescribed to clear the bacterial infection (Reviewed by Lee *et al.*, 2009; Reviewed by Lennon, 2004).

Rheumatic fever can permanently damage the heart leading to rheumatic heart disease (Reviewed by Hahn *et al.*, 2005).

### Post-Streptococcal Glomerulonephritis

Most cases of post-streptococcal glomerulonephritis occur in children between 2 and 6 years old. It typically occurs ten days after pharyngitis or fourteen days after impetigo (Reviewed by Hahn *et al.*, 2005). The disease is characterized by inflammation of the glomeruli of the kidney, bloody urine, and renal failure. It is caused by the deposition of immune complexes in the glomeruli of the kidney leading to complement activation and local coagulation activation which damages the basement membrane causing glomerulonephritis (Reviewed by Rodriguez-Iturbe and Batsford, 2007). These immune complexes contain antibodies directed to streptococcal antigens and non-streptococcal antigens. Although antibiotic therapy for the primary infection seems to reduce rates of rheumatic fever, the same cannot be said for glomerulonephritis, and there is much debate whether antibiotic therapy makes a difference in its development. Greater than 95% of patients recover spontaneously; however, those that do not recover develop severe renal failure, rashes, joint pain, or persistent fever (Reviewed by Hahn *et al.*, 2005). Certain M types, including M49, are considered nephritic, meaning they have a higher propensity to cause disease. These strains are Class II (opacity factor positive). However, more recent studies show that M protein is not the sole determinant for nephritogenicity. Additional bacterial determinants are still speculative, but these factors include streptokinase (Ska), histone-like proteins (HlpA), streptococcal plasmin receptor (NAPlr), and cysteine protease (SpeB) (Reviewed by Rodriguez-Iturbe and Batsford, 2007). Primary treatment consists of antihypertensives and treatment of the carrier state with penicillin (Lang and Towers, 2001).

### Vaccine Development

There are currently 12 different candidates for a *S. pyogenes* vaccine. Four are based upon M protein, and the remaining eight are based on group A carbohydrate, C5a

peptidase, SpeB, binding proteins, opacity factor, lipoproteins, superantigens, and pili (Guilherme *et al.*, 2009). Only one vaccine candidate, based upon M protein, has made it to the clinical trial phase, and it is currently in phase II clinical trials (Guilherme *et al.*, 2009). This vaccine combined the N-terminal residues of 26 strains of *S. pyogenes*. These epitopes represent the most common infectious M-types in the U.S.A. (Dale *et al.*, 2005; McNeil *et al.*, 2005; Hu *et al.*, 2002). Another M-based vaccine is in experimental phase and is based upon the minimum B cell epitope from the C-terminal portion of M protein. Since the C-terminal end of M protein is conserved amongst the *S. pyogenes* strains, this type of vaccine would provide broad-range protection (Olive, 2007; Medagliani *et al.*, 1995). Additionally, a vaccine is being developed that induces both T and B cell responses based on the C-terminal end of M protein (Guilherme *et al.*, 2009). The development of a vaccine against *S. pyogenes* has been difficult since the vaccine must not induce rheumatic fever from cross-reactive antibodies or cross-reactive T cells. There is a higher predisposition among people who express the HLA-DR7 allele, which is HLA class II, for developing rheumatic fever and rheumatic heart disease. *S. pyogenes* vaccines take this peptide specificity into account and use N- and C-terminal peptides that would induce a solely protective response (Guilherme *et al.*, 2006).

### ***S. pyogenes* Carriage and Survival**

In addition to causing active infection, *S. pyogenes* can be asymptotically carried. This usually occurs after an active infection, suggesting the bacteria have escaped both the antibiotic treatment and the immune response. The primary treatment for *S. pyogenes* infection is penicillin, which has had an increasing treatment failure rate in recent years with current estimates of failure rates ranging from 20% to 40% (Reviewed by Pichichero and Casey, 2007). To date, there have been no penicillin-resistant strains of *S. pyogenes* isolated, suggesting that the bacteria are able to avoid the action of antibiotics and to survive because of mechanisms other than antibiotic

resistance, leading to carriage (Reviewed by Pichichero and Casey, 2007). The first of two primary proposed mechanisms of survival in the presence of antibiotics is that *S. pyogenes* are able to invade tonsillar epithelial cells and reside in an altered growth state until reactivation (Morgan and Buttarò, submitted; Reviewed by Molinari and Chhatwal, 1999; LaPenta *et al.*, 1994). The second is biofilm formation which provides a physical barrier from the action of antibiotics (Manetti *et al.*, 2007; Baldassarri *et al.*, 2006; Lembke *et al.*, 2006).

Current *in vitro* systems for the study of *S. pyogenes* survival include long-term stationary phase survival in chemically defined medium (Cook, 1976) and in Todd-Hewitt broth (Wood *et al.*, 2009; Wood *et al.*, 2005), survival in human saliva (Shelburne *et al.*, 2005), survival in mouse phagocytic cells (Medina *et al.*, 2003), and survival in eukaryotic cells in the presence of extracellular antibiotics (A549, Hep2, HUVEC, Detroit-562, J774, BMEC, and human keratinocytes) (Morgan and Buttarò, submitted; Klenk *et al.*, 2007; Oliver *et al.*, 2007; Reviewed by Molinari and Chhatwal, 1999). In addition to the *in vitro* models, *in vivo* animal models include mice (Medina *et al.*, 2003) and baboons (Taylor *et al.*, 1999).

The two primary mechanisms for *S. pyogenes* survival during carriage are discussed below.

### Intracellular Invasion

*S. pyogenes* have been classically considered to be extracellular pathogens, but *in vitro* and *ex vivo* studies in recent years have shown that *S. pyogenes* invade and survive within eukaryotic cells (Reviewed by Molinari and Chhatwal, 1999; Reviewed by Kreikemeyer *et al.*, 2004). In order to internalize, *S. pyogenes* must first adhere to the host's epithelium. Adherence takes place in two steps. The first is a temporary weak attachment via nonspecific binding involving *S. pyogenes* lipoteichoic acid (LTA). Next,

a specific interaction between *S. pyogenes* adhesins and host extracellular matrix occurs. *S. pyogenes* primarily bind to the extracellular matrix proteins fibronectin and collagen. The adhesins that are expressed by *S. pyogenes* are referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Multiple MSCRAMMs have been shown to be involved in the binding and subsequent invasion into phagocytic as well as non-phagocytic cells. These proteins include the fibronectin binding proteins (SfbI, PrtF1, PrtF2, and M protein), streptococcal surface dehydrogenase, hyaluronic capsule, SpeA, and SpeB (Reviewed by Molinari and Chhatwal, 1999). Currently PrtF1 is the most studied MSCRAMM. Only ~70% of *S. pyogenes* isolates express fibronectin binding proteins, and some of the strains lacking expression have been shown to invade into eukaryotic cells. Therefore, there are two pathways that *S. pyogenes* can use to invade into non-phagocytic cells, depending on each strain's expression of fibronectin binding proteins. Strains lacking SfbI induce actin polymerization and the formation of pseudopod-like structures to engulf bacteria in a zipper-like mechanism (Molinari *et al.*, 2000). In strains that express the fibronectin binding protein SfbI (which is an allelic variant of PrtF1), fibronectin is bound and coats the bacterial cell. The fibronectin binds to the host cell integrins  $\alpha_5\beta_1$  or  $\alpha_v\beta_3$  inducing formation of membrane invaginations without actin rearrangement. The presence of intracellular *S. pyogenes* strains that encode an intact PrtF1 leads to host cell apoptosis and the release of *S. pyogenes* (Reviewed by Kreikemeyer *et al.*, 2004).

There are multiple regulatory factors that affect the binding and internalization of *S. pyogenes*. CovR/S (control of virulence) activity suppresses capsule production. Capsule binds collagen and it also binds the eukaryotic cell directly through CD44

allowing for penetration of *S. pyogenes* into deeper tissues (Wessels *et al.*, 1991). The removal of capsule exposes other MSRCRAMMs located on the bacterial surface leading to increased adhesion and internalization. Therefore, CovR/S may represent a switch between eukaryotic cell invasion and invasion into deeper tissues. Other regulators of adhesins include FasBCA, Rgg/RopB, Mga, and RALPs (Reviewed by Kreikemeyer *et al.*, 2004).

The first demonstration of *S. pyogenes* internalization into eukaryotic cells was by Cleary's laboratory (LaPenta *et al.*, 1994). Subsequently, Chhatwal's laboratory showed the role of PrtF1 in invasion (Molinari *et al.*, 1997). Extending the discovery of cellular invasion to the clinical setting, Österlund as well as Podbielski found *S. pyogenes* in surgically removed tonsillar material from patients who suffered recurrent tonsillitis. Histological examination in both studies revealed that the bacteria localized intracellularly (Podbielski *et al.*, 2003; Osterlund and Engstrand, 1997; Osterlund *et al.*, 1997). *S. pyogenes* are able to survive within eukaryotic cells, as seen using the cell lines A549 and Hep-2, for at least 7 days after they have invaded (Morgan and Buttaro, submitted; Reviewed by Molinari and Chhatwal, 1999; Greco *et al.*, 1995; Osterlund and Engstrand, 1995; LaPenta *et al.*, 1994). In order to re-emerge into the environment, *S. pyogenes* needs to cause membrane injury (Marouni and Sela, 2004).

### Biofilm Formation

Approximately 90% of *S. pyogenes* strains can form biofilms, although the thickness and robustness of the biofilms varies significantly between strains (Manetti *et al.*, 2007; Baldassarri *et al.*, 2006; Lembke *et al.*, 2006). Confocal imaging of *S. pyogenes* associated with glycocalyx in a skin infection provided the first evidence of *S.*

*pyogenes* biofilm formation during infection (Akiyama *et al.*, 2003). *S. pyogenes* can form biofilms on a number of inanimate surfaces including polystyrene and matrix-coated surfaces, glass coverslips, PVC, and contact lens cases (Nithyanand *et al.*, 2010; Courtney *et al.*, 2009; Luo *et al.*, 2008; Lembke *et al.*, 2006; Cho and Caparon, 2005; Conley *et al.*, 2003; Yousefi Rad *et al.*, 1998; Wilson *et al.*, 1991). In vitro *S. pyogenes* biofilms are less antibiotic-sensitive than planktonic cultures, which may contribute to antibiotic treatment failures (Conley *et al.*, 2003; Stewart and Costerton, 2001). Additionally, *S. pyogenes* can form biofilms on a number of biologically significant surfaces. Bacterial isolates from a root canal formed biofilms when regrown on gutta percha (the rubber compound used in fillings) (Takemura *et al.*, 2004). Isolates from impetigo and atopic dermatitis cases showed aggregates of microcolonies surrounded by glycocalyx, suggesting biofilm formation (Akiyama *et al.*, 2003). Also, *S. pyogenes* forms biofilms on zebrafish muscle in areas associated with necrosis, making zebrafish a good model for soft-tissue biofilms (Cho and Caparon, 2005). It is important to note that CS101, an M49 strain used in these studies, is poor at forming biofilms compared to other *S. pyogenes* M types (Courtney *et al.*, 2009).

Survival during carriage is an important factor for *S. pyogenes* virulence, especially as ~12% of children are carriers of the bacteria (Reviewed by Leung and Kellner, 2004). In order to survive, however, the bacteria must first be able to attach eukaryotic cells and avoid immune detection. Infection and survival depends on the expression and interaction of multiple virulence factors.

## ***Streptococcus pyogenes* Virulence Factors**

### General

*S. pyogenes* causes a wide range of diseases, and correspondingly produces at least thirteen virulence factors (Musser and Shelburne, 2009). The virulence factors often play a role in more than one disease and the general functions of a few key factors are discussed below.

### M Protein

M protein, encoded by the *emm* gene, extends from the cell surface and is visible by light microscopy. Classification of *S. pyogenes* strains was developed by Rebecca Lancefield and is primarily based upon the M-serotype of each strain. There are >120 M-types to date. M protein is composed of two polypeptide chains in an alpha-helical coiled-coil structure with three domains. The C-terminal region, found at the cell wall and cell membrane is conserved between M-types. The LPXTG domain found within this C-terminal region is cleaved by sortase, which then also functions to attach M protein to the peptidoglycan precursor lipid II. The central region contains semi-conserved repeats. It is this region that contains the myosin cross-reactive epitopes which are important in the development of rheumatic fever. The final region contains hypervariable 11-amino acid repeats at the N-terminus. The antisera for M-typing recognize these variable residues. M protein plays multiple roles in the virulence of *S. pyogenes*. First, it is involved in the attachment and entry of *S. pyogenes* into eukaryotic cells. M protein can bind multiple host proteins including albumin, fibrinogen, and plasminogen allowing for attachment, immune evasion and invasion into deeper tissues (Reviewed by Bisno *et al.*, 2003). When M protein is complexed with fibronectin it binds the  $\alpha_5\beta_1$  receptor

allowing for invasion into eukaryotic cells (Wang *et al.*, 2006; Rezcallah *et al.*, 2005; Cue *et al.*, 2000; Cue *et al.*, 1998). In addition, M protein is anti-phagocytic by interfering with the alternate complement pathway. It binds factor H as well as factor H-like protein, which degrades C3b. It also binds C4b-binding protein, interfering with C3b binding to the bacterial cell and thus inhibiting phagocytosis. Finally, binding of matrix proteins coats the cells reducing complement deposition. There have been additional M-like proteins identified, and the genes encoding these proteins are now part of the *emm* gene superfamily. Included in this superfamily are *enn*, *mrp*, *fcrA*, *arp*, and *protH*. These superfamily proteins have similar functions to M such as anti-phagocytic functions and attachment (Reviewed by Bisno *et al.*, 2003).

### Capsule

*S. pyogenes* strains have differing levels of capsule expression, and those that express high amounts of capsule are mucoid in appearance. This is because of the hydroscopic mucoid composition of the capsule, which is composed of hyaluronic acid. Hyaluronic acid is a polysaccharide of alternating N-acetylglucosamine and glucuronic acid subunits. The genes encoding capsule production are located on the *has* operon which is composed of three genes: *hasA* encoding hyaluronate synthase, *hasB* encoding UDP-glucose dehydrogenase, and *hasC* encoding UDP-glucose pyrophosphorylase. In the laboratory, capsule is produced during exponential growth and shed from the cell upon entrance into stationary phase. Capsule plays a role in eukaryotic cell binding and resistance to phagocytosis. Strains that express very high levels of capsule physically mask M protein, superseding its role in immune evasion, attachment and eukaryotic cell invasion in exponentially growing cells. In stationary phase the capsule is shed, M

protein is re-exposed, and M protein has a dominant role. In vivo capsule and M protein often act in a concerted manner, and strains expressing high levels of both proteins are highly virulent in humans (Reviewed by Bisno *et al.*, 2003). High capsule expression is associated with invasive diseases as well as rheumatic fever. Three percent of acute pharyngitis isolates demonstrated a mucoid morphology, whereas 21% of isolates from infected tissue at normally sterile sites and 42% of rheumatic fever isolates had the mucoid phenotype (Johnson *et al.*, 1992; Reviewed by Bisno *et al.*, 2003).

#### Streptococcal Pyrogenic Exotoxin B (SpeB)

*S. pyogenes* produces a few cysteine proteases, but none are as abundant or varied in function as streptococcal pyrogenic exotoxin B (SpeB). All analyzed *S. pyogenes* strains contain *speB* in their genome. Overall the sequence is highly conserved among the various strains, but there are some single nucleotide polymorphisms (SNPs) that occur in the coding sequence (Kapur *et al.*, 1993; Reviewed by Chiang-Ni and Wu, 2008). Although the gene is present in all genomes, the protein is expressed at only low levels in 25-40% of all *S. pyogenes* strains (Chaussee *et al.*, 1996; Talkington *et al.*, 1993; Reviewed by Chiang-Ni and Wu, 2008). It is secreted as a 42 kDa zymogen which is then autocatalyzed into a 28 kDa cysteine protease (Reviewed by Chiang-Ni and Wu, 2008). SpeB cleaves proteins of all types, both host and bacterial, including host extracellular matrix, immunoglobulins, complement components, *S. pyogenes* surface adhesins, C5a peptidase, and multiple secreted proteins. Its many actions lead to multiple effects such as escaping immune recognition, invasion into deep tissues, and dissemination from the primary infection site. It cleaves host immunoglobulin as well as complement components C3 and C3b. SpeB cleaves and releases a 116 kDa biologically

active fragment of C5a peptidase from the bacterial surface to inactivate C5a. Also, the positive regulator of the alternate pathway of complement activation, properdin, is cleaved by SpeB. All these actions by SpeB play a role in avoidance of opsonophagocytosis (Reviewed by Bisno *et al.*, 2003; Chiang-Ni and Wu, 2008). In addition, SpeB may play a role in invasion into eukaryotic cells, but this finding is controversial. A *speB* mutant of the M49 strain NZ131 was found to have a 2-3 fold decrease in internalization into human A549 lung epithelial cell line (Tsai *et al.*, 1998; Reviewed by Chiang-Ni and Wu, 2008). However, other studies using M49 and M3 strains found an increase in internalization into Hep-2 and Chinese hamster ovary cell lines, respectively, following a mutation in *speB* (Jadoun *et al.*, 2002; Chaussee *et al.*, 2000; Reviewed by Chiang-Ni and Wu, 2008). This contradiction most likely is due to the use of different bacterial strains and cell lines, and it highlights a difficulty in the study of *S. pyogenes*, specifically the variability in virulence factor expression, regulation, and role in disease depending on strain background (Reviewed by Chiang-Ni and Wu, 2008). Approximately 20% of A549 cells and 11-13% of Hep-2 cells undergo apoptosis after *S. pyogenes* infection, and SpeB is implicated in playing a role in epithelial cell apoptosis (Tsai *et al.*, 1999; Reviewed by Chiang-Ni and Wu, 2008).

SpeB is highly regulated, and it is controlled directly or indirectly by at least 5 transcriptional regulators (Fig. 1) (Reviewed by Chiang-Ni and Wu, 2008). Its expression is linked to multiple diseases, in particular invasive diseases such as necrotizing fasciitis. Previous studies have shown that *speB* expression rapidly increases upon the transition from late exponential phase into stationary phase (Kietzman and Caparon, 2010; Reviewed by Bisno *et al.*, 2003; Reviewed by Chiang-Ni and Wu, 2008).

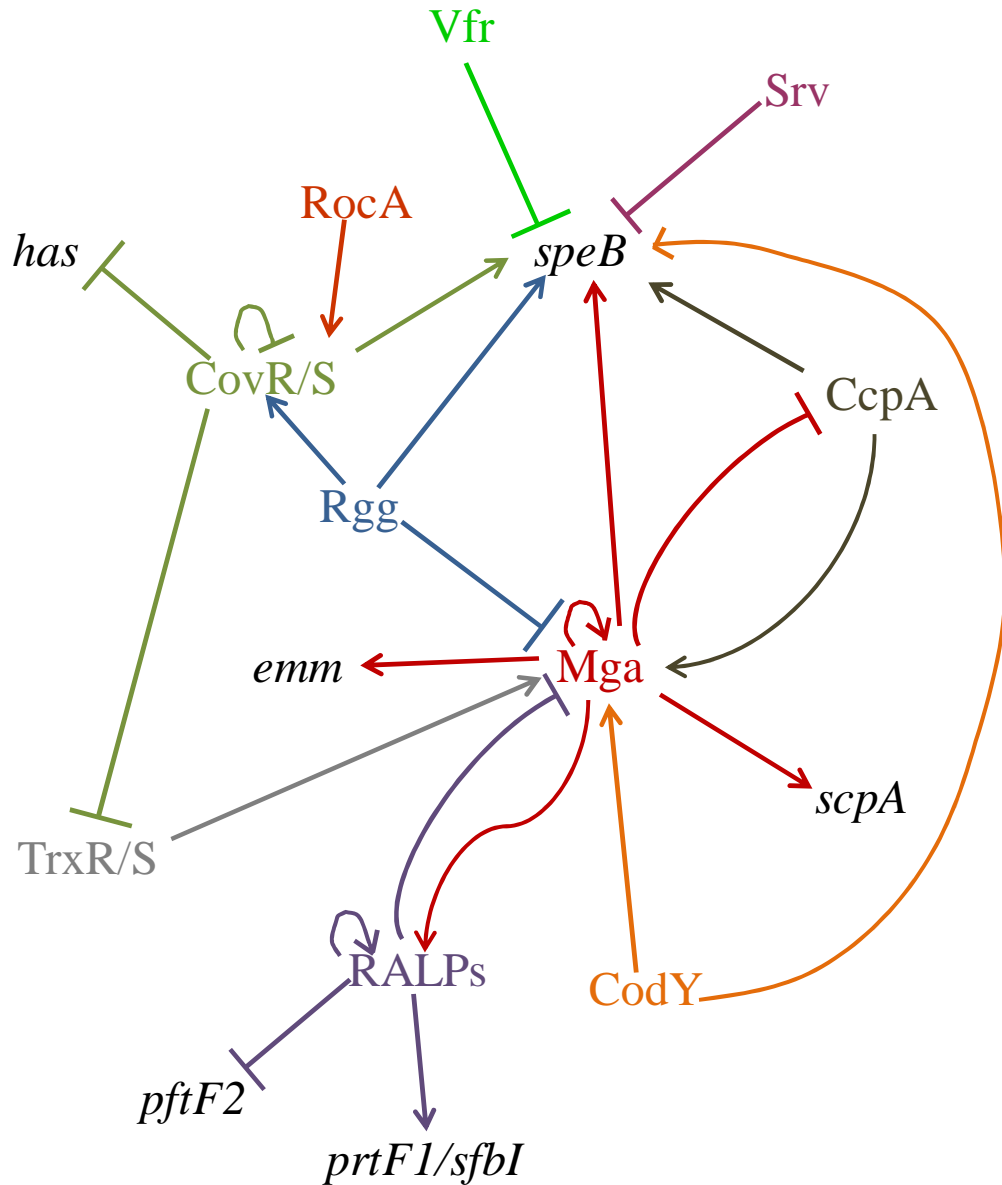


Figure 1. The complex regulatory network of *S. pyogenes*. An overview of known interaction between regulators and virulence factors are shown. Direct and indirect effects are not specified. An arrow represents activation, and a bar represents repression. The genes in black are virulence factors. Each regulator and its actions are colored. CovR/S and TrxR/S are two component response regulators. All other regulators shown are stand alone response regulators.

This growth phase-dependent increase in transcription is regulated by Rgg/RopB, CcpA, and LacD.1 (Kietzman and Caparon, 2010; Reviewed by Kreikemeyer *et al.*, 2003; Chaussee *et al.*, 1999).

#### Streptolysin O (SLO)

Streptolysin O (SLO) is a pore-forming cytolysin that allows for invasion of *S. pyogenes* into eukaryotic cells. The 'O' refers to its sensitivity to oxygen, in that it is inhibited at high oxygen levels. It is cytotoxic to a variety of cell types, and it also allows for the translocation of NAD glycohydrolase, produced by the bacterium, into the host cell resulting in death of the cell (Reviewed by Bisno *et al.*, 2003). *S. pyogenes* are able to induce caspase-mediated apoptosis in macrophages, as well as neutrophils, and this is dependent upon SLO expression (Timmer *et al.*, 2009). In addition to SLO, *S. pyogenes* produces streptolysin S (SLS) which has similar functions in pore forming as SLO and has been suggested to act equivalently to a Type III secretion system for introduction of toxins into eukaryotic cells (Reviewed by Bisno *et al.*, 2003; Madden *et al.*, 2001).

#### Fibronectin Binding Proteins

*S. pyogenes* encodes many proteins involved in the attachment of the bacterium to the host cell. The primary adhesins responsible for attachment bind fibronectin, which acts as a bridge to the eukaryotic cell. *S. pyogenes* encodes at least 5 fibronectin binding proteins (FBPs). Included in these proteins are SfbI (and its allelic variant PrtF1) as well as PrtF2. A full set of FBPs are not present in all *S. pyogenes* strains which may be able to explain tissue tropism differences between strains (Schwarz-Linek *et al.*, 2006). Attachment is a necessary precursor step leading toward invasion of host cells, and there are three mechanisms by which the bacteria can enter eukaryotic cells, two of which are fibronectin dependent (Molinari *et al.*, 2000; Cue *et al.*, 1998).

### Pyrogenic Exotoxins

Streptococcal pyrogenic exotoxins are a family of superantigens that play a role in Streptococcal toxic shock syndrome (STSS). This family includes SpeA, SpeC, SpeG, SpeH, SpeJ, SpeK, SpeL, SpeM, SSA, SmeZ, and SMEZ-2. Superantigens bind MHC-II and TCR in an antigen-independent manner, which activates all T cells with the same V $\beta$  motif. This activation leads to the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-2, and IFN- $\gamma$ . If unrestricted, this overwhelming immune response can cause hypotension and multi-organ failure as seen in STSS patients (Reviewed by Bisno *et al.*, 2003).

### Pili

Gram-negative bacteria have been studied extensively for their pili-mediated role in attachment. In some species this attachment leads to internalization. Pili are much less studied in Gram-positive bacteria. Recently, pili in *S. pyogenes* have been shown to play a role in attachment to cells from tonsil tissue, as well as to keratinocytes. Interestingly, pili-mediated attachment is cell specific. Pili were necessary for attachment to tonsil cells, primary keratinocytes, and HaCaT cells, but pili was not necessary for attachment to Hep-2 or A549 cells (Abbot *et al.*, 2007). Around the same time, it was discovered that pili play a role in biofilm formation. A deletion in pili components led to a reduction in the aggregation of bacteria in culture, suggesting a loss in bacterial attachment. Pili<sup>+</sup> strains were able to grow as a biofilm on polystyrene plastic wells and polylysine coated coverslips, whereas the pili<sup>-</sup> strains were inhibited 5-6 fold in biofilm formation (Manetti *et al.*, 2007).

### Other Virulence Factors

*S. pyogenes* encodes multiple other virulence factors that may play more minor or yet unknown roles in virulence. These virulence factors include streptokinase which complexes with plasminogen, changing the plasminogen activation pocket so that the protein converts into an active serine protease. The activation of plasminogen facilitates microbial dissemination (Sun *et al.*, 2009). Streptococcal C5a peptidase (ScpA) cleaves complement C5a preventing C5a from acting as a chemoattractant for the recruitment of immune cells to the site of infection (Gleich-Theurer *et al.*, 2009). Hyaluronidase cleaves bacterial and tissue hyaluronic acid. This may lead to the spread of large molecules or the use of hyaluronic acid as an energy source (Starr and Engleberg, 2006). At least four DNases have been identified, and they are believed to be involved in disease progression, but their exact mechanism is still unknown (Sumby *et al.*, 2005; Wannamaker *et al.*, 1967; Wannamaker and Yasmineh, 1967). Streptococcal inhibitor of complement inhibits the membrane attack complex of complement and inhibits the mucosal immune response (Ferne-King *et al.*, 2006). A few key virulence factors are necessary for the development of necrotizing fasciitis. In a study by Olsen *et al.*, a single nucleotide insertion in *mtsR* (metal transporter of Streptococcus regulator) led to de-repression of PrsA (peptidyl prolyl isomerase) which is necessary for post-translational maturation of SpeB (Olsen *et al.*, 2010). Also, *S. pyogenes* cell envelope protease (SpyCEP) cleaves host immune factors such as IL-8, GCP-2, GRO $\alpha$ , CXCL1 and CXCL6. Mac1/IdeS was shown to inhibit PMNs by binding to their IgG receptors, and it also functions as a cysteine protease to cleave IgG (Reviewed by Olsen and Musser, 2010).

## Summary of Virulence Factors

The wide range of diseases caused by *S. pyogenes* can be attributed to the range in virulence factor expression between strains. The virulence factors are spatially and temporally expressed at various levels throughout the infectious cycle. Some virulence factors are involved in attachment: PrtF2, M protein, and pili; some are involved in immune avoidance: SpeB, M protein, capsule, SLO, and C5a peptidase; and some factors cause dissemination and damage to other proteins: SpeB, SLO, and hyaluronidase. The interaction of virulence factor expression determines the host range, progression, and severity of disease; therefore, study of features that influence and regulate virulence factors is important for determining the host-pathogen response during *S. pyogenes* infection.

### ***Streptococcus pyogenes* Virulence Factor Regulation**

#### General

*S. pyogenes* possesses an extensive regulatory network composed of stand-alone response regulators (RRs) and two-component signal transduction systems (TCSs) (Fig. 1). Many of these regulators affect transcription of genes encoding proteins involved in metabolism, virulence, or both. TCSs function by recognition of a signal by a sensor histidine kinase which then phosphorylates the response regulator that transcriptionally acts upon the promoter of target genes (Reviewed by Kreikemeyer *et al.*, 2003). The genome sequence of an M1 *S. pyogenes* strain revealed 13 potential TCSs present in the genome (Ferretti *et al.*, 2001). In addition to two component regulators, other Gram-positive microorganisms rely upon alternate RNA polymerase sigma factors for global gene regulation. However, *S. pyogenes* only encodes one alternate sigma factor, ComX

which is encoded by the gene *sigX* (Opdyke *et al.*, 2003; Opdyke *et al.*, 2001; Reviewed by Kreikemeyer *et al.*, 2003). Two copies of *sigX* are present in the *S. pyogenes* genome. *sigX* is transcribed at very low levels and mutation of both genes does not affect growth, but these mutations revealed that ComX regulates the transcription of three genes putatively involved in competence. However, competence has not been demonstrated to date (Opdyke *et al.*, 2003).

### Mga

The first identified regulator of *S. pyogenes* virulence genes is the multi-gene activator (Mga). It regulates ~10% of the genome, and is itself regulated by multiple other regulators (Reviewed by Hondorp and McIver, 2007) (Fig. 1). The genes regulated by Mga include those for adhesins and invasins (including M protein), immune invasion factors, and other factors outside of the core Mga operon. *mga* is transcribed during exponential growth and further activated by elevated CO<sub>2</sub> levels, increased temperature, and iron-limiting conditions. It autoinduces its own expression and it is negatively regulated by RofA/Nra and RopB/Rgg (Reviewed by Kreikemeyer *et al.*, 2003).

### CodY

Under nutrient starvation and stress, bacteria typically upregulate RelA, which converts GTP to (p)ppGpp to inhibit RNA synthesis (stringent response). *S. pyogenes* also possesses a RelA-independent mechanism of activating a stringent response through the action of CodY. This regulator is conserved in low G + C Gram-positive bacteria. CodY is activated by the presence of branched chain amino acids (BCAAs). CodY acts as a transcriptional activator of *pel/sagA* and *mga* (Malke *et al.*, 2006).

### Srv

Streptococcal regulator of virulence (Srv) was originally identified as a homolog of PrfA, which is a member of the Crp/Fnr (cAMP receptor protein/Fumarate and nitrate reduction regulator) family of proteins in *Listeria monocytogenes*. PrfA controls the expression of 73 genes, including virulence factors, in *L. monocytogenes* (Reid *et al.*, 2004). Recent studies show that Srv expression is necessary for virulence and biofilm formation, but its exact role is still being investigated (Doern *et al.*, 2009; Reid *et al.*, 2004).

### RofA-Like Proteins (RALPs)

There are four homologs in the family of RALPs. These proteins share 52% amino acid sequence similarity and 29% identity (Granok *et al.*, 2000). The first regulator in the RALP family to be described was RofA. RALPs autoinduce their own expression and regulate genes encoding MSCRAMMS, hemolysins, proteases, superantigens, and other transcriptional regulators (Reviewed by Kreikemeyer *et al.*, 2003). In particular, RofA and Nra are negative regulators of *mga* and *emm* (Beckert *et al.*, 2001; Reviewed by Kreikemeyer *et al.*, 2003). All strains analyzed have either the *nra* or *rofa* gene, but may have one or more additional RALP genes present (Reviewed by Kreikemeyer *et al.*, 2003). The distribution of the RALP genes is strain specific. NZ131, an M49 strain, does not possess *rofa*, but it does contain *nra* and also two additional RALPs (Kreikemeyer *et al.*, 2002; Kyoto Encyclopedia of Genes and Genomes).

### RopB/Rgg

RopB/Rgg regulates stationary phase genes. Rgg contributes to increased transcription of *speB* as the culture begins to enter stationary phase (Reviewed by Kreikemeyer *et al.*, 2003; Chaussee *et al.*, 1999). Rgg/RopB negatively regulates *mga*, similar to RALPs. It also upregulates genes for a few two component regulators including CovR/S (Reviewed by Kreikemeyer *et al.*, 2003). Chaussee's group has studied this response regulator extensively, and they found that Rgg/RopB affects genes involved in both metabolism as well as virulence. Inactivation of *rgg/ropB* caused M49 strain NZ131 to utilize serine and arginine during exponential phase, even in the presence of glucose (Chaussee *et al.*, 2004; Chaussee *et al.*, 2003).

### CovR/S

Of the ~13 TCS systems that have been identified, CovR/S (control of virulence genes) is the most studied. It negatively regulates capsule expression as well as streptolysin S, streptokinase, streptodornase, and SpeB (Reviewed by Kreikemeyer *et al.*, 2003). CovR/S affects ~15% of the *S. pyogenes* genome (Graham *et al.*, 2002; Reviewed by Kreikemeyer *et al.*, 2003). CovR/S also affects the transcription of genes for response regulators, including RALPs. Its main activity occurs in late exponential phase and stationary phase. CovR/S must be phosphorylated to bind long AT rich sequences in the promoter region, but researchers are still unsure whether there is a conserved binding box (Reviewed by Kreikemeyer *et al.*, 2003). It is generally thought that CovS is the sensor kinase that phosphorylates CovR to transcriptionally regulate gene expression (Churchward, 2007). A study that looked closer at the CovR interaction with CovS found that a mutation in *covS* had no effect upon virulence in mice, but a mutation in *covR* led

to attenuated lethality. This suggests that CovR can be phosphorylated by other factors such as acetyl-phosphate (Dalton *et al.*, 2006). CovR/S has been shown to play a significant role in virulence. A mutation in *covR* decreased the LD<sub>50</sub> in mice by 500 fold (Levin and Wessels, 1998), and it also caused larger lesions in mice, demonstrating the role of CovR/S to repress virulence factors (Engleberg *et al.*, 2001). *covS* mutations have been implicated in animal passage adaptation, where more virulent isolates are recovered from animals (Aziz *et al.*, 2010). In patients, changes in *covRS* sequences have been associated with a switch from a pharyngeal transcriptome profile to an invasive transcriptome profile (Sumbly *et al.*, 2006). Recent studies isolated hypervirulent MIT1 strains after animal passage. Each of these strains had mutations in *covS* and had a different virulence factor profile than the wildtype strain. The authors suggested that *covS* plays a role in CovR/S regulation, but they acknowledge that the phosphorylation system is still unclear (Kansal *et al.*, 2010).

#### Other Response Regulators

In addition to the major response regulators discussed above, *S. pyogenes* expresses numerous other regulators with a variety of functions. Vfr, standing for virulence factor related, is a recently identified repressor of *speB* transcription. The mechanism of action for Vfr is still unknown, but protein sequence analysis suggests it does not function as a DNA binding protein (Ma *et al.*, 2009). RocA, Regulator of Cov, activates transcription of CovR, which is a regulator that was discussed earlier. A mutation in *rocA* caused a threefold decrease transcription of *covR* (Biswas and Scott, 2003). TrxSR, two-component regulatory system X, has recently been identified as an activator of *mga*. This RR is itself repressed by CovR/S, so it can act as a link between

CovR/S activity and *mga* transcription (Leday et al., 2008). CcpA, catabolite control protein A, activates *mga* transcription (Almengor et al., 2007) as well as *speB* transcription (Kietzman and Caparon, 2010).

### **Survival and Persistence**

There are numerous pathogens whose major reservoir is the human. Like *S. pyogenes* these pathogens alternate between carriage and active disease.

#### *Mycobacterium tuberculosis*

*M. tuberculosis* is a pathogen of global significance in that it infects 1 in 3 people worldwide. It is the causative agent in tuberculosis, and it persists in a latent state in alveolar macrophages. *M. tuberculosis* possesses multiple virulence factors that allow for evasion of the immune system as well as its persistence. Upon initial infection, the bacteria multiply until the immune response recognizes them, and the macrophages form granulomas ('tubercles') around the bacteria. It is in these granulomas that *M. tuberculosis* is able to survive. There are >25 genes associated with the persistence of mycobacteria (Reviewed by Honer zu Bentrup and Russell, 2001). The bacteria are acid resistant, and the mechanisms are still under study. Proteins that prevent acidification of the phagosome include Rv3671c (Vandal *et al.*, 2008), OmpA (Raynaud *et al.*, 2002), MgtC (Buchmeier *et al.*, 2000), and proteins involved in peptidoglycan synthesis (Reviewed by Honer zu Bentrup and Russell, 2001). Also, *M. tuberculosis* prevents the maturation of the phagosome and is able to avoid the action of nitric oxide (NO), conferring further resistance to phagocytosis. In addition to these factors, *M. tuberculosis* alters its metabolism during long term persistence. It upregulates isocitrate lyase, an

enzyme involved in the metabolism of fatty acids. Fatty acids are converted to acetyl-CoA by phospholipases and  $\beta$ -oxidation. The acetyl-CoA enters the TCA cycle as citrate. This reaction produces NADH, which can be used as an electron donor for respiration to produce energy. Isocitrate lyase converts isocitrate, an intermediate of the TCA cycle, to succinate and glyoxylate in the glyoxylate bypass. Glyoxalate can be used as a carbon source. This allows *M. tuberculosis* to convert fatty acids to carbohydrates in a carbon-limiting environment (McKinney *et al.*, 2000; Sharma *et al.*, 2000; Reviewed by Honer zu Bentrup and Russell, 2001).

### *Pseudomonas aeruginosa*

Cystic fibrosis (CF) is a hereditary disease caused by a mutation in a cAMP-regulated chloride channel, and it is characterized by lung disease and eventually pulmonary disease. Typically, patients develop chronic bacterial lung infections. Up to 85% of patients are chronically colonized with *Pseudomonas aeruginosa* leading to further lung damage. *P. aeruginosa* is an opportunistic pathogen, and it is found ubiquitously in the environment. It is commonly found in moist areas such as sinks and drains. Upon initial infection, *P. aeruginosa* expresses high levels of adhesins that bind host disaccharide GalNAc $\beta$ 1-4Gal. This disaccharide is more abundant in CF cells compared to wild-type, which may increase attachment of the bacteria in CF patients (Reviewed by Davies, 2002). In chronically infected patients, *P. aeruginosa* becomes hypermutable and alters its protein expression for survival and immune avoidance. The genes for the adhesins are down-regulated upon chronic infection (Oliver *et al.*, 2000; Reviewed by Davies, 2002). *P. aeruginosa* cleaves immunoglobulins, complement, and cytokines by producing elastases and alkaline proteases. Alginate, a mucoid

exopolysaccharide, is produced to protect from mucociliary clearance. The bacteria begin to form biofilms, which protect against phagocytosis (Reviewed by Davies, 2002). Colonizing strains isolated from patients often contain mutations in the alginate synthesis genes resulting in increased production of this major biofilm matrix component. *P. aeruginosa* survives within the biofilm in CF lung mucous, which provides an anaerobic environment. Under oxygen-limiting conditions, *P. aeruginosa* grows in the presence of nitrate and uses nitrate as the final electron acceptor for respiration producing nitrite as an end product. Additionally, *Pseudomonas aeruginosa* increases pyruvate fermentation for long-term survival, producing acetate and lactate as metabolic byproducts (Eschbach *et al.*, 2004).

#### *Staphylococcus aureus*

*S. aureus* is able to colonize the skin in mucous membranes of humans, and it is in a persistent carrier state in ~20% of the population. It causes skin and soft tissue infections, and if it enters the bloodstream it can infect almost any organ. The bacteria can persist in the nasal passages without causing overt disease. The mechanisms of persistence are not fully understood, but it is known that a few factors play a role in its persistence. Teichoic acids, clumping factor B and SasG are adhesin factors, and LsdA is responsible for lactoferrin resistance in nasal secretions allowing for persistence. *S. aureus* has recently been shown to invade eukaryotic cells, but the role of invasion on persistence is still under investigation. *S. aureus* can invade a range of cells including endothelial cells, epithelial cells, osteoblasts, fibroblasts, keratinocytes, PMNs, and macrophages. Upon persistence, *S. aureus* isolates show an altered colony morphology termed small colony variants (SCVs). SCVs are slow growing variants that show

increased, and more frequent, drug-resistant profiles compared *S. aureus* strains with normal colony phenotype. Clinical isolate SCVs were more resistant to antibiotics including gentamycin and ciprofloxacin (Reviewed by Garzoni and Kelley, 2009). SCVs are non-hemolytic, non-pigmented colonies that are ~10 times smaller than colonies of normal strains. The small colony phenotype is because of an acquired auxotrophy for hemin, menadione or thymidine, and the colonies grow as typical colonies in the presence of these three compounds. Hemin is required for the biosynthesis of cytochromes, and the latter two compounds are involved in the electron transport system. This is an unstable phenotype, and the strains eventually revert to the rapid growth rate found in the normal phenotype (Reviewed by von Eiff, 2008). A mutation in *hemB*, a hemin biosynthetic gene, mimics the SCV phenotype. The *hemB* mutant upregulated genes involved in glycolytic and fermentative pathways. It also upregulated purine biosynthesis as well as arginine and proline catabolism. Its use of the arginine deiminase pathway to generate ATP may be used to counteract the acidic environment produced by its fermentative pathways (Seggewiss *et al.*, 2006; Reviewed by von Eiff, 2008). In a septic arthritis mouse model, the SCVs seem to be more virulent per organism based on the high levels of arthritis despite low bacterial numbers present in the joints and spleen (Jonsson *et al.*, 2003; Reviewed by von Eiff, 2008), although studies using other models suggest equivalent levels of virulence (Bates *et al.*, 2003; Reviewed by von Eiff, 2008). In addition, *S. aureus* forms biofilms, serving as an additional mechanism to avoid the action of antibiotics (Reviewed by von Eiff, 2008). Recently, *S. aureus* has been implicated as the primary species in recurrent tonsillitis patients. The isolated strains

were likely due to intracellular persistence rather than persistence within a biofilm (Zautner *et al.*, 2010).

### *Helicobacter pylori*

*H. pylori* is one of few bacteria that are able to survive within the human stomach despite the highly acidic conditions. It is the cause of stomach ulcers, and it has been identified as a bacterial carcinogen, the only bacterium to be identified as such. *H. pylori* survives in the stomach of infected patients, and its extensive genetic diversification is thought to enable survival under changing environmental conditions. *H. pylori* utilizes multiple mechanisms for the generation of diversity including point mutations, intragenomic recombination, and slip-strand mismatching during DNA replication. Intragenomic recombination is facilitated by the natural competence of *H. pylori*. The diversification of its genome is triggered by stress and its diversification plays a role in affecting host adherence as well as host immune evasion (Reviewed by Dorer *et al.*, 2009). Studies looking at isolates 6 years after the sequencing of the parental strain, J99, from the source patient revealed similarity in random amplification of polymorphic DNA PCR profiles for recently obtained strains suggesting diversification within strains of the same PCR profile. Further studies with DNA microarray revealed 3% difference in J99 loci amongst the survivor strains as well as insertions and deletions (Indels) in the genomes of the newly isolated strains (Israel *et al.*, 2001).

### *Streptococcus pneumoniae*

*S. pneumoniae* causes invasive diseases as well as respiratory tract infections. In particular, it causes meningitis, sepsis, and pneumonia. Immune protection is via opsonin dependent phagocytosis. *S. pneumoniae* can colonize the mucosal surfaces of the

nasopharynx of healthy individuals, (Bogaert *et al.*, 2004) and the bacteria can then enter into normally sterile sites such as the lung, middle ear, or bloodstream to cause disease (Reviewed by Weiser, 2010). Carriage is extremely common, and the rate is approximately 10% in younger children and 50% in children 10 years old (Bogaert *et al.*, 2004). In developing countries, the carriage rate goes up to 95% in children under three years old (Lloyd-Evans *et al.*, 1996). *S. pneumoniae* are typically cleared by opsonophagocytosis, but expression of capsule inhibits phagocytosis of the bacteria. Strains that lack capsule production are avirulent. *S. pneumoniae* demonstrates phase shift during the cycles between commensal survival and transition to the bloodstream. While surviving as a commensal organism, the bacteria are transparent with a thin capsule layer to allow binding to host cells (Reviewed by Weiser, 2010). Upon encountering the bloodstream, cells are opaque with high capsule production to avoid phagocytosis. *S. pneumoniae* are naturally competent and take up DNA from the environment through homologous recombination. Additional mutation occurs through error prone mismatch repair following oxidative damage. This mismatch repair has been linked to mutations giving rise to antibiotic resistance (Reviewed by Weiser, 2010). Hiller *et al.* sequenced 17 strains from clinical isolates and found significant differences in genic content between strains, which supports the distributed-genome hypothesis (see below). Fewer than 50% of the orthologous gene clusters were conserved between the strains (Hiller *et al.*, 2007). In addition to causing disease in the upper respiratory tract, *S. pneumoniae* is a leading cause of otitis media in children. 20 to 30% of the pediatric population has recurrent otitis media. This is mostly due to a failure in antibiotic therapy, but only ~50% of bacteria isolated from recurrent infections are antibiotic resistant

(Reviewed by Pichichero, 2000). More recent data have shown that *S. pneumoniae* forms biofilms in the mucosa of the inner ear, which may contribute to antibiotic resistance and recurrent infections, (Moscoso *et al.*, 2009) and it is a predisposing factor to support the distributed-genome hypothesis in this bacteria (Hiller *et al.*, 2007; Ehrlich *et al.*, 2005).

### The Distributed-Genome Hypothesis

Bacterial infections were traditionally thought to be caused by clonal strains of bacteria, in that the infectious agent would share the same genome as the strain isolated after disease. This concept is being challenged with the increasing ease and lower cost of whole genome sequencing. Often, bacterial heterogeneity plays a significant role in the survival and persistence of a bacterial population. In particular, biofilms are composed of a genotypically heterogeneous population of bacterial strains, even in a monospecies biofilm. This leads to the distributed genome hypothesis that states that individual strains within a population have a distribution of genes, meaning that not all organisms have the same set of genes. No one organism has the full complement of genes, and the supragenome is far larger than any one individual genome. The shuffling of genes occurs through horizontal gene transfer. The variability within the population helps ensure the population's survival. For example, in the case of an environmental change, which is often the case for pathogens, certain strains may be more fit for survival and ensure the continued survival of the population as a whole. 10-15% of all genes in *H. influenzae*, *S. pneumoniae*, and *P. aeruginosa* are unique when the sequences are compared to laboratory type strains (Reviewed by Ehrlich *et al.*, 2005). Up to 80% of the population are carriers of *H. influenzae*, and carriage has been linked to horizontal gene transfer. *H. influenzae* acquire DNA of highly related strains that encode an uptake signal sequence.

Analysis of *H. influenzae* strains demonstrated that no two strains encode the same complement of genes, and the supragenome is larger than the genome of any one individual strain, supporting the distributed-genome hypothesis during the carriage of this bacterium (Shen *et al.*, 2005).

#### Diversity of *Streptococcus pyogenes*

Like other pathogens that are both carried and cause various diseases, *S. pyogenes* isolates are genetically variable with both differences in genic content and allelic variations due predominately to single nucleotide polymorphisms (SNPs) and insertions/deletions (Indels). It is generally difficult to associate any one disease with a particular *S. pyogenes* phenotype because of the high level of variation within the *S. pyogenes* genome. Some general attempts at associating M type with disease have been made, but there is variance within each M type. M18 is associated with acute rheumatic fever. M49, M60, and M61 are often found in skin infections. M1, M3, and M28 are associated with pharyngitis and invasive infections. Also, M3 has disproportionately higher occurrence in severe invasive infections (Reviewed by Olsen and Musser, 2010).

Protein serotyping and *emm* sequencing alone are not sufficient enough to detect clonal lineages or chromosomal diversification during infection because of the high mutation rate of certain *S. pyogenes* strains (Scott *et al.*, 2008; Martin *et al.*, 2003). Two studies of *S. pyogenes* isolates from patients with recurrent pharyngitis infection showed 100% (14/14 patients using random amplification of polymorphic DNA, RAPD) and 70% (43/61 patients using restriction fragment length polymorphism, RFLP) of the post-treatment strains were clonal with the strain isolated before antibiotic treatment, suggesting the bacteria survived in the host between outbreaks (Fitoussi *et al.*, 1997;

Osterlund and Engstrand, 1995). However, these techniques do not detect interstrain variation in many cases. Other than full-genome sequencing, modified PFGE and multi-locus sequence typing (MLST) methods are currently the most accurate typing methods employed (Carrico *et al.*, 2006; Enright *et al.*, 2001). Results suggest that polymorphisms may be present even within the same PFGE profile (Desai M, 1998), suggesting that genetic diversification may be occurring within strains that are closely enough related to be considered clonal.

Genetic variation within the *S. pyogenes* genome is extensive and well-documented (Scott *et al.*, 2008; Podbielski, 2007; Burrus and Waldor, 2004; Reviewed by Banks *et al.*, 2002). There is a conserved core genome and a more variable region that has differences in genic content and a higher frequency of single nucleotide polymorphisms (SNPs) (Lefebure and Stanhope, 2007). The core genome of strains from different M genotypes varies by ~14,000 SNPs, whereas many *S. pyogenes* strains of the same M serotype still differ by several hundred SNPs (Reviewed by Olsen and Musser, 2010; Beres *et al.*, 2006). Approximately ten percent of *S. pyogenes* genic content is derived from prophages and integrated conjugative elements (ICEs), and certain regions of the genome are prone to insertions of ICEs, such as the fibronectin-collagen-T-antigen (FCT) region. The FCT region is an 11-16 kb region, with significant variation between strains. It typically includes genes for RALPs, an adhesin, a collagen binding protein (Cpa), the MsmR regulator, and 20 other genes in various combinations (Podbielski, 2007; Reviewed by Banks *et al.*, 2002). In many cases, genes encoding virulence factors are located near 'hotspots' for mobile genetic elements (McMillan *et al.*, 2007) which may alter expression of these virulence factors. Genomic analysis of clinical isolates

from successive *S. pyogenes* epidemics revealed large numbers of SNPs and Indels in the core genome. Even modest changes in the core genome led to large changes in the transcriptome of the isolates (Beres *et al.*, 2010).

#### Diversification during survival

Our laboratory has developed two in vitro models to study survival of *S. pyogenes*. In the first, *S. pyogenes* CS101 was inoculated into Todd-Hewitt (TH) broth and incubated statically at 37°C for >12 weeks without the input of fresh nutrients. The aged culture was plated on TH plates to isolate survivor strains (Wood *et al.*, 2009; Wood *et al.*, 2005). The second model incubated CS101 with A549 human lung epithelial cells in 12-well plates in the presence of penicillin and gentamicin, which do not enter eukaryotic cells and would kill extracellular bacteria. The co-culture was incubated for 5 days, at which time the eukaryotic cells were lysed and plated onto TH plates. The resulting colonies were derived from strains that survived intracellularly (Morgan and Buttaro, submitted).

The survivor strains derived from these two models demonstrated altered colony morphologies. The strains from long-term culture gave either small colonies, which did not express capsule, or atypical large colonies which still expressed capsule (Wood *et al.*, 2005). Co-culture derived strains had only small colony phenotypes or appeared similar to wild-type (Morgan and Buttaro, submitted). These changes in colony morphology were stable and did not change after >200 generations (Wood *et al.*, 2005). Analysis of two of the small colony survivor strains from long-term culture, Alt. 1 and Alt. 2, showed common and unique changes in their proteomes compared to each other and compared to their parental strain CS101 (Wood *et al.*, 2005). These changes appeared to be random

since analysis of the common changes were not detected in other survivor strains (unpublished observations).

During exponential phase growth in TH culture, glucose is abundant, and it is the primary carbon source during this phase. In exponential phase, in the presence of a primary energy source, *S. pyogenes* are homofermentative and they produce predominantly lactic acid. Upon glucose depletion, the cultures enter into stationary phase. During stationary phase, continued metabolism is heterofermentative, with the production of formate, acetate and ethanol. When the survivor strains were regrown in TH broth, the cultures produced large amounts of formate, acetate, ethanol, and ammonia during exponential phase suggesting heterofermentative metabolism is occurring in the presence of glucose. The increase in formate and acetate suggests upregulation of the pyruvate to acetate (PA) pathway (Fig. 2A), the increase in ethanol suggests upregulation of the pyruvate to ethanol (PE) pathway (Fig. 2A), and the increase in ammonia suggests amino acid catabolism (Fig. 2B). Chaussee's group has shown that *S. pyogenes* catabolize arginine, serine, and histidine to generate energy during stationary phase, and this metabolic switch is caused by the growth phase dependent regulator Rgg/RopB (Chaussee *et al.*, 2004; Chaussee *et al.*, 2003). Each survivor strain's metabolic profile is unique when they are compared to each other and to CS101, even between multiple strains isolated from the same culture (Wood *et al.*, 2009). Varied levels of metabolic endproducts suggest altered metabolic flux through the pathways. The metabolic diversity between the survivor strains is important in culture to maintain the pH above the critical threshold of 5.6. Ammonia production by some strains balances out the acid production by other strains. E11 is a survivor strain isolated from co-culture. When

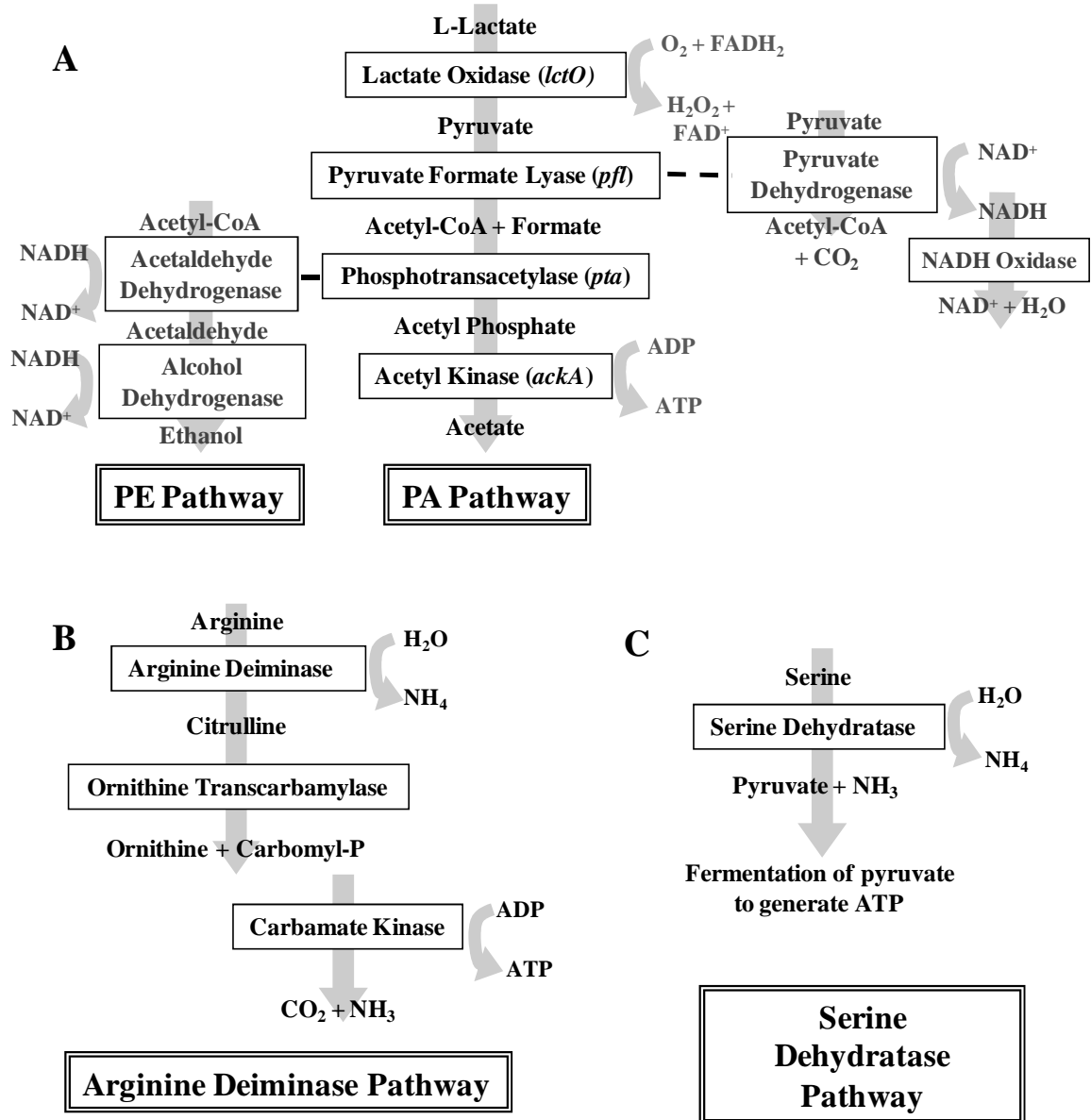


Figure 2. Metabolic pathways upregulated during long term survival. A: The Pyruvate to Acetate (PA) and the Pyruvate to Ethanol (PE) pathways generate ATP and  $NAD^+$  by converting pyruvate to acetate and ethanol. B: The arginine deiminase pathway produces ATP and  $NH_3$ . C: The serine dehydratase pathway produces  $NH_3$  and pyruvate, which can feed into the PA or PE pathway.

regrown in TH broth, E11 upregulates expression of the PA pathway, similar to the survivor strains isolated from long-term stationary phase cultures (Morgan and Buttaro, submitted). Further analysis of multiple co-culture derived strains isolated from the same well showed that these strains did not upregulate PA pathway expression (unpublished data). This observation suggested that metabolic diversification may be occurring not only during long term stationary phase survival, but possibly during intracellular survival, reflecting what may be occurring during carriage in the host.

### **Project Aims**

Previous studies in the laboratory suggested that *S. pyogenes* diversify to form a polyclonal population during survival. Diversification of their proteome and metabolism has been observed during survival in eukaryotic cells and in stationary phase cultures.

The specific aims of this project were

1. To determine whether metabolic diversification occurred during active infection.
2. To determine if in vitro survival leads to diversification in virulence factor expression.
3. To determine whether changes in virulence factor expression affected the survivor strains' level of virulence in the zebrafish model of infection and ability to survive in human blood.
4. To investigate the mechanism leading to strain diversification during in vitro survival.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

#### **Bacterial Strains**

All strains used in these studies were *S. pyogenes* strains. *S. pyogenes* strain CS101 was kindly provided by P. Cleary (University of Minnesota). Clinical strains 221, Sfr321, and MK322 were provided by A. Podbielski (University Hospital Rostock, Germany). Strains 64P, 64BP9L, and 64BP9S were provided by M. Boyle (Juniata College). Strain JRS4 was provided by J. Scott (Emory University). All strains obtained from 14 week cultures of CS101 were designated with 'Alt.' and were randomly chosen colonies. Strains derived from 5 day co-culture with A549 human lung epithelial cells were designated with 'E' before the strain number. The survivor strains Alt. 1 and Alt. 2 were characterized in a previous study (Wood *et al.*, 2005). Survivor strains Alt. 4A through Alt. 5D were partially characterized in a separate study (Wood *et al.*, 2009). The survivor strain E11 was previously characterized (Morgan and Buttaro, submitted). Other survivor strains were previously obtained (Morgan and Buttaro, unpublished data), and are used in this study. All strains are listed in Table 1.

**Table 1. *Streptococcus pyogenes* strains used in this study**

<b>Strain</b>	<b>Strain Characteristics</b>	<b>Source</b>
CS101	Parental Strain Mucoid colony phenotype	(Haanes <i>et al.</i> , 1992)
Alt. 1	CS101 derived survivor strain Small colony phenotype <sup>a</sup>	(Wood <i>et al.</i> , 2005)
Alt. 2	CS101 derived survivor strain Small colony phenotype <sup>b</sup>	(Wood <i>et al.</i> , 2005)
Alt. 4A	CS101 derived survivor strain Atypical large colony phenotype <sup>c</sup>	(Wood <i>et al.</i> , 2009)
Alt. 4B	CS101 derived survivor strain Small colony phenotype <sup>c</sup>	(Wood <i>et al.</i> , 2009)
Alt. 4D	CS101 derived survivor strain Atypical large colony phenotype <sup>c</sup>	(Wood <i>et al.</i> , 2009)
Alt. 5A	CS101 derived survivor strain Atypical large colony phenotype <sup>d</sup>	(Wood <i>et al.</i> , 2009)
Alt. 5B	CS101 derived survivor strain Atypical large colony phenotype <sup>d</sup>	(Wood <i>et al.</i> , 2009)
Alt. 5C	CS101 derived survivor strain Small colony phenotype <sup>d</sup>	(Wood <i>et al.</i> , 2009)
Alt. 5D	CS101 derived survivor strain Small colony phenotype <sup>d</sup>	(Wood <i>et al.</i> , 2009)
E11	CS101 co-culture derived survivor strain Small colony phenotype <sup>e</sup>	Morgan and Buttarro, submitted
E47.5	CS101 co-culture derived survivor strain Large colony phenotype <sup>f</sup>	Morgan and Buttarro, unpublished results
E47.6	CS101 co-culture derived survivor strain Small colony phenotype <sup>f</sup>	Morgan and Buttarro, unpublished results
E47.7	CS101 co-culture derived survivor strain Large colony phenotype <sup>f</sup>	Morgan and Buttarro, unpublished results
E47.8	CS101 co-culture derived survivor strain Small colony phenotype <sup>f</sup>	Morgan and Buttarro, unpublished results
64P	Parental M64 Strain	(Reis <i>et al.</i> , 1984)
64BP9L	Isolate from 9 passages of 64pin human blood Large colony phenotype	(Raeder <i>et al.</i> , 2000)

64BP9S	Isolate from 9 passages of 64pin human blood Small colony phenotype	(Raeder <i>et al.</i> , 2000)
64/14	Isolate from 14 passages of 64P in BALB/c mice	(Reis <i>et al.</i> , 1984)
1 - 18	Strains isolated from 6 week survival cultures of M64 strains	This study
19 - 37	Strains isolated from 7 week survival cultures of M64 strains	This study
15	Isolate from 42 day survival culture of 64/14 Small colony phenotype	This study
26	Isolate from 49 day survival culture of 64p Small colony phenotype	This study
221	M49 clinical isolate from tonsillar material.	(Podbielski <i>et al.</i> , 2003)
Sfr 321	M49 clinical isolate from tonsillar material.	(Podbielski <i>et al.</i> , 2003)
MK322	M6 clinical isolate from tonsillar material.	(Podbielski <i>et al.</i> , 2003)
JRS4	M6 wild type strain	(Scott <i>et al.</i> , 1986)

<sup>a</sup> Randomly chosen colony isolated from an independent 14 week old stationary phase CS101 culture

<sup>b</sup> Randomly chosen colony isolated from a second, independent 14 week old stationary phase CS101 culture

<sup>c</sup> Randomly chosen colony isolated from a third, independent 14 week old stationary phase CS101 culture

<sup>d</sup> Randomly chosen colony isolated from a fourth, independent 14 week old stationary phase CS101 culture

<sup>e</sup> Isolated from an independent well of a 5 day old co-culture of CS101 with A549 human epithelial cells

<sup>f</sup> Isolated from a second, independent well of a 5 day old co-culture of CS101 with A549 human epithelial cells

## **Bacterial Growth Media and Incubation Conditions**

*S. pyogenes* strains were grown in Todd Hewitt (TH) broth (Appendix A; Difco; Detroit,MI). TH agar was made by adding 1.5% w/v agar (Difco; Detroit, MI) to TH broth (Appendix A). A freezer stock of each strain was maintained in 30% glycerol/70% TH stocks at -80°C. Cultures of *S. pyogenes* were inoculated directly from freezer stocks into TH broth. All growth was carried out static at 37°C 5% CO<sub>2</sub>.

## **Survival Assays**

*S. pyogenes* strains were inoculated into 20 to 30 ml of TH broth directly from freezer stocks. The cultures were placed in a 37°C 5% CO<sub>2</sub> incubator static. The cultures were removed at the indicated timepoints for sampling. In a sterile flow hood, the culture was pipette up and down twice with a 1 ml pipettor and 20 µl samples were removed and spotted onto a TH plate. The cultures were placed back in the incubator. The plates were air dried for ~10 minutes in the laminar flow hood. Once the spots were dry, the plates were placed in a 37°C 5% CO<sub>2</sub> incubator overnight. In the morning, plates were scored + or – for growth. If there were no signs of growth, the cultures were removed again and 100 µl samples were spread and incubated onto a TH plate to verify if the cultures had fewer than 10 CFU/ml viable bacteria.

## **Growth Curve**

*S. pyogenes* were inoculated directly from freezer stocks into 20 ml TH broth. The cultures were incubated static in a 37°C 5% CO<sub>2</sub> incubator. Cultures that were no older than 15 hrs were diluted 1:100 in TH broth by adding 0.4 ml of the culture to 39.6 ml of THB. The diluted cultures were placed back in the incubator and growth was monitored by spectrophotometry until OD<sub>600</sub> 0.5. The cultures were then diluted back

1:10 into THB by adding 4 ml to 36 ml THB. 1 ml was removed for immediate OD<sub>600</sub> reading, and 1 ml was removed every half hour to monitor the growth of the cultures. Once the cultures reached OD<sub>600</sub> ~0.6, only 0.5 ml was removed and diluted 1:2 with THB before analysis to prevent saturation of the spectrophotometric readings.

## **Generation of Northern Probes and Sequencing of Regulator Genes**

### Isolation of Genomic DNA From *S. pyogenes*

10 ml of TH broth was inoculated from a freezer stock of *S. pyogenes*. The cultures were placed in 37°C static overnight. The bacteria were harvested by centrifugation at 6000 rpm. The pellets were resuspended in 2 ml of DNA lysis buffer (Appendix A) and 1 ml was added to 0.8 g of zirconia silica beads with 50µl 10% SDS in bead beater tubes. The bacteria were lysed by bead beating 3 times for 1 min at 4800 rpm followed by a 1 min pause (Biospec Products, Bartlesville, OK). The DNA was extracted from the lysate with phenol as follows. 750 µl of the lysate was added to an equal volume of phenol (pH 6.6 Fisher Scientific). The mixture was shaken by hand and centrifuged at 13,000 rpm for 20 min. 600 µl of the aqueous phase (the top layer) was added to an equal volume of phenol/chloroform/isoamyl alcohol (50:49:1, pH 6.7 Fisher Scientific). This was centrifuged at 13,000 rpm for 10 min. The phenol/chloroform/isoamyl alcohol extraction was performed two more times with 50 µl less volume each time. 450 µl of the aqueous phase was mixed with an equal volume of chloroform/isoamyl alcohol (24:1, Acros Organics). DNA was precipitated from 400 µl of the aqueous solution by adding 40 µl of 3 M NaAc (Appendix A) and 800 µl of cold 100% ethanol. The DNA was placed in a -20°C freezer overnight to precipitate the DNA.

The DNA was then isolated by centrifuging the tubes at 13,000 rpm for 20 min at 4°C. The supernatant was discarded, and the pellet was washed with 70% ethanol. The pellet was resuspended in 40 µl of dH<sub>2</sub>O. The DNA was treated with 2 µl of RNase (10mg/ml) for 30 min at 37°C. The DNA was reprecipitated as above and resuspended in 20 µl of dH<sub>2</sub>O. The DNA was stored at -20°C. The isolated DNA was quantitated at OD<sub>260</sub>.

### Polymerase Chain Reaction (PCR)

Oligonucleotide primers for PCR are listed in Table 2. Oligonucleotides were generated by Integrated DNA Technologies (Coralville, IA). PCR reactions were carried out on a Thermocycler 2400 with heated lid (Perkin-Elmer Corp., Foster City, CA) or on a PTC 100 Programmable Thermal controller (MJ Research Inc., Waltham, MA), which does not have a heated lid. A layer of mineral oil (Fisher Scientific, Pittsburgh, PA) was added to the top of the PCR reaction when using the PTC cycler.

### Taq polymerase PCR reaction for production of Northern blot probes

PCR reactions for Northern blot probe generation were carried out with *taq* polymerase. PCR reactions were carried out according to manufacturer's instructions. The final concentrations in each PCR reaction contained: 1X *taq* polymerase buffer (10X, Roche Diagnostics; Indianapolis, IN), 0.2 mM of each dNTP (Promega; Madison, WI), 0.4 µM FWD primer, 0.4 µM REV primer, and 2 U/100 µl *taq* polymerase. The PCR reactions were as follows: an initial step of 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 50°C to 60°C for 30 seconds depending on oligonucleotide T<sub>m</sub>, and then 72°C for 1 minute/kb of product; 72°C for 7 minutes; then a holding step of 4°C until the tubes were removed from the thermocycler.

**Table 2. Oligonucleotides used in this study**

Name	Sequence (5'-3')	Assay used
lctO FWD	AGCACCTGTAGCGGCTCATAAACT	Northern
lctO REV	ATACCTGATGCTCCTGCGTCCAAT	Northern
acoA FWD	GCTTGCTGGTAAAGCAACTGGTGT	Northern
acoA REV	ATAGCTGGACCATTTCCACCACGA	Northern
pfl FWD	TCGTCTTGCTCTTTACGGTGCTGA	Northern
pfl REV	TCTGGCAGTTGGTCAGTCCAAAGA	Northern
pta FWD	TGACACTGTTTCGTCCAGCTCTTCA	Northern
pta REV	GCCATCAAGTGCCAAATCAGGGTT	Northern
ackA FWD	CGTGTTGTTGCTGGTGGTGAACCTT	Northern
ackA REV	AAGTGGTGTAAGCCCATCGAGGT	Northern
gyrA FWD	ACAGGTCGGGAACGTATTGTGGTT	Northern
gyrA REV	GTTCCAAACCAGTCAAACGACGCA	Northern
codY FWD 1	ACAAGCTAGTGCTTATCTCC	Sequencing
codY FWD 2	TATCCAGGAGGTCTAACGAC	Sequencing
codY REV	AAACAGGGAAACCTCTCCCC	Sequencing
ropB FWD 1	AAGCGACTATCATCCGAAAC	Sequencing
ropB FWD 2	ACTTGGAGTCACTATGAGAC	Sequencing
ropB REV	AAGCTAACACCATAAGAGCG	Sequencing
ccpA FWD 1	AAAGTGGTTACAAATCATGC	Sequencing
ccpA FWD 2	ACGCTCTCGTACTCCAGTTG	Sequencing

ccpA REV	AGATGGTGCTCATAATTCAC	Sequencing
srv FWD 1	TACTATCAAAGGGCATTAGC	Sequencing
srv FWD 2	TGTATGTAACTAACGGCCG	Sequencing
srv REV	TGCAGATCCAGATCAAAGCC	Sequencing
SPy1548 FWD	GGAACGTATGAGTTGGTGAC	Sequencing
SPy1548 REV	TTCTTTACCGGGTCTGTGGAGC	Sequencing
relA FWD 1	ACTAAGCGCTTTCTTAGCAG	Sequencing
relA FWD 2	TCGCATCAAATGGGAACTAG	Sequencing
relA REV	TTGACCCGTTGCAAGACAAG	Sequencing
SPy0145 FWD 1	TCAAGAGCAAAGGTGGTGAGAGGA	Sequencing
SPy0145 FWD 2	CCAGTGACAGGTCAATTGTC	Sequencing
SPy0145 REV	GAGGCACAAGCTGCCGAAGC	Sequencing
SPy1630 FWD	AGCTGAGCGGGTTAAGCGTATCAT	Sequencing
SPy1630 Rev	TGGCTTGTCGGTTTGCATCAATGG	Sequencing
CovRS FWD 1	GATAGATTAAGAGGATAAGGGTTGGT	Sequencing
CovRS FWD 2	TGTTTGGAATATGATGAAGCCGT	Sequencing
CovRS FWD3	TGGTCCTATCGGTCGTGTGTATCA	Sequencing
CovRS FWD 4	TGAGGCTGACCGTATGGCAATCAT	Sequencing
CovRS REV	CATCAGCTTCTAACCAGTTGTGGC	Sequencing
RALP 0189c FWD 1	CGCGTGTCTTAGCTCTCTC	Sequencing
RALP 0189c FWD 2	TGTGGACAATACTGTGATCG	Sequencing
RALP 0189c REV	CTGGTGCAAGGCTAAGATGG	Sequencing
RALP 0566c FWD 1	TTAAACACGCGTCACCATTG	Sequencing

RALP 0566c FWD 2	CCATCACACTTAATGATGCC	Sequencing
RALP 0566c REV	CGTTCAGTTCCTCACCTTG	Sequencing
<i>mga</i> FWD seq NZ131	GCGTCGTTGACCGCTTCCCT	Sequencing
<i>mga</i> FWD 2 seq NZ131	ACTGTGAACGCCTATTGAGC	Sequencing
<i>mga</i> FWD 3 seq NZ131	GCCTTTGTGACAAGTGTCGA	Sequencing
<i>mga</i> REV seq NZ131	GACCTTACCTCTATGGGCTG	Sequencing
<i>emm49</i> RT FWD	AAGAGCCTAAGCCGTGACCTTGAA	RT-PCR
<i>emm49</i> RT REV	GACGGCTTGCGTCTGAGATTTGTT	RT-PCR
<i>slo</i> RT FWD	AGCTCCGCCACTCTTTGTGAGTAA	RT-PCR
<i>slo</i> RT REV	CTGCACTAAAGGCCGCTTCAACAT	RT-PCR
<i>prtF2</i> RT FWD	TACATTTGTGCGAAACCGCAGCACC	RT-PCR
<i>prtF2</i> RT REV	AGCGTCACCTTTAGTTGCTTTGCC	RT-PCR
<i>speB</i> RT FWD	CACCAAATCAACCGTGGCGACTTT	RT-PCR
<i>speB</i> RT REV	AGAAGTTACGTCCGTCAGCACCAT	RT-PCR
<i>proS</i> RT FWD	TCTTGATCGCTGGGTGGTTCTTGA	RT-PCR
<i>proS</i> RT REV	CATCCAAGCAGCCAATTCTGCCTT	RT-PCR

---

### *Pfu* polymerase PCR reaction for regulatory gene sequencing

*Pfu* polymerase has a proof reading function and, therefore, is less error-prone than *taq* polymerase, and it was used for sequencing PCRs. The final concentrations in each PCR reaction were as follows: 1x *pfu* polymerase buffer (Aligent; Santa Clara, CA), 0.2 mM of each dNTP (Promega; Madison, WI), 0.4  $\mu$ M FWD primer, 0.4  $\mu$ M REV primer, and 5 U/100  $\mu$ l of *pfu* polymerase (Aligent; Santa Clara, CA). The PCR reactions were as follows: an initial step of 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 50°C to 60°C for 30 seconds, and then 72°C for 2 minute/kb of product; 72°C for 7 minutes; then a holding step of 4°C until the tubes were removed from the thermocycler.

### Agarose Gel Electrophoresis

PCR products were visualized on a 0.8 – 1.2% agarose gel (Fisher Scientific; Fair Lawn, NJ), depending on DNA fragment size. Agarose was prepared in 1X Tris-acetate buffer (TAE; Appendix A). Electrophoresis was run with 1X TAE buffer between 115 and 140 volts (Hoefer Scientific Instruments, San Francisco, CA). The agarose was then submerged in an ethidium bromide bath until bands were detectable by UV light. Pictures were taken using either a Polaroid MP4 land camera (Polaroid, Waltham, MA), or a Geldoc XR molecular imager using Quantity One software (Rio-Rad; Hercules, CA).

### Set-up and Analysis For Sequencing Reactions

DNA sequencing was carried out either by the Kimmel Cancer Center (Thomas Jefferson University School of Medicine, Philadelphia, PA), Seqwright (Fisher Scientific, Houston, TX) or Genewiz (South Plainfield, NJ). The PCR products were cleaned up prior to sequencing using a Qiaquick PCR Cleanup Kit (Qiagen; Germantown, MD)

according to manufacturer's instructions, and the clean PCR products were quantified at OD<sub>260</sub>. The FWD primers used for sequencing are listed in Table 1. Sequence analysis was performed using Chromas 2.33 software. The resulting sequences were aligned using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Sequencing was performed once for most strains except for those that showed a mutation compared to CS101. DNA was isolated, amplified, and sequenced two additional times for any strain that showed a mutation.

## **RNA Isolation, Detection, and Comparison**

### Isolation of Total RNA from *S. pyogenes*

*S. pyogenes* strains were inoculated from freezer stocks into 20 ml of TH broth (Appendix A). The cultures were incubated static at 37°C 5% CO<sub>2</sub>. In the morning (<15 hrs), the cultures were diluted 1:10 by adding 4 ml of culture to 36 ml of TH broth. The diluted cultures were placed back in the incubator and monitored for growth at OD<sub>600</sub>. When the cells reached the indicated OD<sub>600</sub>, the cultures were centrifuged at 6000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 1.5 ml of RNA lysis buffer (Appendix A). This was transferred to two bead beater tubes (Sarstedt; Nümbrecht, Germany) which contained 1.5 g 0.1 mm zirconia silica beads (Biospec Products, Inc.; Bartlesville, OK). The tubes were placed in a bead beater (Biospec Products, Inc.; Bartlesville, OK), and the tubes were beat two times at 4800 rpm for 1 minute, each time followed by 1 minute on ice. The supernatant was removed and 600 µl was added to each of two tubes and heated at 90°C for 1 minute. Total RNA was then isolated using a modified hot phenol extraction method of Shaw and Clewell (Shaw

and Clewell, 1985). An equal volume of hot phenol (pH 4.3, 65°C, Fisher Scientific; Pittsburgh, PA) was added to each tube. The tubes were incubated at 65°C 3 minutes then 5 minutes on ice. The tubes were then centrifuged at 15,000 rpm 15 min 4°C. The extraction was carried out two more times with 50µl less volume each time, and with 10 min centrifuge steps. 450 µl of the aqueous phase was then mixed with an equal volume of chloroform/isoamyl alcohol (24:1, Acros Organics; Morris Plains, NJ) and centrifuged at 15,000 rpm 10 min. RNA was precipitated from 400 µl of the aqueous solution by adding 40 µl of 3 M NaAc (Appendix A) and 800 µl of cold 100% ethanol, and this was placed in a -20°C freezer overnight to precipitate the RNA. The RNA was then harvested by centrifuging the tubes at 15,000 rpm for 20 min at 4°C. The supernatant was discarded, and the pellet was washed with 70% ethanol. The pellet was resuspended in 20 µl of DEPC dH<sub>2</sub>O (Appendix A). The RNA was treated with 1 µl SUPERase-In RNase inhibitor (Ambion; Austin, TX) and 1 µl DNase (Ambion; Austin, TX) in 1x DNase buffer (Ambion; Austin, TX) for 30 min at 37°C. The reaction was then stopped by incubating at 55°C for 5 min. RNA was then stored at -70°C.

RNA for RT-qPCR received an additional DNase reaction after the second phenol extraction. 2 µl of DNase (Ambion; Austin, TX) was added to 500 µl of the aqueous solution in 1X DNase buffer (Ambion; Austin, TX). The reaction was carried out at 37°C. Immediately afterward, hot phenol was added for the third extraction, and the remainder of the protocol was continued.

### Generation of Probes for Northern Blotting

DNA was prepared from CS101 as described above. PCR reactions were set up as described above, using *taq* polymerase. The labeled probes were generated by PCR using the primer pairs listed in Table 2. For each probe, two reaction tubes were setup, with one tube receiving 1  $\mu$ l of Digoxigenin (DIG)-dUTP (Roche Diagnostics; Mannheim, Germany), which incorporates into the PCR product. PCR products were run on an agarose gel and visualized with UV light. Incorporation of DIG-dUTP was confirmed by a slower running band than the non-DIG control. The PCR product was cleaned up with a Qiaquick PCR Cleanup Kit (Qiagen, Germantown, MD). The labeled probe was then quantitated at OD<sub>260</sub> using a Spectronic Genesys 5 spectrophotometer (Milton Roy; Warminster, PA). The probe was then diluted to 10ng/ $\mu$ l for use in Northern blotting.

### Northern Blotting

A 1.4% denaturing agarose gel was prepared by adding 3.5g agarose (Fisher Scientific; Fair Lawn, NJ) to 217.5 ml DEPC H<sub>2</sub>O (Appendix). The suspension was microwaved until the agarose melted, and it was cooled to 55°C. 25 ml of 5X MOPS (Appendix A) and 7.5 ml of 37% formaldehyde (Fisher Scientific; Fair Lawn, NJ) were added to the agarose, and the solution was poured into an RNase Zap (Ambion; Austin, TX) treated gel tray until it solidified. Running buffer (Appendix A) was poured on top of the gel until it covered the surface.

The isolated RNA was quantitated at OD<sub>260</sub>. Denaturing agarose gel electrophoresis and Northern blotting were done on dilutions of the RNA representing 5, 2.5, and 1.25  $\mu$ g of total cellular RNA. The RNA was diluted into DEPC water

(Appendix A). RNA marker was also diluted to its appropriate concentration by adding 3.5  $\mu$ l RNA marker (Promega, Madison, WI) to 1.5  $\mu$ l DEPC H<sub>2</sub>O. The appropriate amount of sample and RNA marker were added to 17.5  $\mu$ l of premix (Appendix A) to denature the RNA at 65°C for 20 minutes. 1.5  $\mu$ l of RNA loading dye (Appendix A) was added to each sample prior to loading. Each RNA sample was then loaded into the wells of the gel and run at 25 V until the samples were clearly within the agarose, at which time the voltage was increased to 70 V.

Even loading of total RNA was first confirmed by visualization of ethidium bromide stained gels, which was present in the loading dye. The RNA was then transferred overnight by capillary action to a MagnaGraph nylon membrane (Osmonics Inc., Minnetonka, MN) using 20X SSC (Appendix A). In the morning, the RNA was crosslinked to the membrane using a Stralinker UV Crosslinker (Stratagene, La Jolla, CA) set for 120,000 mJ.

The membranes were pre-hybridized with 20 ml hybridization solution (Appendix A) at 65°C for 2 hours while rotating end-over-end. After 2 hours, 10  $\mu$ l of the 10 ng/ $\mu$ l probe (described above) was added to 50  $\mu$ l dH<sub>2</sub>O. The probe was boiled 5 minutes then placed on ice for 5 minutes. The probe was then added to the hybridization solution, and the membrane was hybridized overnight at 65°C.

The membrane was removed from the hybridization oven and washed as follows: 5 minutes at room temperature in prewarmed (55°C) wash solution 1 (Appendix A), 10 minutes at room temperature in prewarmed (55°C) wash solution 2 (Appendix A), 20 minutes at 55°C in prewarmed (55°C) wash solution 3 (Appendix A), 2 minutes at room temperature in Boehringer wash buffer (Appendix A). The membrane was then

incubated on a rocker for 30 minutes with 50 ml blocking buffer (Appendix A). 5  $\mu$ l of alkaline phosphatase coupled anti-digoxigenin antibody was added to the solution (Roche, Indianapolis, IN). The membrane was then washed twice for 15 minutes each in Boehringer wash buffer (Appendix A), followed by 3 minutes in Buffer 3 (Appendix A). The membrane was then incubated for 5 minutes with a 1:100 dilution of disodium-3-(4-methoxyspiro[1,2-dioxetane-3'2'-(5-chloro)tricyclo(3.3.1.3<sup>3,7</sup>)decan]-4-yl)phenylphosphate (CSPD) (Roche, Indianapolis, IN), and exposed to X-ray film (Thermo; Rockford, IL). Detection of *gyrA* served as a loading control. The Northern blots represent the result of independent experiments using freshly isolated RNA for each blot.

### **Real Time PCR (RT-qPCR)**

#### Generation of cDNA from RNA

RNA was isolated as described above, using the procedure for RT-qPCR. RNA concentrations were determined by a Nanodrop ND-1000 spectrophotometer at OD<sub>260</sub> (Thermo; Rockford, IL). cDNA was prepared using a Thermo Verso cDNA synthesis kit according to manufacturer's instructions (Thermo; Rockford, IL). 100 ng of RNA was mixed with 1  $\mu$ l (400 ng) random hexamers, 4  $\mu$ l 5X buffer (final 1X), 2  $\mu$ l dNTP mix (final 500  $\mu$ M each), 1  $\mu$ l RT enhancer, and 1  $\mu$ l Verso enzyme mix (Thermo; Rockford, IL) to generate the cDNA. The reaction consists of 1 cycle of 42°C for 30 min then 95°C for 2 minutes. The concentration of cDNA was determined by an ND-1000 Nanodrop spectrophotometer at OD<sub>260</sub> (Thermo; Rockford, IL).

## Real Time PCR

Real Time PCR was run using the 5Prime RealMasterMix SybrROX mix (5Prime; Gaithersburg, MD). Primers were generated that were internal to the gene of interest and are listed in Table 2. All primer sequences, except for that of *emm49*, were based on the SF370 sequence available on the KEGG website (<http://www.genome.jp/kegg/>). The *emm49* primer set was based upon a published CS101 *emm* sequence (Haanes and Cleary, 1989). Real time conditions were as follows: 50 ng cDNA was mixed with the sybrROX mix according to manufacturer's conditions. 11.25 of the SybrRox mix was added to each 25 µl reaction. The FWD and REV primers were added at a final concentration of 250 nM. Real-time PCR was run on an Eppendorf Mastercycler ep. The cycles were run as follows: 1 cycle 95°C 2 min; 40 cycles of 95°C 30 sec, 55°C 30 sec, 68°C 30 sec; and then melting steps of 95°C 15 sec then 60°C to 95°C over 20 minutes. Data analysis was performed using Eppendorf realplex 1.5 software. Each sample was run in triplicate, along with a non-template control for each primer set. Results are reported as the relative expression of the gene of interest to the housekeeping gene *proS*:  $2^{\Delta C_t}$  ( $C_t$  gene of interest -  $C_t$  of *proS* housekeeping gene). Statistical differences in relative expression levels between survivor strains and CS101 were determined using the SPSS statistics program (SPSS Inc., Chicago, IL). Equal variances were assumed for the samples, and they were analyzed by one-way ANOVA using Tukey's post-hoc test.

### **Metabolic Profiles for *S. pyogenes***

Culture supernatants used for metabolite analysis were collected from stationary phase or exponential phase cultures. An overnight (<15 hour) culture of *S. pyogenes* was diluted 1:100 in fresh medium. Growth of newly inoculated cultures was monitored by spectrometry at OD<sub>600</sub> and samples were removed from each tube at the indicated time points. Culture samples were filter sterilized through a 0.22 µm syringe filter (Millipore; Billerica, MA) and subsequently heat inactivated at 90°C for five minutes before being stored at -20°C. The samples were diluted 1:5 in dH<sub>2</sub>O, and L-lactate, formate, and acetate concentrations were determined from culture supernatants using R-Biopharm test kits (Marshall, MI), as described by the manufacturer. Each data point represents the metabolite concentrations from at least two independent cultures.

### **Zebrafish Virulence Assays**

Zebrafish (*Danio rerio*) were maintained according to protocols previously published (Phelps *et al.*, 2009). Zebrafish studies were carried out in the laboratory of Melody Neely, Ph.D. at Wayne State University School of Medicine, Detroit, MI. Fish were maintained in 5 to 25 gallon tanks filled with tap water and treated with Amquel to remove chlorine. The temperature was maintained at 28°C.

#### Mortality

The zebrafish were placed in a solution of Tris-buffered tricaine (3-amino-benzoic acid ethylester, Sigma; St. Louis, MO), pH 7.0, 168 µg/ml to anesthetize them prior to intramuscular injection. 10<sup>5</sup> to 10<sup>6</sup> log phase bacteria were injected into the dorsal muscle of 6 fish with a 10 µl volume, and the fish were maintained at 28°C. The

remaining culture was serially diluted and plated on Columbia colistin-nalidixic acid agar (CNA) plates for CFU enumeration. Survival of the fish was scored each day up to five days. Experiments were done twice with 6 fish for each trial with the exception of CS101.

### Spleen Dissemination

To determine bacterial dissemination, spleens were dissected and enumerated for CFU. First, the fish were placed in 336 µg/ml Tris-buffered tricaine (3-amino-benzoic acid ethylester, Sigma; St. Louis, MO), pH 7.0 for 25 minutes to euthanize. The zebrafish were dissected, and the spleens were removed and placed in a 1.5 ml microcentrifuge tube containing 300 µl phosphate-buffered saline (PBS pH 7.2) (Appendix A). The suspension was homogenized with a microcentrifuge tube tissue grinder (Kontes, Fisher Scientific; Fair Lawn, NJ), and serial dilutions were prepared in THY agar then plated for enumeration on Columbia colistin-nalidixic acid agar (CNA) plates. The protocols for the zebrafish infections were approved by the Wayne State University Institutional Animal Care and Use Committee and followed all federal regulations regarding the care and use of laboratory animals.

### **Whole Blood killing Assay**

The direct test protocol by Lancefield (Lancefield, 1957) was used with modifications. *S. pyogenes* strains were incubated in TH broth for less than fifteen hours at which point the cultures were diluted 1:10 into TH broth. OD<sub>600</sub> was monitored by using a Spectronic Genesys 5 spectrophotometer (Milton Roy; Warminster, PA) and mid-exponential bacteria (OD<sub>600</sub> 0.4 – 0.5) were centrifuged at 8000 rpm for 5 minutes. The

cells were resuspended in phosphate buffered saline (PBS; Appendix A) to a concentration of  $\sim 6 \times 10^3$  CFU/ml. 50  $\mu$ l of the resuspended bacteria was mixed with 850  $\mu$ l heparinized normal human blood to a final concentration of  $3 \times 10^2$  CFU/ml and incubated for 3 hours at 37°C 5% CO<sub>2</sub> with end-to-end rotation. For each bacterial strain, blood from 4 healthy volunteers was used. At T<sub>0</sub> and at 3 hours, 150  $\mu$ l samples were removed. 100  $\mu$ l of this was plated onto TH agar (lower limit of detection 10 CFU/ml). The remainder 50  $\mu$ l was used to make serial 1:10 dilutions in PBS up to 10<sup>-2</sup>. Each dilution was plated onto TH agar and enumerated for CFU after an overnight incubation in a 37°C 5% CO<sub>2</sub> incubator. Change in colony forming units (CFU) was calculated by subtracting CFU at T<sub>0</sub> from CFU at T<sub>3</sub>.

## CHAPTER 3

### RESULTS

#### **Survivor Strains Upregulate Transcription of the PA Pathway Genes**

Previous studies isolated survivor strains from 14 week cultures of *S. pyogenes* CS101 in TH broth (Wood *et al.*, 2009; Wood *et al.*, 2005). In these studies, two survivor strains, Alt. 1 and Alt. 2, grew as small colonies and had unique and common proteomic changes when compared to CS101 and each other. Additionally, nine survivor strains, some isolated from the same culture, were characterized for their exponential phase metabolic activities. Seven of the nine survivor strains had high levels of formate, acetate and ethanol, and ammonia levels were increased for six of the nine strains. The metabolite levels were unique for each strain (Wood *et al.*, 2009). The accumulation of these metabolites suggested an increased expression of the Pyruvate to Acetate (PA) pathway, the Pyruvate to Ethanol (PE) pathway, and/or amino acid catabolism. The Pyruvate to Acetate (PA) pathway converts the lactate produced by fermentation of glucose into acetate and formate (Figure 2A). This pathway produces ATP, and is therefore used for generating energy during stationary phase survival when glucose is depleted. First, lactate is converted to pyruvate through lactate oxidase (LctO). Pyruvate is then converted to acetyl-coenzyme A (AcCo-A) through one of two reactions. In one, pyruvate formate lyase (PFL) produces formate as a byproduct. In the other, the pyruvate dehydrogenase complex (PDH) produces CO<sub>2</sub> as a byproduct. It is worth noting that PFL is an oxygen-sensitive enzyme (Yamada *et al.*, 1985). The AcCo-A produced by either

enzyme is then converted to acetyl phosphate then acetate through phosphotransacetylase (Pta) and acetate kinase (AckA), respectively. This final step produces ATP.

Alternatively, *S. pyogenes* possesses enzymes that can convert AcCoA to ethanol through the PE pathway (Fig 2A), which does not produce ATP, but it does produce reducing potential through the production of NAD<sup>+</sup>.

Additionally, *S. pyogenes* can catabolize amino acids to generate energy during stationary phase survival. Previous studies have shown that *S. pyogenes* can utilize serine, arginine, and histidine during exponential phase when the growth-phase dependent regulator Rgg/RopB is mutated (Chaussee *et al.*, 2004; Chaussee *et al.*, 2003). Studies by Wood *et al.* showed that some survivor strains produce high levels of ammonia during exponential phase, and this ammonia production was necessary to maintain the pH of the culture above the toxic level of 5.6 (Wood *et al.*, 2009). Two amino acid pathways have been proposed to generate energy during stationary phase survival. The arginine deiminase pathway (Fig. 2B) converts arginine to ornithine, CO<sub>2</sub> and ammonia through three enzymes encoded by the *arc* operon. This pathway independently generates ATP. The serine dehydratase pathway (Fig 2C) converts serine to pyruvate and ammonia. The pyruvate can then enter the PA pathway to generate ATP. Both of these pathways generate ammonia, which balances the pH within the long-term stationary phase culture.

To investigate whether the increased metabolite levels corresponded to transcriptional upregulation of the genes of the PA pathway, Northern blotting was performed on CS101, Alt. 1, and Alt. 2. Total RNA was isolated from CS101 and the survivor strains Alt. 1 and Alt. 2 at mid-exponential phase, OD<sub>600</sub> 0.5 to 0.65. The RNA

was used for Northern blotting using probes generated for genes involved in the PA pathway (Fig. 2A). Transcription of *lctO*, *pfl*, *pta*, and *ackA* were up-regulated during exponential phase growth in Alt. 1 and Alt. 2 compared to CS101 (Fig. 3). This upregulation reflects the increased production of formate and acetate in Alt. 1 and Alt. 2 seen in previous studies (Wood *et al.*, 2009), suggesting that this increase corresponds to enzymatic levels. Transcription of *acoA*, a gene encoding one of the subunits in the pyruvate dehydrogenase (PDH) complex, was equal between the three strains. The RNA gels were confirmed for even loading by both visualization with ethidium bromide staining and detection of the transcript for the housekeeping gene gyrase A (*gyrA*).

### **Changes in PA Pathway Expression were Observed Only After Survival and not in Strain Variants Isolated from Passage in Mice or Human Blood**

During survival the bacterial population is in an environment lacking primary energy sources. The selection of strains with altered metabolism in the surviving population may reflect the necessity to use secondary energy sources such as amino acids and lactic acid for survival. During multiple passages in the mouse peritoneum or human blood, strains arise with distinct changes in colony morphology and virulence factor expression patterns (Raeder *et al.*, 2000; Boyle *et al.*, 1994; Raeder and Boyle, 1993; Raeder and Boyle, 1993). Under these conditions, the bacteria may grow rapidly and are probably not under nutrient stress. If PA pathway metabolic diversification is a characteristic selected for during survival, these strains may not have changes in metabolism as seen in the survivor strains.

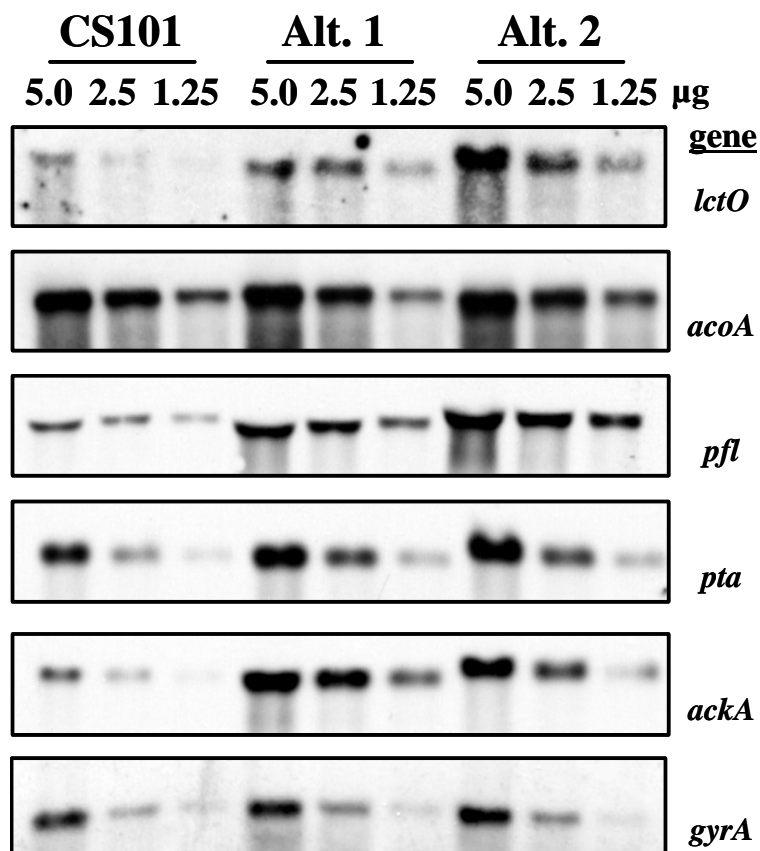


Figure 3. Survivor strains Alt. 1 and Alt. 2 upregulate transcription of the PA pathway genes during exponential phase. Total RNA was isolated from mid-exponential cells ( $OD_{600nm}$  0.50 - 0.65) grown in TH broth. RNA concentrations were determined by spectrophotometric absorbance at 260nm. 5.0, 2.5, 1.25 µg of total RNA was separated on a denaturing agarose gel. RNA gels were Northern blotted and the binding of DIG-labeled DNA probes was detected by CSPD development followed by autoradiography. Equal loading was confirmed by ethidium bromide staining of the gel and by probing for DNA gyrase (*gyrA*). RNA concentrations in µg are noted above the lanes in each image. Fresh RNA was isolated for each experiment, and the results presented here are representative of three independent preparations.

In order to determine whether variants generated from multiple passage expressed the PA pathway as a marker of metabolic diversification, we analyzed PA pathway transcription in four additional *S. pyogenes* strains kindly provided by Michael Boyle. Previous studies in Boyle's laboratory showed that the M64 strain, 64P, changes its morphology and virulence factor expression patterns upon passaging in human blood or by passaging in BALB/c mice (Smith *et al.*, 2005; Raeder *et al.*, 2000; Raeder and Boyle, 1993; Reis *et al.*, 1984). Passaging is very different from survival assays, and it models acute infection rather than carriage. During human blood passaging, 0.1 ml of the bacterial culture was mixed with 0.4 ml heparinized human blood and incubated at 37°C overnight. Every 24 hours, 0.1 ml of the subsequent blood/bacteria mixture was added to 0.4 ml fresh blood. Passaging in mice was performed by injecting 0.5 ml overnight *S. pyogenes* 64P culture IP into a mouse. Spleens were removed after death or euthanization after illness, mashed, and 0.1 ml of the suspension was injected IP into another uninfected mouse. We obtained the parental strain, 64P, and three of its passage-derived strains. Each strain was streaked onto TH agar next to CS101 for a visual comparison (Fig. 4). Strains 64BP9L and 64BP9S were obtained from 9 passages in human blood. 64BP9L has a large colony phenotype, and 64BP9S has a small colony phenotype. Strain 64/14 was isolated from 14 passages in human blood, and its colonies appear large although not as mucoid in appearance as 64BP9L. These passage-derived strains demonstrate an alternate colony morphology without encountering survival conditions.

*S. pyogenes* 64P and its passage-derived strains were regrown in TH broth and RNA was isolated in mid-exponential phase. The RNA was used in Northern blotting

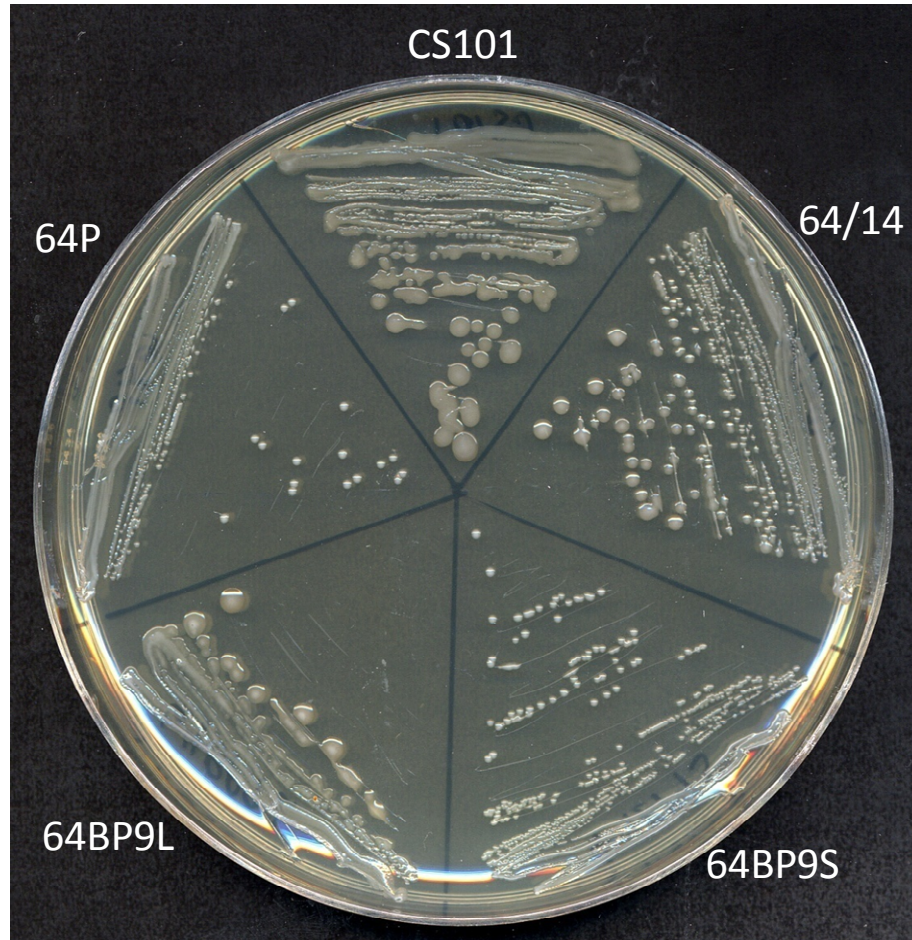


Figure 4. Colony morphologies of *S. pyogenes* 64P and its passage-derived strains. 64P is an M64 strain. 64/14 was isolated from 14 passages in human blood. 64BP9S and 64BP9L were isolated from 9 passages in human blood and display a small and large colony phenotype, respectively. CS101, a parental M49 strain, is shown for comparison. All strains were plated onto TH agar and incubated overnight at 37°C 5% CO<sub>2</sub>.

with probes for three genes in the PA pathway: *pfl*, *pta*, and *ackA*. CS101 was analyzed as a reference control, and Alt. 1 was used as a positive control for PA pathway upregulation. The housekeeping gene encoding for gyrase A (*gyrA*) was detected as a control for even loading. Alt. 1, as already seen in Fig. 3, had increased expression levels in all three genes analyzed. 64P, 64BP9L, 64BP9S, and 64/14 all had similar levels of expression, which were also similar to that of CS101 (Fig. 5). This indicates that passaged strains, despite alternate colony morphology and virulence factor expression patterns, do not upregulate expression of the PA pathway.

To determine if survival of these strains would cause increased expression of the PA pathway, CS101 and each M64 strain was inoculated into TH broth and aged at 37°C 5% CO<sub>2</sub>. Random survivor strains were isolated at 6 and 7 weeks survival and stored as 40% glycerol freezer stocks. 37 isolated survivor strains were regrown, the supernatant was filtered and heated at 90°C for 2 minutes, and formate levels were determined. Formate was detected using a metabolic test kit (R-biopharm), which is a rapid, two step assay. Because of the ease of this assay and its correlation with increased PA pathway activity in previous studies (Wood *et al.*, 2009; Wood *et al.*, 2005), the 37 strains were screened for expression of the PA pathway by detection of formate production. Alt. 1 levels were also analyzed as a positive control for PA pathway upregulation. As expected, some of the survivor strains up-regulated production of formate whereas others did not (Fig. 6). Surprisingly, the percentage of formate producing strains varied, depending upon the survivors' parental strain. 100% of 8 randomly selected survivor strains isolated from a CS101 culture upregulated formate production. CS101-derived survivor strains from previous studies had varied expression levels (Wood *et al.*, 2009).

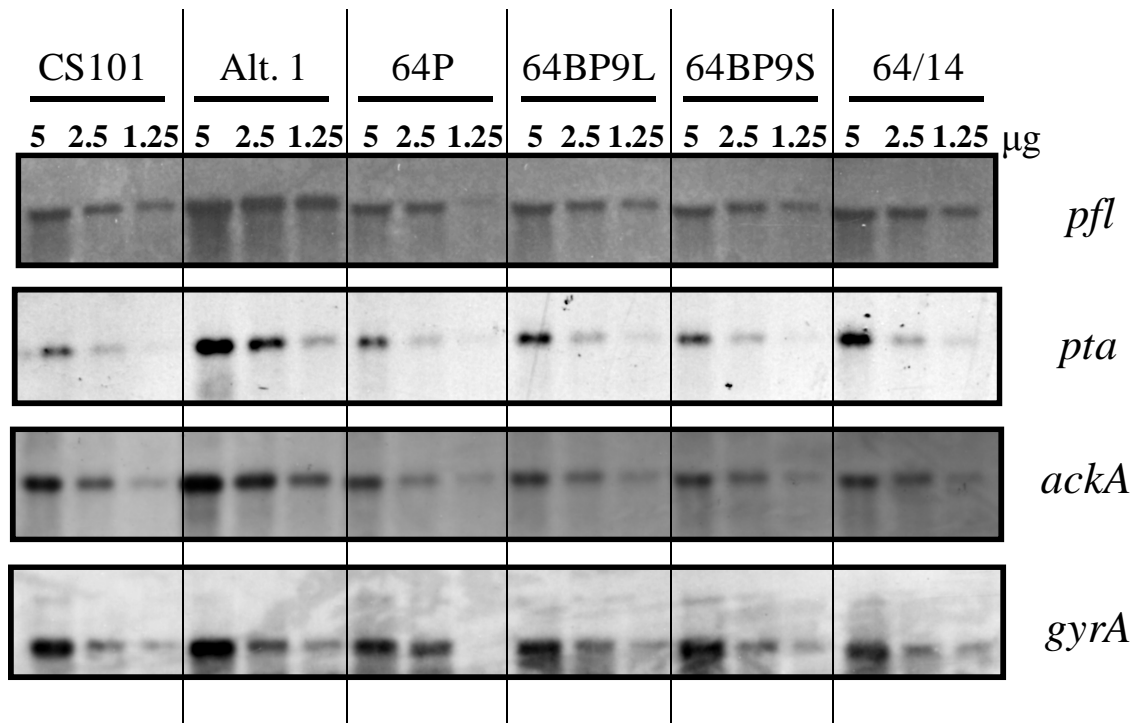


Figure 5. Transcription of genes for the PA pathway was not upregulated in *S. pyogenes* 64P and its passage-derived strains. Total RNA was isolated from mid-exponential bacteria (OD<sub>600nm</sub> 0.50 - 0.65) grown in TH broth. RNA concentrations were determined by spectrophotometric absorbance at 260nm. 5.0, 2.5, 1.25 μg of total RNA was separated on a denaturing agarose gel. RNA gels were Northern blotted and the binding of DIG-labeled DNA probes was detected by CSPD development followed by autoradiography. Equal loading was confirmed by ethidium bromide staining of the gel and by probing for DNA gyrase (*gyrA*). RNA concentrations in μg are noted above the lanes in each image. Fresh RNA was isolated for each experiment, and the results presented here are representative of three independent preparations. CS101 is shown as a comparison for typical parental expression levels. Alt. 1 is shown as a positive control for PA pathway upregulation.

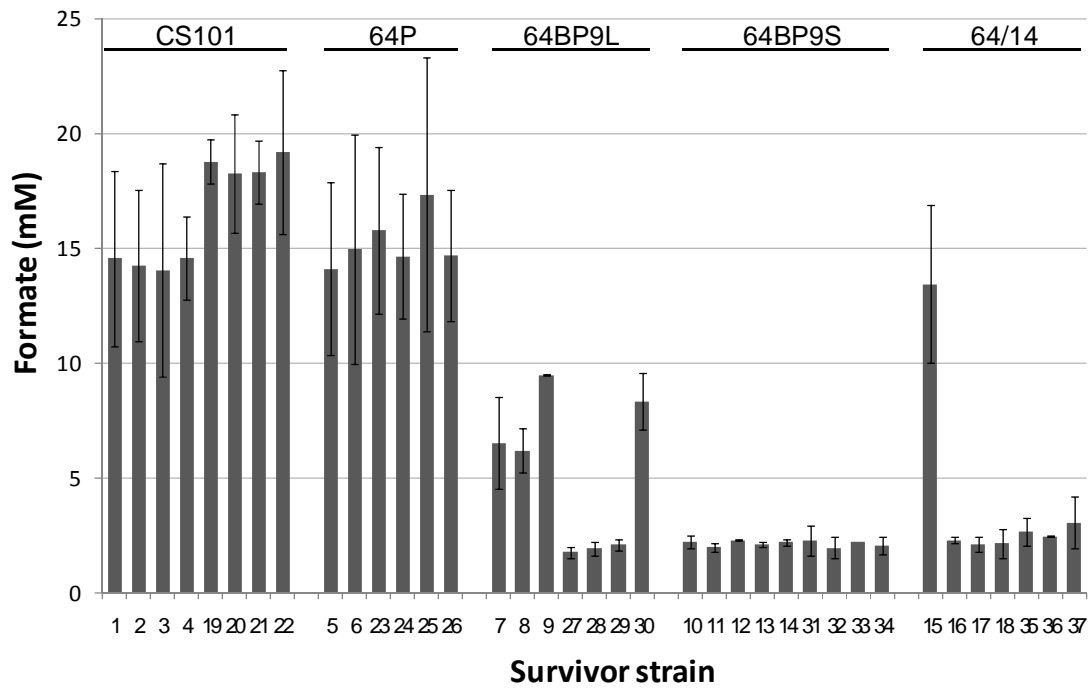


Figure 6. Survivor strains obtained from *S. pyogenes* strains CS101, 64P, and the 64P passaged strains demonstrate variable levels of formate production. The parental strain is shown above the bars. *S. pyogenes* strains were grown statically in TH broth under a 5% CO<sub>2</sub> atmosphere and 24 hour culture samples were removed, filter sterilized, and heated to 90°C for 2 minutes. Formate levels were determined using a formic acid test kit (R-Biopharm). Each concentration is an average of two independent cultures.

100% upregulation of the PA pathway in 6 randomly selected survivor strains was also seen for strains isolated from a 64P culture. 57% of 7 survivor strains from 64BP9L produced increased levels of formate, but this increase was more modest than that of survivor strains from other cultures. No strains from 64BP9S produced high formate levels, and 14% of the 7 strains from 64/14 had formate upregulation. These differences may reflect the random nature of the mutations and the random selection process for survivor strains. Further testing would be required to determine if there is a real difference in the ability of strains to change their regulation of the PA pathway.

To further verify the formate production data, and to investigate whether the increase in formate production was due to an increase at the transcription level, Northern blotting was performed on selected M64 survivor strains. As predicted by increased formate levels (Fig. 6), strain 15 (isolated from a 6-week culture of 64/14) upregulated expression of *pfl*, *pta*, and *ackA* (Fig. 7). Strain 26 (isolated from a 7-week culture of 64P) also upregulated expression of these three genes. In addition, Northern blotting was performed for two strains that did not have increased formate levels: 29 (a 7-week isolate from 64BP9L) and 34 (a 7-week isolate from 64BP9S). Both of these strains showed similar transcription levels of *pfl* to 64BP9S (Fig. 7). These data suggest that increased PA pathway expression during exponential phase occurs after survival, but is not selected by multiple passages in mice or human blood, which mimic acute infection.

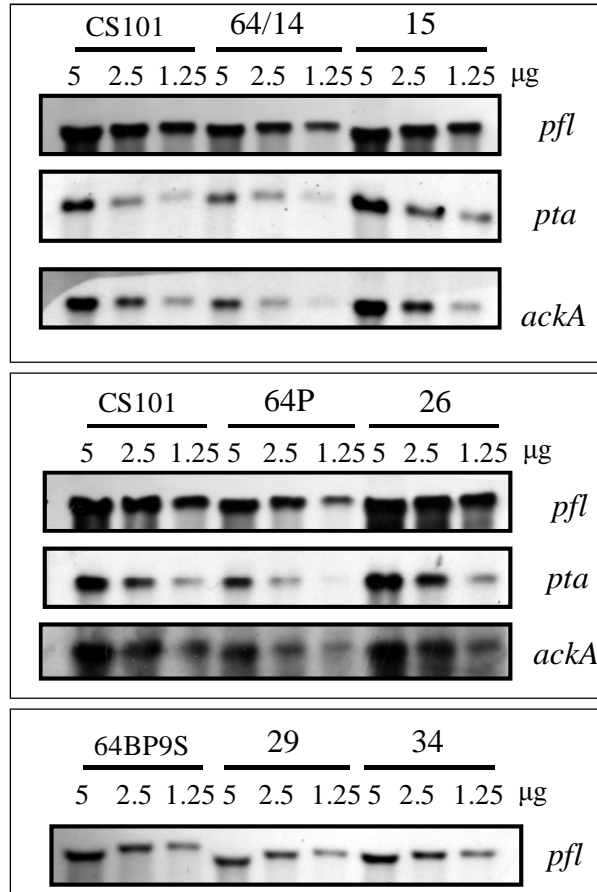


Figure 7. Survivor strains that produce high levels of formate increase transcription of genes for the PA pathway. Total RNA was isolated from mid-exponential cells ( $OD_{600nm}$  0.50 - 0.65) grown in TH broth. RNA concentrations were determined by spectrophotometric absorbance at 260nm. 5.0, 2.5, 1.25 µg of total RNA was separated on a denaturing agarose gel. RNA gels were Northern blotted and the binding of DIG-labeled DNA probes was detected by CSPD development followed by autoradiography. Equal loading was confirmed by ethidium bromide staining of the gel. RNA concentrations in µg are noted above the lanes in each image. The results presented here represent one RNA isolation to verify the results found in Fig. 6. CS101 is shown as a comparison for typical parental expression levels.

### **One of Three Clinical Isolates Upregulated PA Pathway Expression**

In the closed environment of long term stationary phase cultures, metabolic diversity is necessary to maintain the pH above the toxic threshold of 5.6 (Wood *et al.*, 2009; Wood *et al.*, 2005). To see whether metabolic diversification could be detected *in vivo*, PA pathway expression was analyzed in three clinical isolates. The strains were isolated previously from tonsillar material from 3 males 20, 21 and 22 years of age that reported suffering from recurrent tonsillitis (Podbielski *et al.*, 2003). *S. pyogenes* were cultured from the tonsil swab and surgical specimen from one patient (isolate 221) and exclusively from the surgically removed tonsil material of two patients (isolates Sfr321 and MK322) (Podbielski *et al.*, 2003). In all tonsillar samples, intracellular *S. pyogenes* could be visualized by immunohistochemistry and light microscopy with antibodies directed against the streptococcal cell wall (Podbielski *et al.*, 2003). Like CS101, strains 221 and Sfr321 were M serotype 49 strains. MK322 was M serotype 6. One complication of these studies is that only the survivor strains and not the parental strain of the clinical isolates were available, therefore, matched serotype controls were used for the analysis of clinical isolates; CS101 for M49 and JRS4 for M6. Northern blotting was performed on the three clinical isolates and their serotype controls. Probes for transcripts in the PA pathway were used: *lctO*, *pfl*, *pta*, and *ackA*. The gene encoding Gyrase A (*gyrA*) was used as a loading control. Only strain 221 increased transcription of the PA pathway, while Sfr321 and MK322 had similar transcript levels as that of CS101 and JRS4 (Fig. 8).

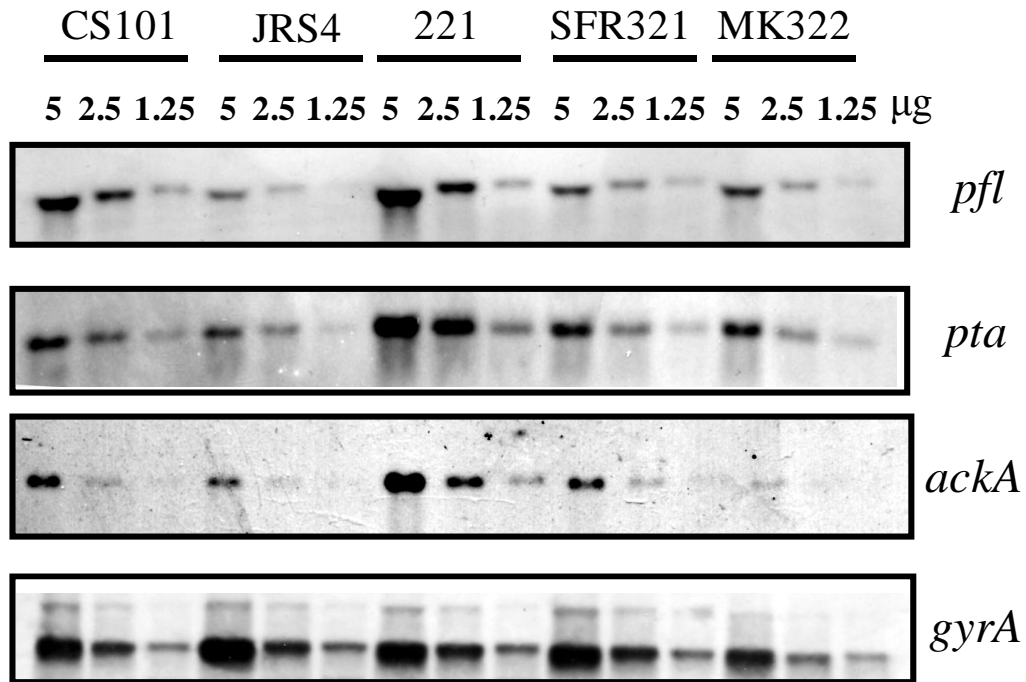


Figure 8. Transcription of genes for the PA pathway was upregulated in one of three clinical isolates. Total RNA was isolated from mid-exponential bacteria ( $OD_{600nm}$  0.50 - 0.65) grown in TH broth. RNA concentrations were determined by spectrophotometric absorbance at 260nm. 5.0, 2.5, 1.25 μg of total RNA was separated on a denaturing agarose gel. RNA gels were Northern blotted and the binding of DIG-labeled DNA probes was detected by CSPD development followed by autoradiography. Equal loading was confirmed by ethidium bromide staining of the gel and by probing for DNA gyrase (*gyrA*). RNA concentrations in μg are noted above the lanes in each image. Fresh RNA was isolated for each experiment, and the results presented here are representative of three independent preparations.

To further verify that strain 221 was the only strain to upregulate PA pathway transcription, metabolite expression was measured. Lactate, formate, and acetate levels were measured during exponential growth using metabolic test kits (R-Biopharm). During PA pathway utilization, lactate is consumed, and formate and acetate are produced. As expected, strain 221 significantly increased formate and acetate production, with a corresponding decrease in lactate (Fig. 9). Both MK322 and Sfr321 had no increase in acetate or formate production, and the levels of lactate were similar to that of the serotype controls. These data suggest that it is possible to isolate intracellular survivors, such as strain 221, that have increased PA pathway expression during exponential growth. In the studies by Podbielski *et al.* only a single strain from each tonsillar sample was saved (Podbielski *et al.*, 2003). Previous in vitro data showed diversity amongst strains in a closed environment, so it would be interesting to obtain multiple strains from one tonsil specimen to analyze for diversity within one organ.

To further confirm that parental, non-surviving strains do not have increased levels of PA pathway expression, multiple parental strains were tested for production of formate. Strains from multiple M-types (M1, M2, M49, and M64) were compared. All of the strains expressed low levels of formate, except for NZ131 (M49) which produced a moderate amount of formate, 7.5 mM (Fig. 10). NZ131 is an isolate from a case of post-streptococcal glomerulonephritis, so it is possible that the bacteria survived within in the host beyond the primary acute infection before isolation of this strain (Simon and Ferretti, 1991).

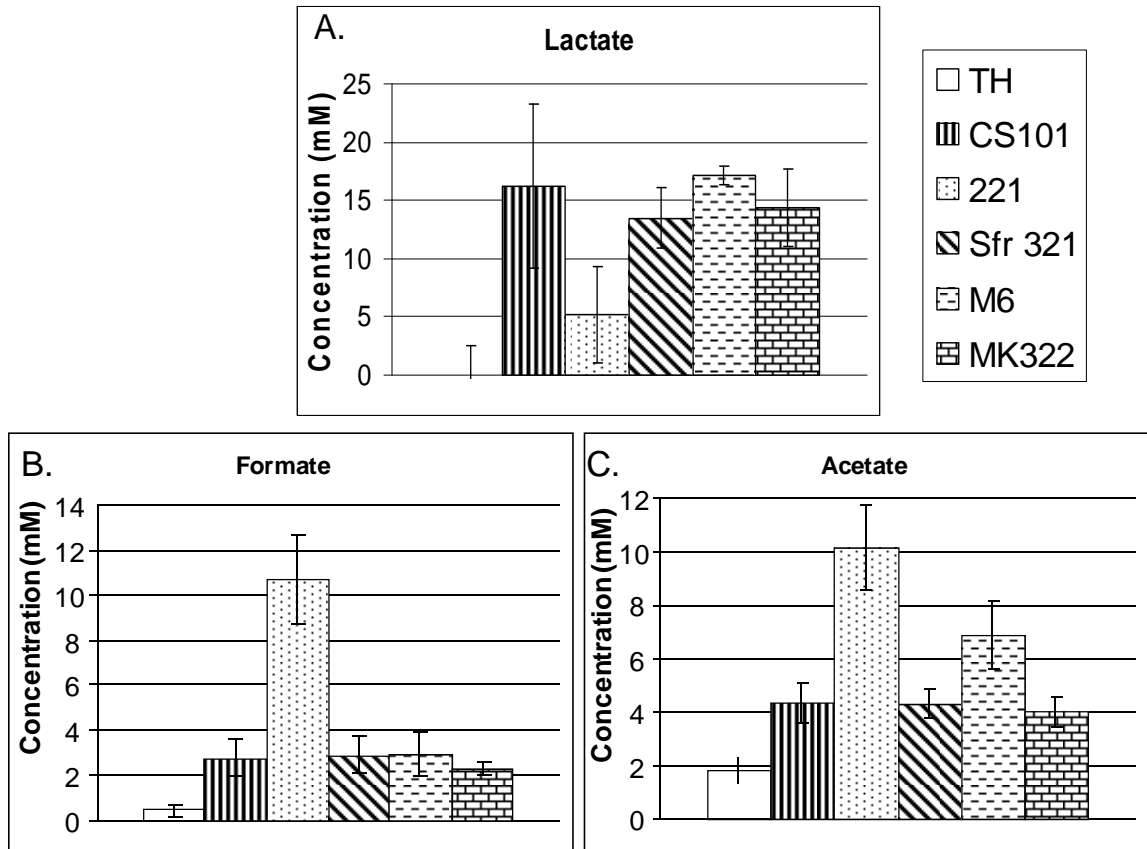


Figure 9. One of three clinical isolates produced increased levels of the metabolites involved in the PA pathway. *S. pyogenes* strains were grown statically in TH broth under a 5% CO<sub>2</sub> atmosphere and exponential cell density was monitored by culture absorbance at 600nm. Late exponential phase (OD<sub>600</sub> 0.80 - 0.90) culture samples were removed, filter sterilized, and (A) lactate, (B) acetate, and (C) formate were determined using metabolic test kits (R-Biopharm). Metabolite concentrations present in sterile TH broth are represented in each graph by the bar at the left. Each bar represents the mean value from at least three independent cultures. The standard deviation of the mean is represented by the error bars.

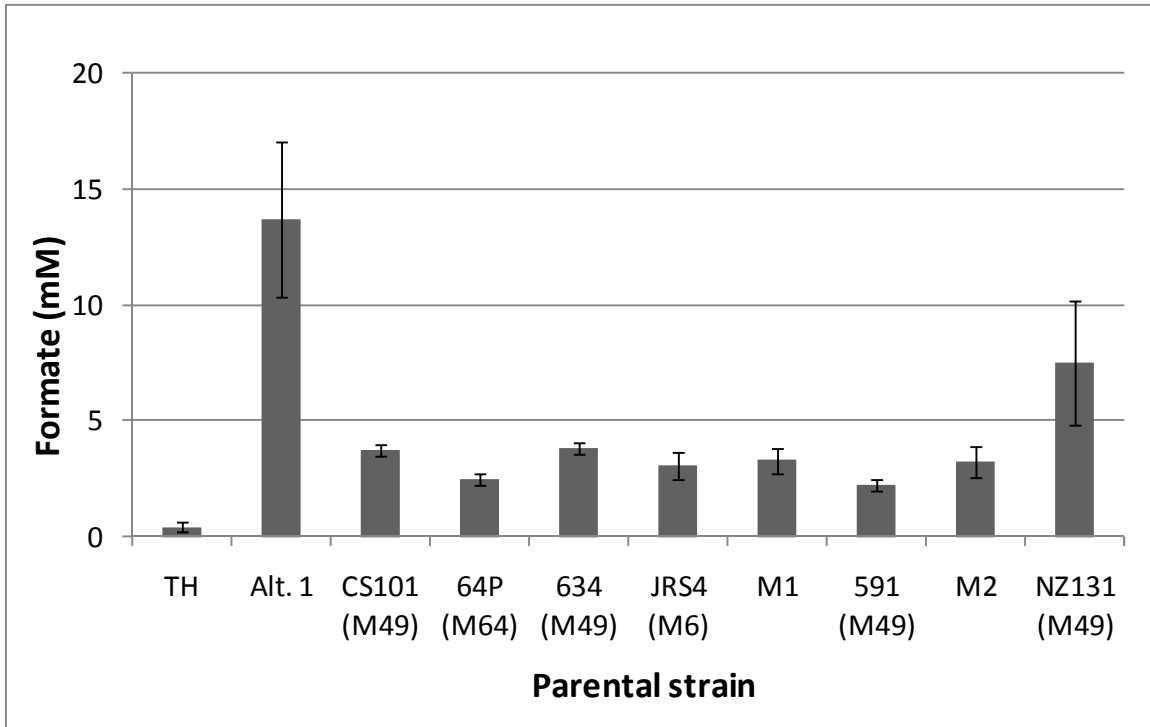


Figure 10. Parental *S. pyogenes* strains produce low levels of formate. *S. pyogenes* strains were grown statically in TH broth under a 5% CO<sub>2</sub> atmosphere, and 24 hour culture samples were removed, filter sterilized, and heated to 90°C for 2 minutes. Formate levels were determined using a formic acid test kit (R-Biopharm). Each concentration is an average of two independent cultures.

### ***S. pyogenes* Survivor Strains Diversify Virulence Factor Expression**

Survivor strains isolated from long term cultures diversify metabolic factors, and this was suggested by the clinical isolates. Therefore, we were interested in investigating whether virulence factor expression was altered upon survival. Survivor strains from both in vitro survival systems were used. Four strains (Alt. 1, Alt. 2, Alt. 4A, and Alt. 4B) were isolates from 14-week CS101 cultures. Five strains (E11, E47.5, E47.6, E47.7, and E47.8) were intracellular isolates from 5 day co-cultures of CS101 with A549 human lung epithelial cells. Alts. 4A and 4B as well as E47.5 – E47.8 were isolated from the same culture or well, respectively. The other survivor strains were each isolated independently.

*S. pyogenes* possess >13 well-characterized virulence factors (Reviewed by Musser and Shelburne, 2009). Four key virulence factors were chosen for characterization; the anti-phagocytic M protein, the cysteine protease streptococcal pyrogenic exotoxin B (SpeB), the pore-forming cytolysin streptolysin O (SLO), and fibronectin binding protein (PrtF2), which plays a role in binding and invasion into eukaryotic cells. The transcription of the genes for these selected key virulence factors during exponential phase was compared between the parental and survivor strains by real-time quantitative PCR (RT-qPCR).

Transcription of *emm*, which encodes M protein, varied between the strains (Fig 11A). Alt. 1, E11, E47.6, and E47.8 showed decreased expression levels compared to the parental strain CS101, while Alt. 2, Alt. 4A, Alt. 4B, E47.5, and E47.7 had similar expression levels as CS101. Expression levels of *prtF2* were similar between each survivor strain and CS101 (Fig. 11B). Expression levels of *slo* in the survivor stains were

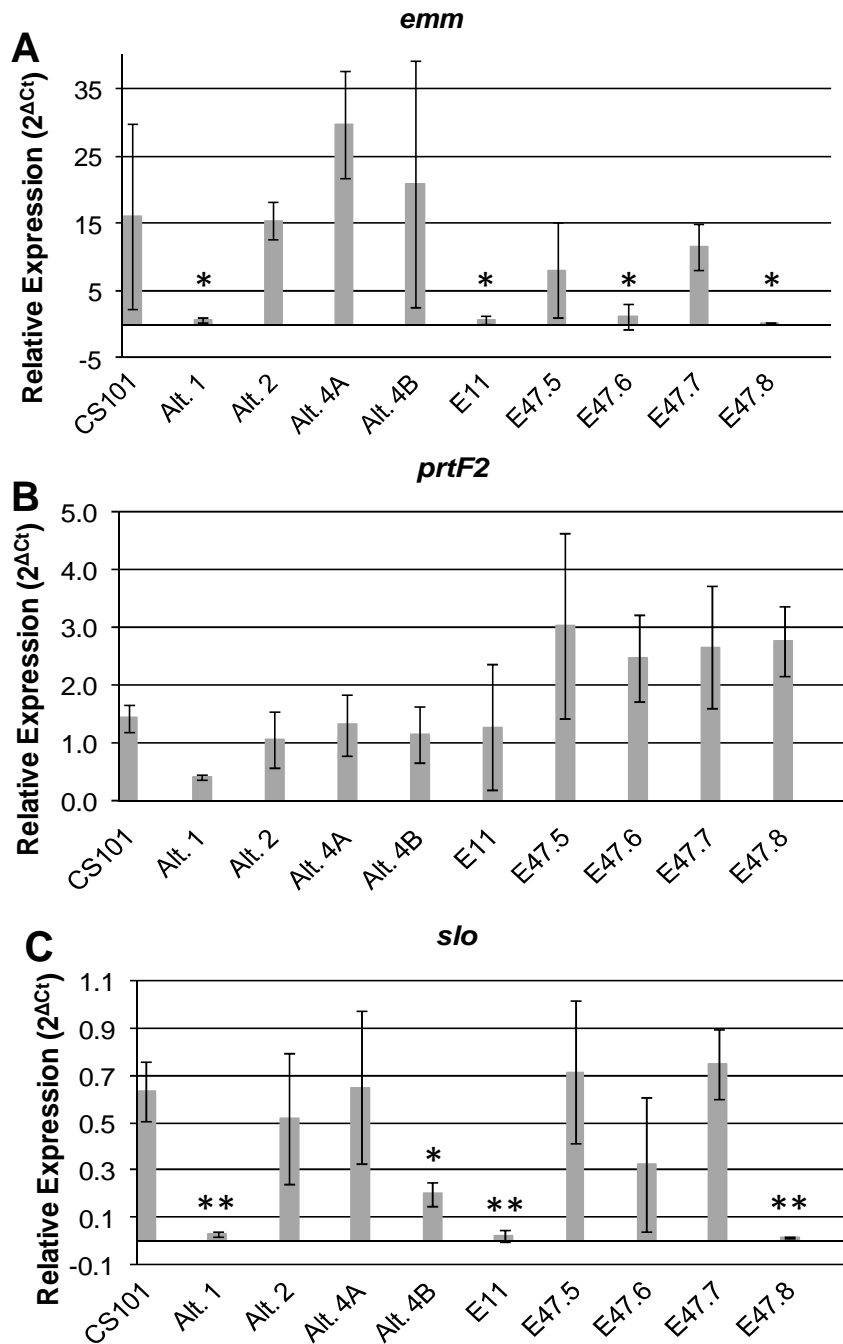


Figure 11. Virulence factor transcription varies between survivor strains. Parental strain CS101 and 9 survivor strains derived from CS101 were analyzed by real-time PCR for expression levels of three virulence factor genes (A) *emm*, (B) *prtF2*, and (C) *slo*. Alt. strains were isolated from 14-week cultures of CS101 in TH (Continued on Page 79)

broth. E11 and E47.5-E47.8 were isolated from 5-day co-cultures of CS101 with the A549 human lung epithelial cell line. The RNA was isolated from mid-exponential cultures in TH broth. Transcription of *proS* was used as a control and data are expressed as relative expression of the gene of interest to *proS* ( $2^{\Delta Ct}$ ). The data shown is an average of 3 independent RNA isolations for each strain. The standard deviation is represented by the error bars. The values for the survivor strains were compared to CS101. (\*) represents a significant difference of  $p < 0.5$ , and (\*\*) represents a significant difference of  $p < 0.05$ .

varied with the strains Alt. 1, Alt. 4B, E11, and E47.8 showing decreased expression, while strains Alt. 2, Alt. 4A, E47.5, E47.6, and E47.7 retained similar expression levels to CS101 (Fig. 11C). Therefore, a large amount of variation in expression levels of genes encoding virulence factors was seen between survivor strains and CS101.

### ***speB* Expression is Variable and Growth-Phase Dependent**

SpeB is a cysteine protease whose exact role in virulence is unclear. SpeB acts upon both host and bacterial proteins. In some strains it has been shown to be important for tissue damage, bacterial invasion, inhibition of phagocytosis, and degradation of complement proteins (reviewed by Chiang-Ni and Wu, 2008). There was no significant change in transcription of *speB* for Alt. 1, Alt. 2, Alt. 4A, and Alt. 4B. All of the survivors from eukaryotic cell co-cultures except E11 displayed a more elevated expression level compared to CS101 and the culture-derived survivors (Fig. 12A). However, at this OD<sub>600</sub>, between 0.5 and 0.65, *speB* transcription was highly variable between experiments. Since *speB* transcription has a rapid increase during the transition from late exponential to stationary phase (Reviewed by Kietzman and Caparon, 2010; Chiang-Ni and Wu, 2008; Loughman and Caparon, 2006), it is possible that the variability was caused by different cultures being at slightly different growth states. To determine if there were other stages of growth that showed less variation in *speB* transcription, CS101 and E47.8 were compared at OD<sub>600</sub> 0.2 and at each 0.1 increment between 0.5 and 1.0, corresponding to early through late exponential phase. E47.8 increased transcription at an earlier growth phase than CS101 (Fig. 12B). *speB* expression is extremely low in CS101 until OD<sub>600</sub> of 1.0. However, expression in E47.8

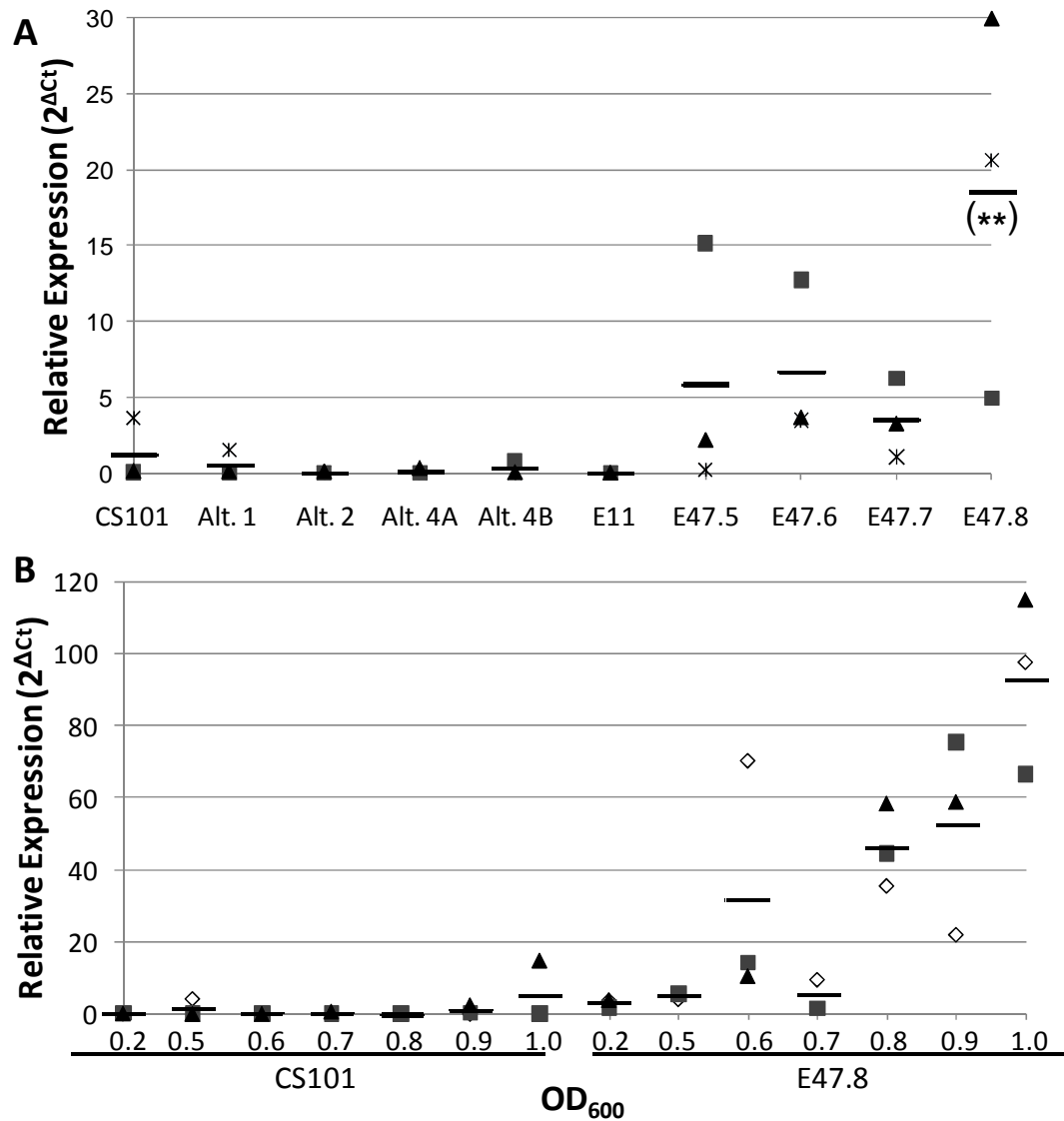


Figure 12. *speB* expression is variable, and its expression increases earlier for survivor strain E47.8 than CS101. (A) Parental strain CS101 and 9 survivor strains derived from CS101 were analyzed by real-time PCR for expression levels of *speB*. The RNA was isolated from mid-exponential cultures, OD<sub>600</sub> 0.5 – 0.65 in TH broth. Transcription of *proS* was used as a control and data are expressed as relative expression of the gene of interest to *proS* ( $2^{\Delta Ct}$ ). The values for the survivor strains were compared to CS101. (\*\*) represents a significant difference of P<0.05. (Continued on Page 82)

(B) Parental strain CS101 and E47.8 were analyzed by real-time PCR for expression levels of *speB* at OD<sub>600</sub> 0.2, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 representing early through late exponential phase. The data shown is an average of 3 independent RNA isolations for each timepoint. Open diamonds represent one experiment, closed squares represent a second experiment, and closed diamonds represents a third experiment.

was already slightly upregulated at OD<sub>600</sub> 0.2 with a massive increase in expression as growth continued. There was still variation between cultures, suggesting some dysregulation of *speB* transcription with a tendency for increased expression in survivors from eukaryotic co-culture (Fig. 12B). As a control for growth kinetics, a growth curve of CS101 and E47.8 revealed that their growth is nearly identical (Data not shown).

### **Survivor Strains Show Diverse Virulence Characteristics**

With a large number of virulence factors and a complex regulation system controlling them, we wanted to see whether the diversity in virulence factor gene transcription applied to changes in virulence. *S. pyogenes* infection of zebrafish (*Danio rerio*) has been established as a simple and accurate model of virulence as it closely mimics results seen in human infections (Miller and Neely, 2004). CS101 and the survivor strains were each injected into the dorsal muscle of zebrafish. Mortality of the fish was scored for survival each day for 5 days. The parental strain CS101 caused 100% mortality by Day 2 (Table 3). In contrast, all of the stationary phase culture-derived survivor strains (Alt. 1, Alt. 2, and Alts. 4A, 4B, and 5A) showed decreased virulence. In addition, the co-culture derived survivor strains E11 and E47.8 showed decreased virulence. However, fish injected with E47.5-E47.7 showed high mortality rates. This study showed that there were changes in virulence between the survivor strains, but this did not correspond to changes in any one virulence factor studied (Figs. 11 and 12, and Table 3).

Table 3. Survivor strains showed variable virulence in zebrafish

Strain	Incidence of zebrafish death <sup>a</sup>					Mortality
	Day 1	Day 2	Day 3	Day 4	Day 5	
CS101	22	8				30/30
Alt. 1		1			3	4/12
Alt. 2		2				2/12
Alt. 4A	2	3				5/12
Alt. 4B		4				4/12
Alt. 5A		1				1/6
E11	1				1	2/12
E47.5	7	5				12/12
E47.6	3	7				10/12
E47.7	3	8				11/12
E47.8		1	1	2		4/12

<sup>a</sup>  $10^5$  -  $10^6$  of log phase bacteria were injected into the dorsal muscle of the zebrafish.

### **Avirulent Survivor Strains Are Cleared**

To further investigate whether the strains with low virulence disseminate and survive in the zebrafish, bacterial load in the spleen was analyzed at 24 hours and 3 days (Table 4). Alt. 1 was not detected in 4/5 animals 24 hours post-infection. E11 and E47.8 were cleared faster than wild-type and essentially eliminated within one day. The fish infected with CS101 had  $\sim 10^4$  CFU in the spleen at Day 1 and were dead by Day 3. Alt. 4A infected fish also had  $\sim 10^4$  CFU in the spleen at Day 1, but the bacteria were cleared by Day 3. Alt. 5A infected fish had  $\sim 10^2$  splenic CFU at 24 hours. Since fish do not have tonsils or nasal associated lymphoid tissues, dissemination to and survival in the spleen are used as measures of virulence.

### **Survivor Strains Are Killed Upon Incubation with Human Blood**

Neutrophils are the primary cells responsible for phagocytosis and clearance of *S. pyogenes* from the human (reviewed by Voyich *et al.*, 2004). Since the strains were cleared in the zebrafish model and many of the strains lacked *emm* expression, a whole blood killing assay was used to determine whether the survivor strains could survive in human blood. CS101 and each survivor strain were incubated with normal human blood from four volunteer donors. CS101 displayed a 4.5-log increase in bacterial numbers upon a 3-hour incubation with the blood (Fig. 13). In contrast Alt. 1, E11, E47.6, and E47.8 had decreased CFU after 3 hours, suggesting that the bacteria were killed. There is a correlation between *emm* expression and the ability of CS101-derived strains to be killed by whole blood (Fig. 11A and Fig. 13).

Table 4. Attenuated survivor strains are cleared from zebrafish spleens

Strain	Spleen CFU <sup>a</sup> 24 hours	Spleen CFU <sup>a</sup> 3 days
CS101	2.5x10 <sup>4</sup> (10/10)	Dead (0/5)
Alt. 1	12 (1/5)	1 (1/5)
Alt. 4A	1.5x10 <sup>4</sup> (5/5)	16 (2/4) <sup>b</sup>
Alt. 5A	2.6x10 <sup>2</sup> (N/A)	N/A
E11	5 (5/5)	0 (0/5)
E47.8	40 (5/5)	14 (1/5)

<sup>a</sup> Spleens were homogenized and plated on CNA medium. The number in parenthesis represents how many fish have remaining CFU, and the average CFU is reported for those fish.

<sup>b</sup> One fish died before harvesting the spleen

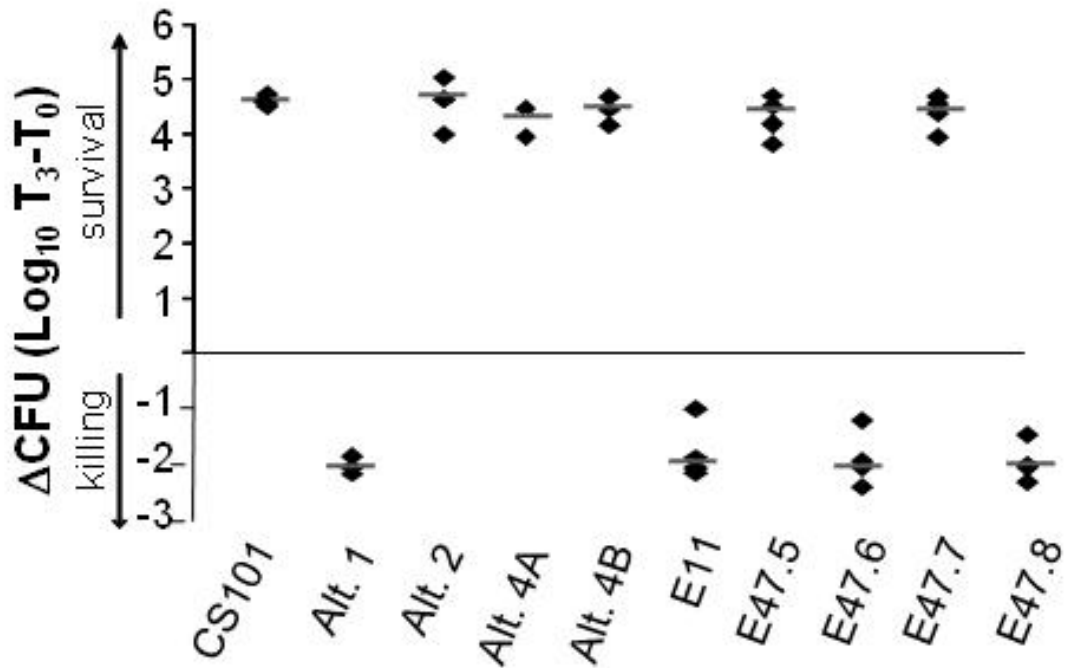


Figure 13. Whole blood killing varies between survivor strains. *S. pyogenes* CS101 and survivor strains were added to heparinized human blood from healthy donors at a concentration of ~300 CFU/ml. The bacteria/blood mixtures were incubated at 37°C for 3 hours. Samples were taken at Time 0 and 3 hours for CFU enumeration. The data show the log<sub>10</sub> difference in CFU (T<sub>3</sub>-T<sub>0</sub>) for each of four donors, and the black bars represent the mean for each strain.

## **The Generation of Diversity in Survivor Strains May Be Due to Random Mutation**

*S. pyogenes* virulence factor expression is controlled by a complex interplay of global regulators, including multiple stand-alone regulators, and 13 two component response regulators (Reviewed by Kreikemeyer *et al.*, 2003). Many of these regulators, such as Mga, CovR/S, and RopB/Rgg regulate multiple factors, including metabolism and virulence factor expression. Figure 1 shows a partial diagram of the complex regulation system, focusing on Mga and the regulation of SpeB.

Strains isolated from the same long-term stationary phase cultures are metabolically diverse when compared to each other, even between strains isolated from the same culture. These phenotypes are stable even after multiple passages, suggesting the changes may be genetic. Accumulation of mutations in long-term stationary phase cultures has been well documented in *E. coli* (Reviewed by Zinser and Kolter, 2004). To determine if mutations in global regulators of *S. pyogenes* could be detected in strains isolated from surviving cultures, the genes of 10 known regulators were amplified by PCR and sequenced in each of the survivor strains. The sequences were then compared to the parental CS101 strain to look for mutations in these genes or their promoter regions. Three strains showed three different types of mutations (Table 5). Alt. 1 had a point mutation in *codY*. This mutation codes for an amino acid change at position 128 of glycine to glutamic acid. CodY is a repressor that responds to levels of branched chain amino acids. Its structure has been determined (Levdikov *et al.*, 2006), and position 128 lies within the N-terminal cofactor binding domain. It is possible that a mutation in this area could affect its regulatory actions. A different mutation was found in Alt. 2. A 12 base pair insertion that is a direct repeat of the sequence in front of it occurred in the

Table 5. Sequencing survivor strain regulators reveals random mutations

	Sequencing Result as Compared to Parental Strain CS101	Function
<i>codY</i>	Point mutation of G to A in Alt. 1. Causes AA #128 to change from G to E.	Controls expression of stationary genes by repressing exponential phase genes (Malke <i>et al.</i> , 2006).
<i>ccpA</i>	No mutations	Repressor that plays a role in catabolite repression (Deutscher <i>et al.</i> , 2005).
<i>srv</i>	No mutations	Homologous to a member of Crp/Fnr family (involved in the acetate switch). Regulates virulence factors in <i>S. pyogenes</i> (Reid <i>et al.</i> , 2004).
<i>SPy1548</i>	Alt 2 showed an insertion of 12 bases. Adds Ile-Val-Val-Ala. Insertion is a repeat of sequence preceding it.	FNR-like protein, which is involved in the acetate switch. FNR is an activator of the Arc operon in <i>S. gordonii</i> (Dong <i>et al.</i> , 2004).
<i>relA</i>	No mutations	Converts GTP to pppGpp during the stringent response (Malke <i>et al.</i> , 2006).
<i>rgg/ropB</i>	No mutations	A mutation in <i>rgg</i> causes utilization of serine and arginine in the presence of carbon. Rgg affects growth phase proteins associated with AA utilization (Chaussee <i>et al.</i> , 2004; Chaussee <i>et al.</i> , 2003).
<i>SPy1630</i>	No mutations	Omega subunit of RNA polymerase. Has a role in stringent response in <i>E. coli</i> (Vrentas <i>et al.</i> , 2005).
<i>SPy0145</i>	No mutations	Homology to AldR, which is a negative regulator of transcription for genes involved in amino acid metabolism in <i>L. lactis</i> (Goupil-Feuillerat <i>et al.</i> , 1997).
<i>covRS</i>	No mutations	Two-component response regulator that acts as a repressor ~15% of the GAS genome. Has a role in stress response and regulation of multiple virulence factors (Churchward <i>et al.</i> , 2009).
<i>mga</i>	Single A insertion in Alt. 5A after a string of As preceding it. Causes a frameshift mutation mid-gene.	Multiple gene activator of GAS, which controls expression of ~10% of the genome. It has effects upon multiple virulence factors, including activation of <i>speB</i> and <i>emm</i> (Hondorp and McIver, 2007).
<i>RALPs</i>	No mutations	RofA-like proteins (RALPs) control multiple virulence factors including <i>prtF2</i> and <i>speB</i> . They also regulate other regulators, including <i>mga</i> (Kreikemeyer <i>et al.</i> , 2003).

SPy1548 gene. SPy1548 is a hypothetical protein in the GAS genome that has homology to an Fnr-family (fumarate nitrate reductase) protein. *Streptococcus gordonii* produces Flp (Fnr-like protein) that activates the arginine deiminase operon (Dong *et al.*, 2004). Fnr proteins are involved in the acetate switch in bacteria such as *E. coli*, in which the cells switch from producing acetate during exponential phase to catabolizing acetate during stationary phase (Kumari *et al.*, 2000). A mutation was also detected in Alt. 5A. A single nucleotide insertion occurred in the middle of the gene, inserting an adenosine. Using a predictive translational website, this mutation at AA229 would introduce numerous stop codons after the mutation. Since this is a mutation mid-gene, this likely would lead to a truncated protein that could be present in the cell or degraded. The Mga protein has two helix-turn-helix (HTH) domains on N-terminal end, upstream of the insertion. The middle of the protein, where the mutation occurs, has a phosphotransferase system (PTS) regulatory domain (PRD). The PRD is responsible for phosphorylation of conserved histidines within its own domain via the PTS phosphorelay (Hondorp and McIver, 2007). It is uncertain what effect the SNP in *mga* may have upon virulence. These mutations were found only in single strains and were not present in other strains, even those isolated from the same stationary phase culture.

## CHAPTER 4

### DISCUSSION

Genic diversification of *Streptococcus pyogenes* has been seen in detailed studies of clinical isolates (Beres *et al.*, 2010), and the mutations seen in successive outbreaks of subclones suggest the strains may be mutating in the host. Although it is well known that *S. pyogenes* strains are diverse, the niche where diversification occurs is still unclear. During studies on two in vitro models of carriage (survival in stationary phase and survival in eukaryotic cells) we observed the formation of polyclonal populations with respect to proteomes and metabolism (Wood *et al.*, 2005; Wood *et al.*, 2009). Metabolic diversification was detected only in strains during survival, suggesting a selection for changes in metabolism during survival. The strains also showed diverse patterns of transcription of genes encoding virulence factors. These changes led to attenuation of a majority of the survivor strains, although the mechanism causing attenuation seemed to vary in the zebrafish model. In human blood, attenuation correlated to changes in M-protein expression. Sequencing of global regulators suggested that the changes are not necessarily due to mutations in global regulators and the mechanism controlling changes in gene expression are likely to be different for each strain. Despite the mutations observed, MLST was not altered in the survivor strains. Taken together, these data suggest that conditions encountered during survival drive random mutation. The nature of the diverse population reflects both random mutation and selective pressures.

### **Metabolic Diversification is Selected During Survival**

Since the metabolic systems in bacteria need to reflect the available nutrients and chemical composition of the environment, bacteria need to adjust metabolism to generate energy, control the pH of the system, create a reducing environment, and to generate molecules for growth and cell maintenance. Metabolic changes allow the bacteria to utilize alternate sources of energy from the environment or utilize molecules, like lactic acid, that are byproducts of exponential phase metabolism. The stresses present in culture and co-culture may drive and select for the diversification of a metabolically heterogeneous population of *S. pyogenes*.

Todd-Hewitt broth is a nutrient rich medium. Glucose is present during exponential phase, and *S. pyogenes* utilizes glycolysis to generate energy followed by fermentation. During exponential phase, *S. pyogenes* is homofermentative and it produces large quantities of lactic acid. *S. pyogenes* lacks the genes that would encode the enzymes of the TCA cycle and electron transport chains, thereby limiting carbon cycling and eliminating the ability to obtain energy by respiration. The primary energy sources such as glucose present in bacterial culture are depleted during long term survival. After depletion of the primary carbon source, *S. pyogenes* can ferment lactate by the PA pathway to obtain energy with the production of formate, acetate and ethanol as byproducts. They can also catabolize amino acids providing both energy and a source of ammonia to help prevent drops in culture pH caused by lactate fermentation..

The intracellular environment of eukaryotic cells is also nutrient-limited, in both phagocytic vesicles as well as in the cytosol (Goetz *et al.*, 2001). Studies with *L.*

*monocytogenes* in macrophages have shown that the metabolism of intracellularly growing bacteria was very different from bacteria growing in a glucose containing medium (Eylert *et al.*, 2008). One study that selected for replication-defective *S. typhimurium* mutants in non-phagocytic cell lines found that the bacterial mutants were auxotrophs for purines, pyrimidines, aromatic amino acids, and histidine. These strains were attenuated in mice, suggesting these components are limited in the intracellular environment (Leung and Finlay, 1991). Additionally, a mutation in *aroA* in *S. typhimurium* gave rise to avirulent strains since mammals do not metabolize the aromatic compounds paraaminobenzoic acid and dihydroxybenzoic acid, for which the bacteria were auxotrophic (Stoker *et al.*, 1983). Therefore, the environment inside eukaryotic cells as well as in long term culture may select for strains with changes in metabolism.

When the isolated survivor strains were regrown in Todd-Hewitt broth, many of the strains displayed a heterofermentative phenotype, even with glucose still present in the medium. These strains had increased transcription of genes for the PA pathway. Although protein levels and activity were not measured, the production of formate, acetate, and ammonia suggested that increased transcription of genes involved in the metabolic pathways corresponded to increased enzyme levels. In addition, *S. pyogenes* can generate energy during stationary phase by catabolizing amino acids. Serine and arginine both contribute to the stationary phase metabolism, and their usage produces ammonia as a byproduct. Some of the survivor strains produced ammonia during exponential phase, suggesting amino acid catabolism in the presence of glucose. In the closed system of long-term survival in TH broth, the pH was greatly affected by the byproducts of these stationary phase metabolites. In order to offset the highly acidic

environment produced by mixed acid fermentation in some strains, ammonia production kept the pH above the critical level of 5.6 (Wood *et al.*, 2009). Therefore, the metabolic diversity of strains generated from a closed in vitro environment may have been driven by the need for pH stabilization.

One strain derived from co-culture of CS101 with A549 human lung epithelial cells, E11, had been previously characterized. E11 survived intracellularly, and mutations in both metabolism and virulence would have a selection in this environment. Theoretically, the intracellular environment of eukaryotic cells possesses its own buffering system, and metabolic diversification would not be necessary to control the pH of the surroundings, in contrast to the closed environment of TH broth. E11 upregulated expression of the PA pathway during exponential phase (Morgan and Buttaro, submitted). Additional co-culture derived survivor strains did not increase expression of the PA pathway, as detected by formate production (data not shown), suggesting that metabolic diversity may be occurring intracellularly. Intracellular nutrient limitation may have selected for diversification of the co-culture derived survivor strains. These strains were not fully characterized for metabolic profile, so it is possible that strains other than E11 may upregulate the PA pathway or amino acid utilization pathways to generate energy during survival.

The metabolically diverse survivor strains also had alterations in colony morphology. The development of *S. pyogenes* strains with alternate colony morphologies have been seen in other studies. When an inducing agent such as penicillin is added to the medium, some *S. pyogenes* become cell wall deficient L-forms. Some of these strains are unstable and revert to parental phenotype, while some strains retain the L-form

morphology upon removal of penicillin, suggesting mutational changes (Reviewed by Domingue and Woody, 1997). Mouse models have shown that L-forms disseminate to the organs, particularly the spleen, after intraperitoneal injection of stable L-form strains of *S. pyogenes* (Kagan *et al.*, 1976). An osmotically stable L-form was able to destroy Girardi human heart cells in human tissue (Leon and Panos, 1976). Intraperitoneal co-infection of rats with penicillin-generated L-forms and the A-49 parental (S) forms of *S. pyogenes* showed that L-forms persisted for more than 30 days whereas the parental strain was only detected up to 15 days post infection (Michailova *et al.*, 2000). In the present studies, alternate colony morphologies were observed upon survival in the two in vitro survival systems, but no L-forms were detected.

*S. pyogenes* M64 strains that were passaged multiple times in blood or in mice resulted in phenotypic changes in colony morphology including small colonies (Smith *et al.*, 2005; Raeder *et al.*, 2000; Raeder and Boyle, 1993; Reis *et al.*, 1984) like those isolated in our survival assays (Wood *et al.*, 2005; Leonard *et al.*, 1998; Morgan and Buttaro, submitted). Alterations in colony morphology in environments of multiple stress factors have been seen in other systems. Upon invasion of eukaryotic cells, *S. aureus* forms small colony variants (SCVs) which, as their name suggests, are small in appearance when compared to the parental strains. SCVs are small because they have accumulated mutations in metabolic genes. Under aminoglycoside selection, such as gentamicin, some of these mutations interfere with production of heme-containing cytochromes. This changes energy production from respiration to predominantly fermentation because of defect in the electron transport system (ETS). Fermentation yields less ATP so cells grow slower such that they form smaller colonies. Since the

electrochemical gradient generated by the ETS is reduced, the import of aminoglycosides is reduced, leading to antibiotic resistance (Chuard *et al.*, 1997). Other mutations result in amino acid auxotrophies. When the amino acids were introduced into the growth medium, the colonies regrew with the characteristics of the parental strain (Reviewed by Garzoni and Kelley, 2009). The *S. pyogenes* strains that were isolated with atypical colony morphology were not an SCV phenotype. The survivor strains grew with similar growth kinetics to the parental strain CS101. The small colony phenotype could be contributed to a reduction in capsule expression.

In addition to having changes in colony morphology, the M64 passaged strains had altered expression of several virulence factors including *speB* and genes in the *mga* regulon (Raeder *et al.*, 2000; Boyle *et al.*, 1994; Raeder and Boyle, 1993; Raeder and Boyle, 1993). The altered *speB* expression led to changes in post-translational modifications of M protein, affecting its expression (Raeder *et al.*, 2000).

However, unlike most strains isolated from late stationary phase cultures or eukaryotic cells, these passaged strains did not express the PA pathway during exponential growth. Only upon inducing survival by regrowing each strain in long term TH cultures did the strains upregulate expression of the PA pathway. This finding suggests that metabolic diversification is probably selected for under conditions of survival but not by sequential passage in animal models.

## **Survival in Stationary Phase and Eukaryotic Cells Led to the Development of Polyclonal Populations With Respect to Virulence Factor Expression**

We observed that there was virulence factor diversification in strains derived from stationary phase culture (Summarized in Table 6). Amongst the culture-derived survivor strains, one had reduced levels of *emm* expression (Alt. 1), and two had reduced levels of *slo* expression (Alt. 1 and Alt. 4B). Alt. 4A and Alt. 4B were both isolated from the same culture. The level of *slo* transcription in these two strains were different from one another, suggesting that diversification in virulence factor transcription occurred between multiple strains from one population. We had already seen that metabolic variation occurred within one population, and this virulence factor variability suggests that conditions encountered during survival allow for the development of a polyclonal population of bacteria.

Levels of *prtF2* and *speB* were similar to those of CS101. Each of the culture-derived strains expressed low levels of *speB* at mid-exponential phase, which is similar to that of CS101. The expression of *speB* typically increases in late-exponential/early-stationary phase, so this low level of expression was not surprising. It was initially unexpected to see changes in virulence factor expression for *emm* and *slo* in these strains since the bacteria were not in contact with eukaryotic cells that would confer a selective advantage for changes in these virulence factors. However, the main selective pressures that are present in this limited, closed environment would include oxidizing conditions and nutrient limitation. These pressures might also be found in environments in the human host such as the intracellular niche. Mutations in regulators that affect both virulence factors and metabolic genes would explain the changes in virulence factor

Table 6. Summary of virulence properties in survivor strains

	Colony Size (capsule)	Virulence Factor Expression				Virulent <sup>c</sup>	Cleared <sup>c</sup>	Growth in Blood <sup>c</sup>
		<i>prtF2</i> <sup>a</sup>	<i>slo</i> <sup>b</sup>	<i>emm</i> <sup>b</sup>	<i>speB</i> <sup>b</sup>			
CS101	Large	=	H	H	L	Y	N	Y
Alt. 1	Small	=	L	L	L	N	Y	N
Alt. 2	Small	=	H	H	L	N		Y
Alt. 4A	Large	=	H	H	L	N	Y	Y
Alt. 4B	Small	=	L	H	L	N		Y
E11	Small	=	L	L	L	N	Y	N
E47.5	Large	=	H	H	H	Y		Y
E47.6	Small	=	H	L	H	Y		N
E47.7	Large	=	H	H	H	Y		Y
E47.8	Small	=	L	L	H	N	Y	N

<sup>a</sup> = represents equal expression amongst the strains analyzed

<sup>b</sup> H represents High expression, L for Low

<sup>c</sup> Y represents Yes, N for No

expression, and there most likely would be no selection in culture against either an increase or decrease in virulence. These regulators, such as CovR/S, CodY, Srv, and Rgg/RopB, sense changes in nutrient availability and are activated or repressed based on these environmental conditions. Therefore, altered regulators generated by random mutation may be positively selected during survival due to nutrient levels, and they may alter the expression level of virulence factors even though the cells are in a closed environment. Indeed, a mutation in CodY was detected in Alt. 1.

The second survival model incubated eukaryotic cells with CS101 in the presence of extracellular penicillin and gentamicin. The isolated strains had parental strain levels of sensitivity to these antibiotics, suggesting they were surviving inside the protected niche of eukaryotic cells (Morgan and Buttarò, submitted).

The levels of *emm* and *slo* expression were variable between strains isolated from the same co-culture well. Two of the four strains, E47.5 and E47.7, showed high levels of *emm* expression. Although it is unknown whether all four strains survived within the same eukaryotic cell, they were isolated from the same well. E47.8 had reduced transcription levels of *slo* compared to the three other co-culture strains isolated from the same well. Thus, virulence factor diversity was generated during survival leading to a polyclonal population.

In addition to nutrient limitation and oxidative stress, invasion efficiency could be a selective force for survival in eukaryotic cells. E11 had a 10-fold increased invasion rate into A549 cells and a 2 fold increase in attachment (Morgan and Buttarò, submitted). Capsule interferes with attachment mediated by attachment proteins such as PrtF2 and M protein. Strains that produce small colonies, like E11, do not produce capsule, so they

would have an increased invasion rate. However, not all the strains isolated from eukaryotic cells gave small colonies. There were no changes in *prtF2* transcription in the co-culture derived strains, despite their increased attachment rate. So while PrtF2 is important in attachment of the bacterium to the host cell, this increased attachment rate must be due to factors other than *prtF2* expression. In addition, three of the co-culture survivor strains, including E11, had decreased levels of *emm* expression. M protein plays a role in the avoidance of phagocytosis as well as in attachment. Therefore, changes in attachment cannot be accounted for by changes in expression of genes encoding for known attachment proteins or capsule.

Strain E11 had the most significant decrease in *slo* expression levels of all the strains studied. Studies have shown that SLO plays a role in internalization. A deletion in SLO resulted in increased internalization of the mutant into human keratinocytes, although the mutant was attenuated in virulence (Bricker *et al.*, 2005; Bricker *et al.*, 2002). These observations would be consistent with the generation of a carriage-adapted strain. In previous studies by our group, E11 had a slightly higher level of cell association with A549 cells, and a greater than 10-fold increase in invasion percentage compared to CS101 (Morgan and Buttaro, submitted). It is possible that the decrease in *slo* expression led to the increased invasion rate of E11, although it could also be an interplay of multiple virulence factors that affected the invasion rate, including SpeB.

Transcription of another virulence factor, *speB*, was also variable in co-culture derived survivors. The expression of *speB* appeared to be dysregulated in many of these strains. Expression of *speB* appeared to be increased in E47.5-E47.8, but due to high variability between experiments, E47.8 was the only strain with a statistically significant

increase. Transcription of *speB* is regulated on multiple levels (Fig. 1). The growth-phase dependent regulators CcpA, LacD.1, and Rgg/RopB lead to increases in *speB* expression during the transition from exponential to stationary phase (Kietzman and Caparon, 2010; Chiang-Ni and Wu, 2008; Chaussee *et al.*, 1999). Comparison of CS101 to the survivor strain E47.8 at early, mid and late exponential phase revealed that E47.8 began to upregulate *speB* expression at an earlier growth phase, and the expression showed high levels of variability. *speB* expression is influenced by at least six additional regulators that could be responsible for variable levels of *speB* transcription (Vfr, Srv, RALPs, Mga, RopB/Rgg, and CovR/S) (Kietzman and Caparon, 2010; Ma *et al.*, 2009; Reid *et al.*, 2006; Kreikemeyer *et al.*, 2003). SpeB production has been implicated in increased epithelial cell invasion in some strains (Tsai *et al.*, 1998), yet also leads to apoptosis in phagocytic cells (Chang *et al.*, 2007; Tsai *et al.*, 1999). The increased levels of *speB* transcription suggest that in the CS101 background SpeB may be partially responsible for increased invasion efficiency. The early expression of *speB* could result in increased invasion throughout growth, allowing better spread of the bacteria between eukaryotic cells.

Therefore, in the intracellular niche, random mutations may have led to alterations in virulence factor transcription leading to the development of a polyclonal population with regard to virulence factors. Phenotypically altered strains would be found within the polyclonal population. Some of these strains may be better adapted for increased invasion and carriage, such as E11. Although not among our randomly selected strains, it is formally possible that other strains may increase virulence properties, and these strains may re-emerge to cause disease.

**Some but not all Survivor Strains were Attenuated in a Zebrafish Model and  
Attenuation was not Correlated with any Particular Virulence Factor Expression  
Pattern**

Since virulence factor transcription was varied, we hypothesized that virulence would also be diverse in survivor strains. Indeed, this is what was observed. However, there was no correlation between virulence factor profiles and virulence in zebrafish (summarized in Table 6). We had looked at transcription levels for four virulence factors, but *S. pyogenes* encodes over 13 virulence factors. It is possible that there may be a correlation between virulence and one of the factors that we had not yet analyzed.

All four of the culture-derived strains were attenuated for virulence in zebrafish despite varying levels of virulence factor expression. Most of the attenuated strains, except Alt. 4A and Alt. 5A, showed low numbers or were not present in the zebrafish spleen at 24 hours post-infection, suggesting clearance from the fish. This is most likely due to clearance, but the bacteria may have been killed by immune cells, such as macrophages or neutrophils, at the site of infection and never made it to the spleen. M protein is a major determinant of survival in the phagocytic cells. Three of the attenuated strains analyzed – Alt. 1, E11, and E47.8 –expressed low levels of *emm* and presumably M protein. The attenuated strains Alt. 2, Alt. 4A and Alt. 4B expressed high levels of *emm*. Both M+ and M- strains can be internalized into human neutrophils, and those with high M expression can avoid phagocytic killing (Staali *et al.*, 2003). It is possible that the M- strains, such as Alt. 1, E11 and E47.8 are killed by neutrophils at the injection site, therefore very few bacteria enter the spleen. However, attenuated strains that express M

protein may be internalized by the neutrophils without immediate killing. The neutrophils containing bacteria would then enter the spleen to be cleared from the fish.

In the case of CS101, the bacterial load within the spleen at 24 hours is  $\sim 10^4$  CFU. CS101 expresses high levels of M as well as capsule, which would prevent its clearance and eventually lead to lethality. All fish infected with CS101 were dead by 3 days post-infection. Alt. 4A gave similar levels of bacterial load to CS101 at 24 hours, and it also expressed high levels of *emm* as well as capsule, as seen by its large colony morphology (Wood et al 2005). However, for unknown reasons, Alt. 4A had attenuated virulence and was cleared by day 3 despite its high splenic load at 24 hours. The other M+ strains, Alt. 2 and Alt. 4B, had attenuated virulence even though they still expressed *emm*.

### **Reduced Survival in Human Blood Correlated with Changes in *emm* Transcription**

A significant characteristic of *S. pyogenes* virulence is its ability to survive in blood. Strains that cause bacteremia in the host need to express virulence factors that would allow for survival inside phagocytic vesicles as well avoidance of the host immune response. We found that there was a drastic variability in the ability of the survivor strains to survive in human blood.

The strains with high *emm* expression, such as CS101, had a  $\sim 4.5$  log increase in bacterial numbers during a three hour incubation in human blood, which implies a rapid doubling rate of approximately 15 min. The strains that were killed in human blood were almost entirely eliminated after a three hour incubation. This difference in survival is striking, and it reflects the rapid and severe onset of disease upon Streptococcal bacteremia. Of the four culture-derived survivor strains, only Alt. 1 was killed in human

blood. Strains E11, E47.6, and E47.8 were derived from co-culture and were killed as well. All killed strains expressed low levels of *emm*. Hyaluronic capsule and M protein often both contribute to phagocytosis resistance and growth in blood. Early studies looking at the role of capsule on growth in human blood showed that an acapsular mutant does not grow in human blood, and it was sensitive to phagocytic killing (Wessels *et al.*, 1994; Wessels *et al.*, 1991). In contrast, later studies showed that capsule is important for resistance to phagocytosis in 10% serum and in mouse infections, but M protein rather than capsule was the major determinant for growth in human blood (Moses *et al.*, 1997). Our results support this conclusion. Although levels of capsule expression were not directly measured, previous studies have shown that capsule expression corresponds directly to colony size (Wood *et al.*, 2005; Leonard (Buttaro) *et al.*, 1998), and colony size did not show a direct correlation with whole blood killing, as can be seen by the difference in survival between the two small colony strains Alt. 1 and Alt. 2. Further, *S. pyogenes* strains have been shown to be internalized by human neutrophils whether they are M+ or M-, but only M+ strain avoid phagocytic killing. This suggests that in addition to the anti-phagocytic properties of M protein, M protein aids in intracellular survival in phagocytic cells (Staali *et al.*, 2003), but it is not essential for survival in epithelial cells like those used for co-culture experiments.

### **Changes in Survivor Strains are Likely Generated by Random Mutation**

We found that random mutations occurred during survival. Sequencing multiple regulator genes in the survivor strains identified unique mutations that occurred in three genes in three separate strains in the limited number of regulators that we analyzed. The

*S. pyogenes* genome is characterized by a high number of genic and allelic changes. Approximately 10% of its 1.9 Mb genome is comprised of prophages and integrated conjugative element (ICEs) (Podbielski, 2007; Banks *et al.*, 2002). A large amount of genic variation occurs in *S. pyogenes* during infection based on analysis of clinical isolates. Even closely related epidemic strains varied by as many as 133 SNPs and Indels. The SNPs occurred at a rate that would be expected for randomly occurring mutations: based on genome sequencing, 69% were non-synonymous (i.e. amino acid-altering) whereas 31% were synonymous, and they were evenly distributed between coding sequences and intergenic regions (Beres *et al.*, 2010). Although these SNPs or Indels have been detected, they haven't been directly analyzed for effects upon regulator activity. A mutation leading to altered function of the regulator proteins could have significant effects upon metabolism and virulence of the strains.

In our survivor strains, the first mutation observed was a SNP in *codY* in Alt. 1 which led to a change in amino acid sequence from a non-polar glycine to the polar, charged glutamic acid. CodY is a global regulator that affects the expression of multiple genes involved in metabolism, stress response, quorum sensing, and virulence. CodY is induced by amino acid starvation via mechanisms separate from the stringent response (Steiner and Malke, 2001). The location of the SNP is a conserved residue in the active site of CodY. The mutation occurred at amino acid 128, which is predicted to be located within the GAF domain based upon the crystal structure of CodY from *B. subtilis*. This domain is present in many signaling and sensory proteins (Levdikov *et al.*, 2006). Since CodY has effects upon metabolic as well as virulence properties, it is possible that this

mutation may have contributed to virulence attenuation and metabolic changes found in Alt. 1.

A second mutation was found in the gene for the multiple gene regulator (*mga*) in Alt 5A. The mutation was a single nucleotide insertion of an adenine after a string of seven adenines. This type of mutation has been seen in other *S. pyogenes* strains. In one study, 85% of the Indels studied among 87 strains were due to a single nucleotide insertion following a homopolymeric nucleotide tract, without regard to the nucleotide type (Beres *et al.*, 2010). This genic insertion would cause a predicted alteration in amino acid sequence in the middle of the phosphotransferase system (PTS) regulation domain at amino acid 229. This regulation domain is located in the central region of the Mga protein downstream of two helix-turn-helix (HTH) domains (Reviewed by Hondorp and McIver, 2007). This stand-alone regulator controls ~10% of the *S. pyogenes* genome, and it is itself regulated by many proteins (Fig. 1) (Reviewed by Hondorp and McIver, 2007). In addition to other virulence factors, Mga influences both *emm* and *speB*, which were not analyzed in Alt. 5A. However, *emm* and *speB* transcription were altered in multiple strains that did not have a mutation in *mga*, so a direct correlation between this mutation and changes in transcription level would need to be tested. Still, the Alt. 5A survivor strain was attenuated in virulence. There are conflicting studies looking at the effect of an *mga* mutation upon virulence. Two studies suggested that an *mga* mutation led to reduced virulence in mouse studies after skin infection (Luo *et al.*, 2008; Kihlberg *et al.*, 1995). However, inactivation of *mga* in strain 64/14 did not reveal any changes in virulence in a mouse skin infection model when compared with the wild-type strain (Boyle *et al.*, 1998). Further, a study by Podbielski's group found that Mga in

CS101, which produces high amounts of capsule, and the strains M2 and 591, low capsule producers, showed conflicting roles in attachment/invasion. Mutations in *mga* in CS101 and M2 led to decreased binding of host matrix proteins as well as serum proteins, but the mutations led to increased attachment and internalization in Hep-2 cells. In M49 strains, Mga activates Nra, which is a repressor of genes for fibronectin binding proteins. Therefore, although an inactivation of *mga* would repress *emm* expression, it may lead to an increase in other fibronectin binding proteins (Fiedler *et al.*, 2010). Overall these studies show that the regulation of- and regulation by- Mga is a complex interaction of virulence factors and at this point it is difficult to determine whether the single nucleotide insertion found in Alt. 5A caused attenuation in zebrafish.

The third mutation observed was a 12 base insertion found in Alt. 2 in the Spy1548 gene. The predicted protein from this ORF has 43% identical residues with the FNR-like protein (Flp) in *S. gordonii*. FNR (fumarate nitrate reductase) is a member of the cAMP receptor protein (CRP)/FNR superfamily, which are active during anaerobic conditions. *E. coli* has the best characterized CRP/FNR proteins, where they act as oxygen sensors. FNR regulators are found in both Gram-negative as well as Gram-positive bacteria. The regulator has been extensively studied in *E. coli*, in which it is the primary protein responsible for the switch between aerobic and anaerobic growth. FNR acts to activate genes in anaerobic metabolism such as nitrate reductase, DMSO reductase, and fumarate reductase. It also represses genes in aerobic growth such as the genes for succinate dehydrogenase and NADH dehydrogenase II. In addition, FNR is sensitive to nitric oxide (NO) which renders it unable to bind DNA (Reviewed by Crack *et al.*, 2008). Alt. 2 was isolated from a 14-week culture of CS101 in TH broth. The

cells were incubated statically in 5% CO<sub>2</sub>, so the cells are in a micro-aerophilic environment. Under these limiting oxygen conditions, FNR would most likely be activated. FNR proteins play a role in inducing the arginine deiminase system in *P. aeruginosa* and *B. licheniformis* (Dong *et al.*, 2004). Stationary phase cultures of CS101 began accumulating high amounts of ammonia by ~12 weeks of culture, suggesting that the bacteria were metabolizing amino acids, such as arginine, to generate energy during stationary phase survival. In exponential phase, however, Alt. 2 did not produce increased amounts of ammonia. The 12 bp insertion in Spy1548 was detected in this strain, but it is not known whether this affects the function of the protein. Transcription analysis was not performed on this regulator gene, so we were not able to determine whether the mutation in Spy1548 or other sources of transcriptional diversification were responsible for the low ammonia production.

In looking at *speB* expression as a function of growth phase, the co-culture survivors expressed high levels of *speB* at an earlier timepoint, suggesting that it was a growth-phase dependent regulator that was affected. Rgg is a growth phase dependent regulator, and it activates *speB* transcription. We had sequenced *rgg/ropB* for all the survivor strains derived from stationary phase cultures, and we did not detect any changes in the nucleotide sequences. However, the strains that showed a large increase in *speB* expression were all co-culture derived survivor strains. We sequenced *rgg/ropB* for mutations in these strains, and no changes were detected in the co-culture derived strains (data not shown). It was surprising to find that there were no mutations in *rgg/ropB*, especially because studies from Musser's group had found that *rgg/ropB* had the highest rate of nucleotide diversification amongst genes in the core genome in a large study of

isolates from invasive disease. The SNPs detected in this study were all nonsynonymous mutations, suggesting that there was a selection for phenotypic variation due to random mutation (Beres *et al.*, 2010). Interestingly, the clinical isolates analyzed were strains isolated from invasive disease, which typically produce high levels of SpeB. Although it would stand to reason that inactivation of *rgg/ropB* via mutation would reduce the amount of *speB* produced (Reviewed by Olsen and Musser, 2010), the regulation system in *S. pyogenes* is complex, especially in relation to *speB* expression (Fig. 1). A loss of Rgg/RopB function can affect other regulators as seen in Fig. 1. In particular, it represses *mga*, which also regulates *speB*; therefore inactivation of *rgg/ropB* could lead to activation of *mga* leading to an upregulation of *speB*. It is possible that additional growth phase-dependent regulators of *speB*, such as CcpA or LacD1, were mutated in one or more of the co-culture derived survivor strains leading to dysregulation of *speB* transcription.

The mechanism and niche for the generation of random mutations in *S. pyogenes* has not been determined. The average mutation rate for most bacteria is approximately  $3 \times 10^{-3}$  mutations/genome/replication. This value is based upon analysis of genetic mutations occurring within the genome, regardless of alteration in phenotype. Some bacteria have higher mutation rates driven by a mutator phenotype (Drake *et al.*, 1998). Certain *S. pyogenes* strains demonstrate a mutator phenotype in stationary phase. The *mutS/mutL* operon is responsible for mismatch repair. In some strains a prophage integrates between these genes causing a lack of *mutL* expression and a mutator phenotype. This was originally observed in strain SF370, where the prophage SF370.4 integrates between these genes. During early exponential phase, the prophage excises, so

that the mismatch repair system is functional with a mutation rate of  $10^{-9}$  to  $10^{-10}$  mutations/generation. These mutation rates are based on a phenotypic change, the resistance to ciprofloxacin, which would have a lower mutation rate than if mutations at the genetic level were analyzed. In late exponential phase and stationary phase, the prophage reintegrates, inactivating the mismatch repair system and increasing the mutation rate to  $10^{-7}$  to  $10^{-8}$  mutations/generation. In general, strains like SF370 and NZ131, which both express the prophage, have bacterial mutations at a rate of  $3.3 \times 10^{-7}$  mutations/generation and  $3.2 \times 10^{-9}$  mutations/generation, respectively. This is as much as 100 fold increase in mutation rate from the non-phage encoding strains like JRS1, with a rate of  $5.3 \times 10^{-10}$  mutations/generation (Scott *et al.*, 2008). In M49 serotypes, approximately 20% of strains examined have the mutator phenotype (Suvorov *et al.*, 2009). Analysis of CS101 revealed that it did not possess the prophage, suggesting that its mutation rate would be that of non-mutator type *S. pyogenes* strains (McShan, personal communication). However, mutation rates can increase due to alterations in other factors.

Random mutation also can occur as a result of environmental stress. During survival in eukaryotic cells and in long term stationary phase cultures, the bacteria encounter adverse conditions such as nutrient limitation, starvation, and immune stress. These conditions would be encountered during intracellular carriage; however, there are stress conditions that could occur during active infection. For example, some strains, especially those that are M-, may be taken up by neutrophils or macrophages. Some additional stresses encountered upon neutrophil uptake include oxidative damage, antimicrobial proteins, antimicrobial peptides, defensins, and proteases (Reviewed by

Urban *et al.*, 2006). *S. pyogenes* has multiple mechanisms of defense against neutrophil stress. It is able to avoid oxidative stress due to the regulator PerR (Brenot *et al.*, 2005; Trainor *et al.*, 1999; Reviewed by Urban *et al.*, 2006), it can avoid phagocytosis, it can survive intracellularly mostly due to expression of H and M proteins, and it can lyse neutrophils through the actions of streptolysin S and streptolysin O (Miyoshi-Akiyama *et al.*, 2005; Sierig *et al.*, 2003; Reviewed by Urban *et al.*, 2006). Random mutation would be beneficial to *S. pyogenes* to generate a subpopulation of strains that increase expression of these defense factors. Passaging of *S. pyogenes* in human blood and the mouse peritoneal cavity gave rise to strains with stable changes in virulence factor expression (Raeder and Boyle, 1993) supporting the idea that multiple stress conditions, including survival, may lead to random mutation.

There are multiple mechanisms by which these stressors can cause mutations within bacteria. The SOS system can drive bacterial mutation rates by inducing an error prone DNA polymerase, such as DinB (Reviewed by Galhardo *et al.*, 2007). *S. pyogenes* encodes a DinB homolog called DinP (Kyoto Encyclopedia of Genes and Genomes). In addition, stress can induce mutations within bacterial populations by affecting mismatch repair, post-replication error correction, or transposon mobility. This stress-induced mutation is prevalent in nature, and bacteria such as *P. aeruginosa* can develop extremely high mutation rates. Further, stress-related mutation is most often selected for within the environmental niche due to stress factors (Reviewed by Galhardo *et al.*, 2007).

Here, we observed three unique mutations in regulators of three different survivor strains. As discussed in the above paragraphs, studies from other groups of these three regulators - CodY, SPy1548, and Mga – showed effects upon virulence properties and/or

metabolic properties. At this point, it is unknown whether the specific mutations we saw had effects upon the function of the regulator proteins. The mutations appeared to be random which may have been induced by stress factors encountered during survival. The changes seen were in three of the nine survivor strains tested. We selected these regulators for analysis since they play a role in metabolism and/or virulence; however, random mutation could occur anywhere within the genome. It is likely that mutations other than those seen in the regulators occurred during survival. The effects of mutations to the phenotypic changes observed were most likely due to an interplay of multiple mutation events, not just a single mutation in a global regulator. Random mutation often aids in the survival of bacterial populations, as may be the case for *S. pyogenes*.

### **Survival Gives Rise to a Phenotypically Polyclonal Population with the Same MLST Type**

We found random mutations within survivor strains, suggesting that survival within the tonsils may provide a protected niche for the generation of diversity in vivo. The ability to diversify may confer phenotypic plasticity to the population. *S. pyogenes* could encounter stress during intracellular survival during antibiotic treatment. The diversification may allow for a subset of bacteria to survive intracellularly, avoiding antibiotics and immune detection, by expressing low levels of virulence factors. Another subset of bacteria may emerge with increased virulence and cause reactivation of disease, or invade systemically causing severe invasive diseases. The genetic diversity can be the result of single nucleotide polymorphisms and insertion/deletion events leading to allelic diversity. As seen by changes in morphology, proteome, metabolism, virulence factor

transcription, virulence, and growth in human blood, *S. pyogenes* acquired random mutations, and the environmental niche selected for phenotypic diversification.

Despite the three random mutations detected in the survivor strains from sequencing only a handful of potential regulators, the multi-sequence locus type (MLST) of the survivor strains did not reveal any mutations, indicating that all of these strains would be considered clonal based upon their MLST-type. MLST is a sequence-based system for determining clonality of clinical isolates. ~500 bp from each of seven genes in the core genome are sequenced, and any sequence divergence would indicate a separate clone. To date, MLST as well as PFGE seem to be the most accurate typing methodologies, short of full-genome sequencing. In addition to the random mutations observed within the genome, these strains had large divergence in phenotype as seen in metabolic as well as virulence properties. This suggests that MLST, although more accurate than many typing methods, has great limitations in determining clonality as defined by strains with an identical genome. Our survivor strains had not only changes in gene transcription, but, in particular, large differences in levels of virulence. In a clinical setting, this could have a significant effect upon disease outcome.

MLST as a predictor of clonal outbreaks obviously lacks in thorough recognition of the small genic changes that lead to large phenotypic diversity within a population. In order to fully determine whether a strain is truly clonal, whole genome sequencing must be performed on the strains. This is demonstrated in a study that analyzed 344 clinical isolates by full genome sequencing and found a large number of SNPs and Indels within population subclones (Beres *et al.*, 2010). Since the first bacterial genome sequenced in 1995, whole genome sequencing in recent years has become relatively cheap and

efficient, and it is emerging as a preferred method for accurate sequence typing (Pearson *et al.*, 2009). Additionally, analysis of genic content can be performed by genome hybridization with a microarray imprinted with each of the distributed genes of a bacterial species (the genes that are separate from the core genome). This allows for a more accurate typing method than MLST, but with similar cost (Hall *et al.*, 2010).

### **General Conclusions**

Heterogeneous bacterial populations are commonly found amongst pathogenic species (Reviewed by Dorer *et al.*, 2009; Hiller *et al.*, 2007; Reviewed by Ehrlich *et al.*, 2005; Shen *et al.*, 2005). Current studies suggest that *S. pyogenes* consists of a mixed population of strains as seen by changes in morphology and virulence in vivo (Aziz *et al.*, 2010; Beres *et al.*, 2010; Beres *et al.*, 2006; Sumbly *et al.*, 2006; Raeder *et al.*, 2000; Boyle *et al.*, 1994). As seen in the results presented here, one of three strains isolated from patients with recurrent pharyngitis up-regulated expression of genes of the PA pathway during exponential phase. These strains were most likely carried within the tonsils of each patient, and therefore were examples of in vivo survivor strains. Although the study was limited in sample number, it does suggest that metabolic and possibly virulence diversity may be occurring in vivo.

Multiple bacterial species have high mutation rates and show diversity upon survival. *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* all are significant human pathogens, and they modify their genome during survival. However, genetic diversification is not a singular predisposing factor for survival. *Mycobacterium tuberculosis* survives within alveolar macrophages. It does this by

altering expression of virulence and metabolic factors, but this alteration is due to stress-sensing transcriptional regulators rather than mutation. The results with other bacterial species have interesting implications for diversification of *S. pyogenes* in the human host.

We observed here that survival gave polyclonal populations in two different in vitro systems, as seen by diversity in metabolism as well as virulence factor expression. We found that these changes led to attenuation in virulence in most survivor strains, but there was no direct association between attenuation and virulence factor expression profile; however, growth in human blood correlates directly with *emm* transcription. The phenotypic and transcriptional changes observed were most likely due to random mutation followed by environmental selection. Lastly, we found that MLST did not change in the survivor strains.

Although it is well documented that *S. pyogenes* clinical isolates are diverse, the in vivo niche that allows for generation of diversity is still unknown. During active infection, such as bacteremia, growth is rapid, there is significant competition from other bacteria in the environment, and the immune system is active. Based on our results, we propose that CS101 could invade eukaryotic cells where the bacteria would be protected from antibiotics such as penicillin and also protected from the immune response. Under these conditions CS101 could mutate into a population of diverse strains which retain their MLST type, but have altered virulence and metabolic properties giving rise to the kind of clinical strain diversification that has been observed by Musser and co-workers when they sequenced sequentially-isolated epidemic strains (Beres *et al.*, 2010).

*S. pyogenes* are not typically intracellular pathogens, so the intracellular environment may be nutrient limiting and cause oxidative stress. These conditions may trigger stress-

induced mutations leading to random mutation. Some mutations may lead to phenotypic changes in the bacteria, and environmental factors within the eukaryotic cell may then select for a polyclonal population with alterations in metabolic properties. Among these polyclonal strains, some strains may be more adapted at continuing to be carried. Other strains may then arise with alterations in virulence properties that could reemerge to cause acute or invasive disease.

## REFERENCES CITED

- Abbot, E.L., Smith, W.D., Siou, G.P., Chiriboga, C., Smith, R.J., Wilson, J.A., *et al* (2007) Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. *Cell Microbiol.* 9: 1822-1833.
- Akiyama, H., Morizane, S., Yamasaki, O., Oono, T. and Iwatsuki, K. (2003) Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. *J Dermatol Sci.* 32: 193-199.
- Almengor, A.C., Kinkel, T.L., Day, S.J., and McIver, K.S. (2007) The catabolite control protein CcpA binds to Pmga and influences expression of the virulence regulator Mga in the Group A streptococcus. *J Bacteriol.* 189: 8405-8416.
- Aziz, R.K., Kansal, R., Aronow, B.J., Taylor, W.L., Rowe, S.L., Kubal, M., *et al* (2010) Microevolution of group A streptococci in vivo: capturing regulatory networks engaged in sociomicrobiology, niche adaptation, and hypervirulence. *PLoS One.* 5: e9798.
- Baldassarri, L., Creti, R., Recchia, S., Imperi, M., Facinelli, B., Giovanetti, E., *et al* (2006) Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. *J Clin Microbiol.* 44: 2721-2727.
- Banks, D.J., Beres, S.B. and Musser, J.M. (2002) The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence. *Trends Microbiol.* 10: 515-521.
- Banks, D.J., Porcella, S.F., Barbian, K.D., Beres, S.B., Philips, L.E., Voyich, J.M., *et al* (2004) Progress toward characterization of the group A *Streptococcus* metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain. *J Infect Dis.* 190: 727-738.
- Bates, D.M., von Eiff, C., McNamara, P.J., Peters, G., Yeaman, M.R., Bayer, A.S. and Proctor, R.A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. *J Infect Dis.* 187: 1654-1661.
- Beckert, S., Kreikemeyer, B. and Podbielski, A. (2001) Group A streptococcal *rofA* gene is involved in the control of several virulence genes and eukaryotic cell attachment and internalization. *Infect Immun.* 69: 534-537.
- Beres, S.B. and Musser, J.M. (2007) Contribution of exogenous genetic elements to the group A *Streptococcus* metagenome. *PLoS One.* 2: e800.
- Beres, S.B., Richter, E.W., Nagiec, M.J., Sumbly, P., Porcella, S.F., DeLeo, F.R. and Musser, J.M. (2006) Molecular genetic anatomy of inter- and intraserotype variation in the human bacterial pathogen group A *Streptococcus*. *Proc Natl Acad Sci U S A.* 103: 7059-7064.

- Beres, S.B., Sylva, G.L., Barbian, K.D., Lei, B., Hoff, J.S., Mammarella, N.D., *et al* (2002) Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc Natl Acad Sci U S A.* 99: 10078-10083.
- Beres, S.B., Carroll, R.K., Shea, P.R., Sitkiewicz, I., Martinez-Gutierrez, J.C., Low, D.E., *et al* (2010) Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. *Proc Natl Acad Sci U S A.* 107: 4371-4376.
- Bharathi, R.S., Agarwal, A., Singh, K., Gambhir, R.P., Mohan, R. and Chaudhry, R. (2009) Necrotizing streptococcal myositis. *ANZ J Surg.* 79: 296-297.
- Bisno, A.L. and Stevens, D.L. (1996) Streptococcal infections of skin and soft tissues. *N Engl J Med.* 334: 240-245.
- Bisno, A.L., Brito, M.O. and Collins, C.M. (2003) Molecular basis of group A streptococcal virulence. *Lancet Infect Dis.* 3: 191-200.
- Biswas, I. and Scott, J.R. (2003) Identification of rocA, a positive regulator of covR expression in the group A streptococcus. *J Bacteriol.* 185: 3081-90.
- Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R., Rumke, H.C., *et al* (2004) Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet.* 363: 1871-1872.
- Boyle, M.D., Hawlitzky, J., Raeder, R. and Podbielski, A. (1994) Analysis of genes encoding two unique type IIa immunoglobulin G-binding proteins expressed by a single group A streptococcal isolate. *Infect Immun.* 62: 1336-1347.
- Boyle, M.D., Raeder, R., Flosdorff, A. and Podbielski, A. (1998) Role of *emm* and *mrp* genes in the virulence of group A streptococcal isolate 64/14 in a mouse model of skin infection. *J Infect Dis.* 177: 991-997.
- Brenot, A., King, K.Y. and Caparon, M.G. (2005) The PerR regulon in peroxide resistance and virulence of *Streptococcus pyogenes*. *Mol Microbiol.* 55: 221-234.
- Bricker, A.L., Carey, V.J. and Wessels, M.R. (2005) Role of NADase in virulence in experimental invasive group A streptococcal infection. *Infect Immun.* 73: 6562-6566.
- Bricker, A.L., Cywes, C., Ashbaugh, C.D. and Wessels, M.R. (2002) NAD<sup>+</sup>-glycohydrolase acts as an intracellular toxin to enhance the extracellular survival of group A streptococci. *Mol Microbiol.* 44: 257-269.
- Buchmeier, N., Blanc-Potard, A., Ehrt, S., Piddington, D., Riley, L. and Groisman, E.A. (2000) A parallel intraphagosomal survival strategy shared by *Mycobacterium tuberculosis* and *Salmonella enterica*. *Mol Microbiol.* 35: 1375-1382.
- Burrus, V. and Waldor, M.K. (2004) Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol.* 155: 376-386.
- Byrne, J.L., Aagaard-Tillery, K.M., Johnson, J.L., Wright, L.J. and Silver, R.M. (2009) Group A streptococcal puerperal sepsis: initial characterization of virulence factors in association with clinical parameters. *J Reprod Immunol.* 82: 74-83.

- Carapetis, J.R., Steer, A.C., Mulholland, E.K. and Weber, M. (2005) The global burden of group A streptococcal diseases. *Lancet Infect Dis.* 5: 685-694.
- Carrico, J.A., Silva-Costa, C., Melo-Cristino, J., Pinto, F.R., de Lencastre, H., Almeida, J.S. and Ramirez, M. (2006) Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus pyogenes*. *J Clin Microbiol.* 44: 2524-2532.
- Chang, C.W., Tsai, W.H., Chuang, W.J., Lin, Y.S., Wu, J.J., Liu, C.C., *et al* (2007) The fate of SPE B after internalization and its implication in SPEB-induced apoptosis. *J Biomed Sci.* 14: 419-427.
- Chaussee, M.A., Callegari, E.A. and Chaussee, M.S. (2004) Rgg regulates growth phase-dependent expression of proteins associated with secondary metabolism and stress in *Streptococcus pyogenes*. *J Bacteriol.* 186: 7091-7099.
- Chaussee, M.S., Ajdic, D. and Ferretti, J.J. (1999) The rgg gene of *Streptococcus pyogenes* NZ131 positively influences extracellular SPE B production. *Infect Immun.* 67: 1715-1722.
- Chaussee, M.S., Cole, R.L. and van Putten, J.P. (2000) Streptococcal erythrogenic toxin B abrogates fibronectin-dependent internalization of *Streptococcus pyogenes* by cultured mammalian cells. *Infect Immun.* 68: 3226-3232.
- Chaussee, M.S., Liu, J., Stevens, D.L. and Ferretti, J.J. (1996) Genetic and phenotypic diversity among isolates of *Streptococcus pyogenes* from invasive infections. *J Infect Dis.* 173: 901-908.
- Chaussee, M.S., Somerville, G.A., Reitzer, L. and Musser, J.M. (2003) Rgg coordinates virulence factor synthesis and metabolism in *Streptococcus pyogenes*. *J Bacteriol.* 185: 6016-6024.
- Chiang-Ni, C. and Wu, J.J. (2008) Effects of streptococcal pyrogenic exotoxin B on pathogenesis of *Streptococcus pyogenes*. *J Formos Med Assoc.* 107: 677-685.
- Cho, K.H. and Caparon, M.G. (2005) Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. *Mol Microbiol.* 57: 1545-1556.
- Chuard, C., Vaudaux, P.E., Proctor, R.A. and Lew, D.P. (1997) Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. *J Antimicrob Chemother.* 39: 603-608.
- Churchward, G. (2007) The two faces of Janus: virulence gene regulation by CovR/S in group A streptococci. *Mol Microbiol.* 64: 34-41.
- Churchward, G., Bates, C., Gusa, A.A., Stringer, V. and Scott, J.R. (2009) Regulation of streptokinase expression by CovR/S in *Streptococcus pyogenes*: CovR acts through a single high-affinity binding site. *Microbiology.* 155: 566-575.
- Conley, J., Olson, M.E., Cook, L.S., Ceri, H., Phan, V. and Davies, H.D. (2003) Biofilm formation by group a streptococci: is there a relationship with treatment failure? *J Clin Microbiol.* 41: 4043-4048.

- Cook, A.R. (1976) A chemically-defined medium for the growth of a ureolytic strain of *Streptococcus faecium*. *J Gen Microbiol.* 97: 235-240.
- Cortes, G. and Wessels, M.R. (2009) Inhibition of dendritic cell maturation by group A Streptococcus. *J Infect Dis.* 200: 1152-1161.
- Costalonga, M., Cleary, P.P., Fischer, L.A. and Zhao, Z. (2009) Intranasal bacteria induce Th1 but not Treg or Th2. *Mucosal Immunol.* 2: 85-95.
- Courtney, H.S., Ofek, I., Penfound, T., Nizet, V., Pence, M.A., Kreikemeyer, B., *et al* (2009) Relationship between expression of the family of M proteins and lipoteichoic acid to hydrophobicity and biofilm formation in *Streptococcus pyogenes*. *PLoS One.* 4: e4166.
- Crack, J.C., Le Brun, N.E., Thomson, A.J., Green, J. and Jervis, A.J. (2008) Reactions of nitric oxide and oxygen with the regulator of fumarate and nitrate reduction, a global transcriptional regulator, during anaerobic growth of *Escherichia coli*. *Methods Enzymol.* 437: 191-209.
- Cue, D., Dombek, P.E., Lam, H. and Cleary, P.P. (1998) *Streptococcus pyogenes* serotype M1 encodes multiple pathways for entry into human epithelial cells. *Infect Immun.* 66: 4593-4601.
- Cue, D., Southern, S.O., Southern, P.J., Prabhakar, J., Lorelli, W., Smallheer, J.M., *et al* (2000) A nonpeptide integrin antagonist can inhibit epithelial cell ingestion of *Streptococcus pyogenes* by blocking formation of integrin alpha 5beta 1-fibronectin-M1 protein complexes. *Proc Natl Acad Sci U S A.* 97: 2858-2863.
- Cunningham, M.W. (2000) Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev.* 13: 470-511.
- Dale, J.B., Penfound, T., Chiang, E.Y., Long, V., Shulman, S.T. and Beall, B. (2005) Multivalent group A streptococcal vaccine elicits bactericidal antibodies against variant M subtypes. *Clin Diagn Lab Immunol.* 12: 833-836.
- Dalton, T.L., Hobb, R.I. and Scott, J.R. (2006) Analysis of the role of CovR and CovS in the dissemination of *Streptococcus pyogenes* in invasive skin disease. *Microb Pathog.* 40: 221-227.
- Davies, J.C. (2002) *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev.* 3: 128-134.
- Desai M, T.A., Efstratiou A, George R, Clewley J, Stanley J (1998) Extensive genetic diversity among clinical isolates of *Streptococcus pyogenes* serotype M5. *Microbiology.* 144: 629-637.
- Deutscher, J., Herro, R., Bourand, A., Mijakovic, I. and Poncet, S. (2005) P-Ser-HPr--a link between carbon metabolism and the virulence of some pathogenic bacteria. *Biochim Biophys Acta.* 1754: 118-125.
- Dicuonzo, G., Fiscarelli, E., Gherardi, G., Lorino, G., Battistoni, F., Landi, S., *et al* (2002) Erythromycin-resistant pharyngeal isolates of *Streptococcus pyogenes* recovered in Italy. *Antimicrob Agents Chemother.* 46: 3987-3990.

- Doern, C.D., Roberts, A.L., Hong, W., Nelson, J., Lukomski, S., Swords, W.E. and Reid, S.D. (2009) Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology*. 155: 46-52.
- Domingue, G.J., Sr. and Woody, H.B. (1997) Bacterial persistence and expression of disease. *Clin Microbiol Rev*. 10: 320-344.
- Dong, Y., Chen, Y.Y. and Burne, R.A. (2004) Control of expression of the arginine deiminase operon of *Streptococcus gordonii* by CcpA and Flp. *J Bacteriol*. 186: 2511-2514.
- Dorer, M.S., Talarico, S. and Salama, N.R. (2009) *Helicobacter pylori*'s unconventional role in health and disease. *PLoS Pathog*. 5: e1000544.
- Drake, J.W., Charlesworth, B., Charlesworth, D. and Crow, J.F. (1998) Rates of spontaneous mutation. *Genetics*. 148: 1667-1686.
- Ehrlich, G.D., Hu, F.Z., Shen, K., Stoodley, P. and Post, J.C. (2005) Bacterial plurality as a general mechanism driving persistence in chronic infections. *Clin Orthop Relat Res*: 20-24.
- Engleberg, N.C., Heath, A., Miller, A., Rivera, C. and DiRita, V.J. (2001) Spontaneous mutations in the CsrRS two-component regulatory system of *Streptococcus pyogenes* result in enhanced virulence in a murine model of skin and soft tissue infection. *J Infect Dis*. 183: 1043-1054.
- Enright, M.C., Spratt, B.G., Kalia, A., Cross, J.H. and Bessen, D.E. (2001) Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. *Infect Immun*. 69: 2416-2427.
- Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D. and Schobert, M. (2004) Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J Bacteriol*. 186: 4596-4604.
- Eylert, E., Schar, J., Mertins, S., Stoll, R., Bacher, A., Goebel, W. and Eisenreich, W. (2008) Carbon metabolism of *Listeria monocytogenes* growing inside macrophages. *Mol Microbiol*. 69: 1008-1017.
- Fernie-King, B.A., Seilly, D.J. and Lachmann, P.J. (2006) Inhibition of antimicrobial peptides by group A streptococci: SIC and DRS. *Biochem Soc Trans*. 34: 273-275.
- Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., *et al* (2001) Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A*. 98: 4658-4663.
- Fiedler, T., Kreikemeyer, B., Sugareva, V., Redanz, S., Arlt, R., Standar, K. and Podbielski, A. (2010) Impact of the *Streptococcus pyogenes* Mga regulator on human matrix protein binding and interaction with eukaryotic cells. *Int J Med Microbiol*. 300: 248-258.
- Fitoussi, F., Cohen, R., Brami, G., Doit, C., Brahim, N., de la Rocque, F. and Bingen, E. (1997) Molecular DNA analysis for differentiation of persistence or relapse from

- recurrence in treatment failure of *Streptococcus pyogenes* pharyngitis. *Eur J Clin Microbiol Infect Dis.* 16: 233-237.
- Galhardo, R.S., Hastings, P.J. and Rosenberg, S.M. (2007) Mutation as a stress response and the regulation of evolvability. *Crit Rev Biochem Mol Biol.* 42: 399-435.
- Garzoni, C. and Kelley, W.L. (2009) *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol.* 17: 59-65.
- Gleich-Theurer, U., Aymanns, S., Haas, G., Mauerer, S., Vogt, J. and Spellerberg, B. (2009) Human serum induces streptococcal c5a peptidase expression. *Infect Immun.* 77: 3817-3825.
- Goetz, M., Bubert, A., Wang, G., Chico-Calero, I., Vazquez-Boland, J.A., Beck, M., *et al* (2001) Microinjection and growth of bacteria in the cytosol of mammalian host cells. *Proc Natl Acad Sci U S A.* 98: 12221-12226.
- Goupil-Feuillerat, N., Coccain-Bousquet, M., Godon, J.J., Ehrlich, S.D. and Renault, P. (1997) Dual role of alpha-acetolactate decarboxylase in *Lactococcus lactis* subsp. *lactis*. *J Bacteriol.* 179: 6285-6293.
- Graham, M.R., Smoot, L.M., Migliaccio, C.A., Virtaneva, K., Sturdevant, D.E., Porcella, S.F., *et al* (2002) Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. *Proc Natl Acad Sci U S A.* 99: 13855-13860.
- Granok, A.B., Parsonage, D., Ross, R.P. and Caparon, M.G. (2000) The RofA binding site in *Streptococcus pyogenes* is utilized in multiple transcriptional pathways. *J Bacteriol.* 182: 1529-1540.
- Greco, R., De Martino, L., Donnarumma, G., Conte, M.P., Seganti, L. and Valenti, P. (1995) Invasion of cultured human cells by *Streptococcus pyogenes*. *Res Microbiol.* 146: 551-560.
- Green, N.M., Zhang, S., Porcella, S.F., Nagiec, M.J., Barbian, K.D., Beres, S.B., *et al* (2005) Genome sequence of a serotype M28 strain of group a streptococcus: potential new insights into puerperal sepsis and bacterial disease specificity. *J Infect Dis.* 192: 760-770.
- Guilherme, L. and Kalil, J. (2007) Rheumatic fever: from innate to acquired immune response. *Ann N Y Acad Sci.* 1107: 426-433.
- Guilherme, L., Fae, K.C., Higa, F., Chaves, L., Oshiro, S.E., Freschi de Barros, S., *et al* (2006) Towards a vaccine against rheumatic fever. *Clin Dev Immunol.* 13: 125-132.
- Guilherme, L., Postol, E., Freschi de Barros, S., Higa, F., Alencar, R., Lastre, M., *et al* (2009) A vaccine against *S. pyogenes*: Design and experimental immune response. *Methods.* 49: 316-321.
- Haanes, E.J. and Cleary, P.P. (1989) Identification of a divergent M protein gene and an M protein-related gene family in *Streptococcus pyogenes* serotype 49. *J Bacteriol.* 171: 6397-6408.

- Haanes, E.J., Heath, D.G. and Cleary, P.P. (1992) Architecture of the vir regulons of group A streptococci parallels opacity factor phenotype and M protein class. *J Bacteriol.* 174: 4967-4976.
- Hahn, R.G., Knox, L.M. and Forman, T.A. (2005) Evaluation of poststreptococcal illness. *Am Fam Physician.* 71: 1949-1954.
- Hall, B.G., Ehrlich, G.D. and Hu, F.Z. (2010) Pan-genome analysis provides much higher strain typing resolution than multi-locus sequence typing. *Microbiology.* 156: 1060-1068.
- Hiller, N.L., Janto, B., Hogg, J.S., Boissy, R., Yu, S., Powell, E., *et al* (2007) Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome. *J Bacteriol.* 189: 8186-8195.
- Hoe, N.P., Fullerton, K.E., Liu, M., Peters, J.E., Gackstetter, G.D., Adams, G.J. and Musser, J.M. (2003) Molecular genetic analysis of 675 group A streptococcus isolates collected in a carrier study at Lackland Air Force Base, San Antonio, Texas. *J Infect Dis.* 188: 818-827.
- Hondorp, E.R. and McIver, K.S. (2007) The Mga virulence regulon: infection where the grass is greener. *Mol Microbiol.* 66: 1056-1065.
- Honer zu Bentrup, K. and Russell, D.G. (2001) Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol.* 9: 597-605.
- Hu, M.C., Walls, M.A., Stroop, S.D., Reddish, M.A., Beall, B. and Dale, J.B. (2002) Immunogenicity of a 26-valent group A streptococcal vaccine. *Infect Immun.* 70: 2171-2177.
- Huang, Y.C., Huang, Y.C., Chiu, C.H., Chang, L.Y., Leu, H.S. and Lin, T.Y. (2001) Characteristics of group A streptococcal bacteremia with comparison between children and adults. *J Microbiol Immunol Infect.* 34: 195-200.
- Israel, D.A., Salama, N., Krishna, U., Rieger, U.M., Atherton, J.C., Falkow, S. and Peek, R.M., Jr. (2001) *Helicobacter pylori* genetic diversity within the gastric niche of a single human host. *Proc Natl Acad Sci U S A.* 98: 14625-14630.
- Jadoun, J., Eyal, O. and Sela, S. (2002) Role of CsrR, hyaluronic acid, and SpeB in the internalization of *Streptococcus pyogenes* M type 3 strain by epithelial cells. *Infect Immun.* 70: 462-469.
- Johnson, D.R., Stevens, D.L. and Kaplan, E.L. (1992) Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. *J Infect Dis.* 166: 374-382.
- Jonsson, I.M., von Eiff, C., Proctor, R.A., Peters, G., Ryden, C. and Tarkowski, A. (2003) Virulence of a hemB mutant displaying the phenotype of a *Staphylococcus aureus* small colony variant in a murine model of septic arthritis. *Microb Pathog.* 34: 73-79.
- Kagan, G., Vulfovitch, Y., Gusman, B. and Raskova, T. (1976) Persistence and pathological effect of streptococcal L-forms in vivo. *INSERM.* 65: 247-258.

- Kansal, R.G., Datta, V., Aziz, R.K., Abdeltawab, N.F., Rowe, S. and Kotb, M. (2010) Dissection of the molecular basis for hypervirulence of an in vivo-selected phenotype of the widely disseminated MIT1 strain of group A Streptococcus bacteria. *J Infect Dis.* 201: 855-865.
- Kaplan, E.L., Chhatwal, G.S. and Rohde, M. (2006) Reduced ability of penicillin to eradicate ingested group A streptococci from epithelial cells: clinical and pathogenetic implications. *Clin Infect Dis.* 43: 1398-1406.
- Kapur, V., Topouzis, S., Majesky, M.W., Li, L.L., Hamrick, M.R., Hamill, R.J., *et al* (1993) A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb Pathog.* 15: 327-346.
- Kietzman, C.C. and Caparon, M.G. (2010) CcpA and LacD.1 affect temporal regulation of *Streptococcus pyogenes* virulence genes. *Infect Immun.* 78: 241-252.
- Kihlberg, B.M., Cooney, J., Caparon, M.G., Olsen, A. and Bjorck, L. (1995) Biological properties of a *Streptococcus pyogenes* mutant generated by Tn916 insertion in *mga*. *Microb Pathog.* 19: 299-315.
- Klenk, M., Nakata, M., Podbielski, A., Skupin, B., Schroten, H. and Kreikemeyer, B. (2007) *Streptococcus pyogenes* serotype-dependent and independent changes in infected HEp-2 epithelial cells. *Isme J.* 1: 678-692.
- Kreikemeyer, B., McIver, K.S. and Podbielski, A. (2003) Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. *Trends Microbiol.* 11: 224-232.
- Kreikemeyer, B., Klenk, M. and Podbielski, A. (2004) The intracellular status of *Streptococcus pyogenes*: role of extracellular matrix-binding proteins and their regulation. *Int J Med Microbiol.* 294: 177-188.
- Kreikemeyer, B., Beckert, S., Braun-Kiewnick, A. and Podbielski, A. (2002) Group A streptococcal RofA-type global regulators exhibit a strain-specific genomic presence and regulation pattern. *Microbiology.* 148: 1501-1511.
- Kreikemeyer, B., Oehmcke, S., Nakata, M., Hoffrogge, R. and Podbielski, A. (2004) *Streptococcus pyogenes* fibronectin-binding protein F2: expression profile, binding characteristics, and impact on eukaryotic cell interactions. *J Biol Chem.* 279: 15850-15859.
- Kumari, S., Beatty, C.M., Browning, D.F., Busby, S.J., Simel, E.J., Hovel-Miner, G. and Wolfe, A.J. (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol.* 182: 4173-4179.
- Lancefield, R.C. (1957) Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. *J Exp Med.* 106: 525-544.
- Lang, M.M. and Towers, C. (2001) Identifying poststreptococcal glomerulonephritis. *Nurse Pract.* 26: 34, 37-42, 44-37; quiz 48-39.
- LaPenta, D., Rubens, C., Chi, E. and Cleary, P.P. (1994) Group A streptococci efficiently invade human respiratory epithelial cells. *Proc Natl Acad Sci U S A.* 91: 12115-12119.

- Lappin, E. and Ferguson, A.J. (2009) Gram-positive toxic shock syndromes. *Lancet Infect Dis.* 9: 281-290.
- Lassalle, S., Hofman, V., Butori, C., Sicard, D. and Hofman, P. (2005) [Histological diagnosis of lung bacterial necrotizing infections: a study of four fulminant cases]. *Ann Pathol.* 25: 349-356.
- Leday, T.V., Gold, K.M., Kinkel, T.L., Roberts, S.A., Scott, J.R., and McIver, K.S. (2008) TrxR, a new CovR-repressed response regulator that activates the Mga virulence regulon in group A Streptococcus. *Infect Immun.* 76: 4659-4668.
- Lee, J.L., Naguwa, S.M., Cheema, G.S. and Gershwin, M.E. (2009) Acute rheumatic fever and its consequences: a persistent threat to developing nations in the 21st century. *Autoimmun Rev.* 9: 117-123.
- Lefebvre, T. and Stanhope, M.J. (2007) Evolution of the core and pan-genome of Streptococcus: positive selection, recombination, and genome composition. *Genome Biol.* 8: R71.
- Lembke, C., Podbielski, A., Hidalgo-Grass, C., Jonas, L., Hanski, E. and Kreikemeyer, B. (2006) Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. *Appl Environ Microbiol.* 72: 2864-2875.
- Lennon, D. (2004) Acute rheumatic fever in children: recognition and treatment. *Paediatr Drugs.* 6: 363-373.
- Leon, O. and Panos, C. (1976) Adaptation of an osmotically fragile L-form of *Streptococcus pyogenes* to physiological osmotic conditions and its ability to destroy human heart cells in tissue culture. *Infect Immun.* 13: 252-262.
- Leonard, B.A., Woischnik, M. and Podbielski, A. (1998) Production of stabilized virulence factor-negative variants by group A streptococci during stationary phase. *Infect Immun.* 66: 3841-3847.
- Leung, A.K. and Kellner, J.D. (2004) Group A beta-hemolytic streptococcal pharyngitis in children. *Adv Ther.* 21: 277-287.
- Leung, K.Y. and Finlay, B.B. (1991) Intracellular replication is essential for the virulence of *Salmonella typhimurium*. *Proc Natl Acad Sci U S A.* 88: 11470-11474.
- Levdikov, V.M., Blagova, E., Joseph, P., Sonenshein, A.L. and Wilkinson, A.J. (2006) The structure of CodY, a GTP- and isoleucine-responsive regulator of stationary phase and virulence in gram-positive bacteria. *J Biol Chem.* 281: 11366-11373.
- Levin, J.C. and Wessels, M.R. (1998) Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. *Mol Microbiol.* 30: 209-219.
- Lloyd-Evans, N., O'Dempsey, T.J., Baldeh, I., Secka, O., Demba, E., Todd, J.E., *et al* (1996) Nasopharyngeal carriage of pneumococci in Gambian children and in their families. *Pediatr Infect Dis J.* 15: 866-871.
- Loughman, J.A. and Caparon, M. (2006) Regulation of SpeB in *Streptococcus pyogenes* by pH and NaCl: a model for in vivo gene expression. *J Bacteriol.* 188: 399-408.

- Luo, F., Lizano, S., Banik, S., Zhang, H. and Bessen, D.E. (2008) Role of Mga in group A streptococcal infection at the skin epithelium. *Microb Pathog.* 45: 217-224.
- Ma, Y., Bryant, A.E., Salmi, D.B., McIndoo, E. and Stevens, D.L. (2009) *vfr*, a novel locus affecting cysteine protease production in *Streptococcus pyogenes*. *J Bacteriol.* 191: 3189-3194.
- Madden, J.C., Ruiz, N. and Caparon, M. (2001) Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. *Cell.* 104: 143-152.
- Malke, H., Steiner, K., McShan, W.M. and Ferretti, J.J. (2006) Linking the nutritional status of *Streptococcus pyogenes* to alteration of transcriptional gene expression: the action of CodY and RelA. *Int J Med Microbiol.* 296: 259-275.
- Manetti, A.G., Zingaretti, C., Falugi, F., Capo, S., Bombaci, M., Bagnoli, F., *et al* (2007) *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. *Mol Microbiol.* 64: 968-983.
- Marouni, M.J. and Sela, S. (2004) Fate of *Streptococcus pyogenes* and epithelial cells following internalization. *J Med Microbiol.* 53: 1-7.
- Martin, J.M., Barbadora, K.A., Wald, E.R. and Green, M. (2003) Classification of M nontypeable group A streptococcus with the use of field inversion gel electrophoresis. *Pediatr Pathol Mol Med.* 22: 303-309.
- McKinney, J.D., Honer zu Bentrup, K., Munoz-Elias, E.J., Miczak, A., Chen, B., Chan, W.T., *et al* (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature.* 406: 735-738.
- McMillan, D.J., Geffers, R., Buer, J., Vlamincx, B.J., Sriprakash, K.S. and Chhatwal, G.S. (2007) Variations in the distribution of genes encoding virulence and extracellular proteins in group A streptococcus are largely restricted to 11 genomic loci. *Microbes Infect.* 9: 259-270.
- McNeil, S.A., Halperin, S.A., Langley, J.M., Smith, B., Warren, A., Sharratt, G.P., *et al* (2005) Safety and immunogenicity of 26-valent group A streptococcus vaccine in healthy adult volunteers. *Clin Infect Dis.* 41: 1114-1122.
- McShan, W.M., Ferretti, J.J., Karasawa, T., Suvorov, A.N., Lin, S., Qin, B., *et al* (2008) Genome sequence of a nephritogenic and highly transformable M49 strain of *Streptococcus pyogenes*. *J Bacteriol.* 190: 7773-7785.
- Medaglini, D., Pozzi, G., King, T.P. and Fischetti, V.A. (1995) Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization. *Proc Natl Acad Sci U S A.* 92: 6868-6872.
- Medina, E., Goldmann, O., Toppel, A.W. and Chhatwal, G.S. (2003) Survival of *Streptococcus pyogenes* within host phagocytic cells: a pathogenic mechanism for persistence and systemic invasion. *J Infect Dis.* 187: 597-603.
- Michailova, L., Markova, N., Radoucheva, T., Stoitsova, S., Kussovski, V. and Jordanova, M. (2000) Atypical behaviour and survival of *Streptococcus pyogenes*

- L forms during intraperitoneal infection in rats. *FEMS Immunol Med Microbiol.* 28: 55-65.
- Miller, J.D. and Neely, M.N. (2004) Zebrafish as a model host for streptococcal pathogenesis. *Acta Trop.* 91: 53-68.
- Miyoshi-Akiyama, T., Takamatsu, D., Koyanagi, M., Zhao, J., Imanishi, K. and Uchiyama, T. (2005) Cytocidal effect of *Streptococcus pyogenes* on mouse neutrophils in vivo and the critical role of streptolysin S. *J Infect Dis.* 192: 107-116.
- Molinari, G. and Chhatwal, G.S. (1999) Streptococcal invasion. *Curr Opin Microbiol.* 2: 56-61.
- Molinari, G., Rohde, M., Guzman, C.A. and Chhatwal, G.S. (2000) Two distinct pathways for the invasion of *Streptococcus pyogenes* in non-phagocytic cells. *Cell Microbiol.* 2: 145-154.
- Molinari, G., Talay, S.R., Valentin-Weigand, P., Rohde, M. and Chhatwal, G.S. (1997) The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect Immun.* 65: 1357-1363.
- Morgan, S. and Buttaro, B. Intracellular persistence and microcolony formation of *Streptococcus pyogenes*. Submitted to *J Bacteriol.*
- Moscoso, M., Garcia, E. and Lopez, R. (2009) Pneumococcal biofilms. *Int Microbiol.* 12: 77-85.
- Moses, A.E., Wessels, M.R., Zalcman, K., Alberti, S., Natanson-Yaron, S., Menes, T. and Hanski, E. (1997) Relative contributions of hyaluronic acid capsule and M protein to virulence in a mucoid strain of the group A Streptococcus. *Infect Immun.* 65: 64-71.
- Musser, J.M. and Shelburne, S.A., 3rd (2009) A decade of molecular pathogenomic analysis of group A Streptococcus. *J Clin Invest.* 119: 2455-2463.
- Nakagawa, I., Kurokawa, K., Yamashita, A., Nakata, M., Tomiyasu, Y., Okahashi, N., et al (2003) Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. *Genome Res.* 13: 1042-1055.
- Nithyanand, P., Thenmozhi, R., Rathna, J. and Pandian, S.K. (2010) Inhibition of *Streptococcus pyogenes* biofilm formation by coral-associated actinomycetes. *Curr Microbiol.* 60: 454-460.
- Olive, C. (2007) Progress in M-protein-based subunit vaccines to prevent rheumatic fever and rheumatic heart disease. *Curr Opin Mol Ther.* 9: 25-34.
- Oliver, A., Canton, R., Campo, P., Baquero, F. and Blazquez, J. (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science.* 288: 1251-1254.
- Oliver, M.A., Garcia-Rey, C., Bosch, R. and Alberti, S. (2007) Evaluation of the ability of erythromycin-resistant and -susceptible pharyngeal group A Streptococcus

- isolates from Spain to enter and persist in human keratinocytes. *J Med Microbiol.* 56: 1485-1489.
- Olsen, R.J. and Musser, J.M. (2010) Molecular pathogenesis of necrotizing fasciitis. *Annu Rev Pathol.* 5: 1-31.
- Olsen, R.J., Sitkiewicz, I., Ayeras, A.A., Gonulal, V.E., Cantu, C., Beres, S.B., *et al* (2010) Decreased necrotizing fasciitis capacity caused by a single nucleotide mutation that alters a multiple gene virulence axis. *Proc Natl Acad Sci U S A.* 107: 888-893.
- Opdyke, J.A., Scott, J.R. and Moran, C.P., Jr. (2001) A secondary RNA polymerase sigma factor from *Streptococcus pyogenes*. *Mol Microbiol.* 42: 495-502.
- Opdyke, J.A., Scott, J.R. and Moran, C.P., Jr. (2003) Expression of the secondary sigma factor sigmaX in *Streptococcus pyogenes* is restricted at two levels. *J Bacteriol.* 185: 4291-4297.
- Osterlund, A. and Engstrand, L. (1995) DNA fingerprinting of *Streptococcus pyogenes* from patients with recurrent pharyngotonsillitis by means of random amplified Polymorphic DNA analysis. *Scand J Infect Dis.* 27: 119-121.
- Osterlund, A. and Engstrand, L. (1995) Intracellular penetration and survival of *Streptococcus pyogenes* in respiratory epithelial cells in vitro. *Acta Otolaryngol.* 115: 685-688.
- Osterlund, A. and Engstrand, L. (1997) An intracellular sanctuary for *Streptococcus pyogenes* in human tonsillar epithelium--studies of asymptomatic carriers and in vitro cultured biopsies. *Acta Otolaryngol.* 117: 883-888.
- Osterlund, A., Popa, R., Nikkila, T., Scheynius, A. and Engstrand, L. (1997) Intracellular reservoir of *Streptococcus pyogenes* in vivo: a possible explanation for recurrent pharyngotonsillitis. *Laryngoscope.* 107: 640-647.
- Park, H.S., Francis, K.P., Yu, J. and Cleary, P.P. (2003) Membranous cells in nasal-associated lymphoid tissue: a portal of entry for the respiratory mucosal pathogen group A streptococcus. *J Immunol.* 171: 2532-2537.
- Passali, D., Lauriello, M., Passali, G.C., Passali, F.M. and Bellussi, L. (2007) Group A streptococcus and its antibiotic resistance. *Acta Otorhinolaryngol Ital.* 27: 27-32.
- Pearson, T., Okinaka, R.T., Foster, J.T. and Keim, P. (2009) Phylogenetic understanding of clonal populations in an era of whole genome sequencing. *Infect Genet Evol.* 9: 1010-1019.
- Phelps, H.A., Runft, D.L. and Neely, M.N. (2009) Adult zebrafish model of streptococcal infection. *Curr Protoc Microbiol.* Chapter 9: Unit 9D 1.
- Pichichero, M.E. (2000) Recurrent and persistent otitis media. *Pediatr Infect Dis J.* 19: 911-916.
- Pichichero, M.E. and Casey, J.R. (2007) Systematic review of factors contributing to penicillin treatment failure in *Streptococcus pyogenes* pharyngitis. *Otolaryngol Head Neck Surg.* 137: 851-857.

- Podbielski, A. (2007) Flexible architecture of the *Streptococcus pyogenes* FCT genome region: finally the clue for understanding purulent skin diseases and long-term persistence? *J Bacteriol.* 189: 1181-1184.
- Podbielski, A., Beckert, S., Schattke, R., Leithauser, F., Lestin, F., Gossler, B. and Kreikemeyer, B. (2003) Epidemiology and virulence gene expression of intracellular group A streptococci in tonsils of recurrently infected adults. *Int J Med Microbiol.* 293: 179-190.
- Raeder, R. and Boyle, M.D. (1993) Association between expression of immunoglobulin G-binding proteins by group A streptococci and virulence in a mouse skin infection model. *Infect Immun.* 61: 1378-1384.
- Raeder, R. and Boyle, M.D. (1993) Association of type II immunoglobulin G-binding protein expression and survival of group A streptococci in human blood. *Infect Immun.* 61: 3696-3702.
- Raeder, R., Harokopakis, E., Hollingshead, S. and Boyle, M.D. (2000) Absence of SpeB production in virulent large capsular forms of group A streptococcal strain 64. *Infect Immun.* 68: 744-751.
- Raynaud, C., Papavinasasundaram, K.G., Speight, R.A., Springer, B., Sander, P., Bottger, E.C., *et al* (2002) The functions of OmpATb, a pore-forming protein of *Mycobacterium tuberculosis*. *Mol Microbiol.* 46: 191-201.
- Reid, S.D., Montgomery, A.G. and Musser, J.M. (2004) Identification of srv, a PrfA-like regulator of group A streptococcus that influences virulence. *Infect Immun.* 72: 1799-1803.
- Reid, S.D., Chaussee, M.S., Doern, C.D., Chaussee, M.A., Montgomery, A.G., Sturdevant, D.E. and Musser, J.M. (2006) Inactivation of the group A *Streptococcus* regulator srv results in chromosome wide reduction of transcript levels, and changes in extracellular levels of Sic and SpeB. *FEMS Immunol Med Microbiol.* 48: 283-292.
- Reis, K.J., Yarnall, M., Ayoub, E.M. and Boyle, M.D. (1984) Effect of mouse passage on Fc receptor expression by group A streptococci. *Scand J Immunol.* 20: 433-439.
- Rezcallah, M.S., Hodges, K., Gill, D.B., Atkinson, J.P., Wang, B. and Cleary, P.P. (2005) Engagement of CD46 and alpha5beta1 integrin by group A streptococci is required for efficient invasion of epithelial cells. *Cell Microbiol.* 7: 645-653.
- Rodriguez-Iturbe, B. and Batsford, S. (2007) Pathogenesis of poststreptococcal glomerulonephritis a century after Clemens von Pirquet. *Kidney Int.* 71: 1094-1104.
- Schwarz-Linek, U., Hook, M. and Potts, J.R. (2006) Fibronectin-binding proteins of gram-positive cocci. *Microbes Infect.* 8: 2291-2298.
- Scott, J., Thompson-Mayberry, P., Lahmamsi, S., King, C.J. and McShan, W.M. (2008) Phage-associated mutator phenotype in group A streptococcus. *J Bacteriol.* 190: 6290-6301.

- Scott, J.R., Guenther, P.C., Malone, L.M. and Fischetti, V.A. (1986) Conversion of an M- group A streptococcus to M+ by transfer of a plasmid containing an M6 gene. *J Exp Med.* 164: 1641-1651.
- Seggewiss, J., Becker, K., Kotte, O., Eisenacher, M., Yazdi, M.R., Fischer, A., *et al* (2006) Reporter metabolite analysis of transcriptional profiles of a *Staphylococcus aureus* strain with normal phenotype and its isogenic *hemB* mutant displaying the small-colony-variant phenotype. *J Bacteriol.* 188: 7765-7777.
- Sharma, V., Sharma, S., Hoener zu Bentrup, K., McKinney, J.D., Russell, D.G., Jacobs, W.R., Jr. and Sacchettini, J.C. (2000) Structure of isocitrate lyase, a persistence factor of *Mycobacterium tuberculosis*. *Nat Struct Biol.* 7: 663-668.
- Shaw, J.H. and Clewell, D.B. (1985) Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn917 in *Streptococcus faecalis*. *J Bacteriol.* 164: 782-796.
- Shelburne, S.A., 3rd, Granville, C., Tokuyama, M., Sitkiewicz, I., Patel, P. and Musser, J.M. (2005) Growth characteristics of and virulence factor production by group A Streptococcus during cultivation in human saliva. *Infect Immun.* 73: 4723-4731.
- Shen, K., Antalis, P., Gladitz, J., Sayeed, S., Ahmed, A., Yu, S., *et al* (2005) Identification, distribution, and expression of novel genes in 10 clinical isolates of nontypeable *Haemophilus influenzae*. *Infect Immun.* 73: 3479-3491.
- Shulman, S.T. (2009) Pediatric autoimmune neuropsychiatric disorders associated with streptococci (PANDAS): update. *Curr Opin Pediatr.* 21: 127-130.
- Sierig, G., Cywes, C., Wessels, M.R. and Ashbaugh, C.D. (2003) Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group a streptococci. *Infect Immun.* 71: 446-455.
- Simon, D. and Ferretti, J.J. (1991) Electrotransformation of *Streptococcus pyogenes* with plasmid and linear DNA. *FEMS Microbiol Lett.* 66: 219-224.
- Sladden, M.J. and Johnston, G.A. (2005) Current options for the treatment of impetigo in children. *Expert Opin Pharmacother.* 6: 2245-2256.
- Smith, T., Sledjeski, D. and Boyle, M. (2005) Selective biological pressure and expression of *S. pyogenes* genes: Lessons from a mouse model of skin infection. *Recent Research Developments in Microbiology.* 9: 183-212.
- Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., *et al* (2002) Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks. *Proc Natl Acad Sci U S A.* 99: 4668-4673.
- Sriskandan, S., Faulkner, L. and Hopkins, P. (2007) *Streptococcus pyogenes*: Insight into the function of the streptococcal superantigens. *Int J Biochem Cell Biol.* 39: 12-19.
- Staali, L., Morgelin, M., Bjorck, L. and Tapper, H. (2003) *Streptococcus pyogenes* expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils. *Cell Microbiol.* 5: 253-265.

- Starr, C.R. and Engleberg, N.C. (2006) Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus. *Infect Immun.* 74: 40-48.
- Steiner, K. and Malke, H. (2001) relA-Independent amino acid starvation response network of *Streptococcus pyogenes*. *J Bacteriol.* 183: 7354-7364.
- Stevens, D.L., Tanner, M.H., Winship, J., Swartz, R., Ries, K.M., Schlievert, P.M. and Kaplan, E. (1989) Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. *N Engl J Med.* 321: 1-7.
- Stewart, P.S. and Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet.* 358: 135-138.
- Stoker, B.A., Hoiseth, S.K., Smith, B.P. (1983) Aromatic-dependent "Salmonella sp." as a live vaccine in mice and calves. *Dev Biol Stand.* 53:47-54.
- Sumbly, P., Whitney, A.R., Graviss, E.A., DeLeo, F.R. and Musser, J.M. (2006) Genome-wide analysis of group a streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathog.* 2: e5.
- Sumbly, P., Barbian, K.D., Gardner, D.J., Whitney, A.R., Welty, D.M., Long, R.D., *et al* (2005) Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response. *Proc Natl Acad Sci U S A.* 102: 1679-1684.
- Sun, H., Wang, X., Degen, J.L. and Ginsburg, D. (2009) Reduced thrombin generation increases host susceptibility to group A streptococcal infection. *Blood.* 113: 1358-1364.
- Suvorov, A.N., Polyakova, E.M., McShan, W.M. and Ferretti, J.J. (2009) Bacteriophage content of M49 strains of *Streptococcus pyogenes*. *FEMS Microbiol Lett.* 294: 9-15.
- Takemura, N., Noiri, Y., Ehara, A., Kawahara, T., Noguchi, N. and Ebisu, S. (2004) Single species biofilm-forming ability of root canal isolates on gutta-percha points. *Eur J Oral Sci.* 112: 523-529.
- Talkington, D.F., Schwartz, B., Black, C.M., Todd, J.K., Elliott, J., Breiman, R.F. and Facklam, R.R. (1993) Association of phenotypic and genotypic characteristics of invasive *Streptococcus pyogenes* isolates with clinical components of streptococcal toxic shock syndrome. *Infect Immun.* 61: 3369-3374.
- Taylor, F.B., Jr., Bryant, A.E., Blick, K.E., Hack, E., Jansen, P.M., Kosanke, S.D. and Stevens, D.L. (1999) Staging of the baboon response to group A streptococci administered intramuscularly: a descriptive study of the clinical symptoms and clinical chemical response patterns. *Clin Infect Dis.* 29: 167-177.
- Timmer, A.M., Timmer, J.C., Pence, M.A., Hsu, L.C., Ghochani, M., Frey, T.G., *et al* (2009) Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. *J Biol Chem.* 284: 862-871.
- Trainor, V.C., Udy, R.K., Bremer, P.J. and Cook, G.M. (1999) Survival of *Streptococcus pyogenes* under stress and starvation. *FEMS Microbiol Lett.* 176: 421-428.

- Tsai, P.J., Lin, Y.S., Kuo, C.F., Lei, H.Y. and Wu, J.J. (1999) Group A Streptococcus induces apoptosis in human epithelial cells. *Infect Immun.* 67: 4334-4339.
- Tsai, P.J., Kuo, C.F., Lin, K.Y., Lin, Y.S., Lei, H.Y., Chen, F.F., *et al* (1998) Effect of group A streptococcal cysteine protease on invasion of epithelial cells. *Infect Immun.* 66: 1460-1466.
- Urban, C.F., Lourido, S. and Zychlinsky, A. (2006) How do microbes evade neutrophil killing? *Cell Microbiol.* 8: 1687-1696.
- Vandal, O.H., Pierini, L.M., Schnappinger, D., Nathan, C.F. and Ehrt, S. (2008) A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat Med.* 14: 849-854.
- Vincent, M.T., Celestin, N. and Hussain, A.N. (2004) Pharyngitis. *Am Fam Physician.* 69: 1465-1470.
- von Eiff, C. (2008) *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. *Int J Antimicrob Agents.* 31: 507-510.
- Voyich, J.M., Musser, J.M. and DeLeo, F.R. (2004) *Streptococcus pyogenes* and human neutrophils: a paradigm for evasion of innate host defense by bacterial pathogens. *Microbes Infect.* 6: 1117-1123.
- Vrentas, C.E., Gaal, T., Ross, W., Ebright, R.H. and Gourse, R.L. (2005) Response of RNA polymerase to ppGpp: requirement for the omega subunit and relief of this requirement by DksA. *Genes Dev.* 19: 2378-2387.
- Wang, B., Yurecko, R.S., Dedhar, S. and Cleary, P.P. (2006) Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. *Cell Microbiol.* 8: 257-266.
- Wannamaker, L.W. and Yasmineh, W. (1967) Streptococcal nucleases. I. Further studies on the A, B, and C enzymes. *J Exp Med.* 126: 475-496.
- Wannamaker, L.W., Hayes, B. and Yasmineh, W. (1967) Streptococcal nucleases. II. Characterization of DNase D. *J Exp Med.* 126: 497-508.
- Weiser, J.N. (2010) The pneumococcus: why a commensal misbehaves. *J Mol Med.* 88: 97-102.
- Wessels, M.R., Moses, A.E., Goldberg, J.B. and DiCesare, T.J. (1991) Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. *Proc Natl Acad Sci U S A.* 88: 8317-8321.
- Wessels, M.R., Goldberg, J.B., Moses, A.E. and DiCesare, T.J. (1994) Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci. *Infect Immun.* 62: 433-441.
- Wilson, L.A., Sawant, A.D. and Ahearn, D.G. (1991) Comparative efficacies of soft contact lens disinfectant solutions against microbial films in lens cases. *Arch Ophthalmol.* 109: 1155-1157.
- Wood, D.N., Chaussee, M.A., Chaussee, M.S. and Buttaro, B.A. (2005) Persistence of *Streptococcus pyogenes* in stationary-phase cultures. *J Bacteriol.* 187: 3319-3328.

- Wood, D.N., Weinstein, K.E., Podbielski, A., Kreikemeyer, B., Gaughan, J.P., Valentine, S. and Buttaro, B.A. (2009) Generation of metabolically diverse strains of *Streptococcus pyogenes* during survival in stationary phase. *J Bacteriol.* 191: 6242-6252.
- Yamada, T., Takahashi-Abbe, S. and Abbe, K. (1985) Effects of oxygen on pyruvate formate-lyase in situ and sugar metabolism of *Streptococcus mutans* and *Streptococcus sanguis*. *Infect Immun.* 47: 129-134.
- Yousefi Rad, A., Ayhan, H., Kisa, U. and Piskin, E. (1998) Adhesion of different bacterial strains to low-temperature plasma treated biomedical PVC catheter surfaces. *J Biomater Sci Polym Ed.* 9: 915-929.
- Zautner, A.E., Krause, M., Stropahl, G., Holtfreter, S., Frickmann, H., Maletzki, C., *et al* (2010) Intracellular persisting *Staphylococcus aureus* is the major pathogen in recurrent tonsillitis. *PLoS One.* 5: e9452.
- Zinser, E.R. and Kolter, R. (2004) *Escherichia coli* evolution during stationary phase. *Res Microbiol.* 155: 328-336.

**APPENDIX A**  
**MEDIA AND SOLUTIONS**

Blocking Buffer	
Boehringer Blocking Reagent	0.5 g
Buffer 1	50 ml
Dissolve by microwaving	
Cool to room temperature before use.	
Boehringer Wash Buffer	
Buffer 1	100 ml
Tween 20	300 $\mu$ l
Make immediately before use.	
Buffer 1 (5X)	
Maleic Acid	58 g
NaCl	43.8 g
NaOH	35 g
Water	to 1.0 L
Adjust pH to 7.5 with NaOH pellets	
Autoclave and store at room temperature.	
Buffer 3	
Tris Base	12.11 g
Mg <sub>2</sub> Cl <sub>2</sub> ·6H <sub>2</sub> O	10.17 g
Water	to 1.0 L
Adjust pH to 9.5 with HCl	
Autoclave and store at room temperature.	
DEPC Water	
DEPC	1 ml
Water	to 1000 ml
Shake well and allow to sit at least 1 hour.	
Autoclave and store at room temperature.	
DNA Lysis Buffer	
Tris	2.4 g
NaCl	11.7 g
0.5 M EDTA, pH 8.0	6.0 ml
Water	to 1000 ml
Autoclave, allow to cool and add the following:	
SDS	5.0 g
Store at room temperature.	
EDTA, 0.5 M, pH 8.0	

EDTA (Disodium salt)	186.12 g
Water	to 1000 ml
Add NaOH until solution reaches pH 8.0	
Autoclave and store at room temperature	
Loading Dye	
EDTA (pH 8.0)	2 ml
Bromophenol Blue	0.25 g
Glycerin	50 ml
DEPC Water	to 100 ml
Autoclave and store at room temperature.	
MOPS Buffer (10X)	
MOPS	20.93 g
NaAcetate	2.05 g
0.5M EDTA (pH 8.0)	10 ml
DEPC	1 ml
Water	to 500 ml
Autoclave and store at room temperature.	
Phosphate Buffered Saline (0.1 M)	
0.2 M $K_2HPO_4$	87.7 ml
0.2 M $KH_2PO_4$	12.3 ml
Water	to 200 ml
Autoclave and store at room temperature.	
Prehybridization Solution	
20X SSC	6 ml
50X Denhardt's (KD Medical, Columbia, MD)	2 ml
10% SDS	1 ml
Sheared Salmon Sperm DNA (10 mg/ml)*	200 $\mu$ l
Water	to 20 ml
*Salmon Sperm is denatured before use: 5 minutes boil, then 5 minutes ice.	
RNA Lysis Buffer	
Tris	1.21 g
EDTA	0.56 g
NaCl	5.84 g
Water	to 500 ml
DEPC	1 ml
Bring to pH 8.0	
Autoclave then add the following	
SDS	2.5 g
Store at room temperature.	
RNA Premix Solution	

10x MOPS	20 $\mu$ l
37% Formaldehyde	35 $\mu$ l
Formamide	100 $\mu$ l
DEPC H <sub>2</sub> O	345 $\mu$ l
Made immediately before use.	
<b>SDS, 10%</b>	
SDS	10.0 g
Water	to 100 ml
<b>Sodium Acetate, 3M</b>	
Sodium Acetate	24.6 g
Water	100 ml
pH to 5.5 with Glacial Acetic Acid	
Store at room temperature	
<b>SSC (20X)</b>	
NaCl	175.3 g
NaCitrate	88.2 g
Water	to 1000 ml
Store at room temperature.	
<b>Todd Hewitt Broth (THB)</b>	
THB (Difco)	30 g
Water	to 1000 ml
Autoclave and store at room temperature.	
Agar: Add 1.5% Agar (Difco) before autoclave and store at 4°C.	
<b>Tris-Acetate Buffer (TAE) (50X)</b>	
Tris Base Solution	242.0 g
Glacial Acetic Acid	57.1 g
EDTA, 0.5M, pH 8.0	100 ml
Water	to 1000 ml
Autoclave and store at room temperature.	
<b>Wash Buffer 1 (2X SSC, 0.5 % SDS)</b>	
20X SSC	50 ml
SDS	2.5g
Water	to 500 ml
<b>Wash Buffer 2 (2X SSC, 0.1 % SDS)</b>	
20X SSC	50 ml
SDS	0.5g
Water	to 500 ml
<b>Wash Buffer 3 (0.1X SSC, 0.1 % SDS)</b>	

20X SSC  
SDS  
Water

0.25 ml  
0.5g  
to 500 ml