DISSERTATION

Titel der Dissertation

Virulence factors in *Streptococcus pyogenes*: horizontal transfer and regulatory aspects

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

Verfasserin:	Zaid Ahmed Pirzada, Mag. rer.nat.
Matrikel-Nummer:	0448737
Dissertationsgebiet:	Genetik – Mikrobiologie (lt. Studienblatt: A 091 441)
Betreuerin:	Emmanuelle Charpentier, Ph.D.

Wien, am 04. March 2009

Dedicated to my parents

Anwer Pirzado

And

Taj Khatoon

It was their dream but unfortunately I lost them during this path

TABLE OF CONTENTS

1a.	**ABSTRACT**	6
1b.	**ZUSAMMENFASSUNG**	8
2.	**INTRODUCTION**	11
2.1.	*Streptococcus pyogenes* (Group A Streptococcus)	11
2.2	Classification of Streptococci	11
2.3	Pathogenesis of *S. pyogenes*	13
2.4	Virulence factors produced by *S. pyogenes*	15
2.4.1	Somatic virulence factors	17
2.4.2	Extracellular virulence factors	19
2.4.3	Regulation of virulence factor expression in *S. pyogenes*	22
2.4.3.1	Two component systems and response regulators	24
2.4.3.2	RNA regulators	29
2.4.3.3	Cell density-dependent regulation	30
2.5	Bacteriophages and horizontal transfer of virulence factors in *S. pyogenes*	34
2.6	RNA regulators	37
2.6.1	Regulatory RNAs: Definition and modes of action	37
2.6.2	Small RNAs in bacterial pathogenicity	39
2.6.3	Small RNAs in *S. pyogenes*	43
2.7	Riboswitches	46
2.7.1	Riboswitches: Definition and modes of action	47
2.7.2	Riboswitches in *S. pyogenes*	50
3	**AIMS OF THIS STUDY**	51
4	**RESULTS**	54

4.1	Project 1: Lysogenic transfer of Group A streptococcus superantigen gene among streptococci……..…	54
4.1.1	Summary.....	54
4.1.2	Manuscript……	56
4.2	Project 2: A bioinformatic screen reveals novel small non-coding RNAs in S*treptococcus pyogenes*…….……...…	67
4.2.1	Summary .....……	67
4.2.2	Manuscript……	69
4.2.3	Additional data…….……	71
4.3	Project 3: Functional analysis of the group A streptococcal *lux*S/AI-2 system in metabolism, adaptation to stress and interaction with host cells……..…	99
4.3.1	Summary .....……	99
4.3.2	Manuscript……	102
4.4	Project 4**:** Group A Streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4 and TLR9……...…	120
4.4.1	Summary .....…….....	120
4.1.	Manuscript……	122
5	**DISCUSSION**……..…	132
5.1	Project 1: Lysogenic transfer of Group A streptococcus superantigen gene among streptococci……..…	132
5.2	Project 2: Novel small RNAs in *S pyogenes*……	134
5.3	Project 3: Functional analysis of the group A streptococcal *lux*S/AI-2 system in metabolism, adaptation to stress and interaction with host cells……..…	138

5.4 Project 4**:** Group A Streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4 and TLR9……...… 141

6 REFERENCES……...… 144
7 ACKNOWLEDGEMENTS……..…… 160
8 CURRICULUM VITAE…… 161

1) ABSTRACT

During my thesis work, I have been interested in extending our understanding of the pathogenicity of the notorious human pathogen *Streptococcus pyogenes*, also called group A streptococcus (GAS). This thesis manuscript is divided into four parts: 1) role of superantigen-encoding lysogenic bacteriophages in the acquisition and spread of virulence genes among streptococci, 2) the identification of small RNAs with potential regulatory functions in GAS, 3) the role of the interspecies communication system *luxS*/AI-2 in GAS metabolism and survival under stress conditions and 4) the role of streptolysins in the innate immune responses of macrophages upon GAS infection.

1) Since the late 1980s, there has been a significant reemergence of severe forms of human diseases caused by GAS. One hypothesis to explain this phenomenon has focused on an enhanced virulence of the pathogen itself. In GAS, prophages encode one or more putative or established virulence factors including superantigens. The first part of my thesis describes the identification of an inducible prophage Φ149 encoding superantigen SSA in an M12 GAS clinical isolate. By lysogenic conversion the phage was transferable *ex-vivo* among GAS clinical isolates of various M serotypes (M1, M3, M5, M12, M19, M28 and M94) as well as of Group C *Streptococcus equisimilis* (GCSE). Our data indicate that horizontal transfer of lysogenic phage among GAS can occur across the M-type barrier and provide further support for the concept of interspecies lysogenic toxin-conversion. Therefore, lysogenic conversion would allow GAS to more efficiently adapt to the changing host conditions, thus potentially leading to fitter and more virulent clones.

2) Recent studies show that in bacteria small RNAs (sRNAs) play a significant role in the regulation of gene expression. However, information regarding sRNAs in *S. pyogenes* is rather limited. Thus, in the second part of my thesis, our aim was to identify novel sRNAs in *S. pyogenes*. For this purpose, we used an *in silico* approach focusing on the intergenic regions of the GAS M1 serotype genome. From 178 identified putative loci, 90 were further selected for analysis and 29 (32%) of these sRNAs showed expression under normal growth conditions. Expression of the candidates was verified in M1, M3, M5 and M49 serotypes, their 5' and 3' ends were mapped, their stability was determined and their *in silico* secondary structures were predicted. We found 5 riboswitches, 6 leader

elements, 4 functional RNAs, 8 T-boxes and 5 novel sRNAs. Two of the sRNA candidates residing in the 5' UTR of the riboflavin and tryptophan transporter genes seemed to control the transporter genes via transcriptional or translational attenuation mechanisms. This shows the importance of sRNAs in the control of the uptake of essential metabolites by regulating gene expression in bacteria. In addition, one sRNA SpyRNA049 identified in the intergenic region, was further analyzed for its potential role in GAS pathogenicity.

3) Previous studies showed that the interspecies *luxS*/AI-2 cell-cell communication system plays a role in metabolism and pathogenicity. In the third part of my thesis we aimed at understanding the role of the *luxS*/AI-2 system in *S. pyogenes* serotypes M1 and M19. Our results indicated a limited role of *luxS*/AI-2 in the activated methyl cycle metabolism. Survival assays with *luxS* mutants as well as *luxS* expression and AI-2 like activity assays under low acidic conditions demonstrated the involvement of *luxS*/AI-2 system in the adaptation of GAS to stress. Interaction analysis with host cells showed that the *luxS* mutants survived in epithelial cells and macrophages to a higher degree compared to the wild-type. Overall, our data show that the *luxS*/AI-2 system is not only involved in the regulation of virulence factor expression but also provides bacteria an advantage to survive in challenging host environments.

4) Bacteria are generally recognized by host immune cells by the pattern recognition receptors like toll-like receptors (TLRs). However, little is known about the recognition of GAS by macrophages. Data described in the fourth part of my thesis suggest that *S. pyogenes* is recognized by macrophages independently of the most commonly TLRs used to recognize gram-positive pathogens, TLR2, TLR4 and TLR9 and their combination. However, the signaling events observed in macrophages upon GAS infection were still dependent on the TLR-adaptor MyD88. Surprisingly, IFN production was independent of the GAS cytolysins, SLS and SLO, often required in other gram-positive bacteria. Overall our data indicate that GAS is recognized by a MyD88-dependent receptor other than those commonly used to detect bacteria and reveal a new type of bacterial recognition for the production of type I IFN.

1b) ZUSAMMENFASSUNG

Während meiner Dissertation lag mein Interesse in der Erweiterung des Verständnisses der Pathogenität des bekannten humanen Pathogens *Streptococcus pyogenes*, auch bekannt als Gruppe A Streptococcus (GAS). Dieses Manuskript ist in vier Teile unterteilt: 1) die Rolle von Superantigen-codierenden lysogenen Bakteriophagen in Erwerb und Verbreitung von Virulenzgenen in Streptococci, 2) der Identifikation von small RNAs mit potentiellen regulatorischen Funktionen in GAS, 3) der Rolle des interspezifischen Kommunikationssystems *luxS*/AI-2 in GAS Metabolismus und Überleben unter Stresskonditionen und 4) der Rolle von Streptolysinen in der angeborenen Immunantwort von Makrophagen auf GAS Infektion.

1) Seit den späten 1980er Jahren, war ein signifikanter Anstieg von schweren Formen durch GAS verursachter Krankheiten im Menschen zu beobachten. Eine Hypothese um dieses Phänomen zu erklären, beruht auf einer gesteigerten Virulenz des Pathogens selbst. In GAS codieren Prophagen für einen oder mehr putative oder bekannte Virulenzfaktoren, einschließlich Superantigenen. Im ersten Teil meiner Dissertation wird die Identifikation des induzierbaren Prophagen Φ149, welcher für das Superantigen SSA in einem klinischen Isolat von GAS des Serotyps M12 codiert, beschrieben. Durch lysogene Konversion war der Phage zwischen klinischen Isolaten von verschiedenen GAS M-Serotypen (M1, M3, M5, M12, M19, M28 und M94), als auch in Gruppe C *Streptococcus equisimilis* (GCSE), *ex-vivo* transferierbar. Unsere Ergebnisse zeigen, dass horizontaler Transfer von lysogenen Phagen zwischen GAS durch die M-Typ-Barriere möglich ist und unterstützen das Konzept der interspezifischen lysogenen Toxin-Konversion. Somit würde lysogene Konversion es GAS ermöglichen, sich effizienter an die wechselnden Bedingungen im Wirt anzupassen, was wiederum zu potentiell konkurrenzfähigeren, virulenteren Klonen führen würde.

2) Kürzlich erschienene Studien zeigen, dass small RNAs (sRNAs) in Bakterien eine signifikante Rolle in der Regulation der Genexpression spielen. Jedoch sind Informationen über die Rolle von sRNAs in *S. pyogenes* relativ begrenzt. Aus diesem Grund war unser Ziel im zweiten Abschnitt meiner Arbeit die Identifikation neuer sRNAs in *S. pyogenes*. Zu diesem Zweck wurde ein *in silico* Verfahren angewandt, fokussierend auf die intergenen Regionen des Genoms von M1-Serotyp GAS. Von 178 identifizierten

putativen Loci wurden 90 zur weiteren Analyse ausgewählt, wobei 29 (32%) dieser sRNAs unter normalen Wachstumsbedingungen exprimiert werden. Die Expression der Kandidaten wurde verifiziert in den Serotypen M1, M3, M5 und M49, ihre 5' und 3' Enden wurden determiniert, ihre Stabilität bestimmt und ihre *in silico* Sekundärstruktur wurde vorhergesagt. Gefunden wurden 5 Riboswitches, 6 leader elements, 4 funktionelle RNAs, 8 T-Boxen und 5 neue sRNAs. Zwei dieser sRNA Kandidaten befinden sich in der 5' UTR von Riboflavin- und Tryptophan- Transportergenen und scheinen diese Gene durch trankriptionelle oder translationelle Attenuierungsmechansimen zu kontrollieren. Das zeigt die Wichtigkeit von sRNAs in der Kontrolle der Aufnahme essentieller Metaboliten in Bakterien durch regulatorische Genexpression. Zusätzlich wurde eine in der intergenen Region identifizierte sRNA SpyRNA049 auf ihre potentielle Rolle in GAS-Pathogenität weiter analysiert.

3) Frühere Studien zeigten, dass das interspezifische Zell-Zell-Kommunikationssystem *luxS*/AI-2 eine Rolle in Metabolismus und Pathogenität spielt. Im dritten Abschnitt meiner Dissertation war das Ziel, die Rolle des *luxS*/AI-2 Systems in den *S. pyogenes* Serotypen M1 und M19 zu verstehen. Unsere Ergebnisse lassen auf eine limitierte Rolle von *luxS*/AI-2 im S-Adenosylmethioninzyklus schließen. Überlebensassays mit *luxS*-Mutanten sowie *luxS*-Expression und AI-2-ähnliche Aktivitätsassays im leicht sauren Milieu demonstrierten die Beteiligung des *luxS*/AI-2 Systems in der Stressadaptation von GAS. Interaktionsanalyse mit Wirtszellen zeigte, dass die *luxS*-Mutanten im Vergleich zum Wildtyp in höherem Ausmaß in Epithelzellen und Makrophagen überlebten. Alles in allem zeigen unsere Daten, dass das *luxS*/AI-2 System nicht nur in die Regulation der Virulenzfaktorexpression involviert ist, sondern auch für Bakterien einen Vorteil darstellt, in herausfordernden Wirtsumgebungen zu überleben.

4) Im Allgemeinen werden Bakterien von den Immunzellen des Wirts durch pattern recognition receptors, wie Toll-like receptors (TLRs), erkannt. Über die Erkennung von GAS durch Makrophagen ist jedoch wenig bekannt. Die Ergebnisse, die im vierten Teil meiner Dissertation beschrieben werden, lassen darauf schließen, dass Makrophagen *S. pyogenes* unabhängig von den TLRs die typisch für gram-positive Pathogene sind (TLR2, TLR4 und TLR9 sowie ihre Kombinationen), erkennen. Jedoch waren die in Makrophagen beobachteten Signalereignisse auf GAS-Infektion immer noch abhängig vom TLR-Adaptor MyD88. Überraschenderweise war die IFN-Produktion unabhängig

von den GAS-Cytolysinen SLS und SLO, die oft von anderen gram-positiven Bakterien benötigt werden. Alles in allem lassen unsere Ergebnisse darauf schließen, dass GAS durch einen MyD88-abhängigen Rezeptor, anders als die üblicherweise verwendeten, erkannt wird und lassen einen neuen Typ der bakteriellen Erkennung für die Produktion von TypI IFN erkennen.

2) INTRODUCTION

2.1 *Streptococcus pyogenes* (Group A Streptococcus)

Group A streptococcus (GAS) or *Streptococcus pyogenes* is a fermentative, facultative anaerobe, nonmotile, nonspore-forming gram-positive coccus, which occurs in chains or pairs, having a diameter of 0.5-1.0 μm. GAS are beta haemolytic streptococci. They require an enriched medium containing blood to grow. They belong to the lactic acid branch of species with low G+C content (Canchaya *et al.*, 2003). The complete genome sequence of 12 GAS clinical isolates have been sequenced so far and the GAS genome size is ~1.8 to 1.9 Mb (Musser & DeLeo, 2005).

2.2 Classification of Streptococci

Streptococci are classified on the basis of their cell wall polysaccharides into different groups: groups A, B, C, G and F when they have N-acetylglucosamine linked to rhamnose polymer backbone and group D on lipoteichoic acids (LTA) presence (Fig 1 A, B). *S. equi* (subspecies *equi* and *zooepidemicus*), *S. dysgalactiae* (subspecies *dysgalactiae* and *equisimilis*), *S. anginosus* and *S. constellatus* belong to beta-hemolytic GCS, while *S. dysgalactiae*, *S. canis*, *S. uberis*, *S. intestinalis*, and *S. anginosus* belong to beta-hemolytic GGS. Some of these bacteria cause various infections mostly in domestic animals and sometimes in humans. Group C and G have been considered as non pathogenic flora of throat, gut and vagina for a long time. Recently, some studies reported cases of streptococcal toxic shock syndrome (STSS) associated with GCS and GGS (Hashikawa *et al.*, 2004).

GAS is further subdivided into different serotypes based on highly variable N terminal sequences of the cell surface M protein as described by Dr. Rebecca Lancefield. Currently, there are over 190 types of M-protein genes (*emm*) identified in GAS (Maripuu *et al.*, 2008).

.

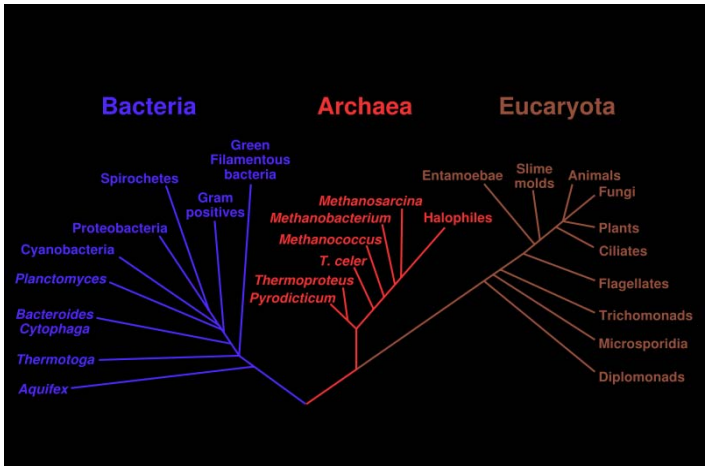


Figure 1 A. Classification of eucaryota, archaea and bacteria (http://www.newjerseymoldinspection.com/images/PhylogeneticTree.jpg)

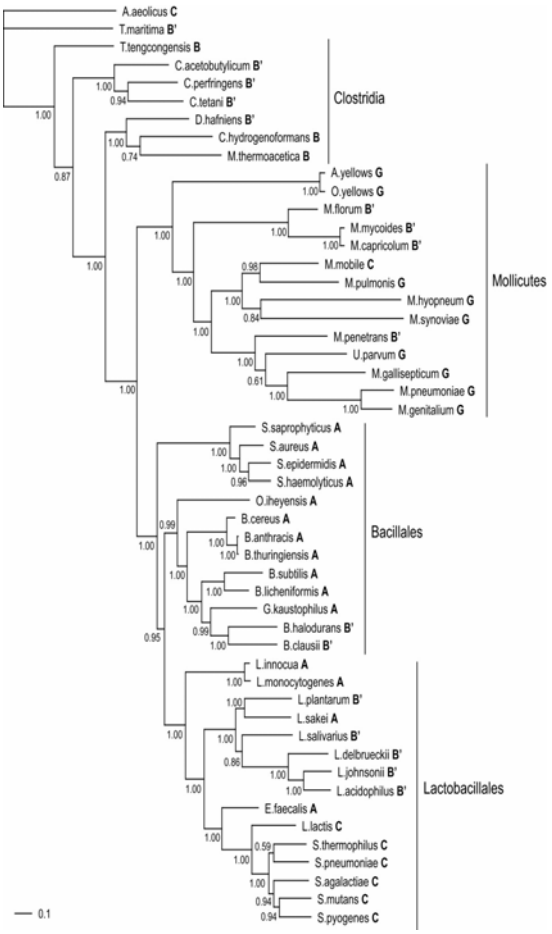


Figure 1 B. Phylogenetic tree of 49 species in the firmicutes (Kochiwa *et al.*, 2007)

2.3 Pathogenesis of *S. pyogenes*

S. pyogenes is an obligate human pathogen. It is a cause of major human morbidity and mortality worldwide. Streptococcal infections have been documented in all races, sexes and age groups. GAS causes 18 million cases of severe diseases resulting in 517,000 deaths each year (Cho & Caparon, 2008). GAS is responsible for a number of diseases ranging from common clinical illnesses such as pharyngitis, impetigo (Figure 2a), cellulitis (Figure 2b) and scarlet fever to severe invasive infections such as puerperal sepsis, myositis, necrotizing fasciitis (NF) (Figure 2c) and streptococcal toxic shock syndrome (STSS) and post infectious sequelae such as rheumatic fever and acute glomerulonephritis (Table 1). NF and STSS are rare but potentially fatal human diseases. NF also called as flesh eating disease is a disease of deeper skin layers and tissues and STSS is caused by bacterial toxin which is responsible for pyrogenicity, hypotension, shock, multi-organ failure and ultimately death.

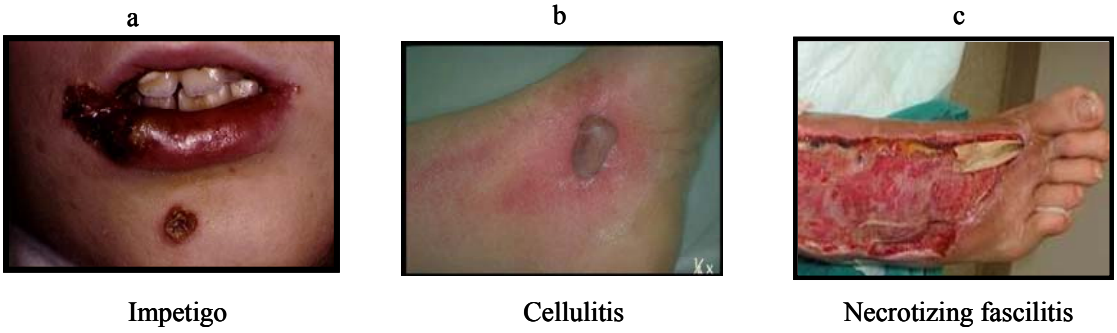


Figure 2. GAS diseases: (a) Impetigo (b) Cellulitis (c) Necrotizing fasciitis. (a:http://pathmicro.med.sc.edu/fox/staph-impetigo.jpg, b:http://www.latrobe.edu.au/podiatry/vascular/vascpics/footcellulitisbig.jpg, c: http://www.bestsyndication.net/images_com/ 2008/02_ february/22/20080222_ hospital_diseases.jpg)

Table 1. Diseases caused by GAS (adapted from Maria Siller thesis)

GAS diseases
Superficial skin and soft- tissue infections
Cellulitis
Tonsillitis
Pharyngitis
Erysipelas
Impetigo
Scarlet fever
Invasive Group A streptococcal diseases
Streptococcal toxic shock syndrome (STSS)
Necrotizing fasciitis (NF)
Bacteremia
Osteomyelitis
Septic arthritis
Pneumonia
Complications of GAS illness - nonsuppurative
Acute Rheumatic fever
Poststreptococcal glomerulonephritis
Complications of GAS illness - suppurative
Cervical lymphadenitis
Endocarditis
Fasciitis/myositis syndrome
Mastoiditis
Meningitis
Otitis media

Since the late 1980s, there has been a significant reemergence of severe forms of diseases (particularly necrotizing fasciitis and STSS) caused by GAS worldwide (Banks *et al.*, 2002; Cunningham, 2000). These outbreaks were associated with new serotypes, especially M1, M3 and M18 (Cleary *et al.*, 1992; Howe *et al.*, 1996; Stevens *et al.*, 1989; Stevens, 1992; Stevens, 2000). One hypothesis to explain this phenomenon has focused on host responses to GAS infections (Howe *et al.*, 1996; Kotb *et al.*, 2002); while others focused on changes in the virulence of the pathogen by acquisition of new virulence factors especially superantigens (Aziz *et al.*, 2004; Aziz *et al.*, 2005; Howe *et al.*, 1996).

S. pyogenes was considered as an extra-cellular organism, but studies have shown bacterial recovery from inside host cells (Medina *et al.*, 2003), indicating that *S. pyogenes* can survive intra-cellularly (Medina *et al.*, 2003). In general, host defense system to bacterial infection involves many mechanisms allowing destroying the incoming pathogen like neutrophil recruitment, opsonization, bacterial entrapment and uptake, intracellular effectors mediated bacterial killing but bacteria use their virulence factors to interfere and impair each of these mechanisms. In the case of GAS, factors which avoid

entrapment by phagocytes include peptidase ScpA, serine protease ScpC, DNAses. Capsule, M and M-like proteins and Sfb1, SIC (Streptococcal inhibitor of complement), SpeB (cysteine protease) inhibit complement and antibody functions. GAS also impairs phagocytic mechanisms with the help of the factors EndoS, Mac (1 and 2) and protein SIC. Cytolysins which promote phagocytic lysis and apoptosis are also used by GAS. Finally, GAS resist effectors of phagocytic killing by D-alanylation of LTA, SpeB, SIC, M protein and GpoA (glutathione peroxidase) (Figure 3) (Kwinn & Nizet, 2007).

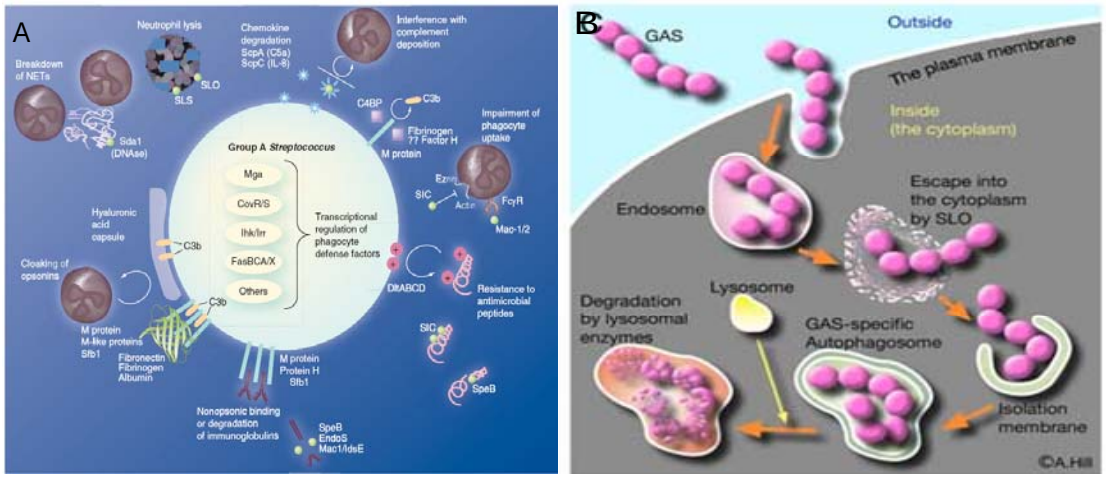


Figure 3. GAS interference with host phagocytic defenses. A (Kwinn & Nizet, 2007), B (http://www.biken.osaka-u.ac.jp/act/act_yoshimori_e.php)

2.4 Virulence factors produced by *S. pyogenes*

GAS survival and pathogenesis is mediated by several virulence factors involved in adhesion, colonization, evasion, persistence and spread in the host (Table 2). GAS virulence factors can be divided into two groups.

Table 2. Virulence factors in *S. pyogenes* (adapted from (Bisno *et al.*, 2003; Commons *et al.*, 2008)

GAS virulence factors
Antiphagocytic
Streptococcal inhibitor of complement (Sic)
Cysteine proteinase (SpeB)
Immunoglobulin G degrading enzyme (IdeS)
Immunogenic secreted protein (Isp)
M protein
M-like protein (Mrp, Enn and others)
Hyaluronic acid capsule
C5a peptidase (ScpA)
Adherence to epithelial cells
Lipoteichoic acid (LTA)
Fibronectin binding proteins
M protein
Hyaluronic acid capsule
Lamin-binding protein (Lmb)
Streptococcal collagen-like surface protein A (SclA)
Streptococcal collagen-like surface protein B (SclB)
Internalisation
M protein
Protein F1
Inavsion
Hyaluronic acid capsule
M protein
Spread through tissues
Hyaluronidase
Streptokinase
SpeB (cysteine protease)
DNAses A-D
Systemic toxicity
Streptolysin O
Streptolysin S
Superantigenic exotoxins
Superantigens
SpeA
SpeC
SpeG
SpeH
SpeI
SpeJ
SpeK
SpeL
SpeM
SSA
SmeZ

2.4.1 Somatic virulence factors

Somatic or surface-exposed virulence factors include M protein, capsule and adhesions.

M protein

The M-protein consists of two polypeptides arranged in a coiled-coil structure that is held in the cell membrane (Figure 4). The C-terminal domain is highly conserved, anchored in the cell membrane via the LPSTGE motif, while the surface-exposed N-terminal portion is highly variable and is responsible for serotype specificity. M protein is a major surface antigen and virulence factor, which is encoded by the *emm* gene. M proteins are involved in various stages of GAS pathogenesis including adhesion, internalization, immune evasion and tissue invasion. It confers resistance to phagocytosis and killing by polymorphonuclear leukocytes by binding to complement control factors and other host proteins to prevent activation of the alternate complement pathway (Bisno *et al.*, 2003).

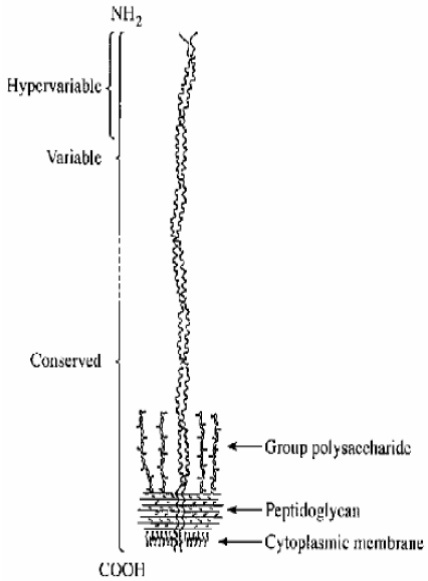


Figure 4. Structure of M protein (Efstratiou, 2000)

Capsule

Capsule is made up of hyaluronic acid (Figure 5) and encoded by an operon composed of *hasA*, *hasB* and *hasC* genes. The capsule has a role in colonization, phagocytic killing and infection, thus encapsulated strains are more pathogenic (Mitchell, 2003; Wessels &

Bronze, 1994). Degree of encapsulation varies among GAS strains. By means of the capsule, bacteria attach to the receptors of host cells. Such binding induces cytoskeleton changes, which help in cellular invasion.

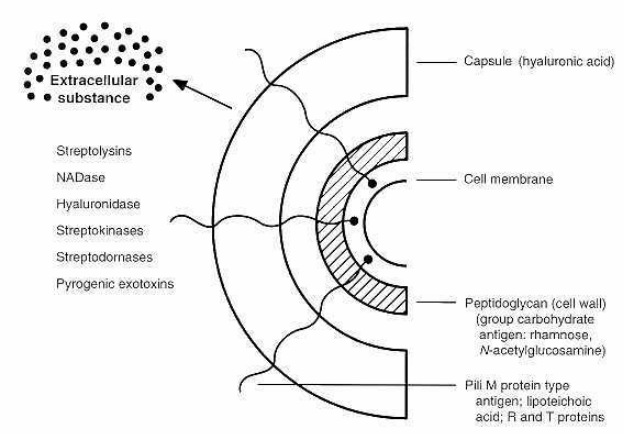


Figure 5. Cell wall structure of *S. pyogenes* (http://gsbs.utmb.edu/microbook/ch013.htm)

Adhesins

Apart from the M protein and capsule, streptococci have at least 17 other adhesion candidates that promote adhesion to host cells. LTA is anchored to the proteins on the bacterial surface. *S. pyogenes* encodes several fibronectin binding proteins (FBPs), which are important for the adhesion to different cell types. Protein F, the fibronectin-binding protein, also mediates streptococcal adherence to the amino terminus of fibronectin on mucosal surfaces. FBPs can help GAS in the internalization of human respiratory epithelial cells. This event may protect streptococci from the host immune responses and antibiotics (Bisno *et al.*, 2003; Cue *et al.*, 1998; LaPenta *et al.*, 1994; Molinari *et al.*, 1997; Osterlund & Engstrand, 1997). The host receptors, which are involved in the adhesion of GAS include fibronectin, vitronectin, fibrinogen, laminin, CD44, CD46 and galactose and fructose containing glycoproteins (Musser & DeLeo, 2005).

.

2.4.2 Extracellular virulence factors

Extracellular virulence factors include Streptococcal hemolysins (SLO, SLS) and Streptococcal pyrogenic exotoxins (Spes). GAS secretes two main types of hemolysins: Streptolysin S (SLS) and Streptolysin O (SLO).

Streptolysin S

SLS is a non-immunogenic, oligopeptide, oxygen-stable, thermolabile cytolysin, responsible for the β-hemolysis on blood agar medium. It is produced when cells are in stationary phase of growth (Sierig *et al.*, 2003). SLS is one of the most potent cytotoxins known. It has a broad spectrum cytolytic activity on the membranes of erythrocytes, leukocytes, platelets and sub-cellular organelles such as lysosomes and mitochondria (Bisno *et al.*, 2003; Nizet, 2002). The genetic locus identified for production of SLS is the *sag* operon, which includes nine genes *sagA-sagI*. SLS is effective in forming transmembrane pores and causing lysis when GAS is in contact with target cells. SLS also promotes resistance to phagocytic killing by exerting cytocidal activity on neutrophils (Kwinn & Nizet, 2007).

Streptolysin O

SLO is an immunogenic, oxygen-labile, 540 amino acid secreted cytolysin that binds to cholesterol in eukaryotic cell membranes, where it oligomerizes to produce large transmembrane pores leading to cell lysis. When grown in culture it is produced during exponential to early stationary phase growth (Sierig *et al.*, 2003). SLO can lyse a number of eukaryotic cell types including erythrocytes, leukocytes, PMNs, macrophages, platelets and various cell culture lines (Palmer *et al.*, 1998). Thus SLO can impair host phagocytic capacity. In an epithelial cell model SLO allows bacteria to avoid lysosomal localization (Kwinn & Nizet, 2007). SLO also translocates NAD-glycohydrolase (SPN) into the cytoplasm of host leading to increase in cytotoxicity in a process called cytolysin-mediated translocation (CMT) (Meehl & Caparon, 2004) and depletes the host cell energy stores.

Streptococcus pyrogenic exotoxins/Superantigens

GAS strains express 11 distinct but structurally related streptococcal pyrogenic exotoxins (SPEs) including 8, which are encoded on prophages: SpeA1-4, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM, streptococcal superantigen SSA and 3, which are encoded on the chromosome: SpeG, SpeJ and the streptococcal mitogenic exoprotein smeZ (Banks *et al.*, 2003; Beres *et al.*, 2002; Commons *et al.*, 2008; Ekelund *et al.*, 2005; Petersson *et al.*, 2004; Sriskandan *et al.*, 2000; Sriskandan *et al.*, 2007).

These SPEs also known as superantigens (SAgs), are roughly 25 kDa secreted proteins that are thought to contribute to the pathogenesis of some GAS diseases, including scarlet fever, streptococcal toxic shock syndrome (STSS) and rheumatic fever. SAgs have the unique ability to cross-link class II major histocompatibility complexes (MHC) on antigen-presenting cells (APCs) to specific variable β-chains of T-cell receptors (TCRVβ) resulting in activation of up to 20% of T cells (Figure 6). Normally peptide antigen activates 0.001% of all T cells. This unspecific massive T-cell proliferation causes release of large amounts of the cytokines, such as interleukin-2 (IL-2), tumour necrosis factor-α (TNF-α) and interferon-γ from the T cells and IL-1 and TNF-α from the APCs. SAgs cause induction of T-cell proliferation in femtomolar concentrations, hence are the most powerful T cell mitogens known. In addition to cytokine production and T-cell proliferation, SAgs are capable of inducing cytotoxicity towards target cells. These exotoxins also induce pyrogenicity, cause capillary leakage, activation of complement, coagulation and fibrinolytic cascades, leading to hypotension through cytokine release and this leading to shock, multi-organ failure and death. Apart from SAgs, proven and potential virulence factors such as hyaluronidase (*hyl*), mitogenic factor (*mf*) and phospholipase A_2 (*sla*) are encoded on prophages.

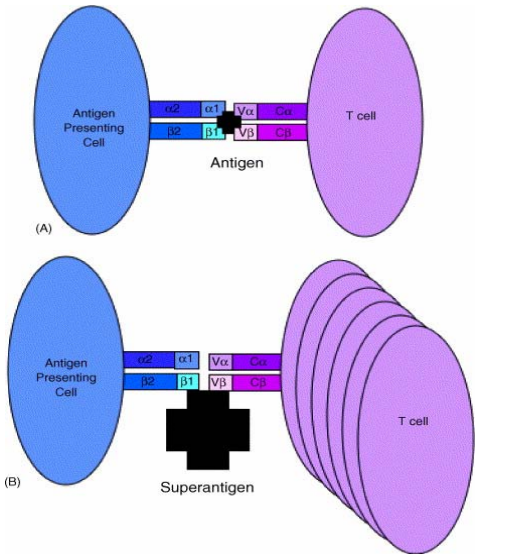


Figure 6. Cross-linkage of superantigens with APC and T cells (Sriskandan *et al.*, 2007)

SSA

The streptococcal superantigen SSA is widely distributed among *S. pyogenes*, found in 12.5% of 80 distinct clonal lineages (Igwe *et al.,* 2003). It is a 260 amino-acid protein that can play a role in symptoms similar to STSS. The amino terminus of SSA displays striking homology to the staphylococcal enterotoxins B, C1, and C3 (SEB, SEC1 and SEC3) rather than to the characterized streptococcal SAgs. In addition to confirmation of SSA mediated activation of T cells, T cell proliferation studies could confirm differences in the stimulation pattern between native and recombinant SSA or even between SSA alleles.

Three *ssa* alleles were found in natural population of *S. pyogenes*. *ssa-1* differs from *ssa-3* by a single synonymous substitution in codon 94. *ssa-2* was present in a single lineage. It is identical to *ssa-3* at codon 94 but has a non synonymous substitution at codon 28 (Reda *et al.*, 1996). Studies of allelic variations at the *ssa* locus, revealed sequence variations upstream of *ssa* in different clonal lineages. All six isolates had a 34 bp insert located at 103 bp 5' end of the *ssa* start codon. This element IS1239, had 30% sequence similarity to insertion sequences and transposases from several other bacterial species. The presence of one copy immediately upstream of *ssa* suggests that the *ssa* gene may be horizontally transferred by a composite transposon in normal bacterial populations

(Kapur *et al.*, 1994). This hypothesis is further supported by the identification of *ssa-3* allele from group C and G streptococci, isolated from patients with invasive streptococcal infections.

2.4.3 Regulation of virulence factor expression in *S. pyogenes*

To establish a successful infection, bacterial pathogens must precisely control the expression of their virulence genes in response to host signals. These signals inform the bacterium that it has reached the proper niche to begin its virulence program. Virulence gene expression in pathogenic bacteria is controlled mainly at the transcriptional level by activation or repression by proteins. Bacteria can also regulate their gene expression via regulatory RNA molecules and in response to their cell density. As in other bacteria, virulence factor expression in GAS is coordinately regulated. Regulation occurs via "stand-alone" response regulators (RRs) and two-component signal transduction systems (TCSs) (Figure 7). The complete genome sequences of 12 GAS strains corresponding to nine serotypes contain 13 TCS and about 30 stand alone transcriptional regulators (Table 3) (Kreikemeyer *et al.*, 2007).

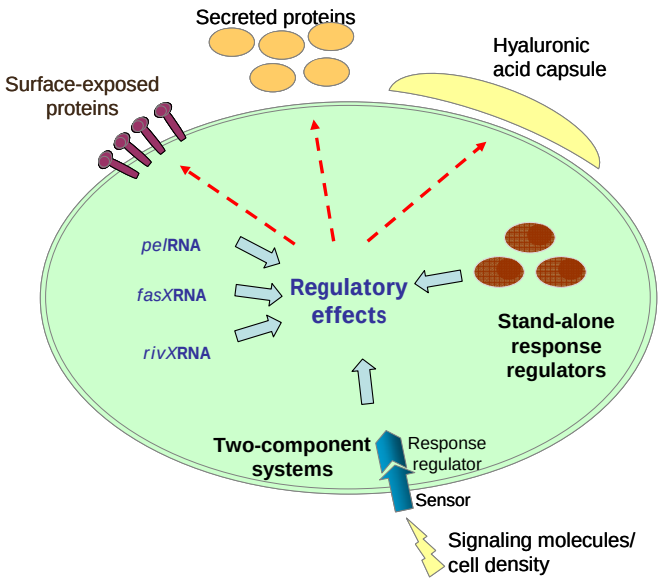


Figure 7. Regulation of virulence factor expression in GAS

Table 3. Regulators in GAS (adapted from Maria Siller thesis)

Stand-alone regulators		
Regulator	**Targets**	**References**
CodY	UR: *pel/sagA*, *nga-slo*, *mga*, *grab*, *scl*, *prtS*, *scpA*, *speH*, *ideS*, *hasA* DR: *covRS, ropB*, *opp*, *ska*, *sda*	Malke *et al.,* 2006; Malke and Feretti, 2007
LacD.1	UR*: manL, slo, sagH, ntpK* DR: *speB, salA, scrA*	Loughman and Caparon, 2006
MalR	DR: transcripts of polysaccharide utilization proteins. MalR contributes to the persistence of GAS in the oropharynx.	Shelburne *et al*., 2007
Mga	UR: *arp*, *emm*, *enn*, *scpA*, *fcrA*, *nra*, *mga*, *ska*, *speB*, *scnA*/*salA*, *opp*, *fbA*, *sic*, *lbp*, *scl1/scl-A*, *sof*, *mrp* DR: genes for sugar utilization	Deutscher *et al.,* 2005; Hondrop and McIver, 2007; McIver *et al*., 1999; Kreikemeyer *et al*., 2003
MsmR	UR: *prtF2, cpa, nra, spy0128, nga, spy0166, slo, spy0170, spy2006, sof, sfbx, hasA*	Nakata *et al*., 2005
MtsR	UR: *htsA* DR: *sia* operon, *mtsA* The *mtsR* mutant is hypersensitive to streptonigrin and hydrogen peroxide.	Bates *et al*., 2005; Hanks *et al*., 2006
RALP3	DR: *hasA, spn, sdal, lsap, speB, mga, slo, covR, sic, sagA, grab, emm, eno* (at logarithmic growth phase)*, ska, scpC* UR: *scpA, cpa, eno* (at stationary growth phase)*, sic* RALP3 contributes to epithelial cell inavasion and bloodstream survival. Counteracts Nra and MsmR regulation.	Kreikemeyer e*t al*., 2007
RALP4 (RivR)	UR: *mga*, *emm*, *scpA*, *fba*, *sic*, *scl*, *grm* (Mga regulated genes), *speB*, spy*1508*/NT01sp*1245,* M5005_spy*0190*/NT01sp*0244* and NT01sp*1815* (hypot. proteins); required for virulence in *covR* deleted mutant.	Roberts and Scott, 2007
Nra	DR: *cpa*, *mga*, *nra*, *prtF2*, operon *orf5-nifR3L-kinL*, operon *cpa-lepAL-egfflSL-orf2, sof/sfbII*, *sagA*, *speA*, *speB*, *ralp3*	Kreikemeyer *et al*., 2003; Kreikemeyer *et al*., 2007; Molinari *et al*., 2001; Podbielski *et al*., 1999
RofA	UR: *prtF, rofA, rpsL, hasB, emm2* DR: *emm6, mga, sagA, speA, speB, ska*	Beckert *et al*., 2001; Fogg and Caparon, 1997; Kreikemeyer *et al*., 2003
Rgg/RopB	UR: *speB, autolysin, clpB, lysozyme, covR, covS, fasBCA, isp1, isp2, ihk, irr* DR*: mf, DNA entry nuclease (orf226), orf953, emm, grab, hasAB, orfX, sagA, scl1, scpA, ska, slo, speH, mac, mga, cpsX, yufM, lytR, spy0875,* ClpE, ClpL	Chaussee *et al*., 2002; Chaussee *et al.,* 2004; Neely *et al*., 2003
PerR	UR: *csp*, *sod*, *czcD* DR: *mtsA*, *mrgA*, *pmtA*, *phtY*, *phtD*, *lsp*, *lrpsN2*	Brenot *et al*., 2005; Brenot *et al*., 2007
P-Ser-HPr	UR: *mga*	Deutscher *et al*., 2005
Srv	UR: *sir*, *spy0044* (zinc containing dehydrogenase), *spy0285* (ATP binding protein), *spy0714* (zinc binding protein), *spy2007*, *sic*, *speB*	Reid *et al*., 2004; Reid *et al*., 2006
Two-component systems		
Regulator	**Targets**	**References**
CovRS	UR: *spy0138*, *spy*1062, *spy1680*, *spy1755*, *spy*533 DR: *covR*, *grab*, *has*-operon, *dppA*, *ideS*, *ihk/irr*, *isp2, lmb*, *mac*, *mspA*, *ralp3*, *ralp4*, *sagA*, *sda*, *ska*, *speB*, *speF*, *speMF*	Federle *et al*., 1999; Gao *et al*., 2005; Gusa and Scott, 2005; Heath *et al*., 1999; Levin and Wessels, 1998; Roberts *et al*., 2007
fasBCAX	UR: *ska*, SLS activity DR: *fbp45*, *mrp*, *sagA*	Kreikemeyer *et al*., 2001
Ihk/Irr	UR: cytokine genes*, fbp, gidB, mf/mf3, mryY, sagA, spy0510, spy1035, spy1093, spy1205, spy1311*	Voyich *et al*., 2003; Voyich *et al*., 2004
SilA/B	UR: *silE/D/CR*, *spyM3-1016* (transposase), bacteriocin like peptide (*blp*) DR: *silC*	Eran *et al*., 2007; Hildalgo-Grass *et al*., 2002
SptRS	UR: carbyhydrate metabolism enzymes, *emm, hasA, perR, rofA, sagA, sic, spd,* *speB, spy0470*	Shelburne *et al*., 2005
VicRK	UR: putative cell wall hydrolase gene *pcsB*, putative phosphotransferase system enzyme II for carbohydrate transport. DR: *spy0183*, *spy0184* (osmoprotectant transporter OpuA) VicRK regulates processes involved in cell wall metabolism, nutrient uptake and osmotic protection.	Liu *et al.*, 2006
TrxSR	UR*: emm1, sic*, *fba*, *scpA*, *sclA*, *grm* (Mga regulated genes), *mga* DR: *rbfA*, *nusA*, *aroB*, *secA*, *dnaJ*, *miaA*, *nagB*, *rplT*, *polA*, *lacE*, *flaR*, *aroD*, *phnA*, *clpL*, *opuAA*, *opuABc*	Leday *et al.,* 2008

Regulatory RNAs		
Regulator	**Targets**	**References**
fasX	UR: *ska*, SLS activity DR: *fbp45*, *mrp*, *sagA*	Kreikemeyer *et al*., 2001
pel	UR: *emm*, *nga*, *sic*, SpeB activity	Mangold *et al*., 2004
RivX	UR: *mga*, *emm*, *scpA*, *fba*, *sic*, *scl*, *grm* (Mga regulated genes), *speB*, spy*1508*/NT01sp*1245,* M5005_spy*0190*/NT01sp*0244* and NT01sp*1815* (hypot. proteins); required for virulence in *covR* deleted mutant.	Roberts and Scott, 2007
UR: up-regulation DR: down-regulation		

2.4.3.1 Two component systems and response regulators

TCSs consist of a sensor histidine kinase, a transmembrane protein, which senses an external signal with its extra cellular domain, and a response regulator located in the cytoplasm, which after activation acts as a transcription factor, to control target gene expression. GAS has 13 common TCSs, however only some of them have been well studied, like CsrRS/ CovRS, FasBCAX, Ihk/ Irr, SilA/B, VicRK and SptRS.

CsrRS/ CovRS

CsrRS/ CovRS is the best described TCS in GAS. CovR controls the expression of ~15% of GAS genome transcripts (Graham *et al.*, 2002). Cov stands for control of virulence and Csr for capsule synthesis regulator. It encodes two proteins similar to response regulator and sensor components. These proteins repress the expression of the *has* operon (capsule). Apart from this CsrR repress transcription of the genes encoding streptokinase (*ska*), streptolysin S (*sagA*), mitogenic factor (*speMF*), streptodornase (*Sda*), cysteine protease (*SpeB*) and CsrR itself (Figure 8) (Darmstadt *et al.*, 2000; Federle *et al.*, 1999; Heath *et al.*, 1999). The system is triggered by Mg2+, which is low in extra-cellular body fluids but high inside host cells or outside the human body. The CovRS system inactivates many invasion related GAS virulence genes outside the human body (and inside a host cell), but activates virulence factors at Mg2+ concentrations encountered in human mucosal secretion and extra-cellular fluids. If CovS is mutated, completely abolishing any activity of this system, GAS becomes significantly more invasive (Bernish & van de Rijn, 1999; Darmstadt *et al.*, 2000; Graham *et al.*, 2002; Gryllos *et al.*, 2003; Levin & Wessels, 1998; Sumby *et al.*, 2006; Wessels, 1999). A phenotypic switch from mucosal to invasive forms of GAS infection was correlated with mutations in the *covR/S* locus (Sumby *et al.*, 2006).

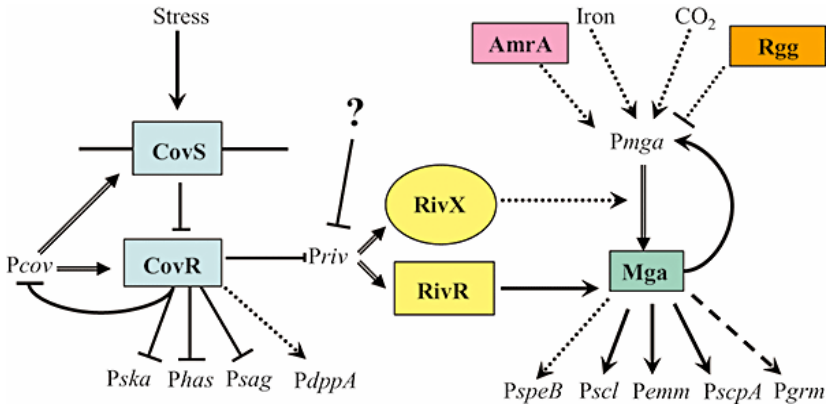


Figure 8. CovRS regulation (Roberts & Scott, 2007)

FasBCAX

FasBCAX is the fibronectin/ fibrogen binding haemolytic activity/ streptokinase regulator. It encodes two sensor histidine kinases (FasBC) and one response regulator (FasA), integrating different signals into one adaptive response. The main effector of the Fas system is the putative untranslated 200 nt regulatory RNA encoded by fasX (Kreikemeyer *et al.*, 2001). The Fas operon upregulates the expression of *sagA* and *ska*, and downregulates the expression of adhesin genes (*fbp54* and *mrp*). FasBCAX shows homology to quorum-sensing TCSs in *S. aureus* and *S. pneumoniae* and assist in tissue invasion and destruction (Klenk *et al.*, 2005). Expression of *fasBCA* genes changes in response to temperature change from 29ºC to 37ºC (Sitkiewicz & Musser, 2006).

Ihk/ Irr

The Ihk/ Irr (*isp*-adjacent histidine kinase/response regulator) TCS has an important role in immune evasion as it controls the expression of genes that facilitate protection from PMN leukocytes mediated killing and assist in GAS survival and pathogenesis response (Federle *et al.*, 1999; Voyich *et al.*, 2003; Voyich *et al.*, 2004). It down regulates genes involved in cell wall metabolism, transcription, oxidative stress and virulence. The Ihk/ Irr mutant was found more susceptible to killing by H_2O_2 and neutrophil primary granules (Musser & DeLeo, 2005).

Sil operon

The Sil locus stands for streptococcus invasive locus and is present in about 18% of clinically relevant GAS isolates. The Sil system consists of the TCS SilA/B, two putative ABC-transporters SilD and SilE and SilCR, which seems to be a quorum sensing effector molecule. Sil upregulates the transcription of its own signalling molecule and the two ABC transporters. The bacteriocin like peptide (*blp*) operon and the *spyM3_1016* transposase have already been identified as targets (Eran *et al.*, 2007; Hidalgo-Grass *et al.*, 2002).

VicRK

VicR inactivation down-regulates the transcription of 13 genes including the putative cell wall hydrolase gene *pcsB* and a putative phosphotransferase system enzyme II for carbohydrate transport, and up-regulates the expression of five genes, including an osmoprotectant transporter OpuA. *vicR* inactivation induced osmotic stress and increased susceptibility to osmotic pressure. VicRK regulates processes involved in cell wall metabolism, nutrient uptake, and osmotic protection (Liu *et al.*, 2006).

SptRS

SptR and SptS encode two component gene regulatory systems, involved in the persistence of GAS in saliva, as the mutant of *sptR* was less able to survive in saliva. Microarray analysis of GAS grown in saliva showed that the expression of several virulence factors in *sptR* mutant was significantly decreased (Shelburne *et al.*, 2005).

TrxSR

The recently described TrxSR (two-component regulatory system X) displays homologies to a virulence related TCS in *S. pneumoniae*. A Trx response regulator mutant is defective in Mga expression and is attenuated in virulence in a murine model of GAS soft tissue infection. TrxR is the first TCS that regulates Mga expression in response to a yet unidentified signal. *trxSR* is a part of CovR-repressed virulence operon, which performs a range of different functions in translation, transcription, replication,

transport and stress. As CovR can influence Mga expression, this shows that important global regulatory networks can influence each other to affect pathogenesis in GAS (Leday *et al.*, 2008).

Response regulators

Apart from TCS, GAS also relies on about 30 stand-alone response regulators, for which sensory elements have not been described. These response regulators control the expression of virulence factors in response to environmental signals.

Mga

Mga stands for multiple gene activator. It is a ubiquitous stand-alone virulence response regulator. It is necessary for adherence, internalization and host immune evasion. Mga activates the expression of virulence genes by directly binding to their promoters. 10% of the three GAS genome transcriptome was affected when *mga* was deleted (Ribardo & McIver, 2006). Mga activates transcription of a number of virulence genes in GAS including its own gene. The Mga regulon is expressed maximally during exponential phase and activates factors involved in early stages of GAS infection. Repression of *mga* transcription is mediated by Rgg/RopB and Nra (RALP), while it is activated in response to CO_2 levels and inhibited by high salt and iron limitation conditions (Caparon *et al.*, 1992; McIver *et al.*, 1995; McIver & Scott, 1997). Mga regulates the expression of M protein (*emm* and *arp*), M-like proteins (*mrp*), C5a peptidase (*scpA*), SIC, SpeB and extracellular matrix binding proteins (*sclA* and *fba*, Figure 9) (Frick *et al.*, 2003; Kreikemeyer *et al.*, 2003; Rasmussen *et al.*, 2000; Roberts & Scott, 2007).

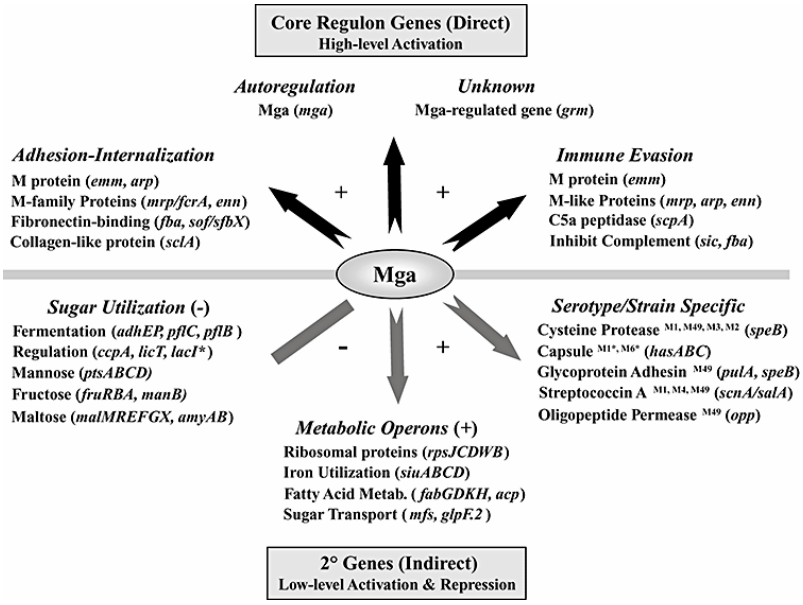


Figure 9. Mga regulon in GAS (Hondorp & McIver, 2007)

RALP (RofA like proteins)

The RALP regulator family of GAS comprises four homologous members: RofA, Nra, RALP-3 and RALP-4 (Granok *et al.*, 2000; Kreikemeyer *et al.*, 2003). These are involved in the control of GAS-host cell interactions, avoidance of host-cell damage and balanced virulence factor expression during stationary phase. Maximal expression of RALP occurs at stationary growth phase. RALP-regulated genes are fibronectin-binding proteins F (s*fbI*) and F2 as well as collagen-binding protein (*cpa*), hemolysins (*sagA*), proteases (*speB*); superantigens (*speA*) and other virulence regulators (*mga*) (Kreikemeyer *et al.*, 2003).

A second well known RALP protein is Nra. It acts as a negative regulator of expression of *cpa*, *prtF*, *speA*, *sagA*, *mga* and *nra* itself conditions (Jaffe *et al.*, 1996; Podbielski *et al.*, 1999). Strain-specific differences are observed with Nra transcriptional circuit as Nra is a negative regulator of pilus gene transcription in M49 serotype but a positive regulator of pilus gene transcription in M53 serotype (Luo *et al.*, 2008). In contrast to RofA, Nra expression is maximal at early stationary phase and its expression is not affected due to environmental conditions (Jaffe *et al.*, 1996; Podbielski *et al.*, 1999).

Rgg/RopB

Rgg also known as RopB, stands for regulation of proteinase. It plays a role in host cell apoptosis, necrosis and dissemination. Rgg controls gene expression during late stationary growth phase. It is a positive regulator of the cysteine protease SpeB. It has a negative influence on the transcription of regulatory factors such as *mga* and *pel/sagA* and a positive influence on transcription of *covRS* and *fasBCA* (Chaussee *et al.*, 2004; Neely *et al.*, 2003).

Srv regulon

Srv stands for streptococcal regulator of virulence. The putative regulator protein Srv is homologous to the transcriptional regulator PrfA of *Listeria monocytogenes*. It belongs to the first GAS member of the Crp/Fnr family of transcriptional regulators. The mortality of mice injected with the *srv* mutant was significantly less as compared to the wild-type (Reid *et al.*, 2004). Another study showed that Sic levels were decreased while SpeB levels were increased in a *srv* mutant (Reid *et al.*, 2006).

CodY

CodY is a nutritional regulator mainly involved in amino-acid metabolism. This protein is highly conserved in low G+C gram positive organisms. It has been identified as a pleiotropic transcriptional regulator, activated by branched chain amino-acids. CodY controls many genes, which are operative in low nutrition conditions. To some extent CodY also regulates a RelA-independent response to amino acid (Steiner & Malke, 2001). CodY induces the expression of *pel*/*sagA* and *mga*, so this suggests a link between nutritional regulation and virulence (Malke *et al.*, 2006).

2.4.3.2 RNA regulators

Virulence gene regulation, as gene regulation in general, was up to recently believed to be exerted mainly by repressor or activator proteins. However, it has become increasingly evident that RNA can control gene expression more commonly than previously

anticipated (Johansson & Cossart, 2003; Shimizu *et al.*, 2002). The RNAs that are involved in gene regulation are discussed in details in section 2.6.

2.4.3.3 Cell density-dependent regulation

Bacteria communicate with each other using small chemical signal molecules called autoinducers. This process involves the production, release, detection and responding to signal molecules by bacteria. These autoinducers are detected when a certain threshold level of these molecules accumulate in the surroundings. Thus this process is called quorum sensing, as it senses a certain number (quorum) of autoinducer molecules. Quorum sensing allows bacteria to modulate gene expression according to the number of bacteria in a community and according to different environmental conditions.

Quorum sensing in gram-negative bacteria

Quorum sensing was first discovered in *Vibrio fischeri*, where it colonizes the light organ of Hawaiian squid *Euprymna scolopes*. Bacteria grow to high density to induce the expression of genes for bioluminescence. Two proteins LuxI and LuxR are responsible for the control of luciferase operon (*luxICDABE*), which is required for the light production. Here, LuxI is the autoinducer synthase, which produces acyl-homoserine lactone (AHL), while LuxR is the cytoplasmic DNA binding transcriptional activator. At first, LuxI is produced and it can freely diffuse out of cell. Once it reaches a certain threshold concentration it can bind to LuxR and then this complex can bind to promoter region of a target operon (*luxICDABE*) and activates its transcription (Figure 10). Activation of this operon also activates *luxI* expression as it is also encoded in this operon, thus leading to a positive feedback loop (Waters & Bassler, 2005a). Hence the whole population acts like a synchronous behavior. In quorum sensing there is extreme specificity between autoinducer molecule and autoinducer receptor, so even in mixed population each species can respond only to its own signal.

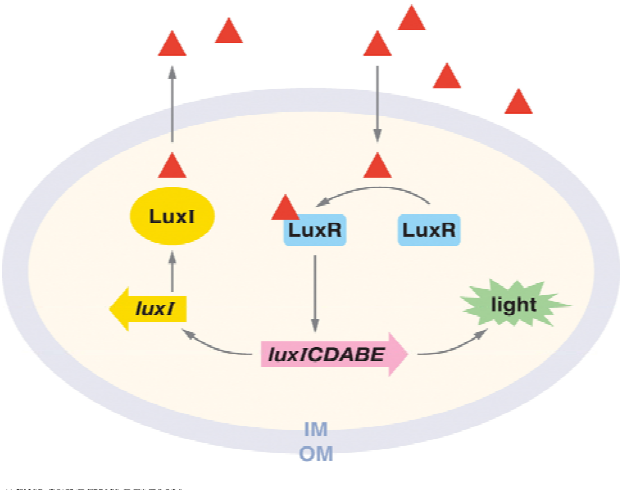


Figure 10. Quorum sensing in gram-negative bacteria (Waters & Bassler, 2005b)

Quorum sensing in gram-positive bacteria

In gram-positive bacteria quorum sensing involves modified oligopeptides as signal molecules instead of AHL. The peptides that are produced are first binding to membrane bound sensor histidine kinases as receptors. Simultaneously they are modified, processed and transported by dedicated oligopeptide transporters. These molecules can not diffuse freely in and out of the cell as AHLs do.

A well known example of quorum sensing system in gram-positive bacteria is the *Staphylococcus aureus* Agr quorum sensing system. At low density bacteria express protein factors that promote colonization and attachment, while at high density bacteria repress these factors and express toxins and proteases required for dissemination (Lyon & Novick, 2004). In *S. aureus,* the autoinducing peptide (AIP) is encoded by *agrD* and signals through the sensor kinase AgrC and response regulator AgrA. The role of AgrB is to modify and transport AIP out of the cell (Saenz *et al.*, 2000). Thereafter, AIP binds to AgrC and phosphorylates AgrA, which induces expression of RNAIII. This regulatory RNA represses the expression of cell adhesion factors and induces expression of secreted factors (Waters & Bassler, 2005a). AgrA also induces the expression of *agrBDCA* thus leading to a positive feedback loop (Figure 11). In *S. pyogenes*, two putative quorum sensing systems have been described: *silABCD* and *fasBCA* (chapter 2.4.3.1). No signaling peptide has been discovered from the *fasBCA* locus, however the putative

pheromone peptide SilCR from the *sil* operon regulates DNA uptake and the ability to cause invasive infection (Eran *et al.*, 2007).

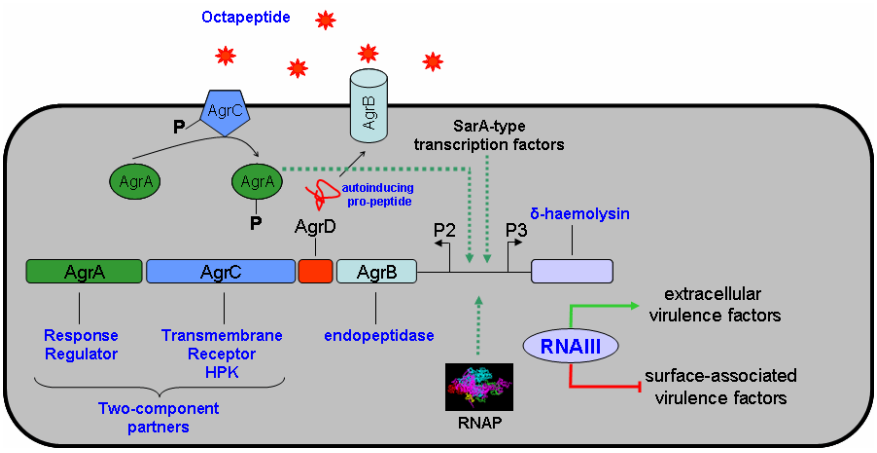


Figure 11. The *agr* system and RNAIII in *S. aureus* (http://www3.imperial.ac.uk/cmmi/research/sivarameshwigneshweraraj)

Interspecies communication

Yet another autoinducing system has been discovered, which detects a furanone-like signaling molecule termed "autoinducer 2" (AI-2). AI-2 regulates bioluminescence in *Vibrio harveyi* (Figure 12) (Bassler *et al.*, 1997; Surette *et al.*, 1999). It is synthesized by the *luxS* gene, which is found in over 55 gram-negative and gram-positive bacterial species. AI-2 is proposed to have a role in interspecies communication. Many studies in gram-positive bacteria have shown the role of *luxS* in pathogenesis. LuxS and AI-2 have potential role in metabolism and quorum sensing. In many oral pathogens, the *luxS* mutant leads to a modified biofilm architecture (Table 4). In some oral bacteria, the *luxS*/AI-2 signaling pathway has been also associated with acid and oxidative stress tolerance, bacteriocin synthesis, competence and antibiotic susceptibility. In *Clostridium perfringens*, transcription of three toxins encoding genes was reduced in a *luxS* mutant strain. However in *S. aureus*, a *luxS* mutant strain had no effect on virulence but showed a growth defect in sulfur-limited defined medium. In *S. pyogenes*, the *luxS*/AI-2 signaling was shown to be involved in the regulation of pathogenicity and *S*-adenosylmethionine (SAM) utilization pathway. Mutation of *luxS* in an M6 serotype showed enhanced expression of *sagA* associated with enhanced haemolytic activity and reduction in SpeB protease proteolytic activity (Lyon *et al.*, 2001). Marouni and Sela (2003) observed a

Table 4. LuxS mutant phenotypes in gram-positive bacteria (adapted from Maria Siller thesis)

Bacterium	Phenotype	References
Bacillus subtilis	Decreased biofilm formation and swarming ability.	Lombardia *et al*., 2006
Clostridium perfringens	Reduced toxin production; reduced *pfo* mRNA.	Othani *et al*., 2002
Listeria monocytogenes	Cell attachment during biofilm formation affected.	Challan Belval *et al*., 2005
Porphyromonas gingivalis	Reduced levels of Tlr (TonB-linked hemin binding protein) and the protease Kgp. Upregulatipon of HmuR (TonB-linked hemin binding protein) and hemin binding lipoprotein FetB, FeoB1 (a Fe2+ ion transporter protein) and ferritin (iron storage protein). Impaired hemin uptake in hemin-depleted medium. Reduced TonB, excinuclease, hemin-regulated receptor. Increased hemin aquisition protein Rgp. Reduced Rgp and Kgp proteases, haemagglutinin levels. Failed to form biofilm with *S. gordonii luxS* mutant.	James *et al*., 2006 Chung *et al.*, 2001 Burgess *et al*., 2002 McNab *et al.*, 2003
Staphylococcus aures	Growth defect in sulfur limited defined media.	Dohorty *et al*., 2006
Straphylococcus epiderimidis	Increased biofilm development and enhanced virulence. Genes involved in sugar, nucleotide, amino acid, and nitrogen metabolism, but also virulence-associated genes coding for lipase and bacterial apoptosis proteins and the pro-inflammatory phenol-soluble modulin (PSM) peptides are altered by LuxS/AI-2 signaling system.	Xu *et al*., 2006 Li *et al*., 2008
Streptococcus anginosus	Increased susceptibility to erythromycin and ampicillin. Deficient in biofilm formation.	Ahmed *et al.*, 2007 Petersen *et al*., 2006
Streptococcus intermedius	Decreased haemolytic actitiy.	Pecharki *et al.*, 2008
Streptococcus gordonii	Altered biofilm morphology. Failed to form biofilm with *P. gingivalis luxS* mutant. Decrease *gtfG*, *fruA*, *lacD.*	Blehert *et al*., 2003 McNab *et al*., 2003
Streptococcus mutans	Impaired bacteriocin mutacin I (*mutA*) and *mutR (activator).* Increased acid sensitivity; increased survival rate in the presence of hydrogen peroxide; downregulation of *ffh*, *brpA, recA*, *smnA*, *nth.*	Meritt *et al*., 2005 Wen and Burne, 2004
Streptococcus oralis	No mutualism with *Actinomyces naeslundii;* spare biofilm formation.	Rickard *et al*., 2006
Streptococcus pneumoniae	Widened competence expression; increased autolysis in cultures entering stationary phase; Altered transcriptional profiles; attenuated in mouse. Altered proteome.	Romao *et al*., 2006 Joyce *et al*., 2004 Stroeher *et al*., 2003
Streptococcus pyogenes	The mutant was internalized by Hep-2 cells with higher efficiency than the wild-type. LuxS mutation reduced *speB* mRNA and increased *emm3* mRNA. Increased streptolysin S; increased *sagA* expression; decreased growth rate (media dependent); decreased SpeB activity.	Marouni *et al*., 2003 Lyon *et al*., 2001

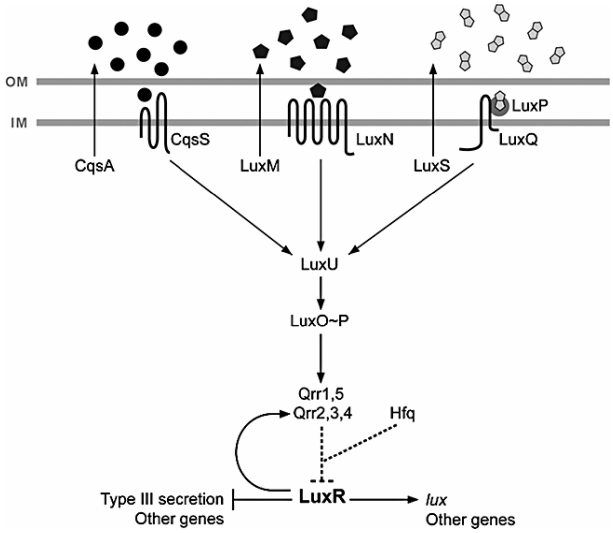


Figure 12. *V. harveyi* AI-2 regulates bioluminescence (Waters & Bassler, 2006)

higher ability of a *luxS* mutant in an M3 serotype to be internalized in pharyngeal epithelial cells as well as an enhanced *emm* transcription and slightly enhanced *covRS* transcription.

2.5 Bacteriophages and horizontal transfer of virulence factors in *S. pyogenes*

Bacteriophages are typically divided into two groups on the basis of their life cycles. The lytic phages do not enter into an extended relationship with their host bacterium. These phages infect, replicate, assemble, rupture and release from the host cells. On the other hand temperate or lysogenic phages can integrate their DNA into the host bacterium's chromosome and replicate with the host DNA thereby altering the host genomes by the introduction of novel genes into their host cells (Figure 13). Thus, bacteria can acquire genes from other strains by lateral gene transfer also referred as horizontal transfer of genes. Horizontal transfer of virulence factors is a major driving force in the emergence and evolution of pathogenic isolates (Boyd & Brussow, 2002). Newly recombinant bacteria can become more fit by gaining new alleles or genes such as immune evasion, antimicrobial agent's resistance or virulence. Three main means for horizontal gene transfer are transformation, conjugation and transduction, which involve several mobile

elements like insertion sequences, plasmids and bacteriophages. As transformation and conjugation appear to play minor role in streptococci, thus bacteriophages are significantly responsible for horizontal gene transfers (Canchaya *et al.*, 2003).

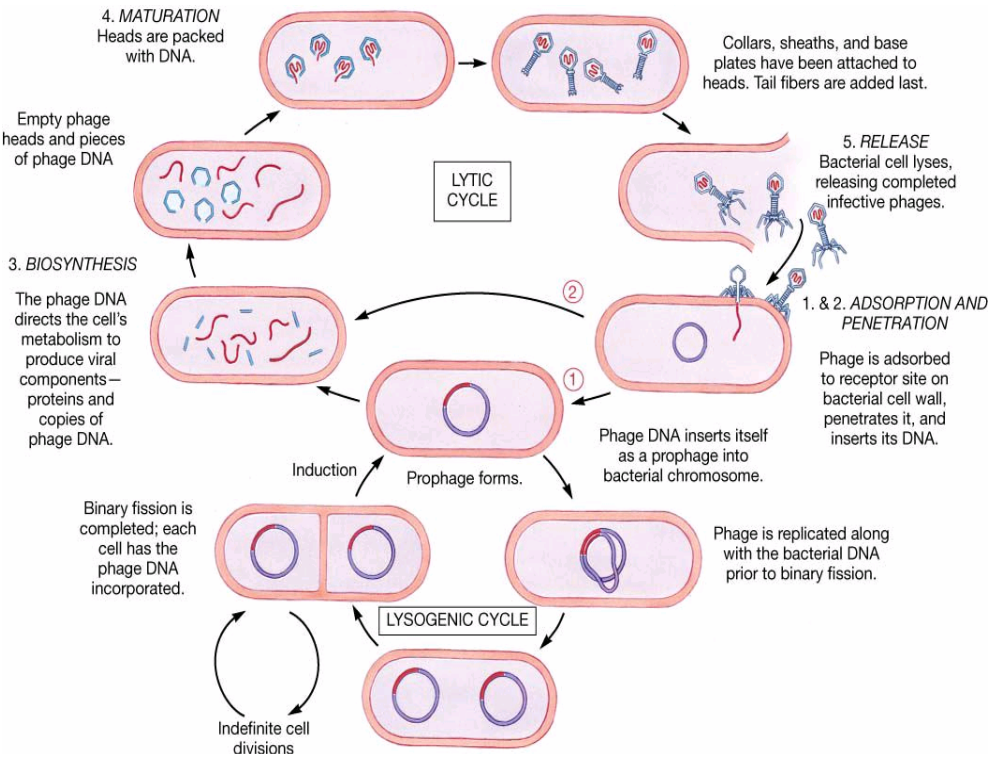


Figure 13. Lytic and lysogenic life cycle of bacteriophages (http://www.bio.miami.edu/dana/pix/bacteriophage_life_cycle.jpg)

Bacteriophage-encoding virulence factors can convert their bacterial host in a process known as phage lysogenic conversion, from a non-pathogenic strain to a virulent strain or a strain with increased virulence. GAS prophage sequences constitute 7-14% of the total genome of sequenced strains. It is a polylysogenic organism, containing many prophages or prophage like elements. The complete genome sequences of twelve GAS strains representing six different M serotypes confirm the presence of two to eight phages per genome (Table 5). Sequence alignments of the completely sequenced GAS genomes showed that ~90 percent of sequences are conserved, thus the significant source of genomic divergence is due to prophage sequences (Fischetti, 2007). All the prophages contain one or two proven or putative virulence factors like SPEs, DNAses, novel

phospholipase A2 (SlaA), efflux pump responsible for resistance to macrolide antibiotics and a novel cell wall anchored protein hypothesized to be an adhesin (Musser & DeLeo, 2005). The gene order of prophages from lactic acid bacteria having low G+C ratio is as follows: left attachment site (*attL*), lysogeny, DNA replication, transcriptional regulation, DNA packaging, head-joining-tail-tail fiber, lysis modules, right attachment site (*attR*) (Canchaya *et al.*, 2003). At one end of phage genome is the gene required for phage integration and on the other end between the attachment site and the gene for cell lysis is the toxin cassette. These toxin cassettes mostly encode GAS mitogens, like streptodornase or superantigens. Since many streptococcal SAgs-encoding genes are located on integrated phage genomes, it is now widely acknowledged that a favorable condition exists for genetic transfer among different GAS M types as well as other streptococcal species. GAS phages are therefore of highly mosaic nature, and exchange of virulence genes between phages seem to be a frequent event (Aziz *et al.*, 2005).

Table 5. Prophages in GAS sequenced strains (adapted from Beres *et al.*, 2006)

Strain	M type	Size (bp)	CDS[a]	Prophage	MLST[b]	GenBank accession no	ATCC no.
SF370	1	1,852,441	1,697	4	28	AE004092	700294
MGAS5005	1	1,838,554	1,865	3	28	CP000017	BAA-947
MGAS10270	2	1,928,252	1,987	5	55	CP000260	BAA-1063
MGAS315	3	1,900,521	1,865	6	15	AE14074	BAA-595
SSI-1	3	1,894,275	1,861	6	15	BA000034	—
MGAS10750	4	1,937,111	1,979	4	39	CP000262	BAA-1066
Manfredo	5	1,841,271	1,803	5	99	—	—
MGAS10394	6	1,899,877	1,886	8	382	CP000003	BAA-946
MGAS2096	12	1,860,355	1,898	2	36	CP000261	BAA-1065
MGAS9429	12	1,836,467	1,878	3	36	CP000259	BAA-1315
MGAS8232	18	1,895,017	1,845	5	42	AE009949	BAA-572
MGAS6180	28	1,897,573	1,894	4	52	CP000056	BAA-1064

[a]CDS= coding sequence, [b]MLST= Multilocus sequence typing

Since the mid 1980s, there is an unexplained resurgence of GAS infections observed with an occurrence of new and more severe infections. Particularly STSS caused by GAS was reported from Europe, USA and Japan (Cunningham, 2000; Stevens *et al.*, 1989). Many theories have tried to explain this phenomenon. Some have focused on host cells factors while others on an increased virulence of strains with a high speculation that phages might play a significant role in the transfer of virulence determinants especially SAgs. Indeed, SPEs were found to be involved in the pathogenesis of the observed severe

streptococcal diseases. However the evidence for the later theory is mostly indirect based on the distribution patterns of superantigens throughout the GAS population or their association with prophage sequences (Broudy & Fischetti, 2003; Kapur *et al.*, 1992; Musser *et al.*, 1993). Besides this, there is also an old theory that M protein acts as a barrier for bacteriophage transfer from one M serotype to another one. This means that M protein would be preventing phage-mediated spread of virulence factor transfer among the GAS population (Cleary & Johnson, 1977; Spanier & Cleary, 1980).

2.6 RNA regulators

As bacteria are facing different environmental conditions in their life time, hence they need to adapt according to the changing environmental conditions. Bacteria have a number of virulence factors, which are needed to be activated or repressed according to the environmental or host signals. Up to recently, it was believed that bacteria regulate mainly their gene expression by means of repressor or activator proteins at the transcriptional level. However, it has been increasingly evident that RNA can control gene expression more commonly than previously anticipated. Some of the described RNAs provide house-keeping functions like RNaseP, tmRNA and 4.5S RNA, while others are regulators of gene expression.

2.6.1 Regulatory RNAs: Definition and modes of action

Many terms have been used for small RNAs like sRNAs (small RNAs), ncRNA (noncoding RNAs), snmRNAs (small non messenger RNAs), fRNA (functional RNAs), regulatory RNAs and riboregulators. sRNAs are mainly confined to control biological functions as bacterial accessory elements (Wagner *et al.*, 2002). sRNAs have emerged as major regulators of adaptive responses and RNA-mediated regulation plays a main role in virulence. Most of the sRNAs are non essential i.e. their absence does not cause cell death.

The most common mode of action of regulatory RNAs is by base pairing to mostly the 5' end of target mRNAs, via a so-called antisense mechanism, thus leading to down-regulation or up-regulation of the gene expression at the post-transcriptional level (Figure

14). Binding of a sRNA to a target mRNA mostly inhibits translation or facilitates the decay of mRNA, however sometimes it can also activates the translation by conversion to a translationally active conformation (Storz *et al.*, 2005). Regulatory RNAs can affect the expression of multiple target mRNA or a single target mRNA encoding a virulence factor. They can also inhibit or activate the expression of single target mRNAs, which decrease or increase the synthesis of single regulatory protein, in turn affecting the expression of a great number of downstream genes. The CsrA protein, regulator of carbon storage, adhesion, cell surface properties and virulence, interacts with target mRNAs and either facilitates their rapid degradation or activates translation (Liu *et al.*, 1997). The third mechanism involves binding of a metabolite to the nascent mRNA thus inducing a structural change to a terminator or a translationally inactive state, called riboswitch (Winkler & Breaker, 2005).

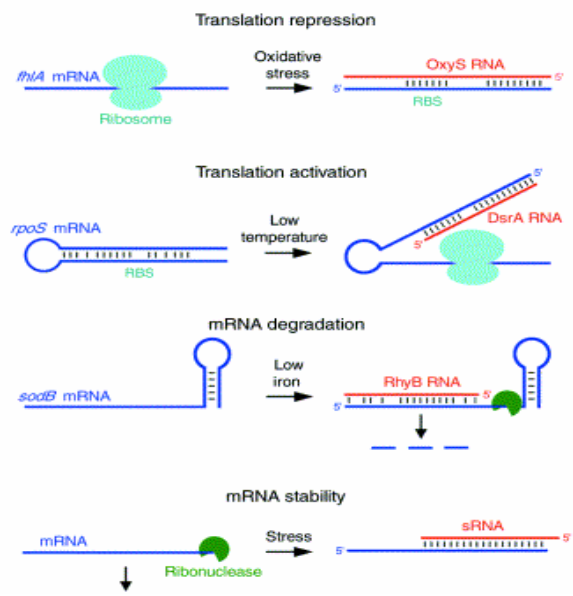


Figure 14. Mechanisms of action of regulatory RNAs (Storz *et al.*, 2004)

Regulatory antisense RNAs can be of two types: cis-encoded or trans-encoded. Cis-encoded RNAs can bind to the genes downstream from the same locus and are fully complementary to their target RNAs. In contrast trans-encoded RNAs and their targets are encoded in separate loci and the regions of complementarity are generally short and often non-contagious (Altuvia & Wagner, 2000; Delihas, 1995). Most of the

chromosomally encoded antisense sRNAs belong to this class. In gram-negative bacteria, many sRNAs that form only a limited complementarity to their targets require the hexameric Sm-like protein Hfq (Valentin-Hansen *et al.*, 2004). Hfq can help in stabilizing sRNAs by promoting antisense-target RNA pairing or by acting in an unfolding or playing chaperone role. Hfq can also act as an adapter between sRNAs and RNase E for degradation (Romby *et al.*, 2006). Many of the sRNAs discovered are stable when induced, exhibiting a half life of 20-60 minutes. Many sRNAs are unprocessed primary transcripts e.g. DsrA, Spot42, MicF, OxyS, while several others are generated from longer precursors and are finally cleaved to their actual sizes e.g. 4.5S RNA, tmRNA, 6S RNA.

Regulatory RNAs regulate gene expression in different growth conditions. For example, in *Listeria monocytogenes*, the transcriptional regulator PrfA is thermo-regulated i.e. its translation initiation only happens when temperature is increased to 37^{o}C (Johansson *et al.*, 2002). This occurs through a riboswitch-like mechanism. A typical non-coding sRNA can bind to several different targets, as it usually forms only a limited number of basepairs to its target mRNA. This limited complementarity not only allows single sRNA to act on multiple targets but also allows the sRNAs to be induced under different growth conditions, allowing multiple sRNAs to act on single targets (Romby *et al.*, 2006).

2.6.2 Small RNAs in bacterial pathogenicity

More than 80 non-coding RNAs have been found in *Escherichia coli*. The majority of these RNAs are present in pathogenic strains and have a role in stress-response regulation and bacterial pathogenicity (Table 6). The first trans-encoded antisense RNA identified is MicF in *E. coli* (Andersen *et al.*, 1987). Expression of MicF is induced by different environmental stresses like osmolarity and temperature (Delihas & Forst, 2001). MicF binds to the translation initiation region (TIR) of *ompF* mRNA, blocks the translation of the OmpF protein (porin protein) and induces rapid RNase E dependent *ompF* mRNA degradation (Rasmussen *et al.*, 2005).

Table 6. Regulatory RNAs in bacterial pathogenicity (adapted from Maria Siller thesis)

RNA (Species)	Target genes	Mechanism of action	References
CsrB (*Salmonella typhimurium*)	UR: genes of the salmonella pathogenicity island 1	Protein-targeting CsrB RNA functions as an antagonist of CsrA by sequestering this protein and preventing its action.	Altier *et al*., 2000
CsrB (*Escherichia coli*)	UR: *glgC* (ADP-glucose pyrophosphorylase)	Protein-targeting CsrB RNA functions as an antagonist of CsrA by sequestering this protein and preventing its action.	Baker *et al*., 2002
CsrB/C/D (*Vibrio cholerae*)	Interact via CsrA with the expression of Qrr sRNAs.	Protein-targeting The three sRNAs control the activity of CsrA, which interacts with the expression of Qrr sRNAs, therefore regulating the entiere quorum-sensing system.	Lenz *et al.*, 2005
csRNA (1-5) (*Streptococcus pneumoniae*)	DR: (csRNA4 and csRNA5) stationary-phase autolysis	Most likley inhibit initiation of transaltion by RNA-RNA base pairing.	Halfmann *et a*l., 2007
DsrA (*Escherichia coli*)	UR: *rpoS* DR: Global regulator H-NS	RNA-RNA basepairing Basepairing to the *rpoS* mRNA leader sequence, opens secondary structure formation so that translation can occur. Inhibits *hnS* mRNA translation by base pairing.	Lease *et al*., 2004; Repoila and Gottesmann, 2001; Wassarman *et al.,* 1999
fasX (*Streptococcus pyogenes*)	UR: SLS, *ska* DR: *mrp*, *fbp54*, *sagA*	unknown	Kreikemeyer *et al*., 2001
gadY (*Escherichia coli*)	UR: *gadX, gadW* (acid response genes)	RNA-RNA basepairing Basepairing with the 3´end of the *gadX* mRNA leads to stabilization of the target transcript.	Opdyke *et al*., 2004
GcvB (*Salmonella typhimurium*)	DR: Seven target mRNAs that encode periplasmic substarte-binding proteins of ABC uptake systems for amino acids and peptides.	RNA-RNA basepairing Represses target mRNAs by binding to extended C/A-rich enhancer motifs. In some cases GcvB maskes directly the S-D-motif to prevent 30 S subunit binding.	Sharma *et al.,* 2007
IstR1/ltsR2 (*Escherichia coli*)	DR: *tisAB*	RNA-RNA basepairing Base pairing of lstR-1 to the *tisAB* mRNA promotes cleavage of the toxic transcript by RNAseIII.	Vogel *et al*., 2004
MicA, MicF, MicC (*Escherichia coli*)	DR: *ompA, ompF, ompC*	RNA-RNA basepairing MicA, MicC and MicF bind regions in their respective target mRNAs leading to repression of translation and induction of rapid RNase E-dependent mRNA decay.	Vogel and Papenfort, 2006
MicX (*Vibrio cholerae*)	DR: *vc0972* (uncharacterized OMP), *vc0620* (ABC transporter)	RNA-RNA basepairing Primary transcripts of MicX are processed in an RNase E- and Hfq-dependent fashion. Processed MicX downregulates transcripts of *vc0972* and *vc0620*.	Davis and Waldor, 2007
OmrA, OmrB (*Escherichia coli*)	DR: several genes encoding outer membrane poteins, including *cirA*, *fecA*, *fepA* and *ompT*.	RNA-RNA base pairing Because OmrA and OmrB bind Hfq, they were predicted that to act by pairing with their target mRNAs; thus changing stability and/or translation potential.	Guillier and Gottesman, 2006
OxyS (*Escherichia coli*)	Represses translation of *fhlA and rpoS*	RNA-RNA basepairing Blocks translation of *fhlA* mRNA by binding across the S-D sequence. Mechanisms is Hfq dependent.	Argaman *et al*., 2000; Wassarman *et al*., 1999; Zhang *et al.,* 1998
pel (*Streptococcus pyogenes*)	UR: *emm*, *sic*, *nga*, SpeB activity	unknown	Mangold *et al*., 2004
PrfA (*Listeria monocytogenes*)	Listeriolysin O, phospholipases PlcA and PlcB	Thermosensor At low temperature the *prfA*-UTR forms a secondary structure wich masks the RBS. At higher temperature the structure melts, permitting binding to the ribosome.	Johansson *et al*., 2002
PrrB (*Pseudomonas fluorescens*)	UR: of 2, 4-diacetylphloroglucinol (Phl) and hydrogencyanide HCN	Protein targeting The structure is similar to the CsrB and RsmB regulatory RNAs in *E. coli and E. carotovora.*	Aarons *et al.*, 2002
PrrF1 PrrF2 (*Pseudomonas aeruginosa*)	DR: *sodB* (superoxide dismutase), *sdh* (succinate dehydrogenase), and bacterioferritin.	RNA-RNA base pairing Functional homologs of RyhB in *E. coli*; Expression is regulated by iron.	Wilderman *et al*., 2004
Qrr1-4 (*Vibrio cholerae; Vibrio harveyi*)	DR: *luxR*/*hapR*	RNA-RNA base pairing The sRNAs act downstram of LuxO-P to destabilize *luxR*/*hapR* mRNA and regulate quorum-sensing dependent gene	Lenz *et al.,* 2004

		expression in *V. harveyi* and *V. cholerae*.	
RatA (*Bacillus subtilis*)	DR: *txpA* (toxic peptide A)	Toxin:antitoxin modul *ratA* is an antisense RNA that overlaps the *txpA* mRNA. Hybridisation results in degradation of the ds-RNA complex.	Silvaggi *et al.*, 2005
RivX (*Streptococcus pyogenes*)	UR: *mga* regulon	unknown	Roberts and Scott, 2007
RNAalpha (*Vibrio anguillarum*)	DR: iron-uptake complex (*fat* operon)	RNA-RNA basepairing The expression of *fatA* and *fatB* is repressed under iron-rich conditions, in which RNAalpha is induced. RNAalpha is homologous to two-thirds of the coding region of *fatB.*	Waldbeser *et al*., 1995
RNAIII (*Staphylococcus aureus*)	UR: toxins and enzymes DR: surface proteins, *spa*, rot	RNA-RNA basepairing Binding to the anti-S-D region of the *hla* mRNA leads to disruption of the intramolecular base-pairing, so that the ribosome can bind. 3´end of RNAIII binds to the *spa* mRNA, sequesters the RBS and further destabilizes the *spa* mRNA. Binding to *rot* mRNA S-D sequence inhibits translation, thus leading to *rot* mRNA procession.	Geisinger *et al*., 2006; Huntzinger *et al*., 2005; Morfeldt *et al*., 1995; Novick *et al*., 1993
RprA (*Escherichia coli*)	UR: *rpoS*	RNA-RNA basepairing Translational regulation of the stationary phase sigma factor RpoS is mediated by the formation of a double-stranded RNA stem-loop structure in the upstream region of the *rpoS* mRNA, occluding the translation initiation site.	Majdalani *et al*., 2001
RsmB (*Pseudomonas aeruginosa*)	UR: production of AHL C4-HSL and C6-HSL	Protein-targeting AHL production increases when the RsmB RNA binds the repressor protein RsmA, which therfore becomes inactivated.	Burrowes *et al*., 2005
RsmB´ (*Erwinia caratovora*)	UR: proteoloytic enzymes, proteases and cellulases	Protein-targeting Inhibits target mRNA degradation by binding to the RNA binding protein RsmA.	Johansson *et al*., 2003; Liu *et al*., 1998
RsmY, RsmZ (*Pseudomonas fluoresecens*)	UR: biocontrol traits	Protein-targeting RsmZ sequesters the RNA-binding protein RsmA, a translational regulator of genes involved in biocontrol. Relieve RsmA-mediated regulation of secondary metabolism and biocontrol traits.	Valverde *et al*., 2003
RyaA/SgrS (*Escherichia coli*)	DR: *ptsG* (encodes the glucose transporter of the phosphoenolpyruvate phosphotransferase system (PTS))	RNA-RNA base pairing RyaA is required for posttranscriptional regulation of *ptsG* in response to phosphoglucose stress. In addition, *ryaA* transcription is activated by YabN, the member of transcriptional regulators.	Vanderpool *et al*., 2004
RyhB (*Escherichia coli*)	DR: *sodB* (encoding superoxide dismutase), *ftn* and *bfr* (encoding ferritin and bacterioferritin), and several iron-sulfur cluster-containing TCA cycle enzyme genes, including the *sdh* operon (encoding succinate dehydrogenase) and *acnA* (encoding aconitase)	RNA-RNA basepairing Base pairing between RyhB and its mRNA target and subsequent Rnase E-mediated degradation of the RyhB-mRNA duplex. Because RyhB is itself negatively regulated by Fur, the net effect is positive regulation of these genes under high-iron conditions.	Afonyushkin *et al.,* 2005; Masse *et al.,* 2002; Masse *et al.,* 2003; Masse *et al.,* 2005
RyhB (*Vibrio cholerae*)	DR: iron storage and utilization genes; motility, chemotaxis and biofilm formation.	RNA-RNA basepairing *V. cholerae* may use a system analogous to the *E. coli* RyhB mechanism for regulating genes encoding iron-containing proteins and those involved in iron metabolism.	Mey *et al*., 2005
SR1 (*Bacillus subtilis*)	unknown	RNA-RNA basepairing Glucose mediated repression of SR1 transcription; regulated by CcpN.	Licht and Brantl, 2005
SurA, SurC (*Bacillus subtilis*)	*yndL* (involved in porulation)	RNA-RNA basepairing Antisense sRNA to *yndL* mRNA.	Silvaggi *et al*., 2006
VR-RNA (*Clostridium perfringens*)	UR: *colA*, *plc*, *ptp*, *cpd* DR: *ycgJ*, *metB*, *cysK*, *ygaG*	unknown	Shimizu *et al*., 2002
UR: up-regulation DR: down-regulation			

The most studied example of a regulatory RNA controlling bacterial virulence is the 512-nucleotide long RNAIII of *S. aureus*, which affects both transcription and translation of virulence genes. It acts as multi-gene regulator, repressing the expression of cell surface proteins like protein A and fibronectin-binding proteins and activating the expression of extracellular toxins and proteases like α-toxin, β-haemolysin, TSST-1, enterotoxin B, leucocidin, staphylokinase, serine protease, metalloproteases (Janzon & Arvidson, 1990; Wolz *et al.*, 1996). In totality, RNAIII regulates the expression of ~100 genes (Dunman *et al.*, 2001). Consequently, the *agr* system functions as a switch from a localized infection to invasive and systemic dissemination (Novick & Muir, 1999). Apart from regulatory functions, RNAIII also acts as an mRNA encoding δ–hemolysin. Unlike most of the antisense RNAs, RNAIII can activate the expression of its target e.g. *hla* mRNA (Morfeldt *et al.*, 1995) instead of inhibiting its expression.

OxyS RNA of *E. coli* is yet another example of a multi-gene regulator, regulating the expression of ~40 genes. It has a role in defense against oxidative damage. It accumulates at high levels when bacteria are treated with hydrogen peroxide (Altuvia *et al.*, 1997). OxyS negatively affects the expression of two target genes: *fhlA* (a transcriptional activator protein) and *rpoS* (stress/ stationary phase σ subunit of RNA polymerase).

DsrA is a 87 nt long sRNA which affects polysaccharide capsule synthesis. It is induced at low temperature. mRNA encoding global transcriptional proteins RpoS and hns are targets of DsrA. One segment of nucleotides binds to the TIR of *hns* mRNA while another one basepairs to nucleotides near the 5' end of the *rpoS* mRNA (Lease & Belfort, 2000).

The RhyB sRNA, 90 nt long, is regulated by iron. At high iron concentration, it is repressed by the ferric uptake protein Fur. Fur dependent activation of *sdh*, genes required for growth on succinate, occurs by repression of RhyB transcription.

2.6.3 Small RNAs in *S. pyogenes*

In *S. pyogenes*, so far, only three putative regulatory RNAs have been discovered: *fasX*RNA, *pel*RNA and *rivX*RNA.

***fasX* RNA**

Fas stands for fibronectin / fibrinogen binding/ haemolytic activity / streptokinase regulator. Downstream of *fasBCA* is a small 200 nucleotide RNA *fasX*. The *fas* operon consists of two genes encoding histidine kinases, FasB and FasC and the single response regulator FasA. Presence of two sensor kinase genes might mean that the sensors are bind to different environmental stimuli. These genes were identified on the basis of homology to the *S. aureus agr* two-component system and *S pneumoniae com* operons, however a quorum-sensing driven regulation process was not demonstrated for *fasX*. Rather, *fasX* controls the growth phase associated expression of GAS virulence factors. It regulates surface fibronectin and fibrinogen binding proteins and certain GAS virulence factors like *mrp*, *fbp45*, streptolysin A (*pel*) and streptokinase (*ska*) transcription (Figure 15). Both *fasX* RNA and the fasA response regulator have a similar regulatory effect on the transcription of virulence factors, however complementation studies showed that the small *fasX* RNA mainly controls virulence factor expression (Kreikemeyer *et al.*, 2001). Another study shows that the wild-type strain adhered to and was internalized by Hep2 cells at a higher degree compared to the *fasX* mutant. In addition, a *fasX* mutant showed reduced aggressiveness of strain as the mutant displayed decreased cytokine production, apoptosis induction and cytotoxicity. These results show that *fasX* expression is important for GAS in tissue colonization and intracellular persistence (Klenk *et al.*, 2005).

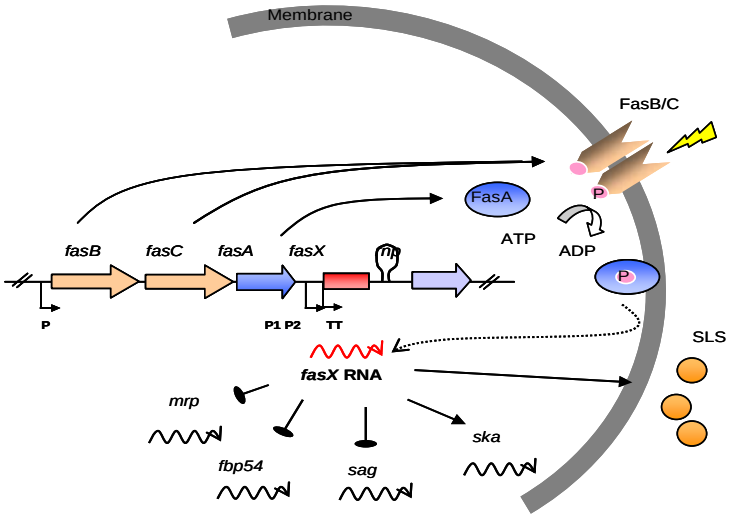


Figure 15. The *fas* operon and *fasX* RNA (from Maria Siller thesis). P1 and P2: putative promoter sequence, TT: putative transcription terminator

pel RNA

pel stands for pleiotropic effect locus. It includes the *sagA* gene, structural gene for SLS, and functions as an effector of virulence factor expression (Nizet *et al.*, 2000). Downstream of the *sagA* gene are *sagB* to *sagI* genes, which are involved in chemical modification, processing and secretion of SLS (Figure 16). Creation of isogenic *sagBC*, *sagDEF* and *sagGHI*-deficient strains and complementation studies proved that the phenotype is due to the *pel* locus and not due to polar effects of downstream genes. *pel* RNA regulates the expression of virulence factors like M protein, SpeB, Ska (Biswas *et al.*, 2001; Li *et al.*, 1999) and SIC (Mangold *et al.*, 2004). *pel* RNA is regulated in a growth-phase dependent manner and is induced by condition media. TCSs or genes encoding signaling molecules have not been identified in the vicinity of the *pel* locus, however addition of conditioned media to early logarithmic cells induced transcription of *pel*.

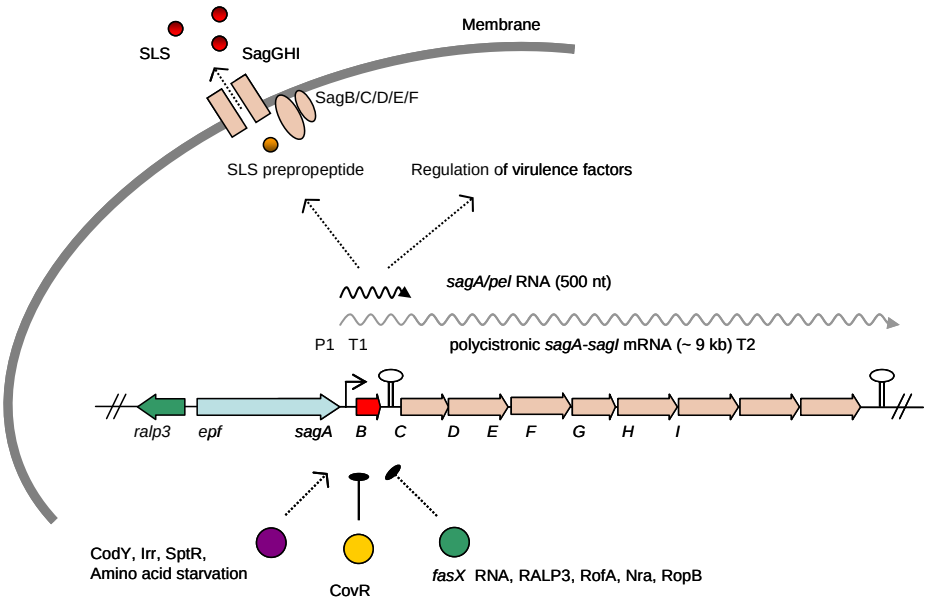


Figure 16. The genetic locus for *pel* RNA and SLS production in *S. pyogenes* (M1 serotype) (from Maria Siller thesis). P1: transcriptional start, T1: putative transcriptional terminator 1, T2: putative transcriptional terminator 2

rivX RNA

RivR, a RofA-like protein, activates the expression of *mga*. RivX is a novel small RNA, encoded within an operon with *rivR*. *rivX* also activates the expression of the Mga regulon via a RivR-independent mechanism. *rivX* is conserved in all GAS genomes sequenced to date. Furthermore, RivRX is directly repressed by CovR (Roberts *et al.*, 2007). This shows a link between two major regulators of GAS virulence, Mga and CovR, where RivR and RivX play intermediaries in pathogenic regulatory circuits (Figure 17). A *rivRX* mutant in the *covR* deletion background was strongly attenuated for virulence in a murine model of invasive soft tissue infection (Roberts & Scott, 2007). *rivRX*-activated virulence factors include M protein, ScpA, Fba and Sic (Ashbaugh *et al.*, 2000; Lukomski *et al.*, 2000; Okada *et al.*, 1994; Terao *et al.*, 2001). The *rivRX* locus does not autoregulate its own expression. Further, the analysis of *rivX* containing non sense mutations proved that the sRNA *rivX* itself, rather than a peptide encoded by *rivX* is responsible for *rivX* activity. However, *rivX* dependent *mga*-activated gene expression requires Mga protein. To note, the presence of *rivX* RNA was not demonstrated by Northern blot analysis but by RT-PCR analysis.

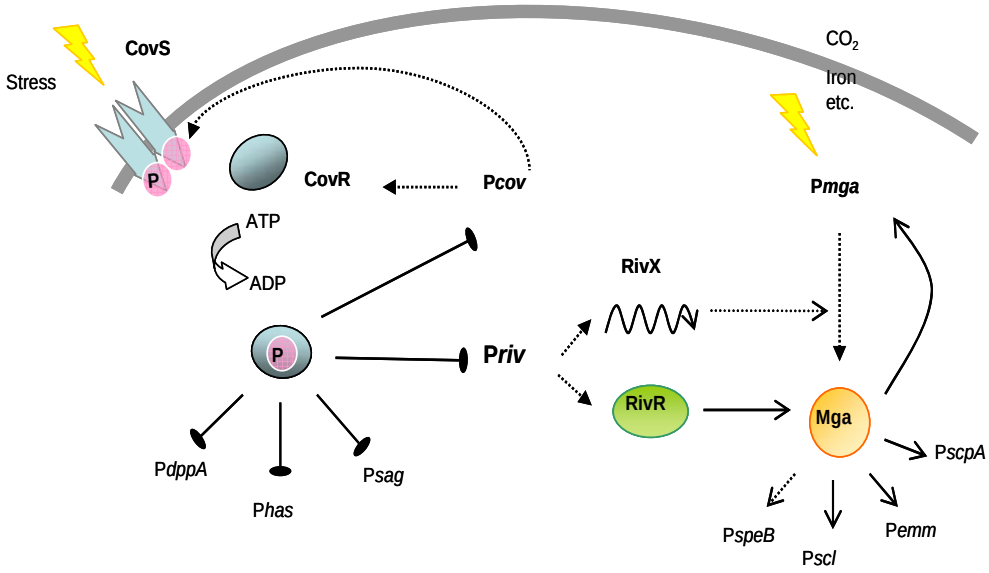


Figure 17. Model for regulation of virulence by RivR and *rivX* RNA (from Maria Siller thesis)

2.7 Riboswitches

Most of the riboregulator sRNAs function *in trans* while there is another class of regulatory RNAs, which are *cis*-acting RNA elements called riboswitches. To date more than a dozen classes of riboswitches are known (Barrick & Breaker, 2007). Although riboswitches were found in the genomes of archea, fungi and plants, most of them were identified in bacteria. Especially in Fermicutes (low G+C gram-positive) bacteria use extensively riboswitches (Table 7). As example, more than 70 *cis*-acting regulatory RNAs were described in *Bacillus subtilis*. In bacteria, the AdoCbl riboswitch is the most prevalent one but TPP, FMN and SAM-I riboswitches are also common in many groups (Barrick & Breaker, 2007).

Table 7. Distribution of riboswitches (Adapted from Winkler and breaker, 2005)

Gram-positive bacteria	Gram-negative bacteria
B12 riboswitch (Cobalamin)	
Bacillus, Listeria, Enterococcus, Clostridium, Thermoanaerobacter	Escherichia, Salmonella, Klebsiella, Yersinia, Vibrio, Pasteurella, Pseudomonas, Shewanella, Xanthomonas
FMN riboswitch	
Bacillus, Oceanobacillus, Staphylococcus, Listeria, Enterococcus, Streptococcus, Lactococcus, Lactobacillus, Clostridium, Thermoanaerobacter	*Coxiella, Shewanella, Escherichia, Klebsiella, Pasteurella, Haemophilus, Vibrio, Xanthomonas, Salmonella, Yersinia, Shigella, Photorhabdus, Actinobacillus, Pseudomonas*
TPP riboswitch	
Bacillus, Listeria, Staphylococcus, Oceanobacillus, Enterococcus, Streptococcus, Lactococcus, Lactobacillus, Clostridium, Thermoanaerobacter	*Pasteurella, Haemophilus, Aeromonas, Coxiella, Shewanella, Vibrio, Xanthomonas, Thiocystis, Escherichia, Salmonella, Shigella, Erwinia, Photorhabdus, Yersinia, Pseudomonas*
Lysine riboswitch	
Bacillus, Staphylococcus, Oceanobacillus, Clostridium, Thermoanaerobacter	*Shewanella, Pasteurella, Haemophilus, Vibrio, Escherichia, Shigella*
SAM riboswitch	
Bacillus, Listeria, Staphylococcus, Oceanobacillus, Clostridium, Thermoanaerobacter	*Xanthomonas*
Purine riboswitch	
Bacillus, Listeria, Staphylococcus, Oceanobacillus, Geobacillus, Enterococcus, Streptococcus, Lactococcus, Clostridium, Thermoanaerobacter	*Vibrio*, *Bdellovibrio*
Glycine riboswitch	
Bacillus, Listeria, Staphylococcus, Oceanobacillus, Streptococcus, Clostridium	*Vibrio*
GlcN6P riboswitch	
Bacillus, Listeria, Staphylococcus, Oceanobacillus, Lactobacillus, Enterococcus, Clostridium, Thermoanaerobacter	
PreQ riboswitch	
Bacillus, Streptooccus, Lactococcus	

2.7.1 Riboswitches: Definition and modes of action

Bacteria are able to sense and quantify intracellular metabolite concentration and regulate relevant gene expression accordingly. Riboswitches are RNAs that bind to cellular metabolites and by this leads to changing secondary or tertiary structures which then can modulate gene expression in *cis* without the involvement of protein factors. Nearly all of the riboswitches are *cis*-acting regulatory elements and are part of the 5' UTRs of mRNAs. Riboswitches can bind to metabolites ranging from very simple amino acids to very complex vitamins (Montange & Batey, 2008). These metabolites are involved in fundamental biochemical processes like coenzyme B12, thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), S-adenosylmethionine (SAM), lysine, guanine and adenine. Riboswitches are almost always located near the genes involved in biosynthesis or transport of their metabolites they sense. When a metabolite is present in the sufficient

quantity, most of the riboswitches function by inhibiting the biosynthesis or transport of a related gene, to avoid overproduction of the metabolites in the cell. Riboswitches show high specificity and affinity to their targets. Most riboswitches are composed of two functional domains. The first portion is the conserved "aptamer" domain, which binds to the metabolite and immediately downstream the second domain is called a "sequence element" or "expression platform", which controls gene expression at the transcriptional or translational level. The aptamer domain size ranges from ~70 to ~200 nucleotides, while the expression platform varies greatly in size, sequence and structures (Winkler & Breaker, 2005).

To date riboswitch mediated control of gene expression is exercised by four modes of actions. The most common mode of action is at the transcriptional level, where metabolite binding favours transcriptional termination or anti-termination conformation, leading to off or on gene expression respectively (Figure 18). Upon metabolite binding, GC-rich intrinsic terminator stems followed by polyuridine tracts are formed within the 5' UTR region of the riboswitch, which destabilizes the elongation complex (Winkler & Breaker, 2003; Yarnell & Roberts, 1999). Another common mode of gene control is at the translational level, where metabolite binding either sequester the ribosomal binding site or present it, thus making the gene either off or on, respectively (Figure 18). Hence riboswitches control gene expression by directly binding to metabolites and upon binding to their specific metabolite, they fluctuate among different conformations. Transcriptional attenuation is the most common mode of action in gram-positive bacteria while gram-negative organisms mostly use translational attenuation (Vitreschak *et al.*, 2004; Winkler & Breaker, 2003). The most common mode of gene expression control is by feedback repression; however there are examples where activation of gene expression is also observed.

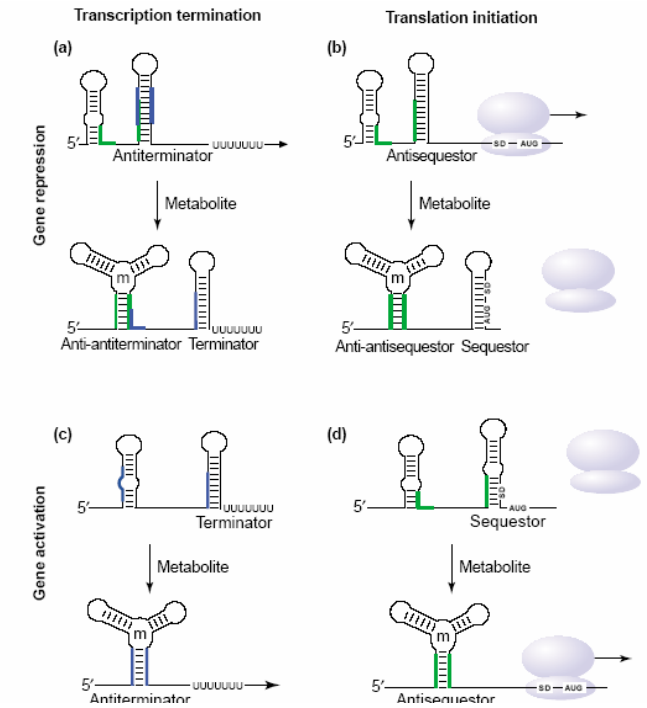


Figure 18. Riboswitch mediated gene control (Nudler & Mironov, 2004)

Alternative modes of riboswitch-mediated gene expression control are by cleavage of nascent RNA and alterations in mRNA stability or splicing (Montange & Batey, 2008). Unlike traditional riboswitches, GlcN6P riboswitch does not induce a conformational change upon binding to its ligand but rather it acts as a ribozyme, which self-cleaves its downstream *glmS* genes (Cochrane & Strobel, 2008). The *glmS* gene encodes the enzyme glutamine-fructose-6-phosphate amidotransferase. This enzyme produces glucosamine-6-phosphate (GlcN6P) and this compound triggers the riboswitch function. For the downregulation of gene expression, ribozyme self-cleavage activity is necessary. The RNA cleavage site resides upstream of the *glmS* open reading frame.

In some cases like in glycine riboswitch there are two aptamers followed by single expression platform (Mandal *et al.*, 2004). Cooperative binding of metabolites to riboswitches helps bacteria to sense even small changes in the metabolite concentrations. Riboswitch from *B subtilis* activates transcription upon glycine exposure. Thus it is rare example of genetic on switch (Tucker & Breaker, 2005).

2.7.2 Riboswitches in *S. pyogenes*

Although many riboswitches have been discovered in gram positive bacteria especially in *B subtilis*, but so far no information is available regarding riboswitches in *S pyogenes*.

3) AIMS OF THIS STUDY

S. pyogenes (GAS) is a notorious human pathogen, causing a number of diseases world-wide. It is a cause of great concern of human mortality and morbidity. A number of important questions related to *S. pyogenes* pathogenicity, virulence factor transfer and regulatory aspect still remains unanswered today. The knowledge about GAS pathogenesis and its control is necessary not only to help finding novel antimicrobial strategies to this pathogen but also to help understanding the molecular mechanisms that could explain the recent reemergence of severe infections caused by this pathogen.

Project 1: Lysogenic transfer of Group A streptococcus superantigen gene among streptococci

Since the mid-1980s there has been a significant reemergence of the severe forms of GAS infections worldwide, particularly necrotizing fasciitis and STSS. Apart from GAS, in the last few years, GCS and GGS, which are normally regarded as commensal organisms, have also been recovered from NF and STSS infections. Many hypotheses have emerged to explain this phenomenon, some focusing on the impaired host defenses to GAS while others focused on the emergence of more virulent strains. Bacteriophage-encoded virulence factors by lysogenic conversion can convert a non pathogenic strain into a pathogenic strain. As GAS is a polylysogenic organism, one can hypothesize that horizontal transfer of virulence factors may play a significant role in increased pathogenicity. On the other hand, a number of studies indicate that M protein can function as barrier to the horizontal gene transfer.

In our lab we found a phage ϕ149 carrying the SSA superantigen-containing gene in M12 GAS strain. We wanted to characterize ϕ149 and determine the broad host range of this phage to find out whether ϕ149 can convert a non-toxigenic strain to a toxigenic strain via lysogenic conversion. To facilitate the study, a kanamycin resistance cassette (*aphIII*) was inserted in the *ssa* coding sequence in phage ϕ149, thus generating the recombinant phage ϕ149::*aphIII* in strain EC516. Strain EC516 would be then used as a donor strain of phage ϕ149::*aphIII* in transfer experiments to other GAS strains of different M serotypes and to group C, G and *S. pneumoniae* strains using kanamycin resistance as a marker for phage transfer. This study would further allow the evaluation of the role of

bacteriophages in horizontal transfer of toxins and emergence of increased virulent strains.

Project 2: A bioinformatic screen reveals novel small non-coding RNAs in S*treptococcus pyogenes*

Bacteria need to control their gene expression according to changing environmental conditions. It has become increasingly evident now that apart from proteins, RNA can also control gene expression more commonly than previously anticipated. A number of small RNAs have been discovered in bacteria. However, only three putative regulatory RNAs have been discovered in *S. pyogenes*: *fasX*RNA, *pel*RNA and *riv*XRNA.

The main goal of this study was to identify and characterize novel ncRNAs in *S. pyogenes* using a large scale bioinformatic approach. Four computational algorithms, 'QRNA', 'RNAz', 'Alifoldz', 'sRNAPredict2' and the Rfam database would be used to predict sRNAs in intergenic regions of *S. pyogenes* M1 strain SF370. Selected predictions would be verified experimentally by Northern blot analysis and the interesting candidates would be characterized further.

This is the first study aiming at uncovering ncRNAs in GAS in a genome-wide fashion. In a long-term perspective, this research would ultimately allow gaining information about the role of ncRNAs in the physiology, metabolism and virulence in GAS.

Project 3: Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress and interaction with host cells

Bacteria have the ability to sense signaling molecules released by similar bacteria. These signaling molecules are extra-cellular, low molecular weight molecules called "auto-inducers" (AIs). Recently, another autoinducing system has been discovered, which detects a furanone-like signaling molecule termed "autoinducer 2" (AI-2) in *V. harveyi*. AI-2 is synthesized by the *luxS* gene, which is found in over 55 gram-negative and gram-positive bacterial species. AI-2 is proposed to have a role in interspecies communication.

Knowledge about quorum sensing systems in *S. pyogenes* is very limited. Two putative quorum-sensing systems, *sil* and *fasBCA*, have been reported in GAS. The *luxS* homologue was found in *S. pyogenes* and shown to play a role in certain pathogenicity

aspects (Lyon *et al.*, 2001; Marouni & Sela, 2003). Because of the dual metabolic and signaling function of *luxS*/ AI-2, which was reported in certain bacteria, we here were interested in the functional analysis of *luxS* in metabolism versus its implication in pathogenicity-linked aspects.

Project 4: Group A Streptococcus activates type I interferon production and MyD88-dependent signalling without involvement of TLR2, TLR4 and TLR9

One of the mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through the toll-like receptors (TLRs). TLRs recognize specific molecular patterns that are present in microbial components like peptidoglycan, flagellin, LPS etc. Stimulation of different TLRs induces distinct patterns of gene expression, which leads to the activation of innate immunity and also instructs the development of antigen-specific acquired immunity.

Streptococcal invasins and protein toxins interact with mammalian blood and tissue components leading to host cell killing and provoke a damaging inflammatory response. *S. pyogenes* possesses two cytolysins: SLS and SLO. Previous data show that the gram-positive pathogen *Listeria monocytogenes* produces listeriolysin O, which allow the bacteria to escape from the phagolysosome and leads to production of interferons. Here my aim was to find whether a similar mechanism of evasion from the immune system occurs in *S. pyogenes* and whether SLS or SLO is responsible for the production of interferons from infected macrophages.

4) RESULTS

4.1 Project 1: Lysogenic transfer of Group A streptococcus superantigen gene among streptococci

4.1.1 Summary

Since the mid-1980s, there has been a significant re-emergence of severe forms of diseases (particularly necrotizing fasciitis and streptococcal toxic shock syndrome, STSS) caused by Group A Streptococcus (GAS) worldwide (Banks *et al.*, 2002; Cunningham, 2000). One hypothesis to explain this phenomenon has focused on changes in the virulence of the pathogen itself. With this respect, fluctuations in the severity and character of GAS virulence have been linked to the plasticity of the streptococcal genome involving mobile genetic elements. In GAS, prophages encode, in addition to their essential viral proteins, one or more putative or established virulence factors including superantigens (Banks *et al.*, 2002; Brussow *et al.*, 2004). Via lysogenic conversion, the toxin-encoding bacteriophages can convert their bacterial host from a non-pathogenic strain to a virulent strain or a strain with increased virulence (Brussow *et al.*, 2004).

Screening GAS clinical isolates for superantigen-encoding bacteriophage transfer revealed a clinical isolate of M12 serotype derived from a patient with STSS that carries a novel bacteriophage, ϕ149, encoding the streptococcal superantigen SSA (Igwe *et al.*, 2003; Reda *et al.*, 1996) (allele *ssa-3*). Sequence analysis of the att-L proximal region of ϕ149 showed a mosaic nature of the phage, which shares characteristics with previously described GAS prophages. Release of ϕ149 from the bacterial chromosome was obtained after induction with the antibiotic and DNA damaging agent mitomycin C, hydrogen peroxide and UV treatment. Bacteriophage titer was estimated as 7×10^4 pfu/mL. Remarkably, lysogenic conversion of GAS clinical isolates of various M serotypes (M1, M3, M5, M12, M19, M28 and M94) as well as of Group C *Streptococcus equisimilis* (GCSE) clinical isolates via transfer of a recombinant phage ϕ149::Km^r was successfully obtained. ϕ149::Km^r from selected lysogenized GAS and GCSE strains could be transferred back to M12 GAS strains.

This is the first direct experimental evidence for toxigenic conversion via lysogeny across a diverse range of GAS clinical isolates of distinct M types and between GAS and GCSE. Our data indicate that horizontal transfer of lysogenic phage among GAS is not obligatory M-type restricted and provide further support for the concept of interspecies lysogenic toxin-conversion. Streptococci might employ this mechanism specifically to allow more efficient adaptation to changing host challenges, potentially leading to fitter and more virulent clones.

My contribution to this study was the determination of bacteriophage titer, analysis of the inducibility of ϕ149 by mitomycin C, ultra-violet radiation and H_2O_2 using lysogenic infections as read-outs. Infection assays were performed to determine the host range of ϕ149 with 34 GAS, 10 GCS and 10 GGS. Retransfer experiments were done from selected M serotypes to M12 serotype and from GCS to GAS. All of the lysogens were checked by PCR and Southern blot analyses.

4.1.2 Manuscript 1

Lysogenic transfer of Group A streptococcus superantigen gene among streptococci

Published manuscript: Journal of Infectious Diseases (2008) 197(2), 225-234

Authors:

Ivo Vojtek, **Zaid Ahmed Pirzada**, Birgitta Henriques-Normark, Markus Mastny, Rajendra Prasad Janapatla and Emmanuelle Charpentier

Lysogenic Transfer of Group A *Streptococcus* Superantigen Gene among Streptococci

Ivo Vojtek,[1] Zaid A. Pirzada,[1] Birgitta Henriques-Normark,[2,3] Markus Mastny,[1] Rajendra P. Janapatla,[1] and Emmanuelle Charpentier[1]

[1]Max F. Perutz Laboratories, University of Vienna, Department of Microbiology and Immunobiology, Vienna, Austria; [2]Swedish Institute for Infectious Disease Control, Department of Bacteriology, and [3]Karolinska Institute, Solna, Sweden

A group A *Streptococcus* (GAS) isolate, serotype M12, recovered from a patient with streptococcal toxic shock syndrome was analyzed for superantigen-carrying prophages, revealing ϕ149, which encodes superantigen SSA. Sequence analysis of the att-L proximal region of ϕ149 showed that the phage had a mosaic nature. Remarkably, we successfully obtained lysogenic conversion of GAS clinical isolates of various M serotypes (M1, M3, M5, M12, M19, M28, and M94), as well as of group C *Streptococcus equisimilis* (GCSE) clinical isolates, via transfer of a recombinant phage ϕ149::Km^r. Phage ϕ149::Km^r from selected lysogenized GAS and GCSE strains could be transferred back to M12 GAS strains. Our data indicate that horizontal transfer of lysogenic phages among GAS can occur across the M-type barrier; these data also provide further support for the hypothesis that toxigenic conversion can occur via lysogeny between species. Streptococci might employ this mechanism specifically to allow more efficient adaptation to changing host challenges, potentially leading to fitter and more virulent clones.

Streptococcus pyogenes, or β-hemolytic group A *Streptococcus* (GAS), is a notorious human pathogen, responsible for a number of different infections that range from mild skin diseases to fulminant, severe, invasive syndromes. Since the mid-1980s, a remarkable increase in the incidence of the severe forms of GAS infections (in particular, necrotizing fasciitis and streptococcal toxic shock syndrome [STSS]) has been observed worldwide [1, 2]. Many hypotheses have emerged to explain this phenomenon; some focus on impaired host defense against specific strains or specific virulence determinants (e.g., immunogenetic background or predisposing factors), whereas others focus on changes in bacterial virulence itself [3–5].

Fluctuations in the severity and character of streptococcal virulence have also been linked to the plasticity of the streptococcal genome. Mobile genetic elements, including lysogenic bacteriophages, that have integrated into the GAS genome over time were found to be almost exclusively responsible for the genetic differences among strains [6–11]. Recent genomic analyses indicate that GAS is a polylysogenic organism in which prophage sequences constitute ~10% of the total genome [1, 3, 7, 12]. In GAS, prophages encode 1 or more putative or established virulence factors, including phospholipases, streptodornases, and superantigens, in addition to their essential viral proteins [1, 12]. It is now widely acknowledged that virulence gene transfer may occur among GAS when favorable conditions are encountered in the human host [13–18]. By means of lysogenic conversion, the toxin-encoding bacteriophages can convert their bacterial host from a nonpathogenic strain to a virulent strain or a strain with increased virulence [12, 13, 19]. Along these lines, prophages have been shown to play a key role in subclone diversification and to be largely responsible for the uniqueness of clinical isolates [3]. Consequently, GAS prophages play a critical role in determining the distinct disease pathologies associated with otherwise similar strains [1].

The critical role of superantigens in severe GAS infection underlines the clinical relevance of prophages [2, 20, 21]. In GAS, the majority of superantigen genes are located on prophage genomes. Experimental ex vivo toxigenic conversion by lysogeny has been demonstrated

Received 30 April 2007; accepted 28 July 2007; electronically published 5 January 2008.

Potential conflicts of interest: none reported.

Presented in part: 7th American Society for Microbiology Conference on Streptococcal Genetics, Saint-Malo, France, 18–21 June 2006 (abstract A126).

Financial support: Austrian Science Fund Project (grant P17238-B09 to E.C.); Austrian National Bank (grant 10802 to E.C.); Higher Education Commission of Pakistan (scholarship to Z.A.P.); Austrian Exchange Service (scholarship to R.P.J.).

Reprints or correspondence: Dr. Emmanuelle Charpentier, Max F. Perutz Laboratories, University of Vienna, Dr. Bohrgasse 9/4, 1030 Vienna, Austria (emmanuelle.charpentier@univie.ac.at).

The Journal of Infectious Diseases 2008; 197:225–34
© 2007 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2008/19702-0009$15.00
DOI: 10.1086/524687

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strain		
E. coli	Host strain for cloning	Lab collection
DH5-α		
TOP10	Host strain for cloning	Lab collection
S. pyogenes		
RDN149	M12, ϕ149 donor, *ssa*+, *speH*+	[29]
EC516	RDN149 (ϕ149::Km^r)	This study
RDN151	M12, indicator strain for ϕ149, *ssa*−, *speH*+	[29]
EC627	RDN151 (pMSP3535)	This study
EC496	RDN151 lysogenized with ϕ149	This study
EC497	RDN151 lysogenized with ϕ149	This study
CS112	M12, ϕCS112 donor, *speC*+	[38]
CS24	M12, indicator strain for ϕCS112 and ϕ149, *speC*−, *ssa*−, *speH*−	[38]
RDN313	CS24 lysogenized with ϕCS112	This study
RDN312	CS24 lysogenized with ϕ149	This study
EC500	CS24 lysogenized with ϕ149	This study
RDN1	M1, clinical isolate	Lab collection
RDN2	M19, clinical isolate, recipient for ϕ149	Lab collection
RDN3	M1, clinical isolate	Lab collection
RDN4	M49, clinical isolate	Lab collection
RDN5	M3, clinical isolate, recipient for ϕ149	Lab collection
RDN6	M3, clinical isolate, recipient for ϕ149	Lab collection
RDN8	M1, clinical isolate, recipient for ϕ149	Lab collection
RDN27	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN29	M1, clinical isolate	Lab collection
RDN57	M49, clinical isolate	Lab collection
RDN60	M5, clinical isolate, recipient for ϕ149	Lab collection
RDN74	M89, clinical isolate	Lab collection
RDN78	M3, clinical isolate	Lab collection
RDN83	M89, clinical isolate	Lab collection
RDN86	M1, clinical isolate	Lab collection
RDN89	M3, clinical isolate	Lab collection
RDN100	M83, clinical isolate	Lab collection
RDN104	M81, clinical isolate	Lab collection
RDN106	M94, clinical isolate, recipient for ϕ149	Lab collection
RDN107	M1, clinical isolate	Lab collection
RDN114	M3, clinical isolate	Lab collection
RDN116	M94, clinical isolate, recipient for ϕ149	Lab collection
RDN119	M1, clinical isolate	Lab collection
RDN138	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN139	M28, clinical isolate	Lab collection
RDN144	M28, clinical isolate, recipient for ϕ149	Lab collection
RDN145	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN146	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN147	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN148	M12, clinical isolate, recipient for ϕ149	Lab collection
RDN152	M22, clinical isolate	Lab collection
RDN156	M28, clinical isolate, recipient for ϕ149	Lab collection
EC1091	RDN148 lysogenized with ϕ149::Km^r	This study
EC1092	RDN148 lysogenized with ϕ149::Km^r	This study

(*continued*)

Table 1. (*Continued.*)

Strain or plasmid	Relevant characteristic(s)	Source or reference
EC1093	RDN148 lysogenized with ϕ149::Km^r	This study
EC749	RDN156 lysogenized with ϕ149::Km^r	This study
EC917	RDN156 lysogenized with ϕ149::Km^r	This study
EC727	RDN116 lysogenized with ϕ149::Km^r	This study
EC1083	RDN116 lysogenized with ϕ149::Km^r	This study
S. dysgalactiae subsp. *equisimilis*		
EC783	Clinical isolate	Lab collection
EC788	Clinical isolate	Lab collection
Plasmid		
pUC19	ColE1ori, Amp^r, *lacZ*	Lab collection
pMSP3535	*ermB*, *repDEG*-pAMβ-1, *nisRK*, ColE1ori, P*nisA*	[37]
pEC61	pUC19Ω*aphIII*	This study
pEC96	pEC61Ω*ssa-up*	This study
pEC97	pEC96Ω*ssa-down*	This study

NOTE. Lab, laboratory.

only for *speA* and *speC* and almost exclusively among strains belonging to the same M serotype. The suggested bacteriophage-mediated transfer of other superantigens is based only on indirect evidence: for example, their association with prophage sequences, their distribution pattern throughout the streptococcal population, or the inducible character of prophages carrying the respective genes [13, 15, 22–25]. Further, a number of studies indicate that M proteins could function, directly or indirectly, as barriers to horizontal gene exchange. A phenotypic correlation between resistance to bacteriophage infection and M-protein surface expression has been described [26, 27]. Nonrandom associations between exotoxin alleles and *emm* patterns were also observed, thus suggesting some direct or indirect biological interactions between M-protein surface structures and bacteriophage-associated properties [28–30]. In this study, we focus on the mobility of superantigen SSA, which has been associated with GAS isolated from patients with STSS and which has highly potent superantigenic activity that has been proven experimentally [31–33]. Presence of the *ssa* gene has been detected across the GAS population, as well as in clinical isolates of group C (GCS) and group G (GGS) streptococci (*Streptococcus dysgalactiae* subspecies *equisimilis* GCSE and GGSE, respectively), thus suggesting the possibility of interspecies *ssa* gene transfer [14, 29, 30, 34–36].

We report here the characterization of an inducible chimeric prophage, ϕ149, carrying the superantigen-encoding *ssa-3* allele from a GAS clinical isolate of serotype M12, RDN149. We describe the host range of this phage by investigating its transferability to GAS strains of various M types, as well as to GCSE and GGSE. This study experimentally confirms previous indications that lysogenic toxin conversion can occur across the M-type barrier in GAS and between GAS and GCSE.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Streptococcal strains (table 1; [29, 37, 38]) were grown at 37°C with 5% CO_2 without agitation in Todd-Hewitt broth either with or without 0.2% yeast extract supplementation and on tryptic soy agar supplemented with 3% sheep blood or THY agar. Transformation of *Escherichia coli* and *S. pyogenes* with plasmid DNA was performed as described elsewhere [39, 40]. Whenever required, antibiotics were added to the media: erythromycin at 3 μg/mL for *S. pyogenes*; and kanamycin at 25 μg/mL for *E. coli* and at 300 μg/mL for streptococcal strains.

General DNA manipulation and analysis. Isolation of streptococcal DNA (genomic and phage) and general DNA manipulations were performed as described elsewhere [16, 39], with minor modifications. VBC-Biotech Services supplied the primers used in this study (table 2) and performed sequencing reactions. General sequence analysis was performed by use of the DNA* (DNAStar) software package and the Basic Local Alignment Search Tool (BLAST) algorithm.

Construction of ϕ149::Km^r. A kanamycin-resistance (Km^r) cassette, *aphIII,* was inserted into the *ssa* gene of phage ϕ149 in strain RDN149, thus generating strain EC516, which carried recombinant phage ϕ149::Km^r. For this purpose, an 856-bp fragment upstream of *ssa* and a 569-bp fragment downstream of *ssa* were amplified by use of RDN149 genomic DNA and primers (containing flanking restriction sites) OLEC154/OLEC155 and OLEC153/OLEC159, respectively. After digestion with appropriate restriction enzymes, fragments were ligated and cloned into pEC61, a suicide plasmid for GAS. The resulting plasmid, pEC97, was digested with *Sca*I (which interrupts the ampicillin-resistance cassette) and used to transform electrocompetent RDN149. Km^r clones were then se-

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Direction	Sequence (5' to 3' direction)	Specific gene target
oliRN70	F	GCTATTTTTTGACTTACTGGG	*aphIII*
oliRN69	R	TCCGTATCTTTTACGCAGCGG	*aphIII*
oliRN110	F	CGCTGCGTAAAAGATACG	*aphIII*
oliRN111	R	AATGGAGTGTCTTCTTCC	*aphIII*
oliRN133	F	TATTCGCTTAGAAAATTAA	*emm*
oliRN134	R	GCAAGTTCTTCAGCTTGTTT	*emm*
oliRN39	F	AAAAATGACATTGCTTGGGC	*hki*
oliRN40	R	TTTAGTAGATATACTTTATTGGC	*hki*
oliRN233	F	AGATTGGATATCACAGG	*speH*
oliRN234	R	CTATTCTCTCGTTATTGG	*speH*
oliRN237	F	GTGTAGAATTGAGGTAATTG	*ssa*
oliRN238	R	TAATATAGCCTGTCTCGTAC	*ssa*
OLEC141	F	AACAAGTCACTGTTCAAG	*ssa*
OLEC142	R	GCTACAACGAGTATTCTT	*ssa*
OLEC166	F	GTACGAGACAGGCTATAT	*ssa*
OLEC211	F	TGCACAATTATTATCGATTAG	*ssa*
OLEC153	F	TAACTTGGATCCGTTCAATAGTAAAGATTTAGCTGC	Insertion of *aphIII* into *ss*
OLEC154	R	TTCTTACTGCAGGCTGACCTGTGGATCTTACATTAG	Insertion of *aphIII* into *ss*
OLEC155	F	AAGTAAGCATGCACCCAATATTTTTCGTTAGTGAAG	Insertion of *aphIII* into *ss*
OLEC159	R	TATGAAGGTACCTTAACTAGTATCATGGATTTGATAC	Insertion of *aphIII* into *ss*
OLEC179	F	TTGCTCATGCGCAATTCG	Spy1278
OLEC497	R	AATATTGGGTGTTTTAGAAGCC	Spy1278
OLEC178	R	ATCGCTTGCTTAAACTCG	*prx*
OLEC395	F	ATGGAGAAGTTGTGACTAAGG	*prx*
OLEC396	F	TGACAATGGATATATCGCAGG	*prx*
OLEC156	R	TTAACTAGTATCATGGAT	Intergenic *ssa-prx*
OLEC167	R	CAATTACCTCAATTCTAC	Intergenic *ssa*-Spy1278
OLEC267	R	AAGAACCTTGTCGCTATC	Intergenic attR-Spy1275
OLEC325	R	GATGACTGACCATTATAG	Intergenic attR-Spy1275
OLEC324	R	GCTACAAAATAACGAAAC	Intergenic attR-*prx*
OLEC500	F	AGAAGCAACTGAATTGA	Spy1329

NOTE. The underlined sequences indicate restriction sites. Spy1275, Spy1278, and Spy1329 refer to annotated open reading frames in strain MGAS10750F. F, forward; *prx*, paratox gene; R, reverse.

lected and analyzed by polymerase chain reaction (PCR) and Southern blot analyses. This ensured that the recombination event by double crossover was correct and did not affect the DNA regions located upstream and downstream of *ssa*.

Phage induction by mitomycin C treatment. Mitomycin C (Sigma) was added (0.2 μg/mL) to cultures grown to early–mid-logarithmic phase [24, 41]. After incubation for 3 h at 37°C, cells were removed by centrifugation and supernatants were sterile-filtered through 0.45-μm pores (Iwaki Glass). Possible bacterial contamination of filtrates was excluded by inoculating an aliquot of the filtrates in liquid and on solid media.

Detection of phage release in mitomycin C–treated bacterial cultures. Two approaches were used. (1) A phage indicator strain culture grown to mid-logarithmic phase was mixed with sterile water, poured over THY agar plates, and allowed to sediment. Superfluous bacterial suspension was carefully removed and plates were left to dry. Supernatants of mitomycin C–treated phage donor cultures were then spotted on the indicator lawn. (2) Supernatants of mitomycin C–treated phage donor cultures were mixed with cultures of phage indicator strain and added to molten top agar. The mixture was then poured over THY agar plates and allowed to dry. In both approaches, the final step was to incubate the plates at 37°C until plaques were observed.

Lysogenization of recipient strains with phage ϕ149. By use of the first approach described above, secondary growth colonies were recovered from the areas of lysis and analyzed for sensitivity to phage infection. Lysogenized strains for which no lysis was observed were then selected and analyzed by PCR and Southern blot analysis for the presence of the phage.

Determination of the host range of ϕ149::Km^r. Supernatants of mitomycin C–treated EC516 cultures were mixed with recipient cultures grown to early–mid-logarithmic phase and further incubated for 3 hours at 37°C. Aliquots of the mixture were then plated on tryptic soy agar that contained kanamycin. Selected Km^r-

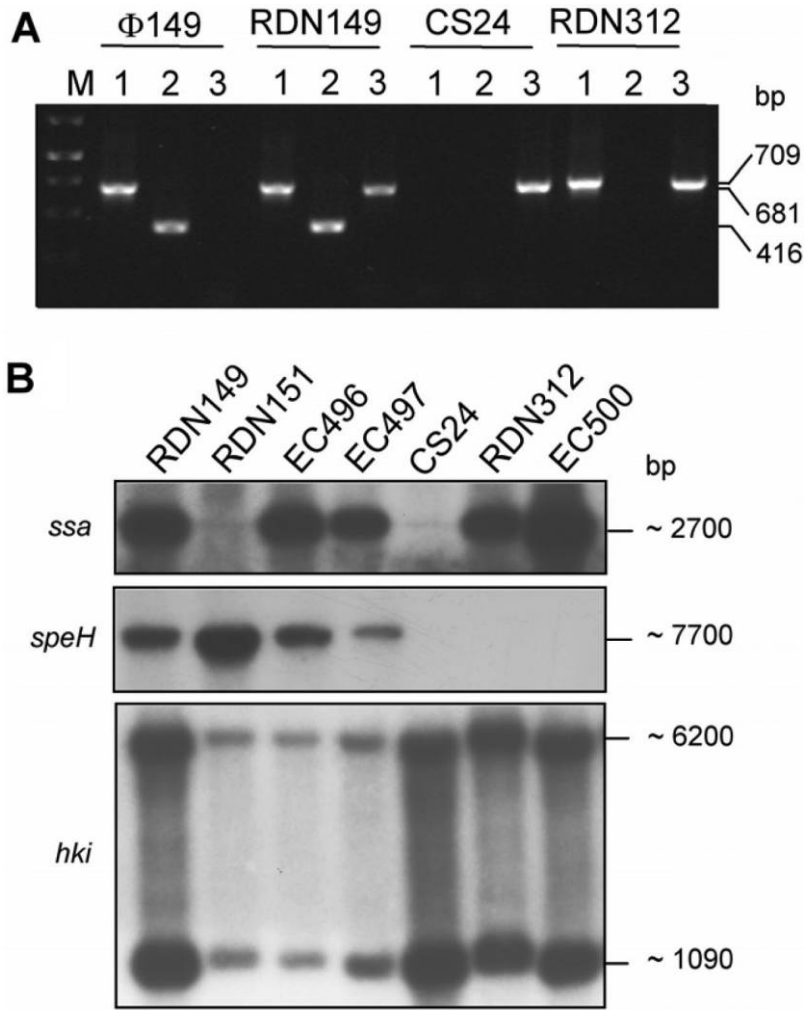


Figure 1. Gel electrophoresis patterns demonstrating ex vivo lysogenic transfer of *ssa-3*–carrying phage ϕ149 to group A *Streptococcus* (GAS) clinical isolates of type M12. *A,* Results of polymerase chain reaction analysis of isolated phage DNA from strain RDN149 and genomic DNA from phage donor strain RDN149, phage indicator strain CS24, and lysogenized strain RDN312 with primers specific to the *ssa* gene *(lane 1),* the *speH* gene *(lane 2)* and the *hki* gene *(lane 3). hki* encoding a putative histidine kinase served as positive control for GAS genomic DNA. *M,* 1-kb DNA ladder (Fermentas Life Sciences). The 709-, 681-, and 416-bp DNA fragments correspond to the *ssa-*, *hki-*, and *speH*-specific amplified products, respectively. *B,* Results of Southern blot analysis of *Hin*dIII-digested genomic DNA of phage donor strain RDN149; phage indicator strains RDN151 and CS24; and lysogenized strains EC496, EC497, RDN312, and EC500, with α-^{32}P labeled DNA probes specific to the *ssa, speH,* and *hki* genes. The approximate sizes of the hybridizing fragments are indicated in bp.

positive colonies were analyzed by PCR and Southern blot analysis to determine whether the recombinant phage was present. Lysogenization was confirmed for 2–3 independent lysogens per infection experiment. Lysogenization experiments were performed independently 3–4 times.

RESULTS

Phage release after mitomycin C treatment of an M12 clinical isolate. The analysis of a type M12 clinical GAS isolate, RDN149, recovered from a patient with STSS, revealed the superantigen genes *ssa* and *speH* (table 1). Subsequent treatment of a bacterial culture of RDN149 with mitomycin C resulted in the release of phage particles in the culture supernatant, which could be demonstrated by plaque detection with the phage indicator strains CS24 and RDN151 (M12 types). The plaques obtained with both indicator strains were turbid, ~2 mm in diameter, and the bacteriophage titer was estimated at 7×10^4 pfu/mL (data not shown). The presence of phage particles in the supernatant of mitomycin C–induced RDN149 cultures was further confirmed by isolation of phage DNA. In contrast, no phage DNA could be detected in the supernatant of cultures that were not subjected to mitomycin C treatment (data not shown). Further characterization of the released prophage(s) by PCR analysis indicated the possible presence of 2 phages in the supernatant (figure 1*A*).

Ex vivo lysogeny of phage ϕ149 associated with* ssa*-toxigenic conversion. To investigate whether phage(s) released from strain

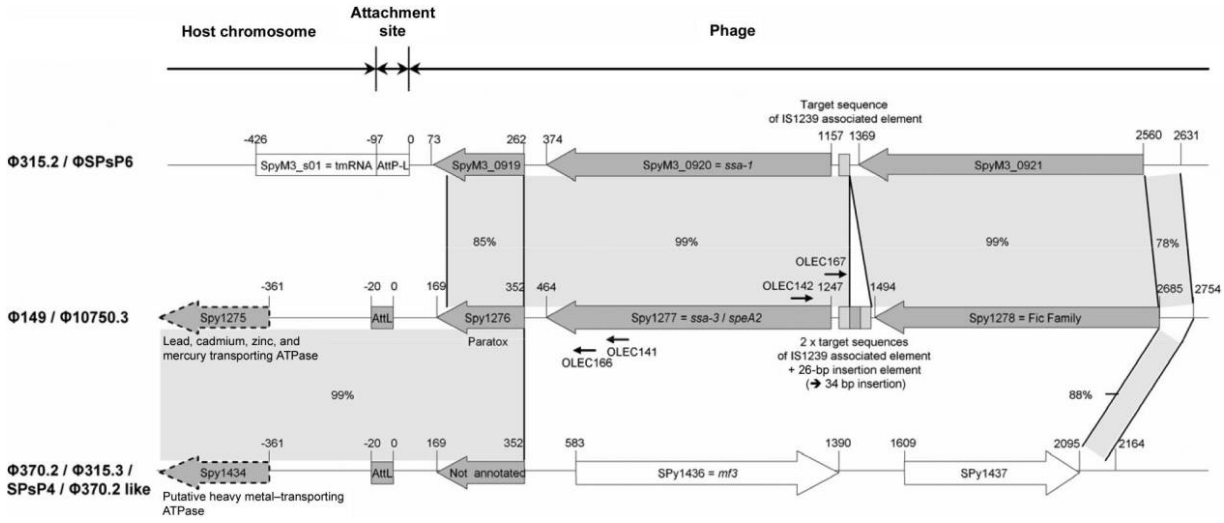


Figure 2. Comparison of the att-L proximal region of *ssa-3*–carrying phage ϕ149 with the att-L proximal regions of *ssa-1*–carrying phages ϕ315.2 (MGAS315; M3) and SPsP6 (SSI-1, M3) and *mf3*-carrying phages ϕ315.3 (MGAS315; M3), SPsP4 (SSI-1; M3), ϕ370.2 (SF370; M1), and ϕ370.2-like (MGAS8232; M18). The shaded areas indicate the regions of similarity with the percentage of nucleotide identity given. The 34 bp IS*1239*-related sequence located 103 bp upstream of the *ssa* coding sequence is shown. Annotated open reading frames refer to the genomes of MGAS315 strain (ϕ315.2) and SF370 (ϕ370.2). *Small, black arrows,* positions of primers.

RDN149 could convert *speH*-negative and *ssa*-negative strains into *speH*-positive and/or *ssa*-positive strains, lysogenization experiments involving the phage indicator strain CS24 with mitomycin C–induced supernatants of RDN149 were conducted. Each lysogenization experiment performed was positively controlled with ϕCS112 lysogenization of recipient strain CS24 with strain CS112 used as phage donor [24]. Lysogenic clones were obtained successfully and further analyzed for transfer of the superantigen genes. PCR analysis of genomic DNA from the phage donor strain (RDN149), phage indicator strain (CS24), and lysogenized CS24 strains (RDN312 and EC500), as well as purified phage DNA from RDN149, showed that only *ssa,* not *speH,* was transferred to the phage indicator strain (figure 1*A*).

These results, confirmed by Southern blot analysis, suggested that in RDN149, *speH* and *ssa* are encoded on 2 distinct prophages (figure 1*B*). The transferable, lysogenic, *ssa*-carrying phage was designated ϕ149. To further verify that lysogeny was responsible for the observed *ssa*-toxigenic conversion, we repeated the experiments with the second phage indicator strain, RDN151. Toxigenic conversion of RDN151 with ϕ149 was also observed (figure 1). Thus, we have identified *ssa*-carrying phage ϕ149 in strain RDN149, which can convert *ssa*-negative clinical strains of type M12 to *ssa*-positive strains via lysogenization.

Characterization of the* ssa*-carrying phage ϕ149. The superantigen SSA shares considerable homology with the *S. aureus* enterotoxins SEB and SEC [33, 42]. Three alleles of *ssa* have already been identified in natural GAS populations [35]. The alleles *ssa-1* and *ssa-3* differ by a single synonymous substitution in codon 94, and both encode SSA-1. The *ssa-2* allele is identical to *ssa-3* at codon 94, but has a nonsynonymous substitution at codon 28, which changes the second amino acid of the mature protein from serine to arginine [35]. PCR amplification of the *ssa* coding sequence in RDN149 with primers oliRN237 and OLEC156, followed by sequencing, revealed the *ssa-3* allele. To determine the attachment site of ϕ149 and its location on the chromosome of strain RDN149, we took advantage of the fact that, in all GAS prophages, the genes encoding virulence factors are located at one extremity of the prophage, between the phage lysis cassette and the phage attachment site [12]. At the time of the study, 2 GAS prophages, ϕ315.2 (strain MGAS315 [M3 type]) and ϕSPsP6 (strain SSI-1 [M3 type]), had already been described that carried the *ssa* gene (*ssa-1* allele) [7, 10].

Southern blot analysis of *Hin*dIII-digested genomic DNA of strain RDN149 revealed an *ssa*-hybridizing DNA fragment, the size of which (~2700 bp) was different from that expected in strains MGAS315 and SSI-1 (5090 bp) (figure 1). There are several possible explanations for this result: (1) ϕ149 is identical to ϕ315.2 or ϕSPsP6 but is integrated at a different attachment site; (2) ϕ149 is identical to ϕ315.2 or ϕSPsP6 and is integrated at the same attachment site, but the chromosomal region surrounding the attachment sites differs among strains; or (3) ϕ149 is a novel GAS prophage that has not yet been characterized.

We performed inverse PCR using religated, *Hin*dIII-digested genomic fragments of strain RDN149 as DNA templates and *ssa*-specific outward primers OLEC141 and OLEC142 (table 2; figure 2). The PCR fragment obtained was purified and used as a DNA template in PCR reactions with embedded primers OLEC166 and OLEC167 (table 2; figure 2). Sequence analysis of the resulting PCR fragment indicated that ϕ149 consisted of a chimeric GAS prophage. The downstream region of the *ssa* cod-

ing sequence, which includes a hypothetical phage protein referred to as paratox, the left ϕ149 attachment site, and the adjacent sequence on the bacterial chromosome showed identity to the DNA region upstream of the streptodornase gene (*mf3*) in 4 GAS prophages that have already been described: ϕ370.2 (SF370 [M1]), ϕSPsP4 (SSI-1 [M3]), ϕ315.3 (MGAS315 [M3]), and ϕ370.2-like prophage (MGAS8232 [M18]) (figure 2) [7, 8, 10, 11].

All 4 prophages, and therefore also ϕ149, are integrated into the GAS bacterial genome at the same attachment site, which is located 341 bp upstream of a putative heavy metal–transporting, ATPase-encoding gene. Nucleotide sequence analysis of the upstream region of *ssa-3* in ϕ149 revealed 99% identity with the upstream region of *ssa-1* in ϕ315.2 (MGAS315 [M3]) and ϕSPsP6 (SSI-1 [M3]). The difference consists of a 34-bp insertion located 103 bp upstream of the *ssa-3* start codon, which is not present upstream of *ssa-1*. This 34-bp insertion comprises a 26-bp insertion element (5'-CTCTTTTAAAATTAAAACATTGATTT-3'), which doubles the sequence 5'-AATTTTAT-3'. The 26-bp insertion element was previously identified upstream of the insertion sequence IS*1239* and, on this basis, it was suggested that it may function as a target sequence [42]. It was shown to be more frequently associated with *ssa-3* than with *ssa-1* or *ssa-2* [35]. In addition, the presence of IS*1239* associated with the 26-bp insertion element was identified upstream of *ssa-3* in several serotypes—M4, M15, M23, M33 and M41—leading to the suggestion that IS*1239* might have contributed to the horizontal transfer of *ssa* among GAS [35]. Taken together, these observations suggest that recombination events may have taken place, leading to the mosaic nature of ϕ149.

Determination of host range of ϕ149 among clinical isolates of GAS. We were interested in the possibility that ϕ149 could be transferred ex vivo to clinical isolates of GAS. As described above, we showed transfer of ϕ149 from the donor strain RDN149 (type M12) to the phage indicator strains CS24 and RDN151 (type M12). To facilitate the detection of toxin-converted strains, a selective marker (the kanamycin-resistance cassette, *apiII*) was inserted into the *ssa-3* coding sequence of ϕ149, thus creating strain EC516. Strain EC516 was then used as a donor strain of ϕ149::Km^r in infection experiments involving other GAS clinical isolates of various M serotypes. Lysogenic strains were selected on the basis of their Km^r phenotype, which was verified by PCR scoring for the presence of the Km^r cassette flanked by the interrupted *ssa-3* sequence and further verified by subsequent Southern blot analysis. In this study, 34 clinical isolates, which represented 12 M types, were analyzed for lysogenic conversion. A total of 17 strains that comprised 7 different M types and were classified as pattern A-C/SOF- strains (M1, M3, M5, M12, and M19) and pattern E/SOF+ strains (M28 and M94) [43, 44] were successfully lysogenized (table 3; figure 3). Further, the transfer of ϕ149::Km^r from selected M1, M12, and M94 lysogens back to clinical isolates of type M12 (RDN151 and RDN138) was obtained. This indicated that the recombinant phage was still functional once inserted in the chromosome of the lysogenic strains. In addition, infection experiments with UV-induced and hydrogen peroxide (H_2O_2)–induced EC516 culture supernatants led successfully to lysogenic clones among M12 strains (data not shown).

Table 3. Ex vivo lysogenic transfer of ϕ149::Km^r between streptococcal species.

Group, M type	Clinical isolates, no.	Serum opacity factor	Lysogenization[a] with ϕ149::Km^r	
			Positive	Negative
Group A *Streptococcus*				
M1	7	−	1	6
M3	5	−	2	3
M5	1	−	1	0
M12	8	+	8	0
M19	1	−	1	0
M22	1	+	0	1
M28	3	+	2	1
M49	2	+	0	2
M81	1	+	0	1
M83	1	−	0	1
M89	2	+	0	2
M94	2	+	2	0
Group C *Streptococcus*	10	. . .	2	8
Group G *Streptococcus*	10	. . .	0	10

[a] Each lysogenization experiment was performed at least 3 times independently. Lysogens were analyzed by polymerase chain reaction and Southern blot analysis for the presence of the recombinant phage.

Ex vivo lysogenic transfer ϕ149 to other streptococcal species. Several studies report the presence of *ssa-3* among the emerging human pathogens GCSE and GGSE, thus suggesting a temporal dynamic for *ssa-3* exchange across streptococcal species [14]. Here, we asked whether ϕ149 could be lysogenically transferred ex vivo from GAS to other streptococcal species. Infection experiments were conducted that used the ϕ149::Km^r phage donor strain EC516 and 10 GCSE clinical isolates and 10 GGSE clinical isolates as recipient strains, by use of the same approach described above. Two GCSE strains were successfully lysogenized (table 3). ϕ149::Km^r was also transferred from selected GCSE lysogens back to clinical isolates of type M12 (RDN151 and RDN138). Taken together, the ability of ϕ149::Km^r to lysogenize GAS strains of different clonal lineages and different M types as well as GCSE isolates indicate that phage transfer most probably played a critical role in mediating the spread of *ssa* across the streptococcal population.

DISCUSSION

The recent reemergence of severe, invasive GAS diseases observed during the mid-1980s has encouraged many researchers to investigate this phenomenon. Some studies questioned whether enhanced virulence could be associated with a particular M type or with cer-

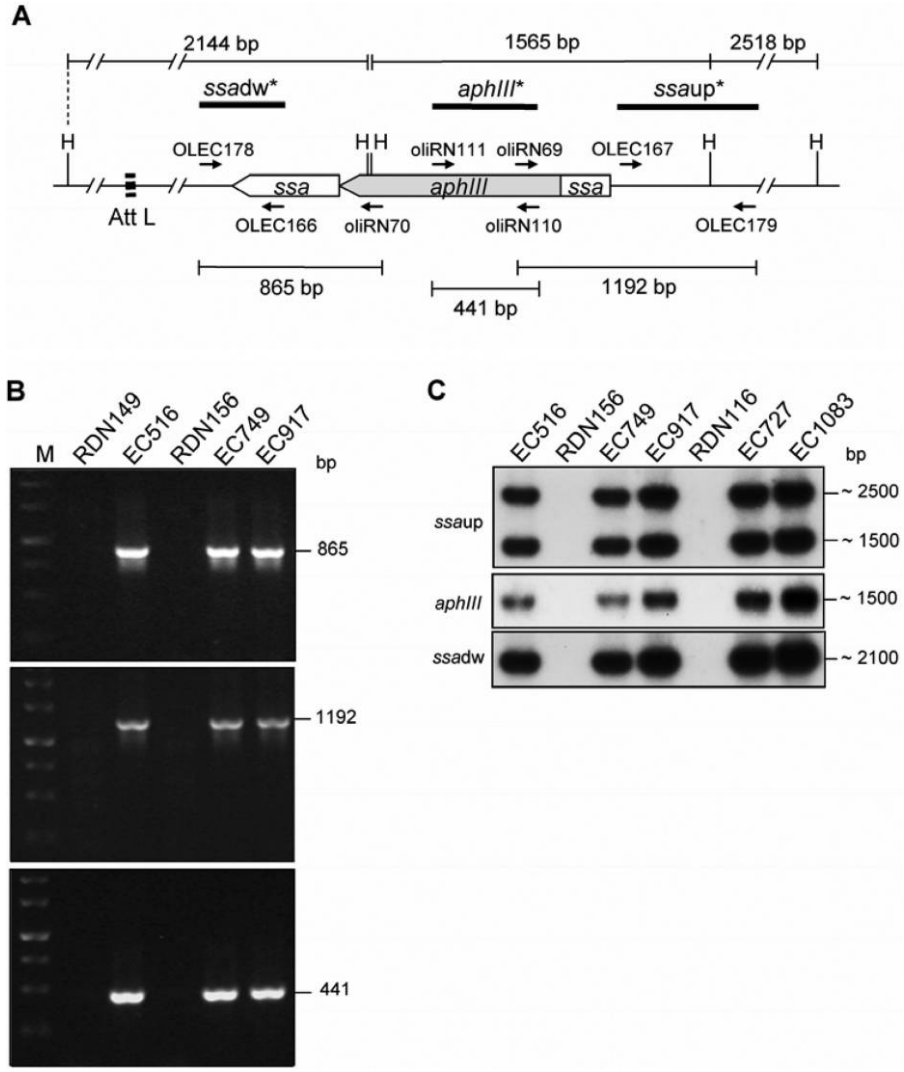


Figure 3. Analysis of the broad host range of recombinant phage ϕ149::Km^r. *A,* Representative restriction map of the att-L proximal region of ϕ149::Km^r. Genes are drawn to scale. *Small, black arrows,* positions of primers. Sizes of PCR and hybridizing restriction fragments are indicated in bp. *B,* Results of polymerase chain reaction analysis of genomic DNA from ϕ149 donor strain RDN149, ϕ149::Km^r donor strain EC516, recipient strain RDN156 (M28), and lysogenized strains EC749 and EC917, with primers OLEC178 and oliRN70 *(top),* oliRN69 and OLEC179 *(middle),* and oliRN111 and oliRN110 *(bottom). M,* 1-kb DNA ladder (Fermentas Life Sciences). *C,* Results of Southern blot analysis of *Hin*dIII-digested genomic DNA from ϕ149::Km^r donor strain EC516, phage recipient strain RDN156 (M28) and corresponding lysogenized strains EC749 and EC917, phage recipient strain RDN116 (M94) and corresponding lysogenized strains EC727 and EC1083 with α-^{32}P labelled DNA probes specific to the *ssa* upstream fragment (*ssa*up), *aphIII* gene, and *ssa* downstream fragment (*ssa*dw).

tain virulence factors. Reports show the existence of particular virulent and invasive clones that have successfully spread worldwide [3, 4, 7, 45]. However, despite the dominance of certain M types, such as M1, M3, M12, and M28, among endemic and invasive GAS isolates, no exclusive link between M type and specific diseases could be demonstrated. Only certain M types seem to exhibit a correlation with invasive diseases (e.g., M1 and M3) or postinfectious sequelae from superficial GAS infection (e.g., M18) [11, 29, 30, 45]. Furthermore, invasive GAS isolates do not seem to be characterized by a common toxin-gene profile. Although reports show the dominance of 1 or 2 toxin-gene profiles in all M types, strains that did not share the predominant profile still showed nonrandom distribution of key toxin genes that were characteristic of the M type [29].

Among bacterial factors that can determine enhanced virulence are superantigen-carrying bacteriophages that can, via lysogenic transfer, convert a nontoxinogenic strain to a toxin-producing strain [19]. In this study, the analysis of a clinical GAS isolate, type M12, to determine its superantigen-encoding bacteriophage profile uncovered prophage ϕ149, which carried the allele *ssa-3* that encodes the streptococcal superantigen SSA. Sequence analysis of the att-L proximal region of ϕ149 indicated that the phage was a mosaic prophage that shared characteristics

of 2 different types of prophages: ϕ370.2, ϕSPsP4, ϕ315.3, and ϕ370.2–like phages (already identified in M1, M3, and M18 strains) and ϕ315.2 and SPsP6 phages (M3 strains). This indicates that phage genomic rearrangements occurred, leading to genetic mosaicism in ϕ149. Recently, the complete genome sequence of an M4-type strain, MGAS10750, revealed that the strain harbors an *ssa-3*–carrying prophage, ϕ10750.3 [46], whose att-L proximal region is 100% identical to that of ϕ149 (figure 2). The contribution that the genetic mosaicism of GAS prophages makes to the emergence and diversification of the globally disseminated clonal M1T1 strain has already been demonstrated, indicating the exchange of genetic material among GAS prophages [1, 3, 12].

Superantigen SSA produced by GAS shares greater homology with staphylococcal enterotoxins SEB and SEC than it does with streptococcal superantigens. Several studies during the past decade have further documented the presence of the *ssa* gene among GAS isolates of different M types, as well as in emerging clinical isolates of GCSE and GGSE [14, 29, 30, 32–35]. In our study, we performed infection experiments on GAS clinical isolates of various M types, as well as experiments on GCSE and GGSE clinical isolates using mitomycin C–induced cell-free culture supernatants of strain EC516 harboring the selective recombinant phage ϕ149::Km^r. Our results show the ability of ϕ149::Km^r to lysogenize GAS clinical isolates of 7 different M types (M1, M3, M5, M12, M19, M28, and M94), as well as the GCSE clinical isolates. To our knowledge, this is the first direct experimental evidence for toxigenic conversion via lysogeny across a diverse range of strains of distinct M types and between GAS and GCSE. However, no lysogenic conversion of GGSE clinical isolates could be observed. We hypothesize that such events could take place, but the experimental conditions in our study might have limited their detection.

Although some studies indicate that M proteins could function as barriers to horizontal gene exchange, recent genomic sequence analysis strongly suggests that extensive lateral gene transfer occurred among GAS isolates of different M types [1]. Along these lines, lysogenic conversion of 1 T25-type strain with SpeC toxin originating from an M12-type isolate was described; however, the lysogenic transfer event was not confirmed by DNA analysis [31]. Reports on intergroup streptococcal bacteriophage transfer remain limited. Intergroup transduction of markers for streptomycin and bacitracin resistance between GCS and GAS using A-25 lytic phage and propagation of a temperate phage isolated from GGS on either GAS or GCS have been reported [47, 48]. GCSE and GGSE, although generally considered to be commensal organisms, have also been isolated from infections in humans that had clinical manifestations similar to those caused by GAS. In particular, reports of GCSE and GGSE isolates recovered from patients with severe forms of infection and of the presence of superantigen genes in these isolates have increased in recent years [49]. Our data strongly support the hypothesis that the emergence of superantigen genes in GCSE and GGSE may occur by means of horizontal transfer from GAS via temperate phage. Accordingly, it has been suggested that housekeeping genes are exchanged among GCSE, GGSE, and GAS via lateral transfer with a strong net directionality of gene movement from GAS donors to GCS and GGS recipients, but only on the basis of phylogenetic studies [50]. Our experimental findings raise questions about the extent to which M type and streptococcal species are barriers to the horizontal transfer of lysogenic phages, as well as questions about the assumption of phage specificity within a given streptococcal group.

Given the critical role of temperate bacteriophages in toxigenic conversion and in the mediation of strain diversity and evolution in GAS, it will be important to obtain additional knowledge of the molecular mechanisms underlying these events [19, 23]. Further investigation is also needed to unravel the complex interplay between strain genotype, in vivo toxin-gene transfer, regulation of toxin expression, and host factors (i.e., genetics and underlying conditions) that is responsible for the development of severe streptococcal disease.

Acknowledgments

We thank Dr. Schlievert for his gift of the ϕCS112 phage donor strain CS112 and indicator strain CS24; Dr. Garnier and Dr. Ploy for their gift of group B, C, and G streptococcal clinical isolates; and Dr. Dunny for his gift of vector pMSP3535. We also thank Dr. Eckert for her help with sequence analyses; Dr. Bläsi, Dr. Novak, and Dr. Witte for helpful discussions; and Dr. Normark for critical reading of the manuscript.

References

1. Banks DJ, Beres SB, Musser JM. The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence. Trends Microbiol **2002**; 10:515–21.
2. Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev **2000**; 13:470–511.
3. Aziz RK, Edwards RA, Taylor WW, Low DE, McGeer A, Kotb M. Mosaic prophages with horizontally acquired genes account for the emergence and diversification of the globally disseminated M1T1 clone of *Streptococcus pyogenes.* J Bacteriol **2005**; 187:3311–8.
4. Aziz RK, Ismail SA, Park HW, Kotb M. Post-proteomic identification of a novel phage-encoded streptodornase, Sda1, in invasive M1T1 *Streptococcus pyogenes.* Mol Microbiol **2004**; 54:184–97.
5. Kotb M, Norrby-Teglund A, McGeer A, et al. An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nat Med **2002**; 8:1398–404.
6. Banks DJ, Porcella SF, Barbian KD, et al. Progress toward characterization of the group A *Streptococcus* metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain. J Infect Dis **2004**; 190:727–38.
7. Beres SB, Sylva GL, Barbian KD, et al. 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 USA **2002**; 99:10078–83.
8. Ferretti JJ, McShan WM, Ajdic D, et al. Complete genome sequence of an M1 strain of *Streptococcus pyogenes.* Proc Natl Acad Sci USA **2001**; 98: 4658–63.
9. Green NM, Zhang S, Porcella SF, et al. Genome sequence of a serotype M28 strain of group A *Streptococcus:* potential new insights into puerperal sepsis and bacterial disease specificity. J Infect Dis **2005**; 192:760–70.

10. Nakagawa I, Kurokawa K, Yamashita A, et al. 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 **2003**; 13:1042–55.
11. Smoot JC, Barbian KD, Van Gompel JJ, et al. Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. Proc Natl Acad Sci USA **2002**; 99:4668–73.
12. Brussow H, Canchaya C, Hardt WD. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev **2004**; 68:560–602.
13. Broudy TB, Fischetti VA. In vivo lysogenic conversion of Tox(−) *Streptococcus pyogenes* to Tox(+) with lysogenic streptococci or free phage. Infect Immun **2003**; 71:3782–6.
14. Igwe EI, Shewmaker PL, Facklam RR, Farley MM, van Beneden C, Beall B. Identification of superantigen genes *speM, ssa,* and *smeZ* in invasive strains of beta-hemolytic group C and G streptococci recovered from humans. FEMS Microbiol Lett **2003**; 229:259–64.
15. Kapur V, Nelson K, Schlievert PM, Selander RK, Musser JM. Molecular population genetic evidence of horizontal spread of two alleles of the pyrogenic exotoxin C gene (*speC*) among pathogenic clones of *Streptococcus pyogenes*. Infect Immun **1992**; 60:3513–7.
16. Ikebe T, Wada A, Inagaki Y, et al. Dissemination of the phage-associated novel superantigen gene *speL* in recent invasive and noninvasive *Streptococcus pyogenes* M3/T3 isolates in Japan. Infect Immun **2002**; 70:3227–33.
17. Musser JM, Hauser AR, Kim MH, Schlievert PM, Nelson K, Selander RK. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. Proc Natl Acad Sci USA **1991**; 88:2668–72.
18. Voyich JM, Sturdevant DE, Braughton KR, et al. Genome-wide protective response used by group A *Streptococcus* to evade destruction by human polymorphonuclear leukocytes. Proc Natl Acad Sci USA **2003**; 100:1996–2001.
19. Zabriskie JB. The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. J Exp Med **1964**; 119:761–80.
20. Proft T, Sriskandan S, Yang L, Fraser JD. Superantigens and streptococcal toxic shock syndrome. Emerg Infect Dis **2003**; 9:1211–8.
21. Schlievert PM. Role of superantigens in human disease. J Infect Dis **1993**; 167:997–1002.
22. Banks DJ, Lei B, Musser JM. Prophage induction and expression of prophage-encoded virulence factors in group A *Streptococcus* serotype M3 strain MGAS315. Infect Immun **2003**; 71:7079–86.
23. Broudy TB, Pancholi V, Fischetti VA. The *in vitro* interaction of *Streptococcus pyogenes* with human pharyngeal cells induces a phage-encoded extracellular DNase. Infect Immun **2002**; 70:2805–11.
24. Johnson LP, Schlievert PM, Watson DW. Transfer of group A streptococcal pyrogenic exotoxin production to nontoxigenic strains of lysogenic conversion. Infect Immun **1980**; 28:254–7.
25. Musser JM, Kapur V, Kanjilal S, et al. Geograpic and temporal distribution and molecular characterization of two highly pathogenic clones of *Streptococcus pyogenes* expressing allelic variants of pyrogenic exotoxin A (scarlet fever toxin). J Infect Dis **1993**; 167:337–46.
26. Cleary PP, Johnson Z. Possible dual function of M protein: resistance to bacteriophage A25 and resistance to phagocytosis by human leukocytes. Infect Immun **1977**; 16:280–92.
27. Spanier JG, Cleary PP. Bacteriophage control of antiphagocytic determinants in group A streptococci. J Exp Med **1980**; 152:1393–1406.
28. Bessen DE, Izzo MW, Fiorentino TR, Caringal RM, Hollingshead SK, Beall B. Genetic linkage of exotoxin alleles and *emm* gene markers for tissue tropism in group A streptococci. J Infect Dis **1999**; 179:627–36.
29. Schmitz FJ, Beyer A, Charpentier E, et al. Toxin-gene profile heterogeneity among endemic invasive European group A streptococcal isolates. J Infect Dis **2003**; 188:1578–86.
30. Vlaminckx BJ, Mascini EM, Schellekens J, et al. Site-specific manifestations of invasive group a streptococcal disease: type distribution and corresponding patterns of virulence determinants. J Clin Microbiol **2003**; 41:4941–9.
31. De Marzi MC, Fernandez MM, Sundberg EJ, et al. Cloning, expression and interaction of human T-cell receptors with the bacterial superantigen SSA. Eur J Biochem **2004**; 271:4075–83.
32. Mollick JA, Miller GG, Musser JM, Cook RG, Rich RR. Isolation and characterization of a novel streptococcal superantigen. Trans Assoc Am Physicians **1992**; 105:110–22.
33. Mollick JA, Miller GG, Musser JM, Cook RG, Grossman D, Rich RR. A novel superantigen isolated from pathogenic strains of *Streptococcus pyogenes* with aminoterminal homology to staphylococcal enterotoxins B and C. J Clin Invest **1993**; 92:710–9.
34. Reda KB, Kapur V, Mollick JA, Lamphear JG, Musser JM, Rich RR. Molecular characterization and phylogenetic distribution of the streptococcal superantigen gene (*ssa*) from *Streptococcus pyogenes.* Infect Immun **1994**; 62:1867–74.
35. Reda KB, Kapur V, Goela D, Lamphear JG, Musser JM, Rich RR. Phylogenetic distribution of streptococcal superantigen SSA allelic variants provides evidence for horizontal transfer of *ssa* within *Streptococcus pyogenes.* Infect Immun **1996**; 64:1161–5.
36. Davies MR, Tran TN, McMillan DJ, Gardiner DL, Currie BJ, Sriprakash KS. Inter-species genetic movement may blur the epidemiology of streptococcal diseases in endemic regions. Microbes Infect **2005**; 7:1128–38.
37. Bryan EM, Bae T, Kleerebezem M, Dunny GM. Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid **2000**; 44: 183–90.
38. Goshorn SC, Schlievert PM. Bacteriophage association of streptococcal pyrogenic exotoxin type C. J Bacteriol **1989**; 171:3068–73.
39. Caparon MG, Scott JR. Genetic manipulation of pathogenic streptococci. Methods Enzymol **1991**; 204:556–86.
40. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, **1989**.
41. Weeks CR, Ferretti JJ. The gene for type A streptococcal exotoxin (erythrogenic toxin) is located in bacteriophage T12. Infect Immun **1984**; 46: 531–6.
42. Kapur V, Reda KB, Li LL, Ho LJ, Rich RR, Musser JM. Characterization and distribution of insertion sequence IS1239 in *Streptococcus pyogenes.* Gene **1994**; 150:135–40.
43. Johnson DR, Kaplan EL, VanGheem A, Facklam RR, Beall B. Characterization of group A streptococci *(Streptococcus pyogenes)*: correlation of M-protein and *emm*-gene type with T-protein agglutination pattern and serum opacity factor. J Med Microbiol **2006**; 55:157–64.
44. McGregor KF, Spratt BG, Kalia A, et al. Multilocus sequence typing of *Streptococcus pyogenes* representing most known *emm* types and distinctions among subpopulation genetic structures. J Bacteriol **2004**; 186: 4285–594.
45. Schwartz B, Facklam RR, Breiman RF. Changing epidemiology of group A streptococcal infection in the USA. Lancet **1990**; 336:1167–671.
46. Beres SB, Richter EW, Nagiec MJ, et al. Molecular genetic anatomy of inter- and intraserotype variation in the human bacterial pathogen group A *Streptococcus.* Proc Natl Acad Sci USA **2006**; 103:7059–64.
47. Wannamaker LW, Almquist S, Skjold S. Intergroup phage reactions and transduction between group C and group A streptococci. J Exp Med **1973**; 137:1338–53.
48. Colon AE, Cole RM, Leonard CG. Lysis and lysogenization of groups A, C, and G streptococci by a transducing bacteriophage induced from a group G streptococcus. J Virol **1971**; 8:103–10.
49. Sachse S, Seidel P, Gerlach D, et al. Superantigen-like gene(s) in human pathogenic *Streptococcus dysgalactiae*, subsp *equisimilis:* genomic localisation of the gene encoding streptococcal pyrogenic exotoxin G *(speG-(dys))*. FEMS Immunol Med Microbiol **2002**; 34:159–67.
50. Kalia A, Enright MC, Spratt BG, Bessen DE. Directional gene movement from human-pathogenic to commensal-like streptococci. Infect Immun **2001**; 69:4858–69.

4.2 Project 2: A bioinformatic screen reveals novel small non-coding RNAs in *Streptococcus pyogenes*

4.2.1 Summary

Small non-coding RNAs (sRNAs) define a novel class of molecules with regulatory functions in basic house-holding, metabolism and virulence of bacteria. Knowledge of sRNAs in the human GAS pathogen remains limited. To date, only three such sRNAs have been described in the human pathogen *S. pyogenes*: *fasX*RNA, *pel*RNA and *rivX*RNA, all of which RNAs have a role in virulence gene expression in GAS.

The objective of the study was to identify novel sRNAs in this species using a large-scale computational approach. Four different algorithms 'QRNA', 'RNAz', 'Alifoldz', 'sRNAPredict2' and the Rfam database were used to identify intergenic regions (IGRs) likely to encode sRNAs in the genome of M1 strain SF370. Expression of sRNA candidates was validated using Northern blot analysis. Characterization of expressed sRNAs was done by studying their expression pattern in different M-types throughout growth and under stress conditions, determining their intracellular metabolic stability, mapping of 5' and 3' extremities and analyzing their putative secondary structure. In total, 218 individual predictions were summarised into 178 loci, of which 90 were further analyzed. Under normal growth conditions, 29 (32%) sRNAs were expressed, of which five were predicted riboswitches, six were leader elements, four were functional RNAs, eight were predicted T-boxes and five were novel sRNAs of unknown function. Three of these novel sRNAs were determined to be 5'UTR-derived, whereas one novel sRNA was 3'UTR-derived. One IGR encoded three novel sRNAs ending at the same rho-independent terminator. Several predictions next to *scpA*, a gene encoding the virulence factor C5a peptidase, resulted in the mapping of an sRNA originating from the 5'UTR of the *scpA* mRNA through a transcription termination mechanism.

Two sRNAs are located in the 5' UTRs of Spy_0373 and Spy_1016 predicted as riboflavin and tryptophan transporters, respectively. Remarkably, *S. pyogenes* lacks the riboflavin synthetic genes and the terminal tryptophan biosynthetic pathway, and therefore bacterial growth depends strictly on uptake of the metabolites from the environment, which shows an essentiality of Spy_0373 and Spy_1016. Northern blot and RT-PCR

analyses showed that the expression of Spy_0373 and Spy_1016 was dependent on the absence of riboflavin and tryptophan, respectively, from the growth medium. Our data suggest a direct or indirect effect of these metabolites on the expression of the downstream genes via transcriptional termination or translational attenuation mechanisms involving the 5' UTR-located sRNAs. Thus, vital components in the regulation of expression of these important genes reside in the 5'UTRs, indicating the importance of riboswitches and leader elements in gene regulation.

This study allowed the identification and characterization of five novel sRNAs and is the first report of riboswitches, T-boxes and functional RNAs in *S. pyogenes*, some of which are currently being further investigated in our laboratory. We also conclude that algorithms need further development to selectively identify trans-acting sRNAs in *S. pyogenes*.

For the above-mentioned results, we worked in a team and my contribution was to analyze a number of predictions by Northern blot analysis in 4 M serotypes under normal growth conditions and stress conditions (pH, salt and temperature) and determining their stability. I also performed the studies to describe the expression of FMN and tryptophan sRNA elements versus that of the FMN and tryptophan transporters in different growth conditions by Northern blot analysis and semi quantitative RT-PCRs.

4.2.2 Manuscript 2

A bioinformatic screen reveals novel small non-coding RNAs in *Streptococcus pyogenes*

Manuscript: In preparation

Authors:

Maria R. Eckert, Doris Veit, Tanja Gesell, **Zaid Ahmed Pirzada**, Karine Gonzales Martin Grabner, Andreas R. Gruber, Ivo Hofacker, Arndt von Haeseler and Emmanuelle Charpentier

A bioinformatic screen reveals small RNAs in *Streptococcus pyogenes*

Maria R. Eckert[1], Doris Veit[1], Tanja Gesell[2], **Zaid A. Pirzada[1]**, Karine Gonzales[1], Martin Grabner[2], Andreas R. Gruber[3], Ivo Hofacker[3], Arndt von Haeseler[2] and Emmanuelle Charpentier[1,4*]

[1]Max F. Perutz Laboratories (MFPL), Department of Microbiology and Immunobiology, University of Vienna, A-1030 Vienna, Austria.

[2]Center for Integrative Bioinformatics Vienna (CIBIV), MFPL, University of Vienna, Medical University of Vienna, Veterinary University of Vienna, A-1030 Vienna, Austria.

[3]Institute for Theoretical Chemistry (TBI), University of Vienna, A-1090 Vienna, Austria.

[4]The Laboratory for Molecular Infection Sweden (MIMS), Umea University, S-90187 Umea, Sweden.

Keywords: computational tools, *Streptococcus pyogenes*, small RNAs, riboswitches, T-boxes, leader RNAs, gene regulation.

* **Corresponding author:**

Emmanuelle Charpentier; The Laboratory for Molecular Infection Sweden (MIMS), Umea University, S-90187 Umea, Sweden; Phone: +46-907850807, Fax: +46-90772630, Email: emmanuelle.charpentier@molbiol.umu.se

4.2.3 Additional data

Introduction

Several studies described a number of small RNAs (sRNAs) regulating gene expression in bacteria. Many of these play role in the pathogenicity of bacteria. However, information regarding regulatory RNAs in *S. pyogenes* is rather limited. So far only three sRNAs have been discovered, i.e. *pel* RNA, *fasX* RNA and *rivX* RNA.

In our lab, a bioinformatic approach to search for novel sRNAs in *S. pyogenes* revealed 5 novel sRNAs. Three of the novel sRNAs are in the 5' UTR, one in the 3' UTR and only one candidate was found truly in the intergenic region i.e. SpyRNA049. We chose to characterize further SpyRNA049 on the following basis: 1) SpyRNA049 has a stable half life as most of the regulatory RNAs described so far have a long half life, 2) It was truly located in the intergenic region as seen in many regulatory RNAs functioning *in trans* and 3) bioinformatic tools predicted *cfa* mRNA a likely target for SpyRNA049. Thus it was interesting to investigate the potential regulatory role of SpyRNA049 in different aspects of pathogenicity. The following questions were asked:

- Verification of SpyRNA049 computational predictions
- Expression analysis of SpyRNA049
- Conservation and alignment of SpyRNA049 in all M serotypes
- Role of SpyRNA049 in regulating *cfa* mRNA
- Role of SpyRNA049 in host cell interaction
- Role of SpyRNA049 in the expression of virulence factors
- Role of SpyRNA049 in other pathogenicity aspects

Materials and Methods

Computational prediction of SpyRNA049

SpyRNA049 in the genome of M1 *S. pyogenes* strain SF370 (AE004092) was predicted by two different programmes, QRNA (Rivas & Eddy, 2001) and sRNA Predict 2 (personal communication, Hanah Margalet).

Secondary structure predictions of SpyRNA049

Predictions of RNA secondary structure were done using Mfold (Zuker, 2003) default parameters at the Rensselaer Polytechnic Institute server (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi). In addition, RNAfold (Hofacker *et al.*, 1994) of the Vienna RNA package was used.

DNA manipulations and cloning

Unless specified otherwise, DNA manipulations (restriction enzyme digestion, gel electrophoresis, PCR reaction, DNA purification, ligation) were done according to standard techniques (Sambrook *et al.*, 1990). Genomic DNA of *S. pyogenes* was prepared according to a previously described protocol (Caparon & Scott, 1991). PCR reactions were done using *Pwo* Polymerase (Roche), primers listed in Table 1 and *S. pyogenes* SF370 (Table 2) genomic DNA as template. PCR product purification and plasmid preparations were done using commercial kits (Qiagen or PeqLab Biotechnologie GmbH). Sequencing reactions were done at VBC-Biotech and results were viewed using Chromas v.2.31.

Bacterial strains and growth conditions

Please refer to manuscript 4.2.2.

Whenever required, suitable antibiotics were added to the medium at the following final concentrations: kanamycin 300 µg/ml for *S. pyogenes* and 25 µg/ml for *E. coli*.

5' and 3' end mapping

Please refer to manuscript 4.2.2.

Computational prediction of mRNA targets for the novel sRNAs

Please refer to manuscript 4.2.2.

Construction of SpyRNA049-deficient mutant

E. coli TOP10 was used as host for cloning. *S. pyogenes* serotype M1 (SF370) was used as wild-type. The isogenic SpyRNA049-deficient mutants were constructed using a thermo-sensitive strategy described previously (Mangold *et al.*, 2004). First, using wild-type genomic DNA as template and primers containing flanking restriction sites, a 864 bp-SpyRNA049-upstream fragment (primers OLEC823/OLEC824) and a 1074 bp-SpyRNA049-downstream fragment (primers OLEC821/OLEC822) were amplified. After digestion with the respective restriction enzymes, the upstream and downstream fragments were cloned into the thermo-sensitive plasmid pEC214. After introduction of the recombinant plasmid into *S. pyogenes* SF370, a series of temperature shifts with appropriate antibiotic selection was performed thus leading to the final deficient mutant (Table 2) in which the entire SpyRNA049 sequence was deleted in a non-polar fashion. The correct deletion event in the mutants was verified by PCR, Southern blot and sequencing analyses.

Preparation of RNA and Northern blot analysis

Please refer to manuscript 4.2.2.

RNA stability

Please refer to manuscript 4.2.2.

CAMP test

Strains were analyzed for CAMP activity using a conventional diffusion test with minor modifications (Gase *et al.*, 1999). Briefly, strains including group B streptococcus as positive control, were streaked on 5% sheep blood agar plates perpendicular to, but not touching, a streak of the beta-toxin-producing strain *S. aureus* RN450. After 12 to 20 h of incubation at 30°C, streptococcal strains were inspected for CAMP hemolysis.

Bacterial aggregation

Bacterial aggregation was determined as described by (Manetti *et al.*, 2007). *S. pyogenes* strains were grown under agitation in THY at 37°C to stationary phase. Tubes were then incubated without agitation and the sedimentation rate was determined by measuring the OD at 620 nm from the upper part of the tubes at regular time intervals during 2 h.

Bacterial adhesion, internalization and survival assays in host cells

HEp-2 cells and RAW 264.7 mouse macrophages were maintained in RPMI medium (HEp-2 cells) or Dulbecco's modified Eagle's medium (D-MEM, Gibco) (RAW 264.7 cells) supplemented with 10% foetal bovine serum (Gibco), penicillin (100 µg/ml) and streptomycin (100 µg/ml), and grown at 37°C in an atmosphere containing 5% CO_2. Prior the infection assay, the cells were cultured overnight to semi-confluency (1.5 x 10^5 cells per well for HEp-2 cells and 2 x 10^5 cells per well for RAW264.7 cells) in 24-well tissue culture plates containing medium without antibiotic. GAS strains were grown to the same OD at 620 nm corresponding to late-logarithmic phase (HEp-2 cells) and mid-logarithmic phase (RAW 264.7 cells), washed with PBS, suspended in fresh cell culture medium and added to the semi confluent host cell monolayer at a multiplicity of infection (MOI) of 5:1 (HEp-2) and 100:1 (RAW 264.7) in triplicate. Times of incubation were 1 h (adhesion, HEp-2 cells), 2 h (internalization, HEp-2 cells) and 30 min (RAW 264.7 cells). For adhesion assays (HEp-2 cells), infected monolayers were washed extensively with PBS before lysing. For internalization assays (RAW 264.7 cells), extracellularly adhered bacteria were killed by incubation with fresh medium containing either 100 µg/ml gentamicin and 5 µg/ml penicillin (HEp-2 cells) or 60 µg/ml penicillin (RAW 264.7 cells). At indicated time points, the infected monolayers were washed extensively with PBS to remove the antibiotics. Cells were then lysed with chilled sterile distilled water. The number of viable intracellular bacteria released from the lysed cells was determined by plating appropriate dilutions of the lysates on TSA blood plates in triplicates followed by 24 h incubation at 37°C. The number of bacteria that survived intracellularly was calculated using the following equation: average number of bacteria recovered (cfu/ml) per well (triplicate) at designated time point/inoculated number of bacteria (cfu/ml)) x 100. Average values were plotted.

In vitro transcription

Primers containing a T7 promoter were designed in order to amplify the 5' region of *cfa* (primers OLEC1168/OLEC1169) and the 3' region of SpyRNA049 (primers OLEC1171/OLEC1173, Table 1). PCR was done using wild-type GAS (EC904) genomic DNA as template and the primers. *In vitro* transcription was done using the Epicentre kit (Epicentre Biotechnologies).

Gel-mobility shift assays

RNA-RNA band-shift assays were done as described by Sorger-Domenigg *et al.,* 2007 (Sorger-Domenigg *et al.*, 2007) with minor modifications. Briefly, *in vitro* transcribed SpyRNA049 and *cfa* RNA were gel purified. Gel-purified RNAs were 5′-end labelled with [γ-^{32}P]-ATP (Amersham) and re-purified on a 10% polyacrylamide-8 M urea gel. Labelled SpyRNA049 (0.15 pmol) was first incubated at room temperature for 5 min to refold, then for 15 min with purified *cfa* (0.75, 1.5 and 2.25 pmol) in 10 µl VD buffer (10 mM Tris, pH 7.4, 60 mM NH_4Cl, 6 mM β-mercaptoethanol, and 2 M Mg-acetate). Immediately before loading, the samples were mixed with 4 µl of loading dye (75% glycerol, 0.01% xylencyanol, 0.01% bromphenol blue), and loaded on a native 4% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 50 V for 1-2 h. The radioactively labelled bands were visualized with a PhosphorImager (Molecular Dynamics).

Exoprotein precipitation

S. pyogenes culture supernatants were collected at early-logarithmic, mid-logarithmic and late-logarithmic time points, filter sterilized (0.45 µm filter), incubated on ice for 30 min with 1/10 volume 4% (w/v) DOC in TCA and centrifuged for 10 min at 14000 *g* at 4°C. The pellet was resuspended in 1 ml of ice-cold acetone and incubated on ice for 15 min. The acetone-extraction step was repeated two more times and finally the pellet was resuspended in 100 ml of 1 M Tris/HCl, pH 8.0 (Mangold *et al.*, 2004).

Results

Identification of SpyRNA049 by computational prediction

One prediction from QRNA and two predictions from sRNAPredict2 identified the 479 bp IGR between the hypothetical proteins encoding genes SPy1044 and SPy1046 as a putative non-coding sRNA locus.

SpyRNA049 consists of three sRNAs

Using total RNA from the M1 strain SF370 in Northern blot analysis, three transcripts of approximately 180, 80 and 60 nt were revealed (Fig. 1 A). Detection using a riboprobe clearly showed that all three sRNAs were derived from the negative strand (data not shown). The 5' and 3' ends of the sRNAs were mapped by self-ligation and primer extension. The data showed that the three sRNAs are 171, 89 and 76 nt in size and end at the same RIT, differing at their 5' ends (Fig. 1A; 2A, B, C). BPROM predicted a promoter only for the 89 nt transcript, the TransTerm RIT prediction had a region of confidence of 100. Rifampicin assays showed that the 171 nt sRNA was stable ($t_{1/2}$ >30 min), whereas the 89 nt sRNA was unstable ($t_{1/2}$ <5 min) and the 76 nt sRNA of intermediate stability ($t_{1/2}$ ~15 min) (Fig. 1B, C). The best mfe Mfold and RNAfold secondary structure predictions were nearly identical. Two main structure variants could be distinguished, the 171 nt sRNA with one long and two short stem loops and the two shorter sRNAs, with two stem loops of approximately equal length (Fig. 3). All three sRNA structures have two stem loops with an 'AGU' motif (Fig. 3). The RNAfold dot plots showed alternative pairing probabilities for all three sRNAs (data not shown), indicating that the mfe structures are likely to change conformation to structures with larger free energy values.

SpyRNA049 is not conserved in all M serotypes

The IGR is 100% conserved in M1, M2, M4, M12 and M28 strains of *S. pyogenes*, all other *S. pyogenes* strains with an available complete genome sequence contain only the first 167 nt of the IGR. This is either due to a different genomic location of the downstream gene (M3 and M4 strains) or its complete absence from the genome (M5,

M6 and M18; Fig. 4A, B). A homologous stretch corresponding to 81 nt of the 171 nt sRNA in M1 strain SF370 is present in the sequenced M5 strain Manfredo (position 996726-996646, 3 mismatches). Northern blot analysis using total RNA of M3, M5 and M49 strains (Table 1) and a PCR-derived probe corresponding to the entire IGR in SF370 failed to detect sRNA expression in these strains. To confirm sRNA expression in other strains, which contain the entire IGR, M1 strain MGAS5005 and M12 strain RDN151 (Table 1) were analyzed. In both strains, the three sRNAs were detected with a similar expression profile as seen in M1 strain SF370 (Fig. 1A). There was no differential expression of the SpyRNA049 sRNA in any of the stress conditions analyzed.

Is *cfa* an mRNA target of SpyRNA049?

The mRNA target analysis returned the same results for the three sRNAs, with only one true outlier (Fig. 5A). According to this prediction, the 5'UTR of the CAMP factor gene (*cfa*) mRNA is the most likely target of SpyRNA049 (Fig. 5B, C). The *cfa* gene is of particular interest, as several studies provide evidence for a role of the CAMP protein in pathogenicity, such as in group B streptococci (GBS, *Streptococcus agalactiae*) (Bernheimer *et al.*, 1979; Jürgens *et al.*, 1987). The predicted interaction (S-score: 340) involves 51 bases (position 1050538-1050588, SF370 genome) of the *cfa* 5'UTR and 54 bases of SypRNA049 (position 854384-854437, SF370 genome).

To investigate whether *cfa* is the target for SypRNA049, we deleted SypRNA049 in M1 SF370 GAS strain from +1 to the terminator including all three RNAs. To examine whether the CAMP activity was affected in the SpyRNA049 mutant, CAMP test was done with mutant and wild type GAS strains. But CAMP factor activity analysis showed no difference between the SpyRNA049 mutant and wild type strains (Fig. 6). To check whether SypRNA049 affects *cfa* expression at the transcriptional level, Northern blot analysis was done. However, no significant difference in the expression of *cfa* mRNA from early logarithmic to stationary phase was observed comparing the SpyRNA049-deficient and wild type strains (Fig. 7A). We then analyzed the possibility that SpyRNA049 could bind to *cfa* mRNA and affects its stability. Therefore, the stability of *cfa* mRNA was analyzed in SpyRNA049-deficient and wild-type strains. Similarly, the half life of *cfa* mRNA was ~5 minutes in both SpyRNA049-deficient and wild-type

strains (Fig. 7B). Furthermore, band shift assays were done to analyze a possible binding of *cfa* mRNA with SpyRNA049. But the band shift assays also failed to show any shift in the band when the 5' end of *cfa* and the 3' end of SpyRNA049 were allowed to bind with each other (Fig. 8). All of these results indicate that most likely the *cfa* mRNA is not the target for SpyRNA049.

Phenotypic assays

To investigate a possible role of SpyRNA049 in pathogenic aspects of *S. pyogenes*, some phenotypic assays were undertaken. The growth curve of the SpyRNA049-deficient strains (EC1417 and EC1418) was compared to that of the wild-type in rich THY medium. SpyRNA049 was not found to be essential for the growth of GAS as no growth defects were observed. Interestingly the SpyRNA049-deficient strains were growing slightly faster, reaching a higher OD at 620 nm when compared to the wild-type strain (Fig. 9).

Then a bacterial aggregation/sedimentation assay was done comparing wild-type and SpyRNA049-deficient strains (Fig. 10). First, we observed a variation in sedimentation in two SpyRNA049-deficient strains (EC1417 and EC1500). The first mutant EC1417 and its corresponding wild-type partner after temperature shifts EC1420 were not aggregating for 2 h whereas the wild-type EC904 was aggregating. On the other hand, we did not observe any difference in the aggregation when comparing the second mutant EC1500, its corresponding wild-type partner after temperature shifts EC1502 and wild-type EC904.

Does SpyRNA049 have a role in host cell interaction?

In order to investigate the role of SpyRNA049 in infection, we conducted adhesion, internalization and survival assays with pharyngeal epithelial HEp-2 cells and RAW macrophages comparing the SpyRNA049-deficient and wild type strains. However, we could not see a significant difference in adhesion and internalization rates between the two strains (Fig. 11A, B, C).

Does SpyRNA049 have a role in the expression of virulence factors?

SDS-PAGE analysis of intracellular, cell-associated and exoproteins was done to examine the effect of SpyRNA049 on virulence factors. Thus far, we could not observe a striking difference in protein patterns when comparing the SpyRNA049-deficient and wild type strain grown in rich medium. This would have to be repeated and analyzed in conditions of growth in minimal medium.

Discussion

A previous *in silico* study conducted in our lab revealed 5 novel sRNA candidates. We chose one of the interesting candidates SpyRNA049 to describe further. Our investigations revealed that SpyRNA049 is not conserved in all M serotypes of *S. pyogenes* and expressed only in M1 and M12 serotypes. Moreover, our data indicated that SpyRNA049 consists of three independent sRNAs varying in size, stability and secondary RNA structures. Expression of three different sRNAs but having a same 3' end might be using a novel mechanism of gene expression control. It would be interesting to find its mechanism of action.

In silico mRNA target analysis showed the CAMP factor gene (*cfa*) mRNA as the most likely target of SpyRNA049. CAMP factor is an extracellular protein involved in erythrocyte lysis. In order to analyze *cfa* maRNA as target of SpyRNA049 we compared SpyRNA049-deficient and wild-type strains for *cfa* expression, stability and CAMP test, but none of these experiments showed a significant difference. Moreover to analyze a possible binding of SpyRNA049 with *cfa* mRNA band shift assay was done, but no binding was observed. Thus our data do not verify the *in silico* prediction i.e. *cfa* mRNA is a possible target of SpyRNA049.

Our data indicated that SpyRNA049 is not essential for GAS as SpyRNA049-deficient mutant had no growth defects. In contrast, the mutants were growing faster and reaching a higher OD when compared to the wild-type strain. If this could be confirmed it would indicate that sRNAs might play a role in GAS to render the pathogen fitter for infection. Therefore, to investigate the possible role of SpyRNA049 in infection, we performed adhesion, internalization and survival assays with pharyngeal epithelial cells and macrophages. However, no significant difference was observed when SpyRNA049-

deficient and wild-type strains were compared. To investigate the role of SpyRNA049 in virulence factor expression, SpyRNA049-deficient and wild-type strains were compared for intracellular, cell-associated and exoproteins, however no difference could be observed thus far. These experiments need to be repeated to confirm the data.

To further investigate the role of SpyRNA049 in different aspects of pathogenity, a sedimentation assay was done. We found a striking difference between SpyRNA049-deficient and wild-type strains but the wild-type after temperature shifts was also behaving like the mutant. In order to verify whether the sedimation phenotype was linked to SpyRNA049, we constructed a fresh SpyRNA049-deficient strain. Therafter, we did not observe any difference in SpyRNA049-deficient, wild-type and wild-type after temperature shift strains. Thus it still remains an open question what is the role of SpyRNA049. Do all three sRNAs expressed from the same locus have similar role or distinct functions?

FIGURE LEGENDS

Figure 1. SpyRNA049 expression profile and stability. (A) Northern blot analysis of total RNA isolated from M1 serotype *S. pyogenes* (SF370 – EC904). Total RNA samples prepared from cell grown in THY were separated on 8% denaturating PAA (10 µg/lane) and submitted to Northern blot analysis. RNAs were hybridized with a radiolabelled PCR generated SpyRNA049 probe. 5S rRNA was used as loading control. EL: early log; ML: mid log; LL: late log; ES early stationary; S: stationary; LS: late stationary phase of growth. **(B)** Northern blot analysis of RNA prepared from rifampicin-treated cells. Treated RNAs (10 µg/lane) were hybridized with a PCR generated probe specific to SpyRNA049. **(C)** Half-life calculation of the three transcripts derived from the SpyRNA049 locus. Before rifampicin addition, the amount of the transcript is 100%. Over the time, decreasing amounts of RNA are normalized with 5S rRNA amounts in the cells. The half-life is defined as the point at which 50% of the RNA was degraded.

Figure 2. SpyRNA049 mapping. (A) Primer extension analysis of SpyRNA049. A, C, G, T: sequencing reactions. OLEC703: primer extension reaction done with primer OLEC703 and 8 µg of TP2 RNA from EC904. Alternative 5' ends are underlined in the sequencing and with arrows aside the primer extension. Only one 5' end was confirmed by self ligation (SL). **(B) SpyRNA049 sequence. (C) SpyRNA049 locus** Panel A: ***Genomic organization of SpyRNA049 locus***. SpyRNA049 is pictured in red, encoded on the (-1) strand of SF370 genome. Locus tag is given above the grey arrows representing the ORFs; start position of these ORFs is annotated underneath. Zoom is made on SpyRNA049. The position of the three 5' ends, the rho-independent terminator and the size of the three RNAs are given. The sequence of SpyRNA049 is from SF370 sequenced genome [NC_002737], position: 854376 to 854546. Putative promoter regions are underlined in blue; 5'ends are underlined in red; *: primer extension result; °: self-circularization result; Putative translational start (TTG) is underlined in black; Rho-independent terminator is underlined in green. Panel B: ***SpyRNA049 locus annotations***. Annotations to NC_002737 of the NCBI database. Hypothetical proteins annotation was

further checked for orthology using the KEGG database [http://www.genome.jp/kegg/] and by Blast analysis [http://blast.ncbi.nlm.nih.gov/Blast.cgi].

Figure 3. SpyRNA049 predicted structure. Structure predictions for the three transcripts expressed from SpyRNA049 locus using the RNAfold, contrafold and RNA structure algorithm. Panel A: SpyRNA049-76 Panel B: SpyRNA049-89 Panel C: SpyRNA049-171.

Figure 4. (A) GeneDoc alignment of SPyRNA049 locus (coordinates M1 SF370 NC_002737: 854279 – 854756, complementary strand). Blue: putative transcriptional promoters; red: alternative 5' ends; green: putative rho-independent terminator (RIT); grey: sequence variation. The serotype of the different sequenced genome is given. M49 serotype is not fully sequenced yet. IGR is not conserved in M5, M6 and M18 seotypes. **(B) SpyRNA049 Blast analysis** Panel A: *Sequence query used for the Blast analysis.* SpyRNA049-171 sequence (from +1 to RIT) has been taken as template. Panel B: *Blast results*. Numbers refer to panel C. Panel C: *Blast partners*. Accession numbers of each Blast partner is given.

Figure 5. mRNA target predictions for SpyRNA049. Panel A: S-scores for potential interaction between 3' UTRs (grey) and 5' UTRs (red) with SpyRNA049. Panel B: RNAcofold predicted duplex structure of SpyRNA049 and the upstream region of the *cfa* gene. SpyRNA049: red. Panel C: RNAfold predicted interaction between full length SpyRNA049 (76 nt) and *cfa* transcript (#150 nt). Yellow: SpyRNA049; Green: *cfa* mRNA 5'end; Blue: *cfa* mRNA AUG; grey: linker; interaction site: red.

Figure 6. CAMP test. *S. aureus* strain RN450 (EC16) was streaked vertically and SpyRNA049-deficient mutant (EC1417) and SF370 WT (EC904) GAS strains were streaked at right angles. GBS (EC770) was streaked as a positive control of CAMP reaction. SF370 WT (EC904) GAS strain and SpyRNA049-deficient mutant (EC1417) are CAMP negative, while GBS (EC770) gives CAMP positive reaction.

Figure 7. (A) Northern blot analysis of total RNA of wild-type strain EC904 and its isogenic SpyRNA049 mutant EC1417. Specific DNA probes were used for *cfa*. The probe for *tmRNA* acted as loading control. **(B)** Determination of mRNA half-lives in EC904 (wild-type) and EC1417 (SpyRNA049 mutant) by inhibiting mRNA synthesis with the addition of rifampicin. Estimation of mRNA decay is based on Northern blot analysis. Total RNA of mid-logarithmic cells was harvested at the indicated time points (min) after addition of rifampicin (250μg/ml). The blot was hybridized with *cfa*-specific probe and with *tmRNA*-probe acting as loading control.

Figure 8. Band shift assay. Labelled SpyRNA049 (0.15 pmol) was incubated with unlabelled *cfa* RNA in increasing molar ratio as indicated.

Figure 9. Growth curve in THY. Growth curve was done using SpyRNA049-deficient strains (EC1417 and EC1418) and SF370 WT (EC904) GAS strains in THY. SpyRNA049-deficient strains reached stationary phase at higher OD (0.59), while SF370 WT reached stationary phase at lower OD (0.49) when monitored at 620 nm.

Figure 10. Aggregation/ sedimentation assay. Aggregation assay was done using SpyRNA049-deficient strains (EC1417 and EC1418) and SF370 WT (EC904) GAS strains in Thy. Strains were grown till stationary phase with shaking and then OD was noted every 30 minutes without shaking tubes. SF370 WT (EC904) GAS strain was aggregating in 30 minutes while SpyRNA049-deficient strains were not aggregating even till 3 hours.

Figure 11. (A) Adhesion assay. Adhesion assay was done using SpyRNA049-deficient strain (EC1417) and SF370 WT (EC904) GAS strains in Hep2 cells. GAS were grown to late-logarithmic phase and added to monolayers at MOI 5:1 in triplicate. After incubation for 1 hour, infected monolayers were washed extensively with PBS before lysing with chilled sterile distilled water. The number of viable bacteria was counted by plating appropriate dilutions of the lysates on TSA-blood plates. Adherence of WT strain was 30% while adherence of SpyRNA049-deficient strain was 39%. **(B) Internalization**

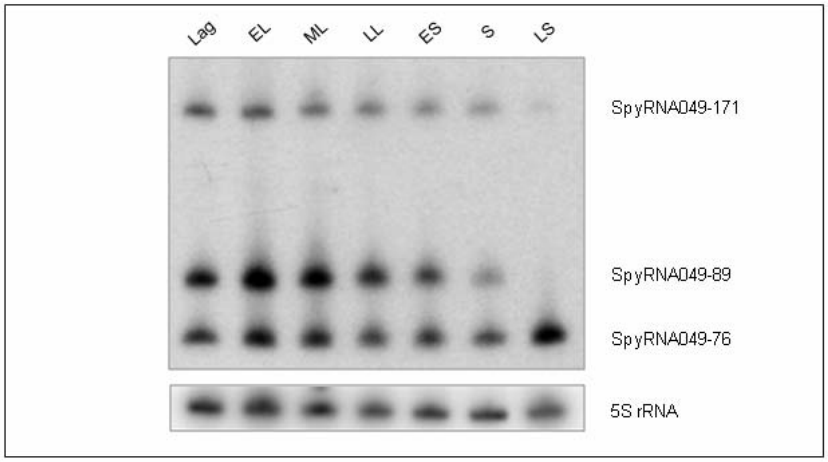
assay with Hep2 cells. Internalization assay was done using SpyRNA049-deficient strain (EC1417) and SF370 WT (EC904) GAS strains in Hep2 cells. GAS were grown to late-logarithmic phase and added to monolayers at MOI 5:1 in triplicate. After incubation for 2 hours, extracellularly adhered bacteria were killed by incubation with medium containing 100 μg/ml gentamicin and 5 μg/ml penicillin for 2 hours. Infected monolayers were washed with PBS before lysing with chilled sterile distilled water. The number of viable bacteria was counted by plating appropriate dilutions of the lysates on TSA-blood plates. Internalization of WT strain was 3.5% while internalization of SpyRNA049-deficient strain was 4.1%. **(C) Internalization assay with RAW cells.** Internalization assay was done using SpyRNA049-deficient strain (EC1417) and SF370 WT (EC904) GAS strains in RAW cells. RAW 264.7 GAS were grown to mid-logarithmic phase and added to monolayers at MOI 100:1 in triplicate. After infection for 30 minutes, extracellularly adhered bacteria were killed by incubation with medium containing 60 μg/ml penicillin for 30 minutes and 1 hour. Infected monolayers were washed with PBS before lysing with chilled sterile distilled water. The number of viable bacteria was counted by appropriate dilutions of the lysates on TSA-blood plates. Internalization of WT strain was 1.9 % while internalization of SpyRNA049-deficient strain was 1.5% at 0 h and 1.5% and 1.1% at 1 hour.

The percent intracellular invasion represents the average CFU/mL number of viable intracellular bacteria recovered at different times following addition of antibiotics versus the average CFU/mL number of inoculated bacteria x 100. The average values and standard deviations are shown.

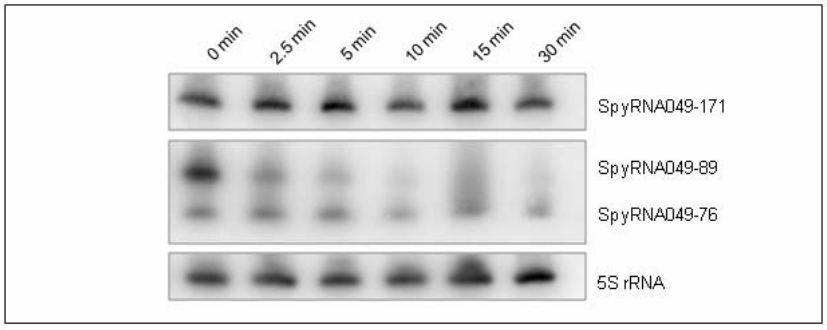
FIGURES

Figure 1

A.



B.



C.

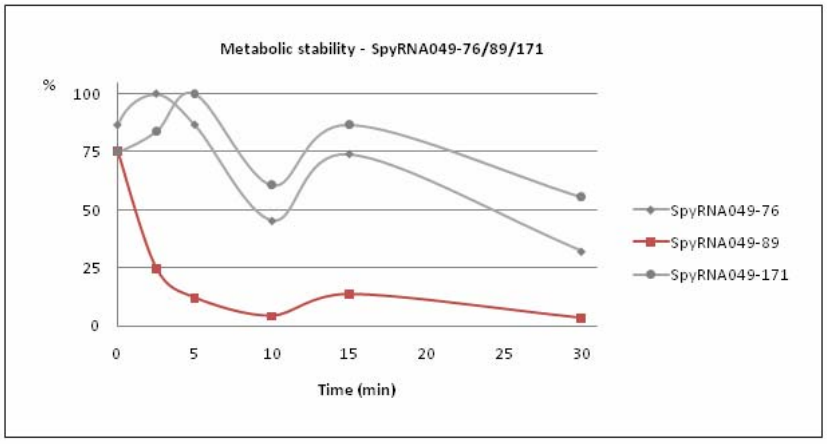


Figure 2A

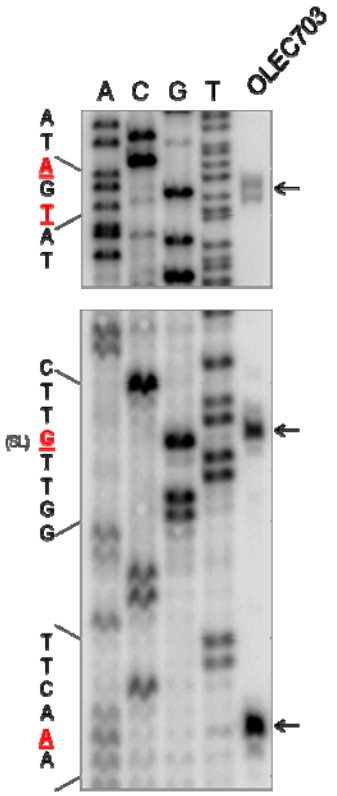


Figure 2B

SpyRNA049-171

AGUAUUAAGUAUUGUUUUAUGGCUGAUAAAUUUCUUUGAAUUUCUCCUUGAUUAUUUGUU
AUAAAAGUUAUAAAAUAAUCUUGUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAA
GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU

SpyRNA049-89

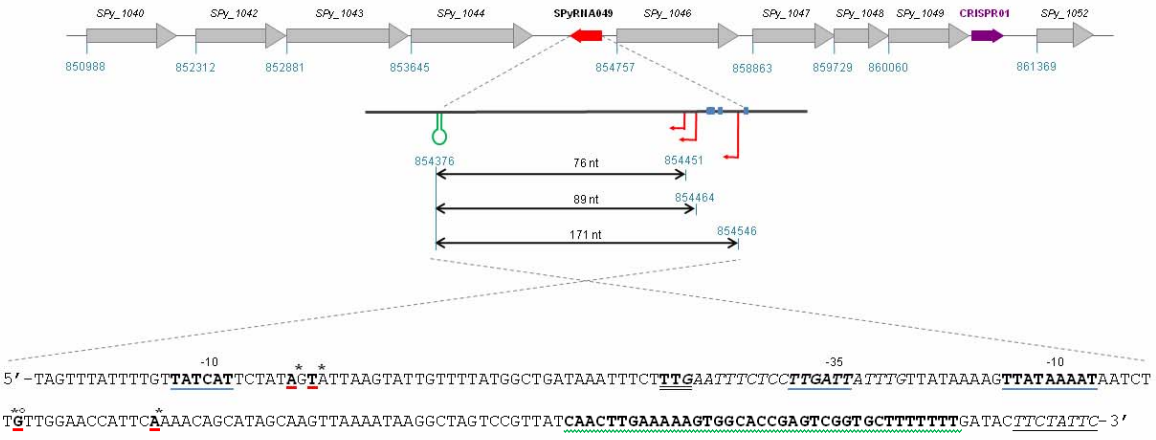
GUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA
AAAAGUGGCACCGAGUCGGUGCUUUUUUU

SpyRNA049-76

AAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCG
AGUCGGUGCUUUUUUU

Figure 2C

A.

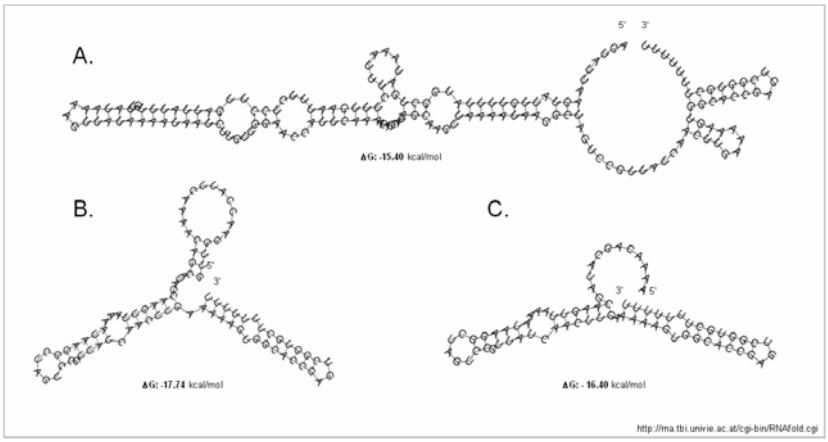


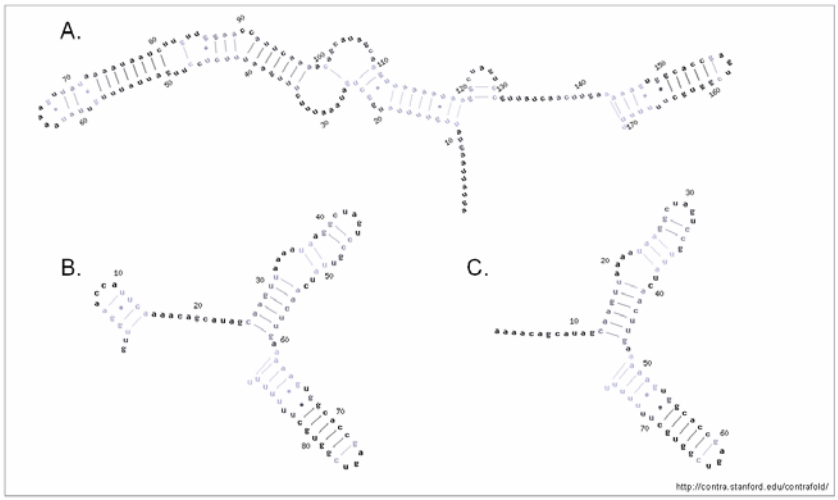
B.

	Name	Start	End	Length (pb)	Length (aa)	Strand	Function
Spy_1040	hemN	850988	852118	1131 nt	376 aa	+1	coproporphyrinogen III oxidase (EC:1.3.3.3)
Spy_1042		852312	852881	570 nt	189 aa	+1	HP (acyl-acyl carrier protein thioesterase [KEGG & NCBIBlast])
Spy_1043		852881	853645	765 nt	254 aa	+1	HP (4-nitrophenylphosphatase (EC:3.1.3.41) [KEGG & NCBIBlast])
Spy_1044		853645	854277	633 nt	210 aa	+1	HP (hypothetical membrane spanning protein [KEGG & NCBIBlast])
SPyRNA049		**854376**	**854546**	**171 nt**			
Spy_1046	csn1	854757	858863	4107 nt	1368 aa	+1	CRISPR-associated protein Csn1
Spy_1047	cas1	858863	859732	870 nt	289 aa	+1	CRISPR-associated protein Cas1
Spy_1048	cas2	859729	860070	342 nt	113 aa	+1	CRISPR-associated protein Cas2
Spy_1049	csn2	860060	860722	663 nt	220 aa	+1	CRISPR-associated protein Csn2
Spy_1052		861369	861776	408 nt		+1	pseudogene

Figure 3

Panel A: **SpyRNA049- 76**
Panel B: **SpyRNA049- 89**
Panel C: **SpyRNA049- 171**





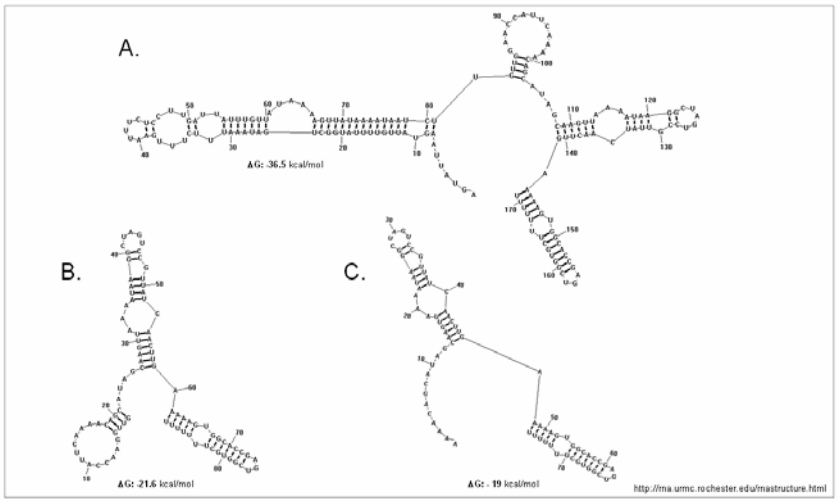
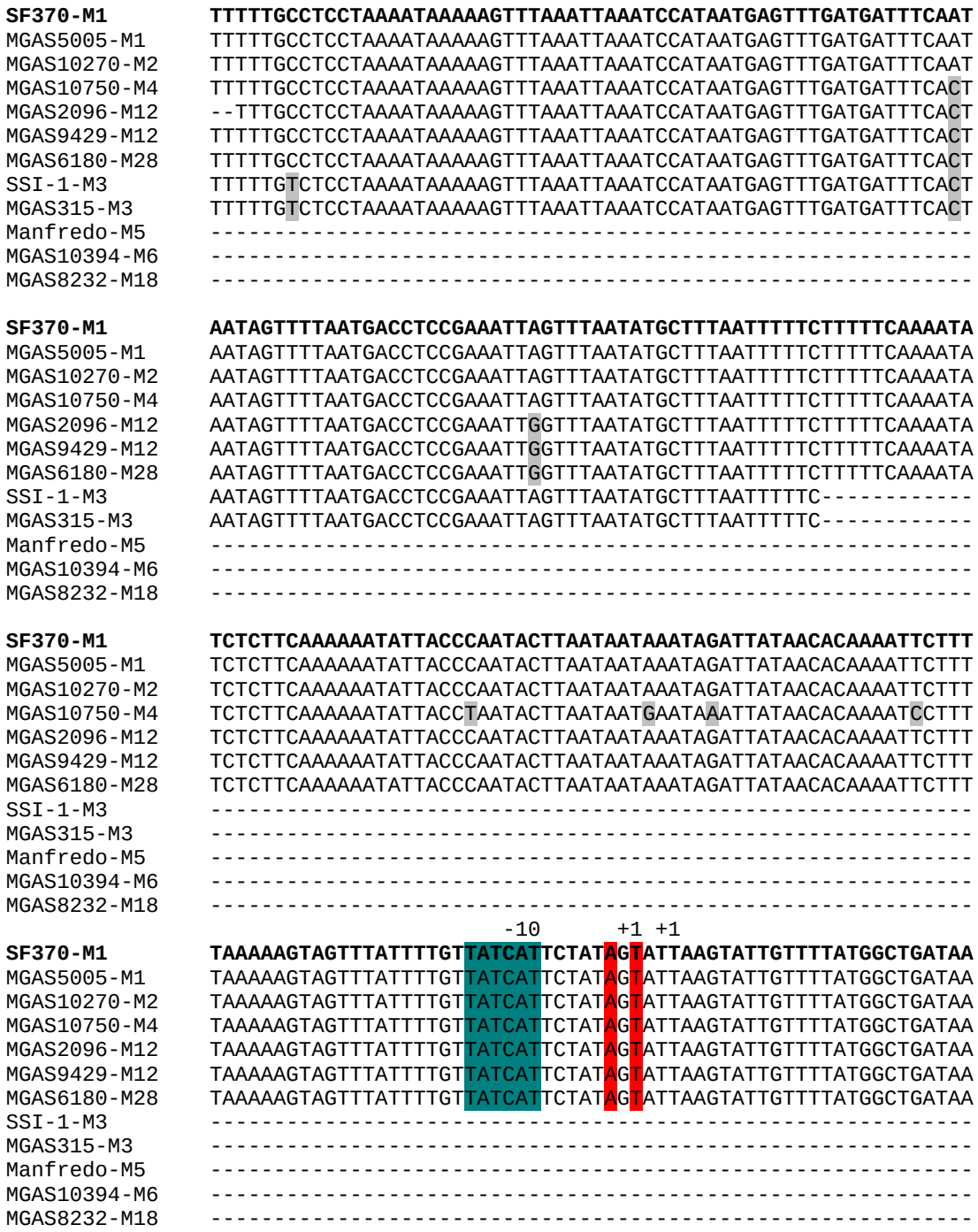


Figure 4A

GenDoc multiple sequence alignment



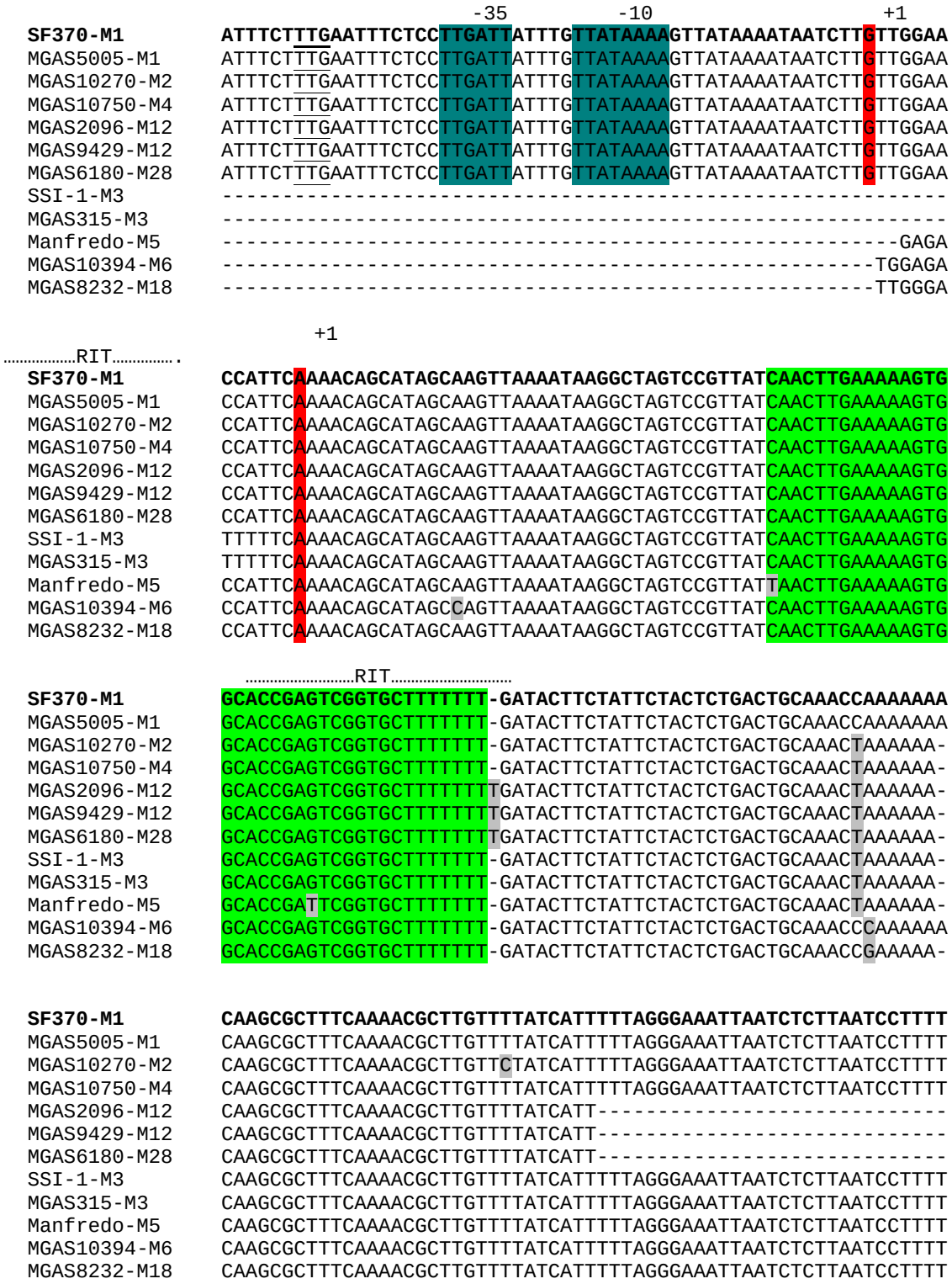

-35
-10
+1
SF370-M1 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS5005-M1 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS10270-M2 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS10750-M4 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS2096-M12 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS9429-M12 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
MGAS6180-M28 ATTTCTTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTTGGAA
SSI-1-M3 ----------
MGAS315-M3 ----------
Manfredo-M5 ----------GAGA
MGAS10394-M6 ----------TGGAGA
MGAS8232-M18 ----------TTGGGA
+1
RIT
SF370-M1 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS5005-M1 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS10270-M2 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS10750-M4 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS2096-M12 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS9429-M12 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS6180-M28 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
SSI-1-M3 TTTTTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS315-M3 TTTTTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
Manfredo-M5 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATTAACTTGAAAAAGTG
MGAS10394-M6 CCATTCAAAACAGCATAGCCAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
MGAS8232-M18 CCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTG
RIT
SF370-M1 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACCAAAAAAA
MGAS5005-M1 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACCAAAAAAA
MGAS10270-M2 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS10750-M4 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS2096-M12 GCACCGAGTCGGTGCTTTTTTTTGATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS9429-M12 GCACCGAGTCGGTGCTTTTTTTTGATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS6180-M28 GCACCGAGTCGGTGCTTTTTTTTGATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
SSI-1-M3 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS315-M3 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
Manfredo-M5 GCACCGATTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACTAAAAAA-
MGAS10394-M6 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACCCAAAAAA
MGAS8232-M18 GCACCGAGTCGGTGCTTTTTTT-GATACTTCTATTCTACTCTGACTGCAAACCGAAAAA-
SF370-M1 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS5005-M1 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS10270-M2 CAAGCGCTTTCAAAACGCTTGTTCTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS10750-M4 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS2096-M12 CAAGCGCTTTCAAAACGCTTGTTTTATCATT----------
MGAS9429-M12 CAAGCGCTTTCAAAACGCTTGTTTTATCATT----------
MGAS6180-M28 CAAGCGCTTTCAAAACGCTTGTTTTATCATT----------
SSI-1-M3 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS315-M3 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
Manfredo-M5 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS10394-M6 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT
MGAS8232-M18 CAAGCGCTTTCAAAACGCTTGTTTTATCATTTTTAGGGAAATTAATCTCTTAATCCTTTT

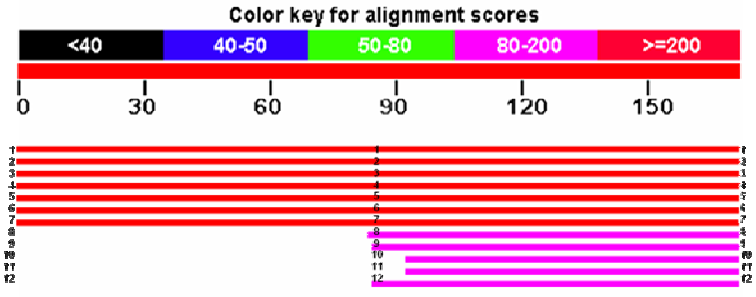
Figure 4B

A.

Query: SpyRNA049-171

agtattaagtattgttttatggctgataaatttctttgaatttctccttgattatttgttataaaagttataaaataatcttgttgga
accattcaaaacagcatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgc
tttttt

B.

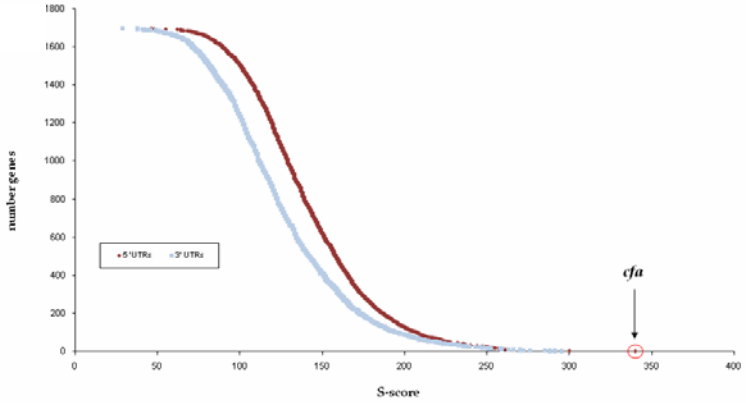


C.

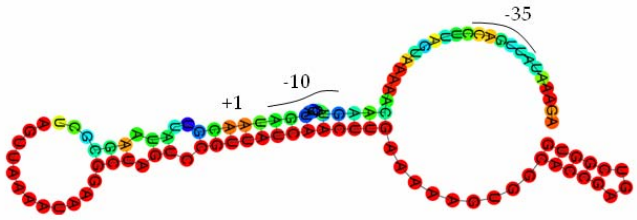
ACCESSION		DESCRIPTION
AE004092.1	1	**Streptococcus pyogenes M1 GAS, complete genome**
CP000262.1	2	Streptococcus pyogenes MGAS10750, complete genome
CP000261.1	3	Streptococcus pyogenes MGAS2096, complete genome
CP000260.1	4	Streptococcus pyogenes MGAS10270, complete genome
CP000259.1	5	Streptococcus pyogenes MGAS9429, complete genome
CP000017.1	6	Streptococcus pyogenes MGAS5005, complete genome
CP000056.1	7	Streptococcus pyogenes MGAS6180, complete genome
AE010030.1	8	Streptococcus pyogenes strain MGAS8232, section 78 of 173
CP000003.1	9	Streptococcus pyogenes MGAS10394, complete genome
AE014074.1	10	Streptococcus pyogenes MGAS315, complete genome
BA000034.2	11	Streptococcus pyogenes SSI-1 DNA, complete genome
AM295007.1	12	Streptococcus pyogenes Manfredo complete genome

Figure 5

A.


number genes
S-score
5'UTRs
3' UTRs
cfa

B.


-35
-10
+1

C.

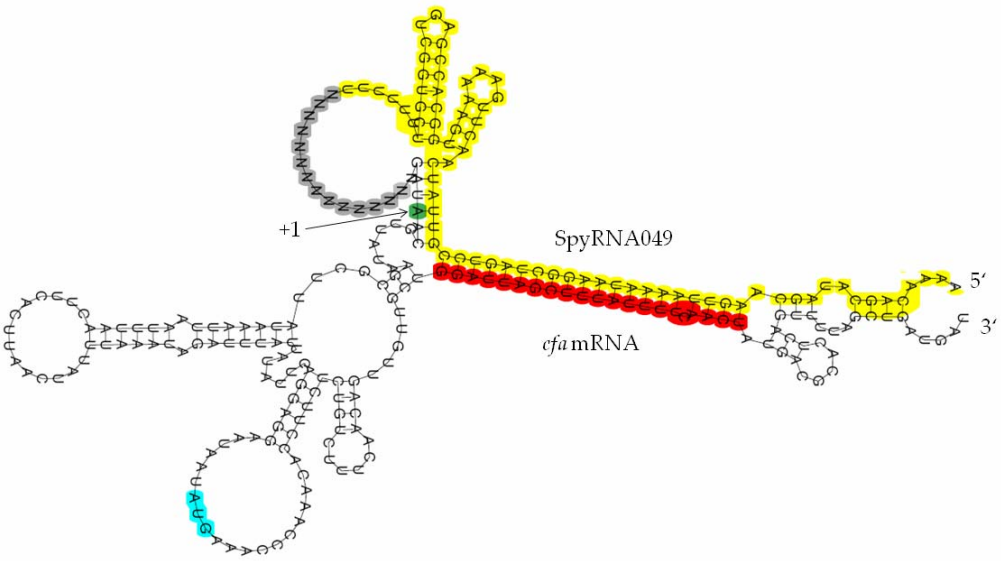

+1
SpyRNA049
cfa mRNA
5′
3′

Figure 6

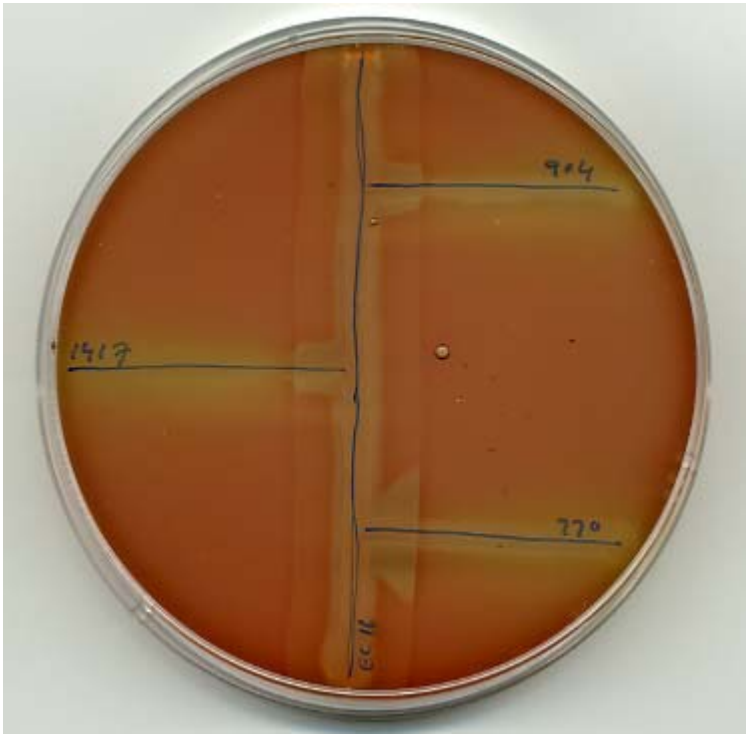


Figure 7A

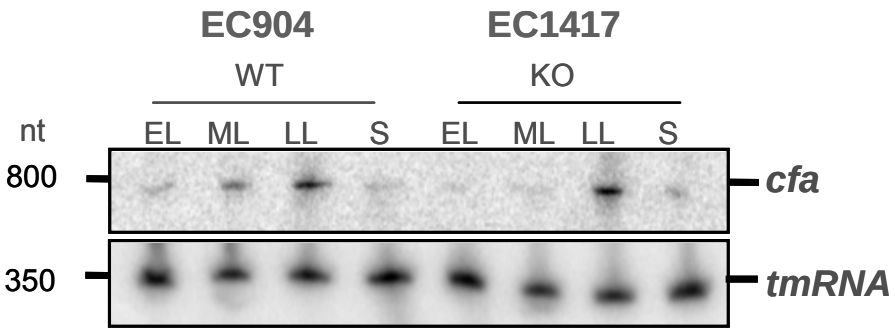


Figure 7B

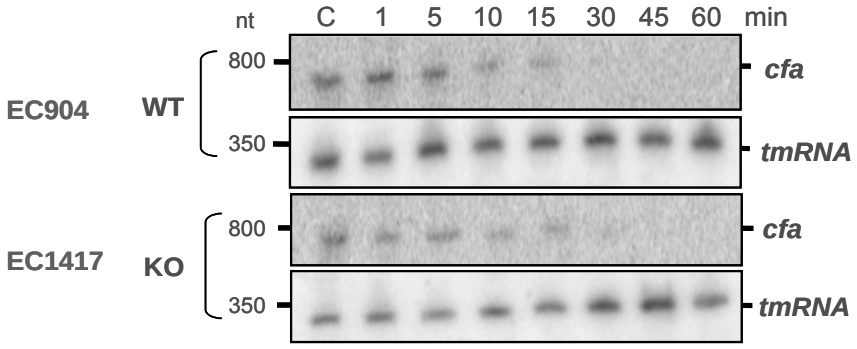


Figure 8

Serial	1	2	3	4
Ratio	-	5	10	15
SpyRNA049	2.3 µL	2.3 µL	2.3 µL	2.3 µL
Cfa		2.2 µL	4.4 µL	6.6 µL
VD	7.7 µL	5.5 µL	3.3 µL	1.1 µL

Labelled SpyRNA049 (84nt) + + + +

Unlabelled Cfa (164nt) molar ratio - 5 10 15

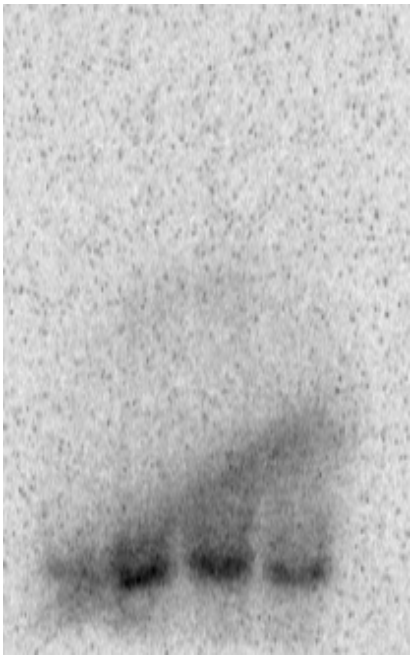


Figure 9

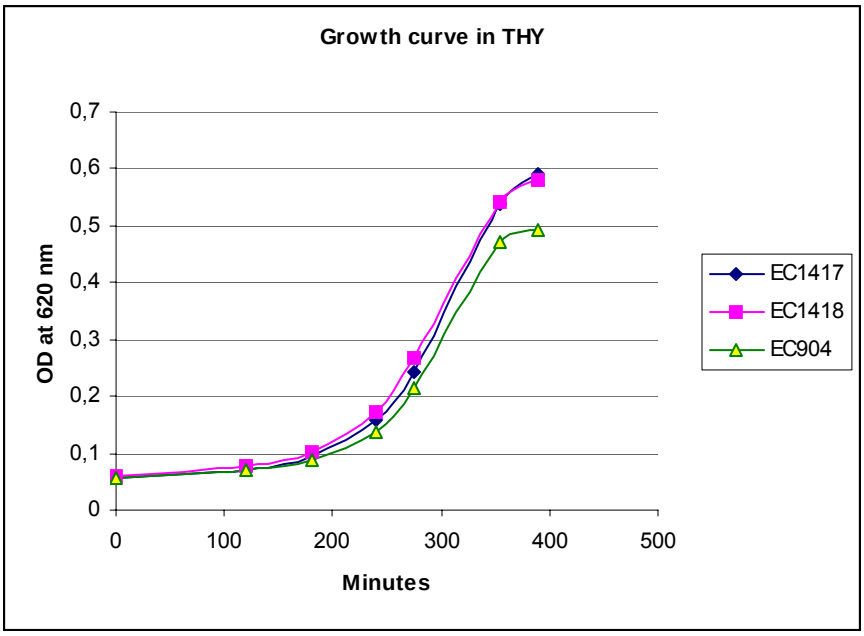


Figure 10

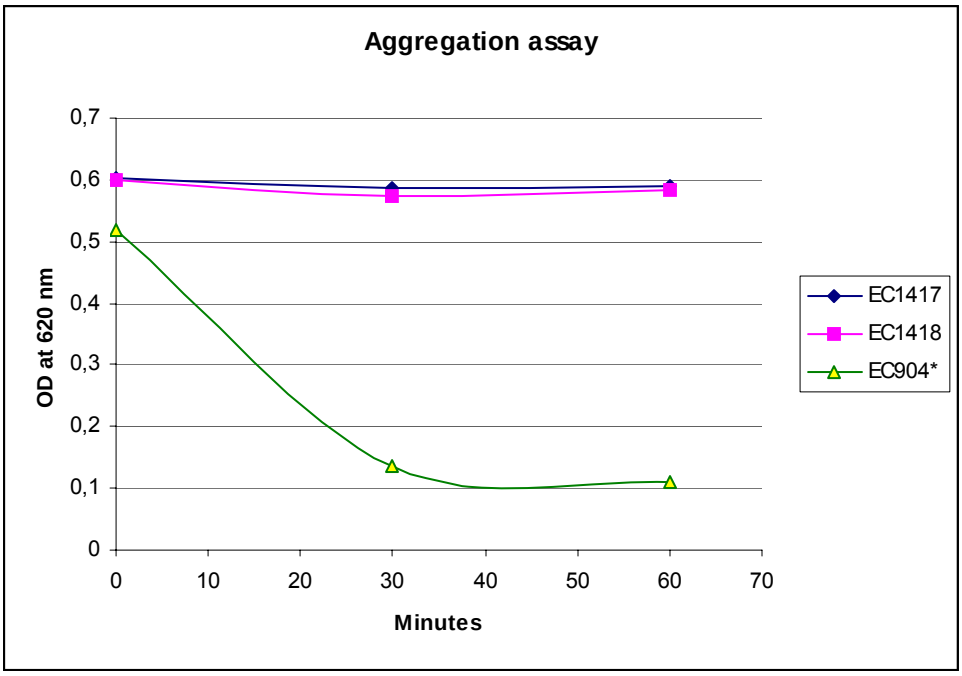


Figure 11A

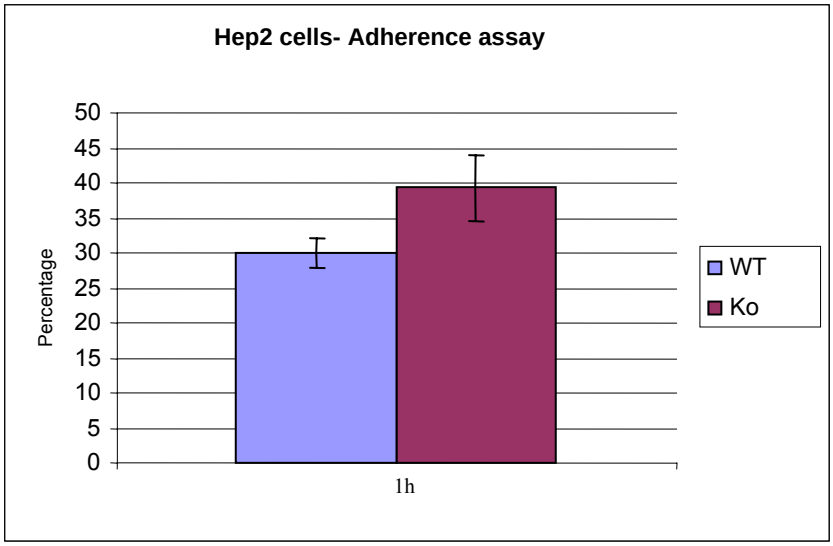


Figure 11B

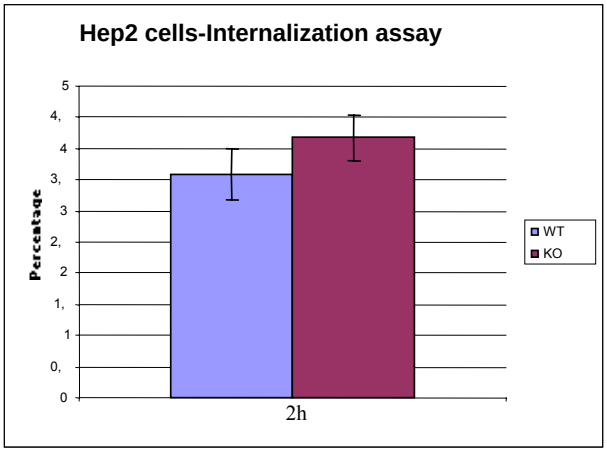
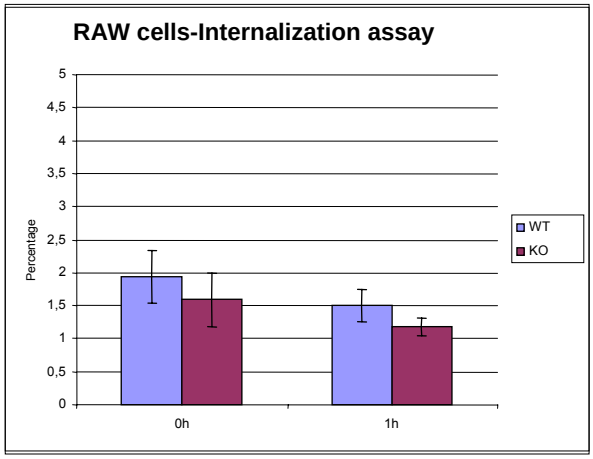


Figure 11C


RAW cells-Internalization assay
Percentage
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0h
1h
WT
KO

TABLES

Table 1 Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Source
Streptococcus pyogenes		
EC904	M1 SF370 wild-type	ATCC 700294
MGAS5005	M1 wild-type	ATCC BAA-947
RDN57	M49 wild-type	Lab collection
RDN60	M5 wild-type	Lab collection
RDN151	M12 wild-type	Lab collection
EC905	M3 wild-type	Lab collection
EC1417	Isogenic SpyRNA049-deficient mutant of M1 SF370	This study
EC1500	Isogenic SpyRNA049-deficient mutant of M1 SF370	This study
EC1420	M1 wild-type after temperature shifts	This study
EC1502	M1 wild-type after temperature shifts	This study
EC1412	EC904 (pEC264)	This study
EC1414	EC904 (pEC264, integrated)	This study
Escherichia coli		
DH5α	Host for cloning	Lab collection
TOP10	Host for cloning	Invitrogen
EC1254	Top10 (pEC262)	This study
EC1409	Top10 (pEC264)	This study
EC1438	Top10 (pEC268, for complementation of 179 bp fragment)	This study
EC1441	Top10 (pEC269, for complementation of 89 bp fragment)	This study
Plasmids		
pEC264	pEC214Ω*SpyRNA049*up-*SpyRNA049*dw	This study
pEC262	pEC214Ω*SpyRNA049*up	This study
pEC268	pEC85Ω*SpyRNA049* (179bp fragment)	This study
pEC269	pEC85Ω*SpyRNA049* (89bp fragment)	This study

Table 2 Oligonucleotides used in this study

Oligo	[a]F/R	Sequence 5'-3'	Specificity
OLEC486	F	TAAAACAAGCGTTTTGAAAGC	SpyRNA049
OLEC487	R	TTAATGACCTCCGAAATTAGT	
OLEC673	F	TGTTGGAACCATTCAAAACAG	primers used for RT-PCR of self-circularized RNA to amplify 5' and 3' ligated ends of SpyRNA049
OLEC674	R	ATTCAAAGAAATTTATCAGCCA	
OLEC702	F	ATACTTCTATTCTACTCTGAC	
OLEC703	R	CGACTCGGTGCCACTTTTTCA	
OLEC740	F	AGTAGTTTATTTTGTTATCA	
OLEC741	R	TGTGTTATAATCTATTTATTA	
OLEC821	F	GACAGCGAGCTCATAGAATGATAACAAAATAAA	SpyRNA049-downstream fragment
OLEC822	R	AAG TGCGAATTCATCAGCATATTGATCTCCAAT	
OLEC823	F	TAGACTCTGCAGGACTGATATCATGGCAGGTAT	SpyRNA049-upstream fragment
OLEC824	R	CTGTAAGAGCTCTTCTATTCTACTCTGACTGCA	
OLEC997	F	GCGTCGGGATCCTTAATGACCTCCGAAATTAGT	Complementation of SpyRNA049 (179bp)
OLEC996	R	TGCAGTCAGAGTAGAATAGAA	
OLEC998	F	CGAGCGGGATCCGAATTTCTCCTTGATTATTTG	Complementation of SpyRNA049 (89bp)
OLEC996	R	TGCAGTCAGAGTAGAATAGAA	
OLEC287	F	AGTTAAGTGACGATAGCCTAG	5S rRNA
OLEC288	R	CTAAGCGACTACCTTATCTCA	
oliRN242	F	CGGTAACTAACCAGAAAGGG	16S rRNA
oliRN243	R	CGTTGTACCAACCATTGTAGC	

[a] F, forward; R, reverse. Underlined sequences indicate restriction sites.

4.3 Project 3: Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress and interaction with host cells

4.3.1 Summary

Quorum sensing mechanism helps bacteria to sense their own number (quorum) in the environment by producing and releasing signaling molecules and then detecting and responding to them. As auto-inducers (AIs) are specific to their sensors so the sensing is restricted within the same species. Recently, a second autoinducing system has been discovered, which detects a furanone-like signaling molecule termed "autoinducer 2" (AI-2) in *V. harveyi*. AI-2 is synthesized by the *luxS* gene, which is found in over 55 gram-negative and gram-positive bacterial species. AI-2 is proposed to have a role in interspecies communication. Knowledge about quorum sensing systems in *S. pyogenes* is very limited. Two putative quorum-sensing systems, *sil* and *fasBCA*, have been reported in GAS. No signaling peptide could be identified within the *fasBCA* locus, but the putative pheromone peptide SilCR from the *sil* operon was shown to regulate DNA uptake and the ability of GAS to cause invasive infection.

Apart from bioluminescence, *luxS*/AI-2 signaling is hypothesized to be involved in the regulation of pathogenicity and *S*-adenosylmethionine (SAM) utilization pathway. In an M6 serotype, Lyon *et al* (2001) observed an enhanced expression of *sagA* associated with enhanced haemolytic activity and a reduction in SpeB protease proteolytic activity in a *luxS* mutant. In an M6 serotype, Marouni and Sela (2003) observed higher ability to be internalized in pharyngeal epithelial cells and an enhanced *emm* transcription in a *luxS* mutant compared to the wild-type. Further they observed that *luxS* expression is under the control of the CsrR regulator.

To investigate the role of *luxS* in AI-2 like activity, our data showed that at late-logarithimic phase AI-2 like activity peaked, but *luxS* transcript levels rapidly decreased, hence *luxS* expression and production of AI-2 like activity are shifted out of phase. Furthermore, an auto-regulatory function of AI-2 like activity on *luxS* expression could not be observed in GAS using conditioned medium. To investigate the role of *luxS* enzyme in AMC metabolism, the growth rates of wild-type and *luxS*-deficient strains in sulfur-limited CDM were analyzed. Overall the growth rates and yields were similar

when wild-type strains were compared to the *luxS*-deficient mutants. These results indicate that *luxS* does not have an essential AMC-related metabolic role in GAS.

The role of *luxS* in adaptation to stress conditions, which bacteria can encounter during the infection was investigated. Exposure of bacterial cultures to acidic conditions revealed a profound increase in acid tolerance in the *luxS*-deficient strains. Exposing cultures to low pH led to reduced expression of *luxS*, thus suggesting a correlation between reduced *luxS* expression and survival under acidic conditions. Accordingly, CM from these cultures showed reduced AI-2 like activity. We observed reduced *luxS* expression at the transcriptional level in wild-type cultures grown in RPMI medium compared to cultures grown in the nutrient rich THY medium. Furthermore, AI-2 activity in CM from wild-type cultures grown in RPMI was reduced to a level similar to CM from *luxS*-deficient cultures. This indicates that under host cell environment mimicking medium (acidic conditions and *in vivo*-like environment), there is an advantage for GAS to lower the expression of *luxS* and the production of AI-2 like activity.

We investigated further whether lowering the expression of *luxS* would be beneficial for bacteria in intracellular host environments. Results from internalization assays showed that the internalization and survival rates of the *luxS* mutant strains of both M1 and M19 serotypes in HEp-2 lung epithelial cells were about 5-fold higher to those of wild-type strains. The *luxS* deficient mutants showed also significantly higher survival rates in macrophages of 2 to 3 fold compared to the wild-type strains. With both serotypes and in both epithelial and macrophages cells, complementing the mutant strains with the *luxS* allele *in trans* restored the intracellular survival rates to wild-type levels. These data indicate that lowering the expression of *luxS* provides GAS an advantage to survive the harsh conditions encountered in host cells. In addition, virulence gene expression in *luxS* mutants showed that *luxS* negatively affects the *fasX* transcript while it positively affects the *sibA* transcript.

Our data indicate a limited role of *luxS* in AMC-related metabolism. However it has a role in adaptation to stress responses and by down-regulation of the expression of *luxS*, *S. pyogenes* has an advantage to survive in a host environment. Hence, *luxS*/AI-2 has an important function in host cell infection.

My contribution to this study was to do the adhesion and internalization assays with HEp-2 and RAW cells, also the internalization assays with the mixed cultures and to check the effect of alkaline pH on *luxS* expression.

4.3.2 Manuscript 3

Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress and interaction with host cells

Published manuscript: BMC Microbiology (2008) 30(8), 188

Authors:

Maria Siller, Rajendra Prasad Janapatla, **Zaid Ahmed Pirzada**, Christine Hassler, Daniela Zinkl and Emmanuelle Charpentier

BMC Microbiology

Research article

Open Access

Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress and interaction with host cells

Maria Siller[†1], Rajendra P Janapatla[†1], Zaid A Pirzada[1], Christine Hassler[1], Daniela Zinkl[1] and Emmanuelle Charpentier[*1,2]

Address: [1]Max F. Perutz Laboratories, University of Vienna, Department of Microbiology and Immunobiology, 1030 Vienna, Austria and [2]The Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, S-90187 Umeå, Sweden

Email: Maria Siller - maria.siller@univie.ac.at; Rajendra P Janapatla - rajanjan@yahoo.com; Zaid A Pirzada - zaid.ahmed.pirzada@univie.ac.at; Christine Hassler - hasslerc@gmx.de; Daniela Zinkl - Daniela.Zinkl@gmx.at; Emmanuelle Charpentier* - emmanuelle.charpentier@univie.ac.at

* Corresponding author †Equal contributors

Published: 30 October 2008

BMC Microbiology 2008, **8**:188 doi:10.1186/1471-2180-8-188

Received: 14 May 2008
Accepted: 30 October 2008

This article is available from: http://www.biomedcentral.com/1471-2180/8/188

© 2008 Siller et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: The *luxS*/AI-2 signaling pathway has been reported to interfere with important physiological and pathogenic functions in a variety of bacteria. In the present study, we investigated the functional role of the streptococcal *luxS*/AI-2 system in metabolism and diverse aspects of pathogenicity including the adaptation of the organism to stress conditions using two serotypes of *Streptococcus pyogenes*, M1 and M19.

Results: Exposing wild-type and isogenic *luxS*-deficient strains to sulfur-limited media suggested a limited role for *luxS* in streptococcal activated methyl cycle metabolism. Interestingly, loss of *luxS* led to an increased acid tolerance in both serotypes. Accordingly, *luxS* expression and AI-2 production were reduced at lower pH, thus linking the *luxS*/AI-2 system to stress adaptation in *S. pyogenes*. *luxS* expression and AI-2 production also decreased when cells were grown in RPMI medium supplemented with 10% serum, considered to be a host environment-mimicking medium. Furthermore, interaction analysis with epithelial cells and macrophages showed a clear advantage of the *luxS*-deficient mutants to be internalized and survive intracellularly in the host cells compared to the wild-type parents. In addition, our data revealed that *luxS* influences the expression of two virulence-associated factors, the *fasX* regulatory RNA and the virulence gene *sibA* (*psp*).

Conclusion: Here, we suggest that the group A streptococcal *luxS*/AI-2 system is not only involved in the regulation of virulence factor expression but in addition low level of *luxS* expression seems to provide an advantage for bacterial survival in conditions that can be encountered during infections.

Background

Bacterial cell density-dependent signaling, also termed "quorum sensing", is used by bacteria to collectively modulate gene expression in response to changes in the population density [1-6]. Although signaling can be achieved through a variety of regulatory mechanisms, all systems described to date involve the production, secretion and detection of extracellular low-molecular-weight signaling molecules called "autoinducers" [1,3,7,8]. Due to the specificity of their respective sensors, recognition of acylated

homoserine lactones (AHLs) (gram-negative bacteria) and peptide autoinducers (gram-positive bacteria) is restricted for communication within the same species. More recently, a novel quorum sensing system involving a furanone-like signaling molecule termed "autoinducer 2" (AI-2) was described to regulate bioluminescence in *Vibrio harveyi* [9,10]. AI-2 is synthesized by the *luxS* gene product, which has been identified in the genome of over 55 gram-negative and gram-positive bacterial species [1,11,12]. Since culture supernatants of several bacterial species had a complementary effect on *luxS*-deficient *V. harveyi*, AI-2 has been proposed to function as a "universal" signaling molecule for interspecies communication [1,9,10]. In addition to controlling bioluminescence, recent studies show that the *luxS*/AI-2 signaling is involved in the regulation of pathogenicity in several organisms [5,12].

Apart from its role in quorum sensing, the enzyme LuxS is tightly coupled to the *S*-adenosylmethionine (SAM) utilization pathway [11-13]. SAM is an essential donor of methyl groups for DNA, RNA and other methylation reactions. Its utilization by methyl transferases yields *S*-adenosylhomocysteine (SAH), which is toxic for the cells and is eliminated through hydrolysis by the nucleosidase Pfs to produce *S*-ribosylhomocysteine (SRH) and adenine. Finally, LuxS cleaves SRH into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD spontaneously forms the cyclic pro-AI-2 molecule, which in *V. harveyi* reacts with borate to form a stable cyclic furanosyl borate diester [1,11,13].

Knowledge about quorum-sensing systems in the gram-positive human pathogen *Streptococcus pyogenes* is rather limited. *S. pyogenes* (Group A Streptococcus, GAS) is responsible for a broad range of diseases including mild illnesses such as pharyngitis, impetigo and scarlet fever and more severe invasive infections such as necrotizing fasciitis, streptococcal toxic shock syndrome and post-infectious rheumatic fever [14,15]. In GAS, like in other bacteria, pathogenicity is multifactorial and requires the coordinated temporal regulation of virulence factor expression in response to changing environmental conditions and cell population density [14,16,17]. Studies have confirmed that expression of several GAS virulence genes is temporal and dependent on growth phase [16-20]. Furthermore, a number of specific regulators (e.g. two-component systems and response regulators) modulate virulence gene expression in a growth phase dependent manner [16,21-24]. Based on similarities with previously described peptide signaling regulons of *S. pneumoniae* (*com* system) and *S. aureus* (*agr* system), two putative quorum-sensing systems, *sil* and *fasBCA*, have been reported in GAS [21,25]. Although no signaling peptide could be identified within the *fasBCA* locus, the putative pheromone peptide SilCR from the *sil* operon was shown to regulate DNA uptake and the ability of GAS to cause invasive infection [21,25]. In addition, the observation of a *luxS* homologue in the GAS genome suggested that quorum sensing via the *luxS*/AI-2 signaling could have a relevant function in the pathogenesis of this organism [26,27]. Two recent studies report a role of *luxS*/AI-2 in the regulation of virulence gene expression in M3 and M6 serotypes [26,27]. However, the role of the *luxS*/AI-2 in the AMC-related metabolism and adaptation to stress conditions in GAS remains unknown.

Here, we were interested in investigating further the function of the *luxS*/AI-2 system in GAS serotypes M1 and M19. Expression of *luxS* in connection with production of AI-2 like activity was analyzed. The metabolic role of *luxS* in the activated methyl cycle (AMC) in GAS was examined. Wild-type and isogenic *luxS*-deficient strains were compared in regard to their adaptation to diverse growth and stress conditions as well as diverse aspects of pathogenicity including interaction with epithelial cells and macrophages. In summary, our data suggest an important function of the *luxS*/AI-2 system in survival and growth of GAS under conditions that are relevant during infections.

Results

Construction of luxS*-deficient mutants*

As reported previously, GAS possesses a *luxS* homolog (coding sequence: 483 bp), the predicted translational product of which shares 36% identity with the LuxS protein from *V. harveyi* [26]. Analysis of the *luxS* coding sequences in clinical isolates of two different serotypes, RDN29 (M1 type) and RDN02 (M19 type), revealed 100% amino acid sequence identity. In a previous study, a GAS strain of serotype M6 (JRS4) was shown to contain in its genome the insertion element, IS *1239*, inserted 111 bp upstream of the *luxS* translational start codon [26]. This insertion was assumed to have an influence on *luxS* expression [26]. Further analysis of the sequence up to 300 bp upstream of the *luxS* coding sequence in RDN29 and RDN02 did not reveal the presence of IS *1239* (data not shown). To analyze the function of the *luxS*/AI-2 system in GAS, we deleted the *luxS* gene in RDN29 and RDN02, thus creating EC478 and RDN306, respectively (Table 1).

Temporal luxS *expression and production of AI-2 like activity*

First, we investigated *luxS* expression at the transcriptional level. Northern blot analysis of RNA extracts from wild-type strains revealed a monocistronic *luxS*-specific transcript of approximately 650 bases in size (Fig. 1A). In both strains, the *luxS* transcript levels were abundant from lag to early-logarithmic phase, after which gradual decrease of the signal intensity towards stationary phase was observed

Table 1: Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
Streptococcus pyogenes		
RDN29	M1 type	ATCC 700294
RDN02	M19 type	Lab strain collection
EC478	Isogenic *luxS*-deficient mutant of RDN29	This study
RDN306	Isogenic *luxS*-deficient mutant of RDN02	This study
EC460	RDN29 (pEC82)	This study
EC489	EC478 (pEC82)	This study
EC490	EC478 (pEC83)	This study
EC480	RDN02 (pEC82)	This study
EC481	RDN306 (pEC82)	This study
EC482	RDN306 (pEC83)	This study
Escherichia coli		
DH5α	Host for cloning; AI-2 deficient (contains a frame-shift mutation in *luxS*)	Lab strain collection
TOP10	Host for cloning	Invitrogen
EC467	DH5α (pEC82)	This study
EC464	DH5α (pEC83)	This study
Vibrio harveyi		
BB170	AI-1 sensor deficient, AI-2 sensor positive	[9]
Plasmids		
pAT21	Ap^r Km^r; pBR322Ω 1.5-kb pJH1 *Cla*I (*aphIII*)	[61]
pUC19	ColE1ori, Amp^r, *lacZ*	New England Biolabs
pEC131	pUC19Ω*luxS*up-*aphIII*-*luxS*down	This study
pEC82	*repDEG*-pAMβ1, *ermAM*/*B*, ColE1ori	This study
pEC83	pEC82ΩP*luxS*-*luxS*-TT	This study

(Fig. 1A). Primer extension analysis further identified the transcription initiation site (+1) as a guanine located 20 nucleotides upstream of the translation start codon (ATG) (Fig. 1B). Then, we investigated whether the growth phase-dependent expression of *luxS* transcripts correlated with production of AI-2 like activity. Analysis of conditioned media (CM) from the *luxS*-deficient mutants demonstrated a loss of AI-2 like activity production in a bioluminescence reporter assay described earlier [9]. In contrast, CM from the wild-type parental strains induced luminescence in the AI-1 sensor defective reporter *V. harveyi* strain BB170 with AI-2 like activity peaking at late-logarithmic phase (Fig. 2A, B). CM prepared from the complemented *luxS*-deficient strains restored luminescence production in the reporter *V. harveyi* strain to a level comparable to that of wild-type CM (Fig. 2A). Furthermore, we showed that the *S. pyogenes luxS* gene complemented successfully the lack of AI-2 production in *E. coli* DH5-α (data not shown), thus confirming that the *luxS* homologue in both M1 and M19 strains is required for production of AI-2 like activity. We then addressed the question whether CM with AI-2 like activity has an autoregulatory effect on *luxS* expression in *S. pyogenes*. CM from wild-type and *luxS*-deficient cultures grown to different phases was added to early-logarithmic wild-type cultures. No significant differences in the intensity of the *luxS* transcript signal were observed by Northern blot analysis when comparing induction with CM from both wild-type and mutant strains and control media (data not shown). Taken together, our data demonstrate that *luxS* transcription and production of AI-2 like activity are shifted out of phase. AI-2 like activity peaked at a time point – late-logarithmic phase – when *luxS* transcript levels rapidly decreased. Importantly, an auto-regulatory function of AI-2 like activity containing CM on *luxS* expression could not be observed in GAS.

Role of luxS *in growth and metabolism*

Growth rates and yields, colony and chain morphology of wild-type and *luxS*-deficient strains in complex THY medium, THY medium supplemented with 10% fetal bovine serum, C-medium and CDM were similar (data not shown). In addition, wild-type and *luxS*-deficient strains did not significantly differ in their ability to form primary adhesion to uncoated polystyrene surfaces (initial step in biofilm formation) when grown in THY or CDM (data not shown). To investigate whether inactivation of *luxS* and therefore disruption of the AMC would lead to a metabolic burden that might influence growth or fitness, we analyzed the growth rates of wild-type and *luxS*-deficient strains in sulfur-limited CDM (CDM-S1 and CDM-S2). Although overall growth rates and yields were significantly reduced in the restricted media compared to those in CDM, they were similar when wild-type strains

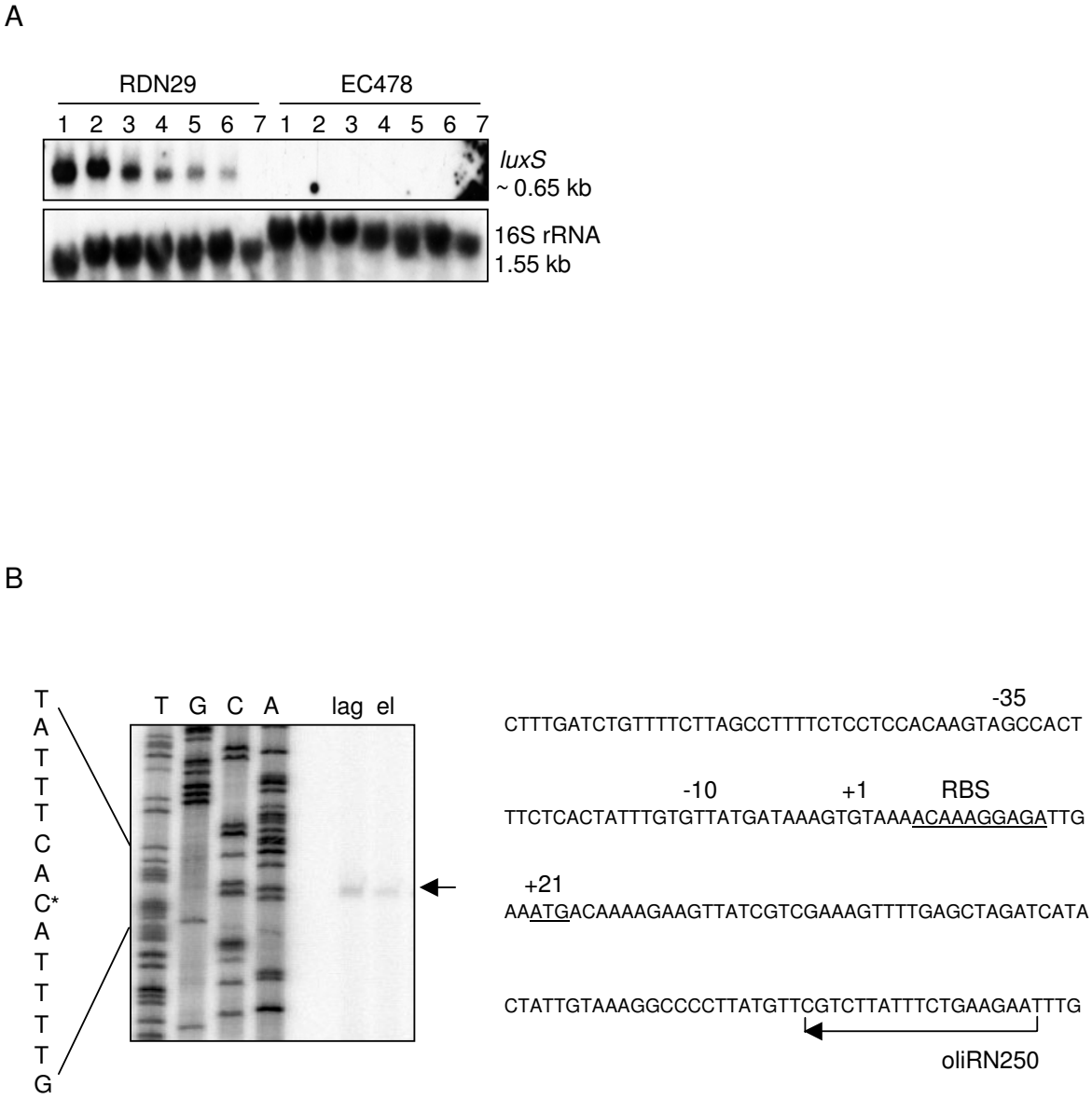


Figure 1

Analysis of the *luxS* transcript in *S. pyogenes*. (A) Temporal analysis of *luxS* expression. Northern blot analysis of total RNA isolated from RDN29 (wild-type M1 strain) and EC478 (isogenic *luxS*-deficient mutant) cultures at (1) lag, (2) early-logarithmic, (3) mid-logarithmic, (4) mid-late-logarithmic, (5) late-logarithmic, (6) early-stationary and (7) stationary (O/N) phase of growth. Blots were hybridized with probes specific to *luxS* and rRNA16S (acting as loading control). The estimated sizes of transcripts are indicated. The blots shown are representative of four independent experiments. Identical *luxS* expression profiles were observed in RDN02 (wild-type M19 strain) and RDN306 (isogenic *luxS*-deficient mutant). (B) Determination of the transcriptional start site of *luxS*. Left: Primer extension analysis using total RNA isolated from RDN29 wild-type cultures at lag (lag) and early-logarithmic (el) phase and the γ-^{32}P-radiolabelled reverse primer oliRN250. As a reference ladder, a DNA sequencing reaction using plasmid pEC83 as template and reverse primer oliRN250 is shown (lanes TGCA). The complementary base (C*) of the transcriptional start site (G), located 20 nucleotides upstream of the translational start codon (ATG), is indicated. The picture shown is representative of three independent experiments. Right: Sequence of the 5' part of the *luxS* locus. The putative -35 and -10 promoter regions and +1 transcriptional start site are represented in bold. The putative ribosomal binding site (RBS) and translational start codon (ATG) are underlined. The arrow indicates the direction of primer oliRN250, which was used for primer extension. An inverted repeat sequence located 97 bp downstream of the translation stop codon could serve as a rho-independent transcriptional terminator (not shown).

were compared to the *luxS*-deficient mutants (data not shown). These results indicate that *luxS* does not have an essential AMC-related metabolic role in GAS.

Role of *luxS* in adaptation to host-induced stress conditions. Further, we investigated whether *luxS* is involved in the adaptation of *S. pyogenes* to stress conditions that can be encountered during infection. Oxidative, acid and salt stresses, which have been shown to interfere with virulence factor expression in GAS [28-30], were studied. Challenging wild-type and *luxS*-deficient strains with hydrogen peroxide (4 mM) and high salt concentrations (150 and 650 mM NaCl) did not induce any significant change in survival rate (data not shown). However, exposure of bacterial cultures to acidic conditions (pH 4) for 2, 4 and 6 h revealed a profound increase in acid tolerance in the *luxS*-deficient strains (Fig. 3A). Complementing the *luxS*-deficient strains with *luxS in trans* resulted in restoration of acid sensitivity to a wild-type level (Fig. 3A, shown only for the 6 h time point), clearly linking *luxS* to the observed phenotype. Importantly, we could show that exposing *S. pyogenes* cultures to low pH led to reduced expression of *luxS* (Fig. 3B), thus suggesting a correlation between reduced *luxS* expression and survival under acidic conditions. Accordingly, CM from these cultures showed reduced AI-2 like activity (Fig. 3C).

Then, we analyzed the role of the *luxS*/AI-2 system in adaptation to an environment that resembles *in vivo* conditions by using RPMI medium (used for culturing eukaryotic cells) and 10% serum. We observed reduced *luxS* expression at the transcriptional level in wild-type cultures grown in RPMI medium compared to cultures grown in the nutrient rich THY medium (Fig. 4A). Furthermore, AI-2 activity in CM from wild-type cultures grown in RPMI was reduced to a level similar to CM from *luxS*-deficient cultures (Fig. 4B). During growth in RPMI, due to the presence of buffer salts and cultivation in the presence of CO_2, no acidification occurs. Therefore, under this growth condition, regulation of *luxS* expression cannot be attributed to low pH. Since low concentration of iron in the RPMI medium could be responsible for the observed phenotype, we supplemented RPMI medium with iron and depleted THY medium from iron. In both cases, no change in *luxS* expression could be observed (data not shown). Our data thus indicate that under specific conditions encountered in the host (acidic conditions and *in vivo*-like environment), there is an advantage for GAS to lower the expression of *luxS* and the production of AI-2 like activity.

Role of *luxS* in internalization and survival in human epithelial cells and macrophages. These results prompted us to determine whether a lack of *luxS* expression gives a beneficial advantage for GAS to survive in an intracellular environment. Interactions of wild-type and *luxS*-deficient strains of both serotypes M1 and M19 with monolayers of human pharyngeal epithelial cells were analyzed. No differences in the number of bacteria adhering to the epithelial cells (30 min, 1 h and 2 h after infection) were observed when comparing wild-type and mutant strains (data not shown). Using an antibiotic protection assay, the internalization and survival rates of the *luxS* mutant strains of both M1 and M19 serotypes were significantly higher (about 5-fold) at 2 h and 5 h after the addition of antibiotics compared to those of wild-type strains (Fig. 5A). Analyzing the number of bacteria that survived intracellularly in macrophages led to the same results. The *luxS* deficient mutants showed significantly higher internalization and survival rates (about 2.5-fold) in macrophages at 0 h, 1 h and 2 h after the addition of antibiotics compared to the wild-type parents (Fig. 5B). With both serotypes and in both epithelial and macrophage cells, complementing the mutant strains with the *luxS* allele *in trans* restored the intracellular survival rates to almost wild-type levels. Since the role of *luxS* in the AMC-related metabolic pathway seems limited, we analyzed the possibility of a signalling function of the *luxS*/AI-2 system in GAS survival in host cells. Antibiotic protection assays were performed with mixed cultures of wild-type and *luxS*-deficient cells (ratio 1:1) comparing with non-mixed wild-type and *luxS*-deficient cultures. The survival rates of the mixed culture in both eukaryotic cell types were almost identical to those of the wild-type strain (data not shown), thus suggesting a potential complementation effect of the *luxS*-deficient cells by the wild-type parent cells possibly by AI-2 production and signaling. In summary, the data indicate that lowering the expression of *luxS* provides GAS an advantage to survive the harsh conditions encountered in host cells.

Virulence gene expression in luxS *mutants*

We also studied whether *luxS* had regulatory effects on pathogenicity functions other than those already described (e.g. effect on cysteine protease, SLS and hyaluronic acid production) [26,27]. Expression of a number of genes encoding virulence factors and their regulators [16,21,28,31-36] was then studied by Northern blot analysis throughout the entire growth phase (Table 2). A consistent decrease in the *fasX* (encoding the effector molecule of the *fasBCA* regulatory system [21]) transcript and a consistent enhancement of the *sibA* (*psp*) (encoding a secreted immunoglobulin binding protein [32]) transcript were detected in the *luxS*-deficient RDN02 (M19) strain compared to the wild-type parent, however not in the M1 background (Fig. 6A, B). This finding does not only add *fasX* and *sibA* to the list of genes being influenced by *luxS* but underlines the fact that regulatory pathways linking the *luxS*/AI-2 pathway to virulence-associated

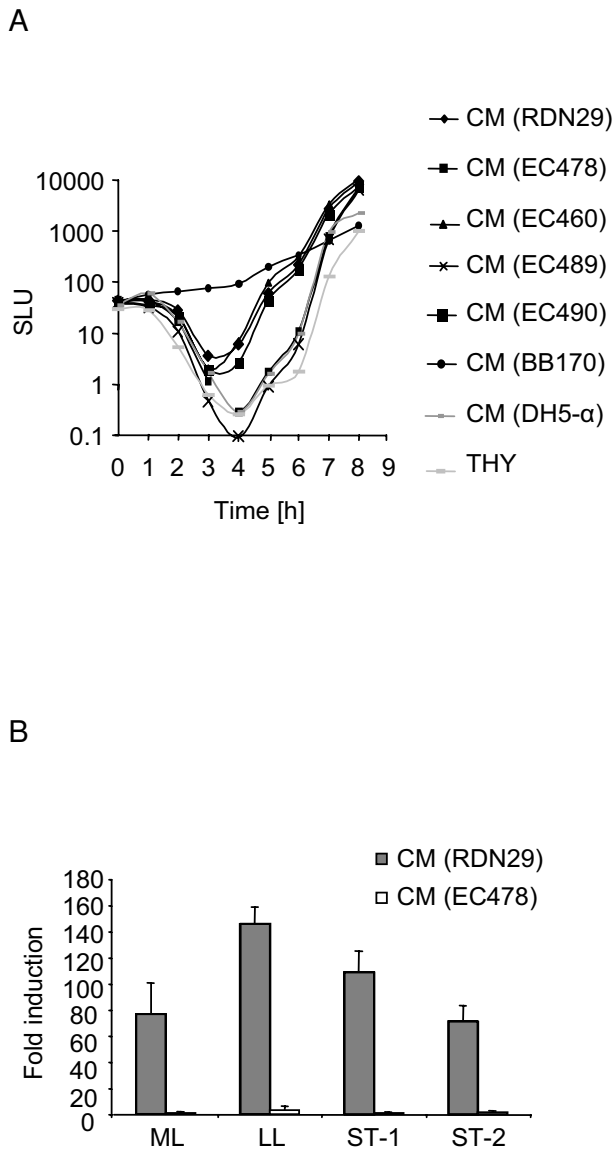


Figure 2

Induction of luminescence in *V. harveyi* by CM from *S. pyogenes*. (A) Induction of luminescence in *V. harveyi* reporter strain BB170 by CM of wild-type and *luxS*-deficient mutants. RDN29: wild-type M1 strain, EC460: RDN29 (pEC82), EC478: isogenic *luxS*-deficient mutant, EC489: EC478 (pEC82), EC490: EC478 complemented with pEC83. THY medium and CM of DH5-α (AI-2 deficient) were used as negative controls; CM of BB170 was used as positive control. CM were prepared from cultures at late-logarithmic phase of growth. The luminescence values expressed in specific light units (SLU = RLU (relative light units)/ $OD_{620\ nm}$) (y axis) are plotted versus time (h) (x axis). Data shown are representative of three independent experiments. Similar data were obtained with CM of the RDN02 (M19 serotype) derivative strains. (B) Fold induction of *V. harveyi* BB170 luminescence by CM of RDN29 and EC478. CM was prepared from cultures at mid-logarithmic (ML), late-logarithmic (LL), early-stationary (ST-1) and stationary phase (ST-2) of growth. Fold induction corresponds to the SLU values obtained with the CM of the analyzed strains versus those obtained with the CM of the negative control THY. Fold induction was calculated after ~4 h of induction when the cell density begins to rise. Induction of luminescence with late-logarithmic CM from the *luxS*-deficient mutant was ~60 fold lower compared to CM from the parent wild-type strain. The average values and standard deviations of three independent experiments are shown. Similar data were obtained with CM of the RDN02 derivative strains.

gene expression can differ among different GAS strains or serotypes.

Discussion

Among the quorum sensing systems described to date, the *luxS*/AI-2 pathway has been shown to be involved in the regulation of virulence in a number of gram-negative and gram-positive bacteria [1,5,11,12]. In this study, we analyzed the expression of the streptococcal *luxS*/AI-2 system, its possible role in the AMC metabolic pathway and its function in adaptation to diverse host-induced stress conditions in two GAS clinical isolates of serotypes M1 and M19.

Expression analysis of *luxS* indicated that the *luxS* transcript is monocistronic (644 bases) in GAS serotypes M1 and M19. In a previous report, which was based on sequence analysis, the *luxS* transcript in GAS (M6 strain) was predicted to be polycistronic as a distal member of the fatty acid metabolism operon [26]. In our study, Northern blot analysis of *luxS* expression did not reveal any additional transcript of higher size. Furthermore, we showed that the monocistronic transcript is expressed in a growth phase-dependent manner, a finding already reported for bacterial species like *Streptococcus bovis*, whereas transcription of *luxS* in species like *Salmonella typhimurium* and *Vibrio fischeri* has been shown to be constitutive [37-39]. Accordingly, production of AI-2 like activity occurred in a temporal fashion in the serotypes analyzed, however, shifted out of phase compared to *luxS* expression. AI-2 like activity peaked at late-logarithmic phase when the *luxS* transcript had already declined being just above the detectable expression level. We also investigated the possibility of an auto-regulation mechanism of *luxS* expression in GAS. Induction experiments by Northern blot analysis failed to show a regulation of *luxS* expression by AI-2 dependent CM. These findings are in accordance with previous work in *S. typhimurium*, where AI-2 production and *luxS* transcription were not in phase either, also suggesting that *luxS* expression was not regulated by AI-2 [38].

Despite intensive research on *luxS* and AI-2 during the last years, a clear separation of the possible metabolic function of AI-2 from its possible signaling activity could not be achieved. A recent comparative genomic and phylogenetic analysis of synthesis and signal transduction pathways showed that *S. pyogenes* contains the two-step enzymatic pathway catalyzed by the Pfs and LuxS enzymes to detoxify SAH leading to AI-2 production [13,40]. Interestingly, in *S. pyogenes* the AMC is incomplete as it lacks the homocysteine methyltransferases (MetE or MetH) that convert the SAH-derived metabolic product homocysteine to methionine. Nevertheless, *S. pyogenes* encodes the SAM synthetase ortholog (MetK) that permits conversion of methionine to SAM [40]. Therefore, either GAS cannot recycle homocysteine to methionine (as it has been described in *Borrelia burgdorferi* [41]) or recycling occurs through other types of methyltransferases. In the former case of a *de novo* methionine synthesis defect, bacterial growth would exclusively depend on sulfur provided from external sources. In our study, decreasing media concentrations of cysteine and methionine reduced to a similar extent bacterial growth and yields in both wild-type and *luxS*-deficient strains. These data indicate that GAS, as a poly-auxotrophic organism, is unable to recycle homocysteine to methionine. Moreover, we showed that inactivation of *luxS* does not lead to a metabolic burden that would influence growth or fitness. Although AI-2 can certainly be considered as a metabolite, the viability and lack of growth defects of the investigated *luxS*-deficient mutants in various media including minimal media depleted of sulfur sources argues against an essential role of *luxS* in AMC-related metabolism.

The previously described regulatory role of *luxS* in the production of virulence factors in GAS [26,27] prompted us to investigate the role of the *luxS*/AI-2 system in the adaptation to host-induced stress conditions. During an ongoing infection, bacteria often have to face challenging conditions in particular niches of the host, including changes in pH, and therefore are forced to develop quickly an adaptive response, which requires fine-tuning of pathogenicity gene expression. Although we could not identify a possible involvement of the GAS *luxS* system in adaptation to oxidative and salt stress, we showed an increased survival of *S. pyogenes* under acidic conditions when the *luxS*/AI-2 system was down-regulated. This is in accordance with *luxS* expression and AI-2 production being significantly lowered when GAS cells were grown in low pH conditions. Interestingly, previous studies in *S. mutans* showed that acid sensitivity was enhanced in *luxS* deficient mutants [42,43] and that *luxS* expression was increased at low pH [44,45]. These reverse effects of *luxS* on tolerance to acidic conditions in *S. pyogenes* and *S. mutans* need to be considered in regard to their different living habitats and pathogenesis. Thus, our data reveal a link between the *S. pyogenes luxS*/AI-2 system and pathways involved in adaptation of the organism to stress [46,47]. In addition, we also observed a reduction of *luxS* expression and AI-2 like activity in GAS cells grown in serum enriched RPMI, a medium with a composition similar to that of human plasma. In a previous study, Marouni et al. reported that in an M6 serotype, the survival of a *luxS*-deficient mutant in epithelial cells at 4 h after infection was higher compared to the wild-type parent [27]. Here we analyzed further the role of *luxS* in interaction of *S. pyogenes* with host cells. No differences in adhesion rates to human pharyngeal epithelial cells were observed when comparing wild-type and *luxS*-deficient

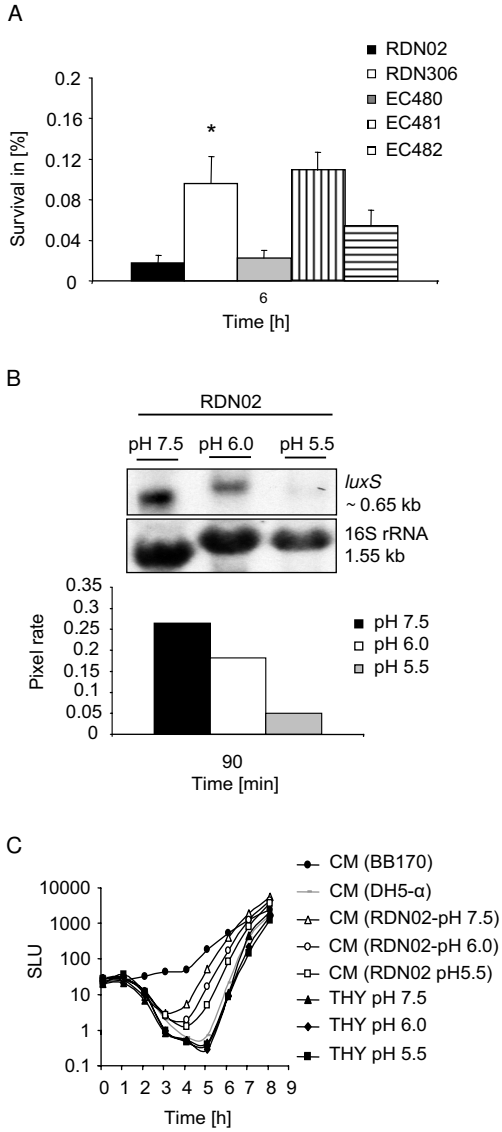


Figure 3

(A) Acid tolerance assay. The ability of RDN02 (wild-type M19 strain), RDN306 (isogenic *luxS*-deficient mutant), EC480 (RDN02 (pEC82)), EC481 (RDN306 (pEC82)) and EC482 (RDN306 (pEC83)) to survive an acid challenge (pH 4) is shown as survival in percent (y axis) at the 6 h time point. The average % survival values and standard deviations of three independent experiments are shown. The asterisk indicates that the difference in survival rates between the *luxS*-deficient mutant and the wild-type strain was statistically significant ($P < 0.05$). Similar data were obtained with the RDN29 (M1 type) derivative strains. (B) Effect of low pH on *luxS* expression. Northern blot analysis was performed with total RNA isolated from RDN02 cultures grown to early-logarithmic phase and then incubated for 90 min with THY medium at pH 5.5, 6.0 and 7.5. Blots were hybridized with probes specific to *luxS* and rRNA16S (acting as loading control). The estimated sizes of transcripts are indicated. Pixel rates of signals (pixel counts obtained with the *luxS* probe versus pixel counts obtained with the rRNA16S probe) are shown. The blot shown is representative of three independent experiments. Similar data were obtained with the RDN29 strain. (C) Induction of luminescence in *V. harveyi* reporter strain BB170 by CM of RDN02. CM was prepared from RDN02 cultures grown to early-logarithmic phase and then incubated for 90 min with THY medium at pH 5.5 (RDN02-pH 5.5), 6.0 (RDN02-pH 6.0) and 7.5 (RDN02-pH 7.5) for 90 min. THY medium at pH 7.5, 6.0 and 5.5 and CM of DH5-α (AI-2 deficient) were used as negative controls; CM of BB170 was used as positive control. The luminescence values expressed in specific light units (SLU = RLU (relative light units)/$OD_{620\ nm}$) (y axis) are plotted versus time (h) (x axis). Data shown are representative of three independent experiments. Similar data were obtained with CM of the RDN29 derivative strains.

mutants in both M1 and M19 serotypes. However, the *luxS*-deficient mutants had a significant advantage to survive intracellularly in both epithelial cells (over a period of 7 h after infection) and macrophages (over a period of 3 h after infection). Taken together, we show that low level of *luxS* and AI-2 expression seems to provide a competitive advantage for GAS survival under specific conditions encountered during infection.

With *luxS* being linked to stress and adaptation to the host, we were interested in investigating additional effects of *luxS* on virulence factor expression in GAS. In an M6 serotype, *luxS* was shown to regulate streptolysin S (SLS) expression at the transcriptional level and SpeB cysteine protease activity [26]. In an M3 serotype, *luxS* was reported to regulate expression of SpeB and M protein at the transcriptional level and hyaluronic acid capsule at the post-transcriptional level [27]. In our report, we show that *luxS* had a positive effect on *fasX* expression and a negative effect on *sibA* (*psp*) expression at the transcriptional level. *fasX* is a small RNA molecule, effector of the *fasBCA* operon, which has regulatory functions on virulence factor expression. *fasX* was also suggested to be involved in local tissue destruction and general bacterial aggressiveness towards host cells [21,48]. *sibA* (*psp*) is a virulence gene encoding a secreted immunoglobulin binding protein [32]. Remarkably, the above described regulatory effects were only observed in the M19 strain, thus suggesting a strain or serotype dependent effect of *luxS* on the expression of these two targets. The strain- or serotype-dependent effect of *luxS* observed in this study emphasizes differences in regulatory pathways among different GAS isolates. Along these lines, previous reports demonstrated that mutations in GAS regulators might alter disease progression [49,50]. This is well illustrated with the two-component regulatory system CovRS where mutations in the sensor encoding gene *covS* have been shown to correlate with human disease severity [51,52]. Mutations in this gene can occur under selective pressure encountered in the host and can generate hypervirulent GAS variants with increased risk of systemic dissemination [51,52]. In the case of *luxS*, strain specificity manifestation of virulence-associated gene regulation by *luxS* has been reported previously in *Neisseria meningitidis* and *Serratia marcescens* [53,54]. Additionally, Marouni et al. attributed the dissimilar results of the effect of *luxS* on bacterial growth and the level of SpeB regulation in GAS to a possible strain-specific effect [27]. The finding that *luxS* can affect the expression of *fasX* RNA also provides an additional evidence for the notion that small RNAs have the ability to integrate cell density signals together with other environmental stimuli to affect gene expression [55].

Conclusion

Our data together with previous reports suggest a complex role of the *luxS*/AI-2 system in *S. pyogenes*. Here, we showed that expression of both *luxS* and AI-2 occurs in a temporal fashion but AI-2 like activity does not seem to have a regulatory effect on *luxS* expression. Analysis of the role of *luxS* in metabolism demonstrated a limited role of *luxS* in the AMC-related metabolism. However, studying the possible function of the *luxS*/AI-2 system in adaptation to stress revealed that a down-regulation of *luxS* expression provides an advantage for *S. pyogenes* to tolerate acidic conditions and grow in a host environment-mimicking medium. Accordingly, there was an increased ability of the *luxS*-deficient mutants to survive intracellularly in both epithelial cells and macrophages. Altogether, our data suggest an important function for the *luxS*/AI-2 system in survival and growth of GAS under conditions that are relevant during infections. Based on the data outlined in this article, it is tempting to speculate that in GAS, *luxS* is not exclusively a key part of a detoxification pathway but rather modulation of *luxS* expression levels would allow adjusting bacterial fitness in response to changing host conditions. Finally, our study revealed two novel virulence-associated targets of *luxS* in *S. pyogenes*: the regulator *fasX* RNA and the virulence gene *sibA*.

Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. pyogenes* was routinely cultured in Todd Hewitt Broth (THB, Bacto, Becton Dickinson) supplemented with 0.2% yeast extract (Oxoid) (THY) without agitation or on trypticase soy agar supplemented with 3% sheep blood (TSA, BBL, Becton Dickinson). C-medium (peptide rich and carbohydrate poor), THY or RPMI medium supplemented with 10% foetal bovine serum (Gibco), metal ion-restricted medium, chemically defined medium (CDM) and sulfur-restricted CDM (CDM-S) were used in specific experiments [56,57]. For the eukaryotic-like environment, RPMI 1640 (PAA) was supplemented with 10% foetal bovine serum (Gibco) or 10% foetal bovine serum and 50 μM $FeCl_3$. For metal-ion restricted medium, THY was treated overnight with 30 g of chelating resin Chelex 100 (Sigma)/liter, supplemented with 1 mM $MgCl_2$ or 1 mM $MgCl_2$and 1 mM $FeCl_3$. Sulfur-restricted CDM (CDM-S) consisted of CDM with sulfur containing salts replaced with their chloride equivalents [58]. CDM contained L-methionine (670 μM), L-cysteine (4,125 μM) and L-cystine (208 μM). In sulfur-limiting conditions, L-cystine was eliminated and only L-cysteine (10 μM or 350 μM) and L-methionine (1 μM or 100 μM) were provided as sulfur source. All *S. pyogenes* cultures were incubated at 37°C in an atmosphere supplemented with 5% CO_2. *Escherichia coli* was grown aerobically at 37°C in Luria-Bertani (LB) medium either in liquid with

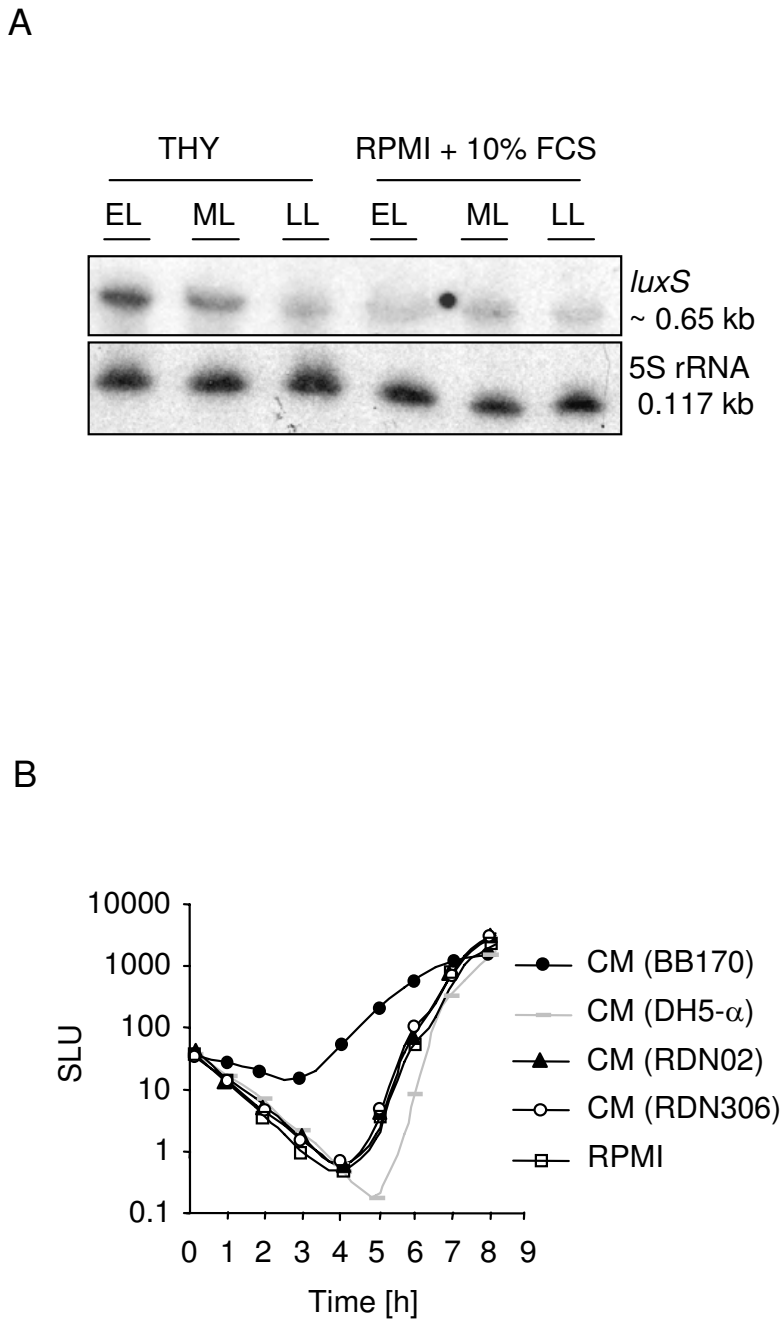


Figure 4

(A) Analysis of *luxS* expression in RPMI medium. Northern blot analysis of total RNA isolated from RDN02 (wild-type M19 strain) cultures at early-logarithmic (EL), mid-logarithmic (ML) and late-logarithmic (LL) phase of growth. Blots were hybridized with probes specific to *luxS* with 5S rRNA as loading control. The estimated sizes of transcripts are indicated. The blots shown are representative of three independent experiments. Identical *luxS* expression profiles were observed with RDN29 (wild-type M1 strain). (B) Induction of luminescence in *V. harveyi* reporter strain BB170 by CM of *S. pyogenes*. RDN02: M19 wild-type, RDN306: isogenic *luxS*-deficient mutant. RPMI medium and CM of DH5-α (AI-2 deficient) were used as negative controls; CM of BB170 was used as positive control. CM was prepared from cultures at late-logarithmic phase of growth in RPMI medium. The luminescence values expressed in specific light units (SLU = RLU (relative light units)/$OD_{620\ nm}$) (y axis) are plotted versus time (h) (x axis). Data shown are representative of three independent experiments. Similar data were obtained with CM of derivative strains of the RDN29 background.

shaking or on agar plates. *V. harveyi* was cultured aerobically at 28°C in an Autoinducer Bioassay (AB) medium [9]. Transformation of *E. coli* and *S. pyogenes* was performed as previously described [59,60]. Whenever required, suitable antibiotics were added to the medium to the following final concentrations: erythromycin 300 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*; kanamycin 25 μg/ml for *E. coli* and 300 μg/ml for *S. pyogenes*. Bacterial cell growth was monitored periodically by measuring the optical density of culture aliquots at 620 nm using a microplate reader (SLT Spectra Reader).

DNA manipulations

DNA manipulations including DNA preparation, amplification, digestion, ligation, purification, agarose gel electrophoresis and Southern blot analysis were performed according to standard techniques [60]. Synthetic oligonucleotides used as primers in this study (Table 2) were supplied by VBC-Biotech Services GmbH. Sequencing reactions were performed at VBC-Biotech Services GmbH.

Construction of the luxS*-deficient mutants*

Replacement of the *luxS* coding sequence with a kanamycin resistance cassette, *aphIII* [61], was performed selecting for a double cross-over event. For this purpose, a 1209 bp fragment upstream of *luxS* (*luxS*-up) and a 1107 bp fragment downstream of *luxS* (*luxS*-down) were amplified using wild-type genomic DNA as template and primers (containing flanking restriction sites) oliRN22/oliRN21 and oliRN20/oliRN19, respectively. The *aphIII* cassette was amplified using plasmid pAT21 as template and primers oliRN23/oliRN24. After digestion with the respective restriction enzymes, the three fragments were ligated and cloned into pUC19 (suicide vector for *S. pyogenes*). The resulting plasmid pEC131 was purified, linearized with the restriction enzyme *Sca*I (which cuts within the ampicillin resistance cassette) and used to transform electro-competent wild-type *S. pyogenes*. Kanamycin resistant clones were selected and the correct replacement event was checked by PCR analysis using combinations of primers oliRN267 and oliRN268 derived from flanking regions upstream and downstream of the *luxS*-up and *luxS*-down fragments and primers oliRN69 and oliRN70 specific to the *aphIII* cassette. Southern blot analysis was done to further confirm that the recombination events had not affected the DNA regions located upstream and downstream of *luxS*. The *S. pyogenes* mutants were grown in liquid medium without antibiotic unless otherwise specified.

Construction of plasmids for complementation studies

Plasmid pEC82 contains *repDEG*-pAMβ1 (the origin of replication of pAMβ1), *ermAM* (an erythromycin resistance gene with its own promoter and transcriptional terminator), ColE1 (a pUC19-based ColE1 origin of replication for *E. coli*) and an expanded MCS (multiple cloning site). To create the pEC83 *luxS*-complementation vector, a 1152 bp large DNA fragment (P*luxS*-*luxS*-TT) containing the *luxS* coding sequence, its putative promoter region and putative transcriptional terminator, was amplified from wild-type genomic DNA using primers (containing flanking restriction sites) oliRN205/oliRN280 and cloned into pEC82. Plasmids pEC82 and pEC83 were introduced in competent *E. coli* and *S. pyogenes* strains selecting for erythromycin resistant clones.

RNA analysis

Total RNA was prepared from culture samples harvested at different time points during growth and further processed for normalization and Northern blot analysis as previously described [62] with minor modifications. Specific α-^{32}P-dATP labeled DNA probes corresponding to internal fragments of genes were created by amplification of wild-type genomic DNA using primers described in Table 2. Primer extension analysis of the *luxS* transcript and DNA sequencing reactions were carried out according to standard protocols [60].

Phenotypical studies

Assays for bioluminescence, acid killing, hydrogen peroxide killing, salt stress and biofilm formation were performed as described previously [9,28,29,43,63] with some minor modifications. For the acid tolerance assay, mid-logarithmic phase bacterial cultures were harvested by centrifugation and washed once with 0.1 M glycine buffer (pH 7.0). Culture aliquots were removed for CFU determination and the remaining cultures were subjected to killing by incubating the cells in 0.1 M glycine buffer (pH 4) for 6 h. The number of viable bacteria was determined by plating appropriate dilutions in triplicate on TSA media. For each individual experiment, the ability of strains to survive the acid challenge was reported as survival in percent, defined by the ratio of average CFU/ml (triplicate measurements) recovered after challenge versus the average number of CFU/ml (triplicate measurements) present immediately before challenge × 100.

Preparation of conditioned medium for induction assays

Conditioned medium (CM) was prepared from bacterial cultures grown in THY buffered with 0.1 M HEPES (Sigma) (pH 7.5) to early-, mid- and late-logarithmic phase. Culture supernatants were separated from bacterial pellets by centrifugation at 9,500 × g for 30 min at 4°C, filter sterilized (0.45 μm) and stored at -20°C. For induction assays, early-logarithmic wild-type cultures were induced for 60 and 90 min with THY adjusted to pH 7.5, 6.0 and 5.0 or with CM of wild-type and *luxS*-deficient cultures from early-, mid- and late-logarithmic growth phase and then subjected to Northern blot analysis.

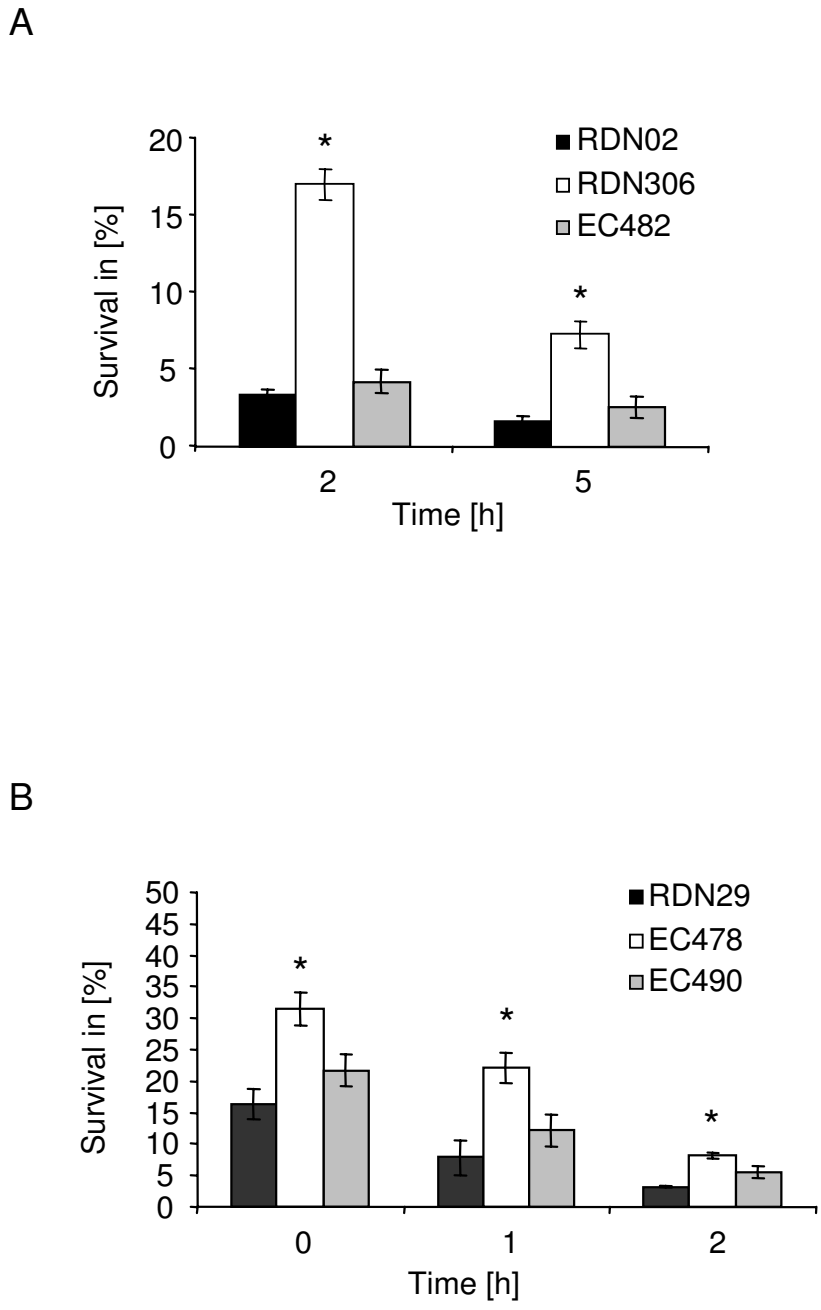


Figure 5

Internalization assays of *S. pyogenes* in epithelial cells and macrophages. RDN29 (wild-type strain), EC478 (isogenic *luxS*-deficient mutant) and EC490 (RDN29 (pEC85)); RDN02 (wild-type strain), RDN306 (isogenic *luxS*-deficient mutant) and EC482 (RDN29 (pEC85)). Bacterial cultures were used to infect (A) HEp-2 human pharyngeal epithelial cells and (B) RAW 264.7 macrophages in an antibiotic protection assay. For this, bacterial cultures were grown to mid-logarithmic (RAW 264.7) and late-logarithmic (HEp-2) phases, growth time points showing optimal internalization rates in RAW 264.7 and HEp-2, respectively. The percent intracellular invasion represents the average CFU/ml number of viable intracellular bacteria recovered at different times following addition of antibiotics versus the average CFU/ml number of inoculated bacteria × 100. The average values and standard deviations of three independent assays are shown. The asterisks indicate that the differences in survival rates between the *luxS*-deficient mutants and the wild-type strains were statistically significant ($P < 0.01$ for Hep-2 epithelial cells, $P < 0.05$ for RAW 264.7 macrophages). Similar data were obtained for both M1 and M19 serotypes.

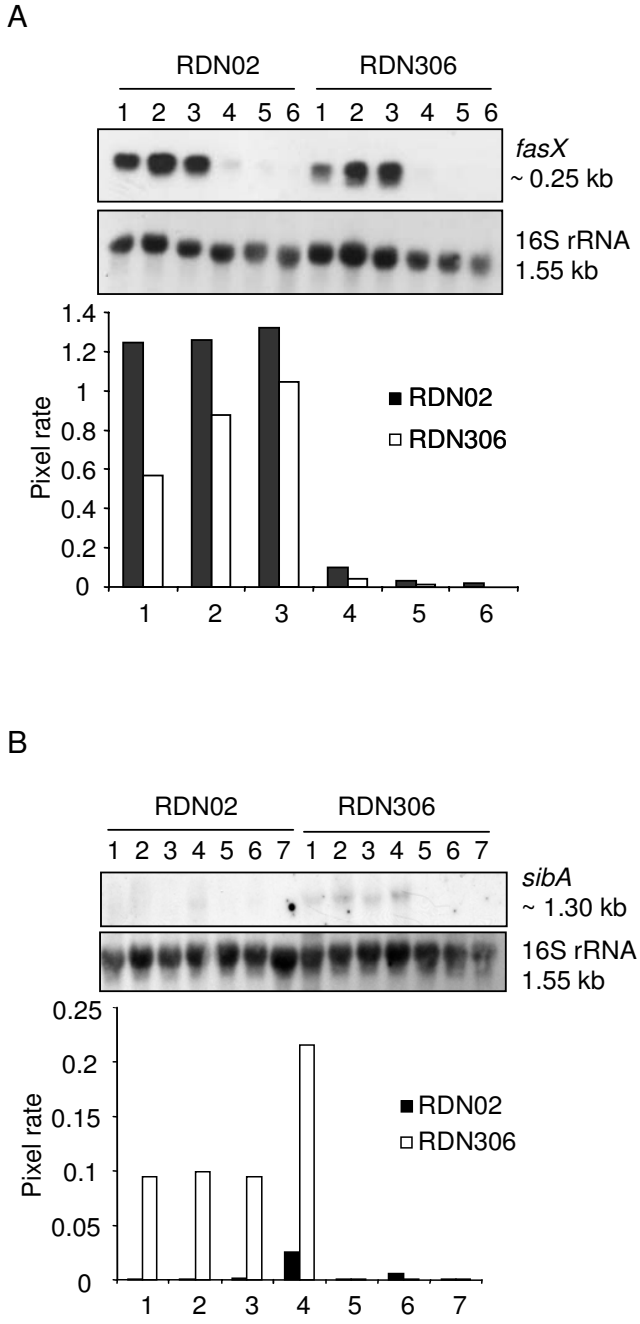


Figure 6

Transcriptional expression of virulence-associated genes in RDN02 (wild-type M19 strain) and RDN306 (isogenic *luxS*-deficient mutant). Northern blot analysis was performed with total RNA isolated from strains grown to (1) lag, (2) early-logarithmic, (3) mid-logarithmic, (4) mid-late-logarithmic, (5) late-logarithmic, (6) early-stationary and (7) stationary phase (O/N). Blots were hybridized with probes specific to *fasX* (A) and *sibA* (*psp*) (B) with a probe specific to 16S rRNA as loading control. The estimated sizes of transcripts are indicated. Pixel rates of signals (pixel counts obtained with probe of interest versus pixel counts obtained with the 16S rRNA probe) are shown for the different time points during growth. The blots shown are representative of three independent experiments.

Table 2: Oligonucleotides used in this study

Oligo	[a]F/R	Sequence 5'-3'	[b]Specificity
oliRN110	F	CGCTGCGTAAAAGATACG	*aphIII*
oliRN111	R	AATGGAGTGTCTTCTTCC	
oliRN130	F	TTGAAATGACAAAAGAAGTT	*luxS*
oliRN129	R	AAAGAGGCTATGATCCTTAT	
OLEC287OLEC288	F	AGTTAAGTGACGATAGCCTAG	5S rRNA
	R	CTAAGCGACTACCTTATCTCA	
oliRN242	F	CGGTAACTAACCAGAAAGGG	16S rRNA
oliRN243	R	CGTTGTACCAACCATTGTAGC	
			Virulence genes
OLEC137	F	GACCTACTCAGGCAAATC	*fbp54*
OLEC138	R	CTTGTCTAAGGCAATCTC	
oliRN204oliRN203	F	TGGTAAAAATGCCTATACCC	*isp2*
	R	GACCTCGTGAAAACTTAGCC	
oliRN248	F	AGGAATAAATTGGTCCTCTT	*nga*
oliRN249	R	GGCAGTTTCAATAAACTCAT	
OLEC268	F	AAGAGATCGAAGAAAGTCTT	*scl*
OLEC269	R	TTTGGTTAGCTTCTTTGTCA	
oliRN202oliRN201	F	AGTTAAGGCACAAGAACAGG	*sibA* (*psp*)
oliRN246	R	TACTTGAGGCACTTTGAACC	
oliRN247	F	ACTAGGAGCTACACAACCAG	*sic*
oliRN263	R	TACCCTGTACCTAATGCTTC	
oliRN264	F	ACATCTCAAGAATTACTAGC	*ska*
oliRN279	R	TTGTTGTAGAGTAGTTTAGC	
oliRN50	F	ATGGAAAATATTCTGATATCTTAG	*slo*
oliRN47	R	CCCAAAGGATTTCATATTGAGC	
	F	CATAATTACAGTCACTGATT	*speC*
oliRN48	R	ATCGAAATGACTAAAGTTCTTCAT	
oliRN55	F	TTTTCAATGGTAGCTCTTG	*speF (mf)*
oliRN56	R	TTCTTGAGCTCTTTGTTCG	
			Stand-alone regulators
OLEC274	F	ATAGCCTAGAAACAGAATTA	*codY*
OLEC275	R	TTCTGAATAAGAAAGCGTAT	
oliRN170	F	TAAGTAAGTTGTTTACAAGTCAAC AGTGGAG	*mga*
oliRN136	R	AAAGGCGTAGATCAATTGG	
OLEC337	F	ATGACGCCTAATAAAGAAGA	*mtsR*
OLEC338	R	CTGTGACATAAAGTTGCTTA	
OLEC315	F	TGGACATTCATTCACATCAG	*perR* (*spf*)
OLEC316	R	ATCAGGTTGGTCTTTTGCTT	
oliRN73	F	AAATACTTGGAATCATCAATCG	*rofA*
oliRN74	R	TTTTCTTGAGCTAATGCAACCG	
OLEC278	F	TAGACAACTTGAATGTCAAT	*ropB*
OLEC279	R	AAGCTTTATCATACTCTTGT	
OLEC276	F	ACATTTTGCAACGGTATATT	*srv*
OLEC277	R	AATAGGGTCATTAAGTCATA	
			Two-component systems
oliRN175	F	CTGCTTGACTTAATGTTACC	*covR* (CovRS)
oliRN176	R	TTTGACAATAATTCTTCACG	
OLEC182	F	GAGCAATAACATTTTAGG	*fasX* (effector molecule, FasBCA)
OLEC183	R	TTACAATCAGCTGATGTG	
OLEC270	F	ATTCGAAAGACATCAGATGT	*Irr* (Ihk/Irr)
OLEC271	R	CATATGTCATCCATCAATTG	

Table 2: Oligonucleotides used in this study *(Continued)*

OLEC339	F	GATATCATGATGCCGATTAA	*sptR* (SptRS)
OLEC340	R	TAGCTAGTATCTTTATCACC	
OLEC280	F	TACTTATTGTGGATGATGAA	*vicR* (VicRK)
OLEC281	R	AATCATATCCCCAAACAATT	
oliRN23	F	AATTGTGAATTCTATCATAATTGTGG (*Eco*RI)	Cassette *aphIII* for *luxS*- deficient mutants
oliRN24	R	TAAATCAAGCTTCTAAAACAATTCATCCAG (*Hin*dIII)	
oliRN20	F	ACTAGTGAATTCTCAAACATAACAATCC (*Eco*RI)	Fragment *luxS*down for *luxS*- deficient mutants
oliRN19	R	TTCTGAGGATCCCCACCATCCAGCC (*Bam*HI)	
oliRN22	F	AATCAAGCATGCTTACTTGGAAAAGAACCCAACC (*Sph*I)	Fragment *luxS*up for *luxS*- deficient mutants
oliRN21	R	AAGTGGAAGCTTGTGGAGGAGAAAAGGC (*Hin*dIII)	
oliRN267	F	CATCGCTGCGCCTCTTGCTAC	Confirmation of the *luxS*- deficient mutants
oliRN268	R	GGGTCATTCCAGAACCACAGA	
oliRN70	F	GCTATTTTTTGACTTACTGGG	
oliRN69	R	TCCGTATCTTTTACGCAGCGG	
oliRN205	F	GTCAATGGATCCCCAGCTCTATTGCACC (*Bam*HI)	P*luxS-luxS*-TT insert for *luxS* complementation plasmid pEC83
oliRN280	R	TATCTAGAGCTCTAGATTACTGAGAAAATC (*Sac*I)	
oliRN250	R	ATTCTTCAGAAATAAGACG	Primer extension

[a] F, forward; R, reverse. Underlined sequences indicate restriction sites.
[b] *fbp54* (fibronectin-binding protein), *isp2* (immunogenic secreted protein homologue), *nga* (NAD-glycohydrolase), *scl* (streptococcal collagen-like protein), *sibA* (secreted immunoglobulin binding protein), *sic* (streptococcal inhibitor of complement), *ska* (streptokinase), *slo* (streptolysin O), *speC* (streptococcal pyrogenic exotoxin C), *speF* (*mf*) (mitogenic factor).

Bacterial adhesion, internalization and survival assays in host cells. HEp-2 cells and RAW 264.7 mouse macrophages were maintained in RPMI medium (HEp-2 cells) or Dulbecco's modified Eagle's medium (D-MEM, Gibco) (RAW 264.7 cells) supplemented with 10% foetal bovine serum (Gibco), penicillin (100 μg/ml) and streptomycin (100 μg/ml), and grown at 37°C in an atmosphere containing 5% CO_2. Prior the infection assay, the cells were cultured overnight to semi-confluency (1.5×10^5 cells per well for HEp-2 cells and 2×10^5 cells per well for RAW264.7 cells) in 24-well tissue culture plates containing medium without antibiotic. GAS strains were grown to the same $OD_{620\ nm}$ corresponding to mid-logarithmic (RAW 264.7) or late-logarithmic (HEp-2) phase, washed with PBS, suspended in fresh cell culture medium and added to the semi-confluent host cell monolayers at a multiplicity of infection (MOI) of 5:1 (HEp-2) or 100:1 (RAW 264.7) in triplicate. Times of incubation were 30 min, 1 h and 2 h (adhesion, HEp-2 cells), 2 h (internalization, HEp-2 cells) and 30 min (RAW 264.7 cells). For adhesion assays (HEp-2 cells), infected monolayers were washed extensively with PBS before lysing. For internalization assays (RAW 264.7 cells), extracellularly adhered bacteria were killed by incubation with fresh medium containing either 100 μg/ml gentamicin and 5 μg/ml penicillin (HEp-2 cells) or 60 μg/ml penicillin (RAW 264.7 cells). At indicated time points, the infected monolayers were washed extensively with PBS to remove the antibiotics. Cells were then lysed with chilled sterile distilled water. The number of viable intracellular bacteria released from the lysed cells was determined by plating appropriate dilutions of the lysates on TSA-blood plates in triplicates followed by 24 h incubation at 37°C. The number of bacteria that survived intracellularly was calculated using the following equation: average number of bacteria recovered (cfu/ml) per well (triplicate) at designated time point/inoculated number of bacteria (cfu/ml)) × 100. Average values were plotted.

Authors' contributions

MS, RPJ, ZAP, CH and DZ carried out the experimental work. MS, RPJ and CH participated in the design of the study. MS helped to draft and critically revised the manuscript. MS designed the figures. EC conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Bonnie Bassler (Princeton University, USA) for her generous gift of *V. harveyi* strains. We thank Nina Gratz and Pavel Kovarik for their help with the macrophage work. We are grateful to Dr. Eszter Nagy (Intercell AG, Vienna, Austria) for helpful discussions and critical review of the manuscript. We thank Dr. Jason Rosch (St. Jude Children's Research Hospital, Memphis, USA) for helpful discussions. This work was supported by grant No. P17238-B09 from the Austrian Science Fund (Fonds zur

Förderung der wissenschaftlichen Forschung, FWF; to EC), grant No. H-1020-2004 from the University Jubilee Foundation of the City of Vienna (Hochschuljubilaümsstiftung der Stadt Wien, HJST; to EC) and grant No. 10802 from the Austrian Nationalbank (die Österreichische Nationalbank, ÖNB; to EC). RPJ and ZAP were recipients of scholarships from the Austrian Exchange Service (Der Österreichische Austauschdienst, ÖAD) and the Higher Education Commission of Pakistan (HEC), respectively.

References

1. Henke JM, Bassler BL: **Bacterial social engagements.** *Trends Cell Biol* 2004, **14(11):**648-656.
2. Lazazzera BA: **Quorum sensing and starvation: signals for entry into stationary phase.** *Curr Opin Microbiol* 2000, **3(2):**177-182.
3. Lyon GJ, Novick RP: **Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria.** *Peptides* 2004, **25(9):**1389-1403.
4. Parsek MR, Greenberg EP: **Sociomicrobiology: the connections between quorum sensing and biofilms.** *Trends Microbiol* 2005, **13(1):**27-33.
5. Walters M, Sperandio V: **Quorum sensing in *Escherichia coli* and *Salmonella*.** *Int J Med Microbiol* 2006, **296(2–3):**125-131.
6. Williams P, Winzer K, Chan WC, Camara M: **Look who's talking: communication and quorum sensing in the bacterial world.** *Philos Trans R Soc Lond B Biol Sci* 2007, **362(1483):**1119-1134.
7. Podbielski A, Kreikemeyer B: **Cell density-dependent regulation: basic principles and effects on the virulence of Gram-positive cocci.** *Int J Infect Dis* 2004, **8(2):**81-95.
8. Suntharalingam P, Cvitkovitch DG: **Quorum sensing in streptococcal biofilm formation.** *Trends Microbiol* 2005, **13(1):**3-6.
9. Bassler BL, Greenberg EP, Stevens AM: **Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*.** *J Bacteriol* 1997, **179(12):**4043-4045.
10. Surette MG, Miller MB, Bassler BL: **Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production.** *Proc Natl Acad Sci USA* 1999, **96(4):**1639-1644.
11. De Keersmaecker SC, Sonck K, Vanderleyden J: **Let LuxS speak up in AI-2 signaling.** *Trends Microbiol* 2006, **14(3):**114-119.
12. Vendeville A, Winzer K, Heurlier K, Tang CM, Hardie KR: **Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria.** *Nat Rev Microbiol* 2005, **3(5):**383-396.
13. Winzer K, Hardie KR, Williams P: **LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria.** *Adv Appl Microbiol* 2003, **53:**291-396.
14. Bisno AL, Brito MO, Collins CM: **Molecular basis of group A streptococcal virulence.** *Lancet Infect Dis* 2003, **3(4):**191-200.
15. Cunningham MW: **Pathogenesis of group A streptococcal infections.** *Clin Microbiol Rev* 2000, **13(3):**470-511.
16. Kreikemeyer B, McIver KS, Podbielski A: **Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions.** *Trends Microbiol* 2003, **11(5):**224-232.
17. Salim KY, de Azavedo JC, Bast DJ, Cvitkovitch DG: **Role for *sagA* and *siaA* in quorum sensing and iron regulation in *Streptococcus pyogenes*.** *Infect Immun* 2007, **75(10):**5011-5017.
18. Beyer-Sehlmeyer G, Kreikemeyer B, Horster A, Podbielski A: **Analysis of the growth phase-associated transcriptome of *Streptococcus pyogenes*.** *Int J Med Microbiol* 2005, **295(3):**161-177.
19. Chaussee MS, Phillips ER, Ferretti JJ: **Temporal production of streptococcal erythrogenic toxin B (streptococcal cysteine proteinase) in response to nutrient depletion.** *Infect Immun* 1997, **65(5):**1956-1959.
20. Smoot LM, Smoot JC, Graham MR, Somerville GA, Sturdevant DE, Migliaccio CA, Sylva GL, Musser JM: **Global differential gene expression in response to growth temperature alteration in group A *Streptococcus*.** *Proc Natl Acad Sci USA* 2001, **98(18):**10416-10421.
21. Kreikemeyer B, Boyle MD, Buttaro BA, Heinemann M, Podbielski A: **Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas) with homologies to two-component-type regulators requires a small RNA molecule.** *Mol Microbiol* 2001, **39(2):**392-406.
22. Mangold M, Siller M, Roppenser B, Vlaminckx BJ, Penfound TA, Klein R, Novak R, Novick RP, Charpentier E: **Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule.** *Mol Microbiol* 2004, **53(5):**1515-1527.
23. Neely MN, Lyon WR, Runft DL, Caparon M: **Role of RopB in growth phase expression of the SpeB cysteine protease of *Streptococcus pyogenes*.** *J Bacteriol* 2003, **185(17):**5166-5174.
24. McIver KS, Scott JR: **Role of *mga* in growth phase regulation of virulence genes of the group A streptococcus.** *J Bacteriol* 1997, **179(16):**5178-5187.
25. Eran Y, Getter Y, Baruch M, Belotserkovsky I, Padalon G, Mishalian I, Podbielski A, Kreikemeyer B, Hanski E: **Transcriptional regulation of the *sil* locus by the SilCR signalling peptide and its implications on group A streptococcus virulence.** *Mol Microbiol* 2007, **63(4):**1209-1222.
26. Lyon WR, Madden JC, Levin JC, Stein JL, Caparon MG: **Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*.** *Mol Microbiol* 2001, **42(1):**145-157.
27. Marouni MJ, Sela S: **The *luxS* gene of *Streptococcus pyogenes* regulates expression of genes that affect internalization by epithelial cells.** *Infect Immun* 2003, **71(10):**5633-5639.
28. Brenot A, King KY, Caparon MG: **The PerR regulon in peroxide resistance and virulence of *Streptococcus pyogenes*.** *Mol Microbiol* 2005, **55(1):**221-234.
29. Dalton TL, Scott JR: **CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*.** *J Bacteriol* 2004, **186(12):**3928-3937.
30. Loughman JA, Caparon M: **Regulation of SpeB in *Streptococcus pyogenes* by pH and NaCl: a model for *in vivo* gene expression.** *J Bacteriol* 2006, **188(2):**399-408.
31. Bates CS, Toukoki C, Neely MN, Eichenbaum Z: **Characterization of MtsR, a new metal regulator in group A streptococcus, involved in iron acquisition and virulence.** *Infect Immun* 2005, **73(9):**5743-5753.
32. Fagan PK, Reinscheid D, Gottschalk B, Chhatwal GS: **Identification and characterization of a novel secreted immunoglobulin binding protein from group A streptococcus.** *Infect Immun* 2001, **69(8):**4851-4857.
33. Liu M, Hanks TS, Zhang J, McClure MJ, Siemsen DW, Elser JL, Quinn MT, Lei B: **Defects in *ex vivo* and *in vivo* growth and sensitivity to osmotic stress of group A Streptococcus caused by interruption of response regulator gene *vicR*.** *Microbiology* 2006, **152(Pt 4):**967-978.
34. Malke H, Steiner K, McShan WM, Ferretti JJ: **Linking the nutritional status of *Streptococcus pyogenes* to alteration of transcriptional gene expression: the action of CodY and RelA.** *Int J Med Microbiol* 2006, **296(4–5):**259-275.
35. Reid SD, Montgomery AG, Musser JM: **Identification of *srv*, a PrfA-like regulator of group A streptococcus that influences virulence.** *Infect Immun* 2004, **72(3):**1799-1803.
36. Shelburne SA, Sumby P, Sitkiewicz I, Granville C, DeLeo FR, Musser JM: **Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva.** *Proc Natl Acad Sci USA* 2005, **102(44):**16037-16042.
37. Asanuma N, Yoshii T, Hino T: **Molecular characterization and transcription of the *luxS* gene that encodes LuxS autoinducer 2 synthase in *Streptococcus bovis*.** *Curr Microbiol* 2004, **49(5):**366-371.
38. Beeston AL, Surette MG: ***pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar *Typhimurium*.** *J Bacteriol* 2002, **184(13):**3450-3456.
39. Lupp C, Ruby EG: ***Vibrio fischeri* LuxS and AinS: comparative study of two signal synthases.** *J Bacteriol* 2004, **186(12):**3873-3881.
40. Sun J, Daniel R, Wagner-Dobler I, Zeng AP: **Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways.** *BMC Evol Biol* 2004, **4:**36.
41. von Lackum K, Babb K, Riley SP, Wattier RL, Bykowski T, Stevenson B: **Functionality of *Borrelia burgdorferi* LuxS: the Lyme disease spirochete produces and responds to the pheromone autoinducer-2 and lacks a complete activated-methyl cycle.** *Int J Med Microbiol* 2006, **40:**92-102.
42. Sztajer H, Lemme A, Vilchez R, Schulz S, Geffers R, Yip CY, Levesque CM, Cvitkovitch DG, Wagner-Dobler I: **Autoinducer-2-regulated**

genes in *Streptococcus mutans* UA159 and global metabolic effect of the *luxS* mutation. *J Bacteriol* 2008, **190(1):**401-415.
43. Wen ZT, Burne RA: **LuxS-mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation.** *J Bacteriol* 2004, **186(9):**2682-2691.
44. Hasona A, Zuobi-Hasona K, Crowley PJ, Abranches J, Ruelf MA, Bleiweis AS, Brady LJ: **Membrane composition changes and physiological adaptation by *Streptococcus mutans* signal recognition particle pathway mutants.** *J Bacteriol* 2007, **189(4):**1219-1230.
45. Lemos JA, Brown TA Jr, Burne RA: **Effects of RelA on key virulence properties of planktonic and biofilm populations of *Streptococcus mutans*.** *Infect Immun* 2004, **72(3):**1431-1440.
46. Azcarate-Peril MA, McAuliffe O, Altermann E, Lick S, Russell WM, Klaenhammer TR: **Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*.** *Appl Environ Microbiol* 2005, **71(10):**5794-5804.
47. DeLisa MP, Bentley WE: **Bacterial autoinduction: looking outside the cell for new metabolic engineering targets.** *Microb Cell Fact* 2002, **1(5):**1-9.
48. Klenk M, Koczan D, Guthke R, Nakata M, Thiesen HJ, Podbielski A, Kreikemeyer B: **Global epithelial cell transcriptional responses reveal *Streptococcus pyogenes* Fas regulator activity association with bacterial aggressiveness.** *Cell Microbiol* 2005, **7(9):**1237-1250.
49. Kreikemeyer B, Nakata M, Koller T, Hildisch H, Kourakos V, Standar K, Kawabata S, Glocker MO, Podbielski A: **The *Streptococcus pyogenes* serotype M49 Nra-Ralp3 transcriptional regulatory network and its control of virulence factor expression from the novel *eno ralp3 epf sagA* pathogenicity region.** *Infect Immun* 2007, **75(12):**5698-5710.
50. Luo F, Lizano S, Bessen DE: **Heterogeneity in the Polarity of Nra Regulatory Effects on Streptococcal Pilus Gene Transcription and Virulence.** *Infect Immun* 2008, **76(6):**2490-2497.
51. Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM: **Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity.** *PLoS Pathog* 2006, **2(1):**e5.
52. Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, *et al.*: **DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection.** *Nat Med* 2007, **13(8):**981-985.
53. Coulthurst SJ, Kurz CL, Salmond GP: ***luxS* mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapenem and prodigiosin.** *Microbiology* 2004, **150(Pt 6):**1901-1910.
54. Dove JE, Yasukawa K, Tinsley CR, Nassif X: **Production of the signalling molecule, autoinducer-2, by *Neisseria meningitidis* : lack of evidence for a concerted transcriptional response.** *Microbiology* 2003, **149(Pt 7):**1859-1869.
55. Bejerano-Sagie M, Xavier KB: **The role of small RNAs in quorum sensing.** *Curr Opin Microbiol* 2007, **10(2):**189-198.
56. Lyon WR, Gibson CM, Caparon MG: **A role for trigger factor and an *rgg*-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*.** *EMBO J* 1998, **17(21):**6263-6275.
57. Rijn I van de, Kessler RE: **Growth characteristics of group A streptococci in a new chemically defined medium.** *Infect Immun* 1980, **27(2):**444-448.
58. Doherty N, Holden MT, Qazi SN, Williams P, Winzer K: **Functional analysis of *luxS* in *Staphylococcus aureus* reveals a role in metabolism but not quorum sensing.** *J Bacteriol* 2006, **188(8):**2885-2897.
59. Caparon MG, Scott JR: **Genetic manipulation of pathogenic streptococci.** *Methods Enzymol* 1991, **204:**556-586.
60. Sambrook J, Fritsch EF, Maniatis T: **.** In *Molecular Cloning: a Laboratory Manual* 2nd edition. Cold Spring Harbor, NY edn: Cold Spring Harbor Laboratory Press; 1989.
61. Trieu-Cuot P, Courvalin P: **Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3'5"-aminoglycoside phosphotransferase type III.** *Gene* 1983, **23(3):**331-341.
62. Herbert S, Barry P, Novick RP: **Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*.** *Infect Immun* 2001, **69(5):**2996-3003.
63. Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E, Kreikemeyer B: **Characterization of biofilm formation by clinically relevant serotypes of group A streptococci.** *Appl Environ Microbiol* 2006, **72(4):**2864-2875.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

BioMedcentral

4.4 Project 4: Group A Streptococcus activates type I interferon production and MyD88-dependent signalling without involvement of TLR2, TLR4 and TLR9

4.4.1 Summary

One of the mechanisms by which the innate immune system senses the invasion of pathogenic micro-organisms is through the toll-like receptors (TLRs). TLRs have a crucial role in early host defence against invading pathogens. Pathogen-associated molecular patterns (PAMPs) in bacteria are recognized by host cell-encoded pattern-recognition receptors (PRRs) to develop immune responses. TLRs are the best studied PRRs. Stimulation of different TLRs induces distinct patterns of gene expression, which leads to the activation of innate immunity and also instructs the development of antigen-specific acquired immunity. All pathogenic bacteria are recognized by one or more TLRs. TLR4 is critical for LPS recognition, TLR2 for lipoteichoic acids, TLR5 for flagellin and TLR9 recognizes bacterial DNA containing unmethylated CpG sequences. The MAP kinase cascade is an evolutionary conserved signaling pathway. There are three major groups of MAP kinases: the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun NH2-terminal kinases (JNK). After ligand mediated dimerization, TLR recruits an adapter protein MyD88 (Myeloid differentiation factor 88). MyD88 then assembles a signalosome containing IRAK (interleukin receptor associated kinase), TRAF6 (tumor-necrosis-factor-receptor associated factor 6) and ECSIT. Together they mediate activation of NF-kB and MAP kinases like p38 and JNK, which leads to the production of inflammatory cytokines such as TNFα,IL-1 and IL-2.

In this study the activation of p38MAPK and NF-κB in WT and TLR2$^{-/-}$ BMDMs was compared, since many gram-positive pathogens are recognized by this receptor. The data demonstrate that TLR2 was not required for GAS-induced signalling. Other TLRs frequently engaged by bacteria are TLR4 and TLR9. To examine whether a combination of TLR2, TLR4 and TLR9 was used for GAS recognition, GAS-induced signalling in TLR2/4/9 triple-deficient BMDMs was analyzed. The signalling events in triple-deficient BMDMs were comparable to those triggered by WT GAS. These data exclude a redundant function of TLR2, TLR4 and TLR9 in GAS recognition, and demonstrate that

macrophages sense GAS using a PRR other than the most common bacterial receptors. GAS-induced activation of p38MAPK was almost completely abolished and the degradation of IκB was strongly diminished in MyD88$^{-/-}$cells when compared to WT cells. These data show that GAS-induced proinflammatory cytokine production is absolutely dependent on MyD88. It was further demonstrated that GAS also elicits MyD88-independent signaling that results in type I IFN production. Analysis of Stat1 activation revealed that neither the deletion of the single cytolysin genes (*sagA* or *slo)* nor the double deletion of cytolysin genes (*sagA-slo)* caused a reduction of GAS-induced IFN signalling. The inflammatory signalling was also not dependent on the expression of the GAS cytolysins as shown by p38MAPK activation. Hence it is surprising that IFN production did not require cytolysins because in other gram-positive pathogens either cytolysins itself or cytolysin-mediated escape of bacteria from phagocytic vesicles trigger IFN production.

This is the first description of activation of MyD88-dependent and -independent pathways upon GAS infection. This data indicate that first, GAS is recognized by an unknown receptor upstream of MyD88, and second, the MyD88-independent signaling proceeds without cytolysins that are often required for IFN production by other gram-positive bacteria. In agreement with our results, a recent study showed that dentritic cells deficient in TLR1, 2, 4, 9 or 2/6 were not impaired in the secretion of the proinflammatory cytokines and argues for the role of a multi-model recognition that is the combination of several different TLR-mediated signaling in *S. pyogenes* (Loof *et al.*, 2008). The activation of multiple signaling pathways may contribute to the known high potential of GAS to induce toxic shock and other GAS-derived pathologies**.**

This work was done in collaboration with a.o. Prof. Pavel Kovarik's lab. I constructed two mutants: the *slo* (encoding Streptolysin O)-deficient and the double *slo-sls* (encoding Streptolysin O and Streptolysin S)-deficient mutants for this study. These mutants were analyzed by PCR, Southern blot and DNA sequencing, in order to verify the correct deletion events in the mutants as well as by doing hemolysis assays.

4.4.2 Manuscript 4

Group A Streptococcus activates type I interferon production and MyD88-dependent signalling without involvement of TLR2, TLR4 and TLR9

Published manuscript: Journal of Biological Chemistry (2008) 283(29), 19879-19887

Authors:

Nina Gratz, Maria Siller, Barbara Schaljo, **Zaid Ahmed Pirzada**, Irene Gattermeier, Ivo Vojtek, Carsten J Kirschning, Hermann Wagner, Shizuo Akira, Emmanuelle Charpentier and Pavel Kovarik

Supplemental Material can be found at:
http://www.jbc.org/cgi/content/full/M802848200/DC1

⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 29, pp. 19879–19887, July 18, 2008
© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Group A Streptococcus Activates Type I Interferon Production and MyD88-dependent Signaling without Involvement of TLR2, TLR4, and TLR9*[S]

Received for publication, April 14, 2008 Published, JBC Papers in Press, May 14, 2008, DOI 10.1074/jbc.M802848200

Nina Gratz[‡], Maria Siller[‡], Barbara Schaljo[‡], Zaid A. Pirzada[‡1], Irene Gattermeier[‡], Ivo Vojtek[‡], Carsten J. Kirschning[§], Hermann Wagner[§], Shizuo Akira[¶], Emmanuelle Charpentier[‡2], and Pavel Kovarik[‡3]

From the [‡]Max F. Perutz Laboratories, Department of Microbiology and Immunobiology, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria, the [§]Institute of Medical Microbiology, Immunology, and Hygiene, Technical University of Munich, 81675 Munich, Germany, and the [¶]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Bacterial pathogens are recognized by the innate immune system through pattern recognition receptors, such as Toll-like receptors (TLRs). Engagement of TLRs triggers signaling cascades that launch innate immune responses. Activation of MAPKs and NF-κB, elements of the major signaling pathways induced by TLRs, depends in most cases on the adaptor molecule MyD88. In addition, Gram-negative or intracellular bacteria elicit MyD88-independent signaling that results in production of type I interferon (IFN). Here we show that in mouse macrophages, the activation of MyD88-dependent signaling by the extracellular Gram-positive human pathogen group A streptococcus (GAS; *Streptococcus pyogenes*) does not require TLR2, a receptor implicated in sensing of Gram-positive bacteria, or TLR4 and TLR9. Redundant engagement of either of these TLR molecules was excluded by using TLR2/4/9 triple-deficient macrophages. We further demonstrate that infection of macrophages by GAS causes IRF3 (interferon-regulatory factor 3)-dependent, MyD88-independent production of IFN. Surprisingly, IFN is induced also by GAS lacking *slo* and *sagA*, the genes encoding cytolysins that were shown to be required for IFN production in response to other Gram-positive bacteria. Our data indicate that (i) GAS is recognized by a MyD88-dependent receptor other than any of those typically used by bacteria, and (ii) GAS as well as GAS mutants lacking cytolysin genes induce type I IFN production by similar mechanisms as bacteria requiring cytoplasmic escape and the function of cytolysins.

Group A streptococcus (GAS[4]; *Streptococcus pyogenes*) is an important human Gram-positive pathogen responsible for a wide spectrum of infections, ranging from mild diseases (*e.g.* tonsillitis) to serious illness (*e.g.* necrotizing fasciitis, sepsis, or severe poststreptococcal sequelae) (1). The persistence of GAS in the human population and the severity of some GAS diseases are the result of activities of a number of virulence factors that enable the pathogen to escape immune surveillance or, on contrary, induce an overreaction of the immune system (2, 3). Although GAS is generally regarded as an extracellular pathogen, recent findings suggest that GAS can survive (although not multiply) within various host cells, such as neutrophils, macrophages, epithelial cells, and fibroblasts (4–7). The surviving bacteria may serve as a reservoir for recurrent GAS diseases.

Immune responses to bacteria are initiated by recognition of bacterial components called pathogen-associated molecular patterns through host cell-encoded pattern recognition receptors (PRRs) (8, 9). Typically, pathogen-associated molecular patterns are components of the bacterial cell wall (*e.g.* lipopolysaccharide and lipoteichoic acid), but they may also be derived from the inside of bacteria (*e.g.* DNA). The primary function of PRRs is to trigger signaling cascades that activate antimicrobial defense programs. The best studied class of PRRs is the Toll-like receptor (TLR) family, which consists of 13 transmembrane glycoproteins in mammals (8). Virtually all pathogenic bacteria are recognized by one or more TLRs, with TLR2 being the receptor for lipoteichoic acid of Gram-positive bacteria, TLR4 the receptor for lipopolysaccharide of Gram-negative bacteria, and TLR9 the receptor for bacterial DNA containing unmethylated CpG sequences. Ligand binding to TLR3, TLR4, TLR7, and TLR9 but not to TLR2 launches two distinct signaling pathways that result in the production of proinflammatory cytokines (*e.g.* TNFα and IL-1) and type I IFNs, respectively. Five TIR domain-containing adaptor molecules are involved in signaling downstream of TLRs: MyD88, TIRAP (or MAL), TRIF (or TICAM1), TRAM (or TICAM2),

* This work was supported in part by Austrian Research Foundation Grants P16726-B14, I27-B03, and SFB F28 (to P. K.) and Grant P17238-B09 (to E. C.); the University Jubilee Foundation of the City of Vienna (Hochschuljubiläumsstiftung der Stadt Wien, HJST) Grant H-1020-2004 (to E. C.); and the European Science Foundation under the EUROCORES Programme EuroDYNA through Contract ERAS-CT-2003-980409 of the European Commission, DG Research, FP6 (to the laboratory of P. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] Recipient of a scholarship from the Higher Education Commission of Pakistan (laboratory of E. C.).

[2] To whom correspondence may be addressed. Tel.: 431-4277-54608; Fax: 431-4277-9546; E-mail: emanuelle.charpentier@univie.ac.at.

[3] To whom correspondence may be addressed. Tel.: 431-4277-54608; Fax: 431-4277-9546; E-mail: pavel.kovarik@univie.ac.at.

[4] The abbreviations used are: GAS, group A streptococcus; BMDM, bone marrow-derived macrophages; IFN, interferon; IRF, interferon regulatory factor; MAPK, mitogen-activated protein kinase; PRRs, pattern-recognition receptors; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; IL, interleukin; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; qRT, quantitative reverse transcription.

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

and SARM, a negative regulator of TRIF (10). MyD88, an essential signaling component of all TLRs except TLR3, is required for activation of MAPKs and NF-κB that cause production of proinflammatory cytokines. Signaling components that are required for the production of type I IFNs are less uniform, and they are partially TLR- and cell type-specific. Recently, much attention has been paid to the elucidation of pathways leading to TLR-independent production of type I IFN in response to intracellular bacteria and/or intracellular DNA (11–14). Although the identity of the critical PRR remains unknown, it is generally believed that it is a cytoplasmic molecule that recognizes bacterial products (possibly bacterial DNA) in the cytoplasm. Signaling downstream of this unknown PRR employs the serine threonine kinase TBK1 or, less frequently, its relative IKK (15–18), which phosphorylates primarily the transcription factor IRF3, causing the activation of the IFN-β gene, the first type I IFN to be expressed (19, 20). Other cytoplasmic PRRs, such as the NOD proteins, recognize bacterial products in the cytoplasm and activate MAPKs and NF-κB but not IFN production (21).

Despite a considerable knowledge of GAS virulence factors, the PRRs that are responsible for recognition of this pathogen by the host are unknown. Remarkably little information is available also about the signaling pathways triggered by GAS in infected innate immune cells that are known to be required for defense against GAS (22). Here we show that GAS activates p38 MAPK, NF-κB, TNFα, and IL-6 production in infected bone marrow-derived mouse macrophages (BMDMs). Similar to other bacteria, these responses depend on MyD88. However, in contrast to most other pathogenic bacteria, the MyD88-mediated signaling was independent of TLR2, the receptor for Gram-positive bacteria, and the other bacterial receptors TLR4 and TLR9. We also rule out the involvement of IL-1 receptor signaling. We further demonstrate that GAS also elicits MyD88-independent signaling that results in type I IFN production. The IFN production did not require the presence of the GAS-encoded cytolysins SLO and SLS. This finding is surprising, since during infections with other Gram-positive bacteria, either the cytolysin itself or cytolysin-mediated cytoplasmic escape of bacteria from phagocytic vesicles was implicated in triggering IFN production (12, 23–25). However, the requirement for IRF3 in the IFN production suggested that other aspects of the GAS-induced IFN production resembled the well established TBK1/IRF3 signaling pathway. This is the first description of activation of MyD88-dependent and -independent pathways upon GAS infection. Our data implicate that GAS is recognized by a yet unknown receptor upstream of MyD88 and establish GAS as a Gram-positive pathogen capable of inducing type I IFN synthesis without molecules usually required for entry of bacterial products into the cytoplasm of infected cells.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Escherichia coli DH5-α and TOP10 were used as hosts for cloning. *S. pyogenes* serotype M1 (ATCC 700294) is a clinical strain originally isolated from an infected wound. The isogenic *sagA*- and *slo*-deficient mutants were constructed using a thermosensitive strategy described previously (26). First, using wild-type genomic DNA as template and primers containing flanking restriction sites, a 1111-bp *sagA* upstream fragment (primers OLEC120/OLEC143), a 1200-bp *sagA* downstream fragment (primers OLEC123/OLEC144), a 1159-bp *slo* upstream fragment (primers OLEC248/OLEC249), and a 1033-bp *slo* downstream fragment (primers OLEC250/OLEC251) were amplified. After digestion with the respective restriction enzymes, the upstream and downstream fragments were cloned into thermo-sensitive plasmids pRDN18 (*sagA*-deficient mutant) and pEC84 (*slo*-deficient mutant) (26). After introduction of the recombinant plasmids into *S. pyogenes* ATCC 700294, a series of temperature shifts with appropriate antibiotic selection was performed, thus leading to the final deficient mutants (strain EC548 (*sagA*-deficient mutant) and EC997 (*slo*-deficient mutant)) in which the entire coding sequence of the gene was deleted in a nonpolar fashion. To create the *sagA*/*slo*-deficient strain (EC1142), the deletion of *slo* was performed as mentioned above in the background of the *sagA*-deficient strain. The correct deletion event in the mutants was checked by PCR, Southern blot, and sequencing analysis. SLS and SLO hemolysis assays further confirmed that the *sagA*-, *slo*-, and *sagA*/*slo*-deficient mutants were defective in SLS and/or SLO activity as described (27, 28). DNA manipulations are described in the supplemental materials.

Bacterial Culture—S. pyogenes strains were grown at 37 °C with 5% CO_2 without agitation in Todd-Hewitt broth (BD Biosciences) supplemented with 0.2% yeast extract and on tryptic soy agar supplemented with 3% defibrinogated sheep blood. Transformation of *E. coli* and *S. pyogenes* was performed as previously described (29, 30). Whenever required, antibiotics were added to the medium at the following final concentrations: erythromycin, 300 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*; spectinomycin, 100 μg/ml for both *E. coli* and *S. pyogenes*. Bacterial cell growth was turbidimetrically monitored at 620 nm with a microplate reader.

Macrophage Cell Culture—Primary BMDMs were obtained from the femur bone marrow of 6–10-week-old mice. Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of L cell-derived CSF-1, as described (31). MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, TLR2/4/9$^{-/-}$, IFNAR1$^{-/-}$, IRF3$^{-/-}$, IL1-RI$^{-/-}$, and control WT mice, all on a C57Bl/6 background, were housed under specific pathogen-free conditions (32–36).

GAS Infections—For infection assays, BMDMs were seeded at 5×10^6 cells/dish in 10-cm dishes containing medium without antibiotics. The next day, *S. pyogenes* cultures grown in THY were harvested at midlogarithmic phase, washed with phosphate-buffered saline, and added to the BMDM monolayers at a multiplicity of infection (MOI) of 100. After 30 min of incubation at 37 °C, nonadherent extracellular bacteria were eliminated by removing the culture medium, and adherent extracellular bacteria were subsequently killed by incubation with fresh medium (without fetal calf serum) containing 60 μg/ml penicillin. At specific time points after infection, supernatants were collected for ELISA, and whole cell extracts were prepared for Western blot analysis. At least three mice of each genotype were used in all infection experiments.

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

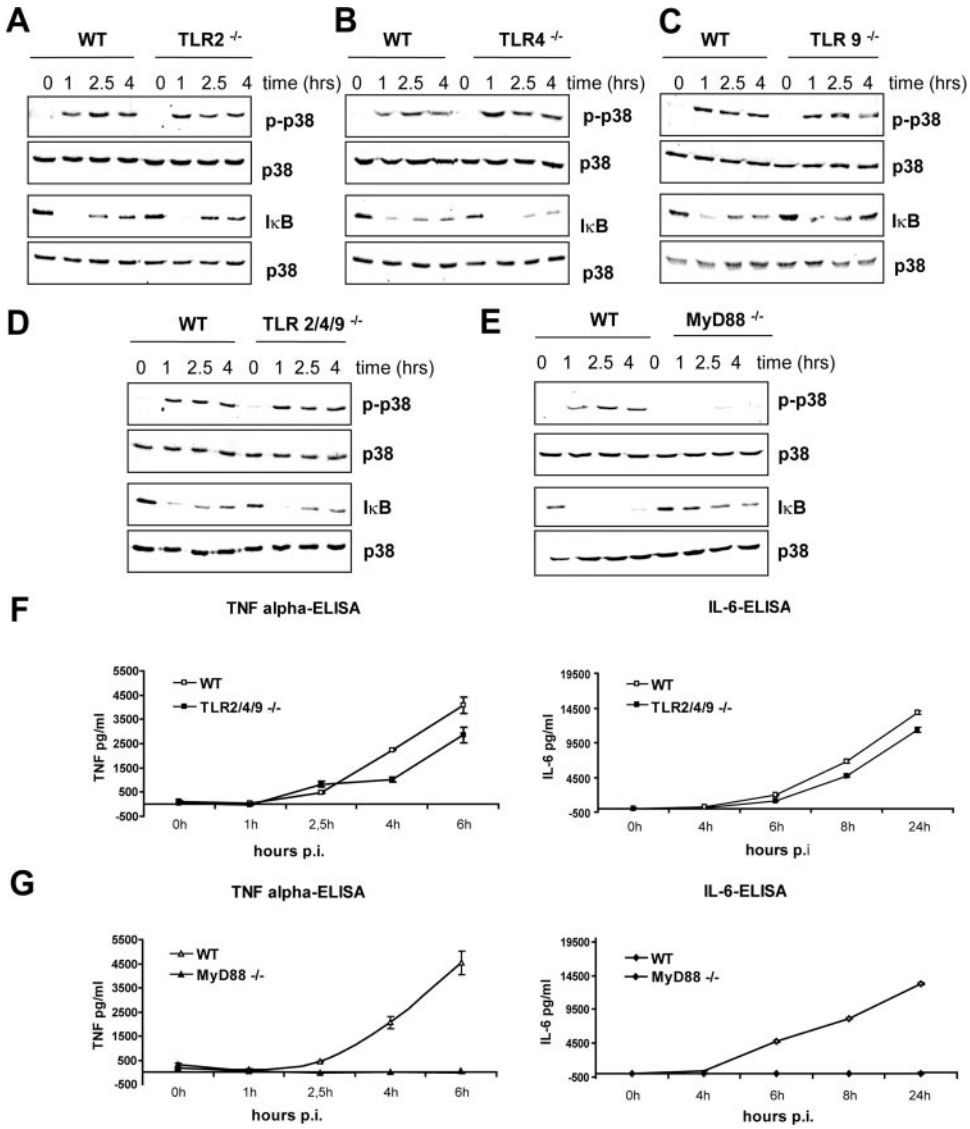


FIGURE 1. **GAS-induced inflammatory signaling and the production of TNFα and IL-6 depends on MyD88 but not on TLR2, TLR4, and TLR9.** *A–E*, BMDMs from TLR2$^{-/-}$ (*A*), TLR4$^{-/-}$ (*B*), TLR9$^{-/-}$ (*C*), TLR2/4/9$^{-/-}$ (*D*), MyD88$^{-/-}$ (*E*), and WT control mice were infected with GAS (MOI 100). At the indicated time points, whole cell extracts were prepared and analyzed by Western blotting using antibodies to phosphorylated p38 MAPK (*pp38*; *top*) and to IκB (*bottom*). Equal loading was controlled by reprobing the membrane with antibody to total p38 MAPK. *F* and *G*, BMDMs derived from TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) (*F*), MyD88$^{-/-}$ (*G*), and control mice were infected with GAS (MOI 100). At the indicated time points, supernatants were collected, and TNFα (*left*) and IL-6 release (*right*) was measured by ELISA. The data represent one of at least three infection experiments carried out independently with different mice of each genotype.

Antibodies—Antibodies to Tyr^{701}-phosphorylated Stat1 (pY701-S1) and phosphorylated p38 (pp38) were purchased from Cell Signaling (Frankfurt/Main, Germany). Antibodies to IκB-α and p38 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to Stat1-α C terminus was previously described (37).

ELISA and Western Blot Analysis—For ELISAs, supernatants of infected macrophages were collected and diluted 1:5 in reagent diluent. TNFα and IL-6 were assayed using DuoSET ELISA kits (R&D Systems, Minneapolis, MN). Western blot analysis was performed using fluorophore-linked secondary antibodies (Molecular Probes-Invitrogen (Lofer, Austria) and Rockland (Gilbertsville, PA)) and an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE).

qRT-PCR—Total RNA was isolated using Trizol® LS reagent (Invitrogen). Reverse transcription of total RNA was performed using oligo(dT)$_{18}$ as primer and Moloney murine leukemia virus reverse transcriptase (Fermentas, St. Leon-Rot, Germany). The cDNA of the IFN-β and Mx2 genes was subsequently analyzed by qRT-PCR as described in the supplemental materials.

RESULTS

GAS Activates Inflammatory Signaling Independently of TLR2, TLR4, and TLR9—To investigate the role of bacteria-recognizing TLRs in responses to GAS infection, we examined the activation of the transcription factor NF-κB by assaying the degradation of its inhibitor (IκB) and the phosphorylation of p38 MAPK in infected BMDMs. Activation of both NF-κB and p38 MAPK was shown in numerous studies to be essential for production of proinflammatory cytokines (38–42). Furthermore, macrophages express all components of the TLR signaling cascade and are known to be required for defenses against GAS infection (22). We first compared the activation of p38 MAPK and NF-κB in WT and TLR2$^{-/-}$ BMDMs, since many Gram-positive pathogens are recognized by this receptor. GAS induced in both WT and TLR2$^{-/-}$ BMDMs a rapid and sustained activation of p38 MAPK (Fig. 1*A*). The degradation of IκB was maximal after 1 h of infection. IκB gradually reappeared at later time points but did not reach the original level during the time frame of observation (up to 4 h). The internalization of GAS (monitored by fluorescence microscopy) was equally efficient in cells of both genotypes (data not shown). These data demonstrate that TLR2 was not required for GAS-induced signaling. Other TLRs frequently engaged by bacteria are TLR4 and TLR9. TLR4 is the receptor for lipopolysaccharide of Gram-negative bacteria, but it has been shown to recognize also cell wall components of mycobacteria and several cytolysins of Gram-positive bacteria (25, 43, 44). TLR9, a CpG DNA receptor localized in endosomal and lysosomal membranes of macrophages and, most prominently, dendritic cells, can bind DNA from both Gram-positive and Gram-negative species (35, 45). Similar to TLR2$^{-/-}$ BMDMs, the activation of p38 MAPK and degradation of IκB in TLR4$^{-/-}$ and TLR9$^{-/-}$ BMDMs was

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

indistinguishable from WT BMDMs (Fig. 1, *B* and *C*). To examine whether a combination of TLR2, TLR4, and TLR9 was used for GAS recognition, we analyzed GAS-induced signaling in TLR2/4/9 triple-deficient BMDMs. As depicted in Fig. 1*D*, the signaling events in triple-deficient BMDMs were comparable with WT cells. These data exclude a redundant function of TLR2, TLR4, and TLR9 in GAS recognition and demonstrate that macrophages sense GAS using a PRR other than the most common bacterial receptors.

GAS-induced Inflammatory Signaling Depends on MyD88—All TLRs except for the double-stranded RNA receptor TLR3 require MyD88 for activation of MAPKs, NF-κB, and the subsequent proinflammatory cytokine production. To investigate whether MyD88 was involved in GAS-induced inflammatory signaling, we examined the activation of p38 MAPK and degradation of IκB in infected MyD88$^{-/-}$ BMDMs. The experiment revealed that GAS-induced activation of p38 MAPK was almost completely abolished, and the degradation of IκB was strongly diminished in MyD88$^{-/-}$ cells when compared with WT cells (Fig. 1*E*). A weak p38 MAPK activation and IκB degradation were observed in MyD88$^{-/-}$ cells at later time points of infection. Thus, the GAS-induced inflammatory signaling was markedly reduced and delayed in MyD88$^{-/-}$ cells. The activation of MAPKs and NF-κB plays a fundamental role in induction of proinflammatory cytokines, such as TNFα or IL-6. To estimate the effect of reduced GAS-induced inflammatory signaling in MyD88$^{-/-}$ BMDMs, we determined the amounts of secreted TNFα and IL-6 in supernatants of GAS-infected MyD88$^{-/-}$ and control WT cells. In parallel, we also measured TNFα and IL-6 production by infected TLR2/4/9 triple-deficient cells. Infection of WT BMDMs with GAS resulted in a robust cytokine production that was only slightly reduced (by less than 20%) in TLR2/4/9 triple-deficient cells (Fig. 1*F*). In marked contrast, TNFα and IL-6 production was completely abolished in GAS-infected MyD88$^{-/-}$ BMDMs (Fig. 1*E*). These data show that GAS-induced proinflammatory cytokine production is absolutely dependent on MyD88. The slight but reproducible reduction of cytokine production by TLR2/4/9 triple-deficient BMDMs indicates that one of these TLRs or a combination of them plays a minor role in GAS-induced proinflammatory cytokine production. However, the largest part of GAS-induced inflammatory cytokine production is mediated by a receptor (or receptors) different from the most common bacteria-specific TLRs. Importantly, this receptor signals via MyD88. The only other known pathways that use the adaptor MyD88 for signaling are the IL-1/IL-18 pathways (32). Although IL-18 is most relevant for the activation and IFN-γ production by Th1 cells (46), IL-1 has been reported be released by innate immune cells upon infection with various pathogens that are sensed in the cytoplasm by components of the inflammasome (47–49). To investigate whether IL-1 release by GAS-infected macrophages is responsible for the MyD88-dependent inflammatory signaling, we infected cells deficient in IL1-RI, the most important IL-1 receptor chain (50). As shown in Fig. 2, the activation of p38 MAPK, the degradation of IκB, and TNFα production were not affected by the deficiency in IL-1 signaling. These data further strengthen our notion that GAS is sensed by a yet unidentified receptor upstream of MyD88.

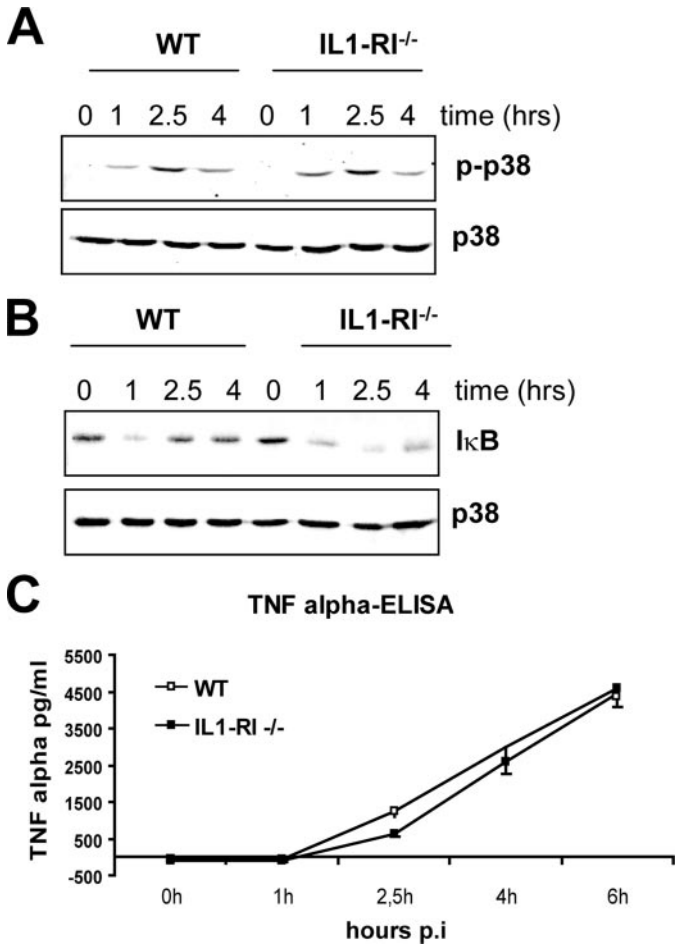


FIGURE 2. **IL-1 signaling is not required for responses of BMDM to GAS infection.** *A* and *B*, BMDMs from IL-1RI$^{-/-}$ and control (WT) mice were infected with GAS (MOI 100) for the indicated periods. p38 MAPK activation (*pp38*) (*A*) and degradation of IκB (*B*) were determined by Western blot analysis. Equal protein loading was confirmed by reprobing the membrane with antibody to total p38 MAPK. *C*, BMDMs derived from IL-1RI$^{-/-}$ and control mice were infected with GAS (MOI 100). At the indicated time points, supernatants were collected, and TNFα release was measured by ELISA. The data represent one of at least three infection experiments carried out independently with different mice of each genotype.

GAS Induces Type I Interferon—Gram-negative and intracellular bacteria are known to elicit MyD88-independent signaling that causes activation of the transcription factor IRF3 and subsequent transcription of the type I IFN-β. The mechanisms of IFN production induced by these two types of bacteria differ upstream of IRF3. Whereas Gram-negative bacteria use TLR4 to stimulate IFN production, intracellular bacteria are recognized by a still unknown cytoplasmic receptor. Engagement of TLR9 can also trigger IRF3/IRF7-mediated type I IFN expression. GAS has been previously reported to induce IFN production in primary human macrophages (51). We asked whether GAS can exert similar effects in mouse macrophages and what the role of MyD88 or the bacteria-specific TLRs therein is. First, tyrosine phosphorylation of the crucial IFN-activated transcription factor Stat1 was analyzed. Tyrosine phosphorylation of Stat1 is a good indicator for autocrine IFN-β production, since this cytokine is the first IFN to be synthetized upon challenge of macrophages with bacteria or their products (52–54). Infection of BMDMs with GAS revealed induction of Stat1

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

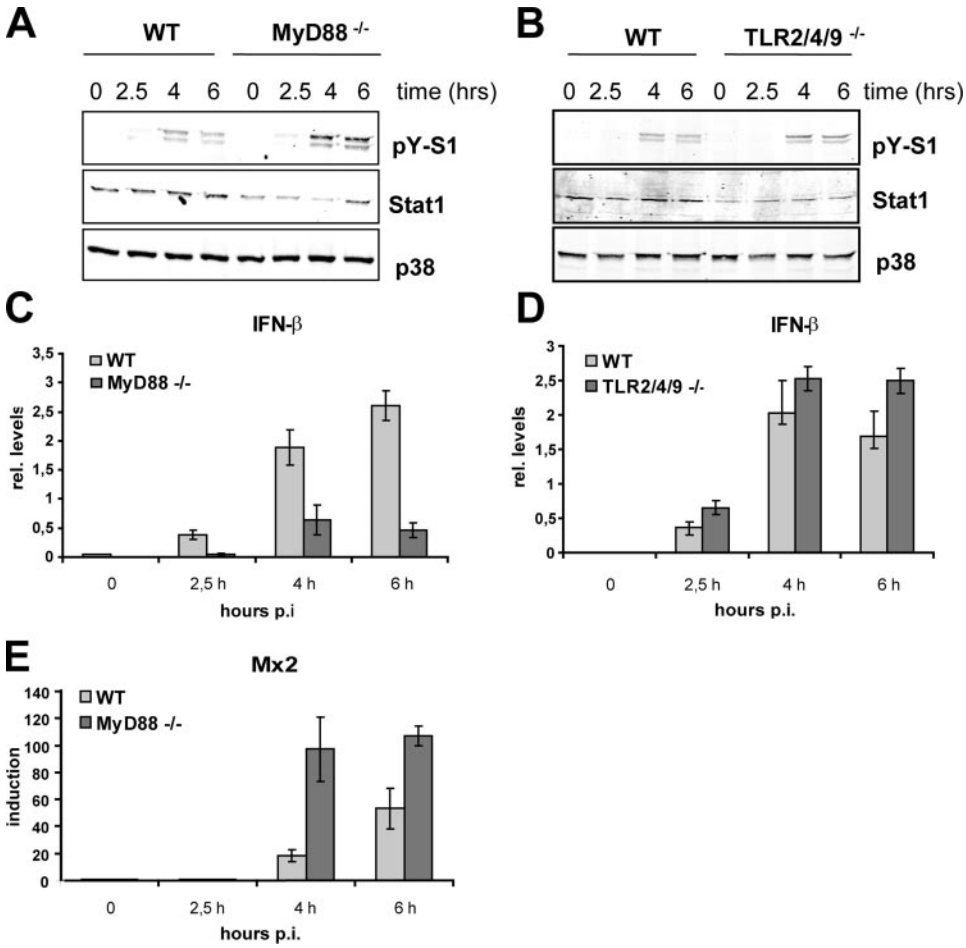


FIGURE 3. **GAS induces IFN-β production and Stat1 activation independently of MyD88, TLR2, TLR4, and TLR9.** *A* and *B*, BMDMs from control (WT), MyD88$^{-/-}$ (*A*), and TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) (*B*) mice were infected with GAS (MOI 100), and whole cell extracts were prepared after the indicated time periods. Stat1 Tyr^{701} phosphorylation and expression were determined by Western blotting using antibody to phosphorylated Stat1 (pY-S1) and total Stat1. Differences in Stat1 expression levels were revealed by reprobing the membrane with antibody to total p38 MAPK (p38). Note the double band on the pY-S1 blot represents the phosphorylated forms of both Stat1 splicing isoforms Stat1-α and Stat1-β. Loading control (S1) was performed with antibody directed to the C terminus of Stat1, which is absent in the Stat1-β isoform. *C* and *D*, BMDMs from wild type, MyD88$^{-/-}$ (*C*), and TLR2/4/9 triple-deficient (TLR2/4/9$^{-/-}$) mice (*D*) were exposed to GAS (MOI 100). After the indicated time points, total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for IFN-β and GAPDH (for normalization) expression. Note that the data show relative IFN-β mRNA levels normalized to GAPDH but not to uninfected samples. *E*, GAS-induced IFN-β stimulates transcription of type I IFN target gene *Mx2*. BMDMs from MyD88$^{-/-}$ and control (wild type) mice were exposed to GAS, and 2.5, 4, and 6 h postinfection (*hours p.i.*), total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for *Mx2* after normalizing to GAPDH and uninfected samples. *Bars*, S.D. of three experiments.

tyrosine phosphorylation after 4 h of infection of WT BMDMs (Fig. 3, *A* and *B*). This late tyrosine phosphorylation is similar to challenges with other bacteria (*e.g. Listeria monocytogenes*) that do not induce IFN synthesis through direct engagement of an IFN-inducing TLR (23). Consistently, Stat1 activation proceeded independently of MyD88 and TLR2, TLR4, and TLR9 signaling, as shown by GAS infection of BMDMs from MyD88$^{-/-}$ (Fig. 3*A*), TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$ (all in Fig. S1), or TLR2/4/9 triple-deficient (Fig. 3*B*) mice. In fact, Stat1 tyrosine phosphorylation was slightly but reproducibly increased in MyD88$^{-/-}$ and TLR2/4/9 triple-deficient BMDMs (Fig. 3, *A* and *B*) when normalized to total Stat1 levels. Since the Stat1 levels were generally slightly lower in BMDMs derived from the gene-targeted mice compared with WT controls, the Western blots were reprobed with p38 antibody to allow a more accurate normalization. Reduced Stat1 levels have been already previously observed in cells that are deficient in IFN or TLR/MyD88 signaling (11, 55, 56). To directly assess IFN-β expression, we performed qRT-PCR to determine IFN-β mRNA levels in cells infected with GAS. The data shown represent relative IFN-β mRNA levels normalized to GAPDH mRNA. We did normalize to uninfected cells, since the IFN-β mRNA was very often below the levels that could be reliably detected by qRT-PCR. The experiment confirmed that the IFN-β expression was independent of TLR2, TLR4, and TLR9 (Fig. 3*D*). The IFN-β mRNA was also induced upon infection of MyD88$^{-/-}$ cells with GAS, albeit the amount was reduced if compared with WT cells (Fig. 3*C*). The reduced IFN-β mRNA levels in MyD88$^{-/-}$ did not result in diminished IFN signaling, since Stat1 activation was increased in these cells if compared with the controlled BMDMs (Fig. 3*A*). The functional activation of IFN/Jak/Stat signaling in MyD88$^{-/-}$ and WT cells was further confirmed by the induction of the type I IFN target gene *Mx2* (57). The transcription of *Mx2* was induced in both the control and MyD88$^{-/-}$ BMDMs with a kinetics that correlated well with the activation profile of Stat1 (Fig. 3*E*). Moreover, *Mx2* transcription was more strongly induced in MyD88$^{-/-}$ cells that also display increased Stat1 activation. Thus, despite the decreased IFN-β mRNA, the IFN/Jak/Stat1 signaling was more efficiently turned on in MyD88$^{-/-}$ cells, suggesting that the increased Stat1 phosphorylation was caused by a more efficient phosphorylation or a lower dephosphorylation rate. To prove the autocrine/paracrine role of the type I IFN in Stat1 activation, we examined Stat1 activation and IFN responses using BMDMs lacking the type I IFN receptor chain 1 (IFNAR1$^{-/-}$). As shown in Fig. 4*A*, Stat1 tyrosine phosphorylation was completely abolished in GAS-infected IFNAR1$^{-/-}$ cells despite robust induction of IFN-β gene transcription in these cells (Fig. 4*A*). Consistently, the induction of the type I IFN-responsive gene *Mx2* was entirely dependent on signaling by the IFNAR1 receptor (Fig. 4*C*). In most cell types, activation of the transcription factor IRF3 launches a feed forward type I IFN amplification loop through induction of IFN-β that drives the expression of

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

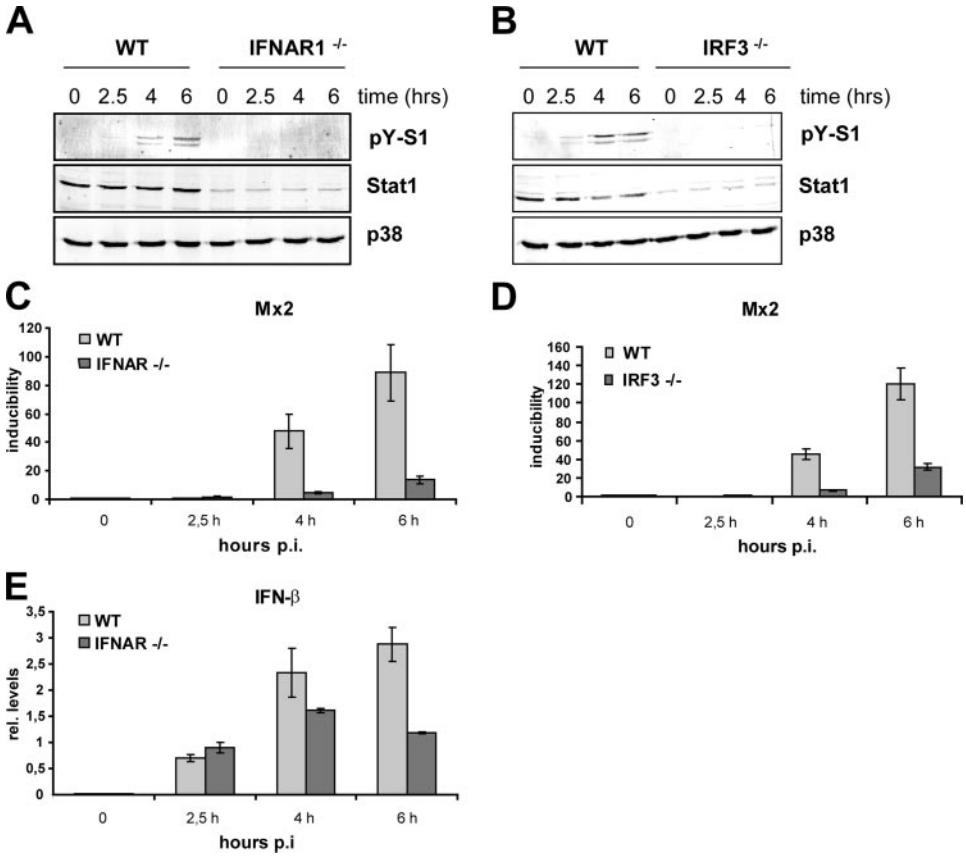


FIGURE 4. **GAS-induced IFN signaling depends on IRF3 and IFNAR1.** *A* and *B*, BMDMs from control (WT), IFNAR1$^{-/-}$ (*A*), and IRF3-deficient (*IRF3*$^{-/-}$) (*B*) mice were infected with GAS (MOI 100), and whole cell extracts were prepared 2.5, 4, and 6 h postinfection. Stat1 Tyr^{701} phosphorylation and expression were determined by Western blotting using antibody to phosphorylated Stat1 (pY-S1) and total Stat1. Differences in Stat1 expression levels were revealed by reprobing the membrane with antibody to total p38 MAPK (p38). *C* and *D*, BMDMs from IFNAR1$^{-/-}$ (*C*), IRF3$^{-/-}$ (*D*), and control (WT) mice were exposed to GAS for the indicated times, and total mRNA was extracted, reverse-transcribed, and analyzed by qRT-PCR for *Mx2* after normalization to GAPDH. *E*, BMDMs from IFNAR1$^{-/-}$ and control (WT) mice were infected with GAS (MOI 100). IFN-β expression was analyzed by qRT-PCR after normalization to GAPDH. *Bars*, S.D. of three experiments.

IFN-αs (19, 20, 58). Thus, a failure in activation of IRF3 results in a deficient production of both the immediate IFN-β and the late phase IFN-αs. To further elucidate the mechanism of GAS-induced IFN production, we infected IRF3$^{-/-}$ BMDMs with GAS and examined the activation of the transcription factor Stat1 and induction of the Stat1 target gene *Mx2*. Both the tyrosine phosphorylation of Stat1 and the transcription of the type I IFN-inducible gene *Mx2* were diminished in IRF3$^{-/-}$ (Fig. 4, *B* and *D*), whereas the MyD88 pathway was not affected by the IRF3 deletion, as revealed by p38 MAPK activation. These data demonstrate that GAS has the ability to induce IFN-β synthesis and IFN signaling in a way that mechanistically resembles the IFN-β activation by intracellular Gram-positive pathogens.

GAS Stimulates IFN Signaling Independently of Cytolysins—The TLR-independent IFN-β induction by GAS was surprising, since GAS is generally regarded as an extracellular pathogen that has only a limited capacity to survive in host cells (4–7). For the so far characterized induction of IFN-β production by Gram-positive bacteria, the expression of cytolysins was required (12, 23, 24). Cytolysins are thought to enable cytoplasmic escape of phagocytosed bacteria. In addition, cytolysins were also shown to directly stimulate IFN production (25). The GAS genome contains genes for two cytolysins. The *slo* gene encodes SLO (streptolysin O), which resembles other known cytolysins (*e.g.* listeriolysin, pneumolysin, and anthrolysin) in terms of sequence, sensitivity to oxygen, and cholesterol binding (59, 60). The other cytolysin, SLS (streptolysin S), is encoded by the *sagA* gene, which is unrelated to the cholesterol-binding cytolysins (60). To examine whether the GAS-derived cytolysins play a role in GAS-induced IFN signaling, *sagA* (SLS)-deficient, *slo* (SLO)-deficient, and double *sagA*/*slo*-deficient mutants were generated and used in infection assays. Analysis of Stat1 activation revealed that neither the deletion of the single cytolysin genes (data not shown) nor the double deletion (Fig. 5*A*) caused a reduction of GAS-induced IFN signaling. The inflammatory signaling was also not dependent on the expression of the GAS cytolysins, as shown by p38 MAPK activation (Fig. 5*B*). These findings suggest that the ability of GAS to induce IFN-β synthesis may not require a contact of GAS components with the still unknown cytoplasmic receptor that is used by other Gram-positive bacteria for IFN-β induction.

DISCUSSION

GAS is the etiological agent of a variety of human diseases. The heterogeneity of GAS diseases arises in part from the high diversity of GAS-mediated host-pathogen interactions in which virulence factors, GAS genome composition, prophage DNA, and plasticity of the GAS transcriptome play key roles (2, 61–64). A major factor influencing the severity of GAS infections is also the genetic inventory of the host. In humans, the differences in susceptibility to severe GAS diseases were mapped to the major histocompatibility complex locus (65). More severe infections and a generalized toxic shock syndrome appear to be associated with higher expression of inflammatory cytokines in both humans and mice (66). Cytokine production in GAS responses is regulated by T cells through GAS superantigens and the as yet poorly understood activation of innate immune cells. Our findings demonstrate that GAS is able to induce cytokines in macrophages through MyD88-dependent and -independent pathways. The identity of the GAS-recognizing receptor upstream of MyD88 poses an intriguing question,

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

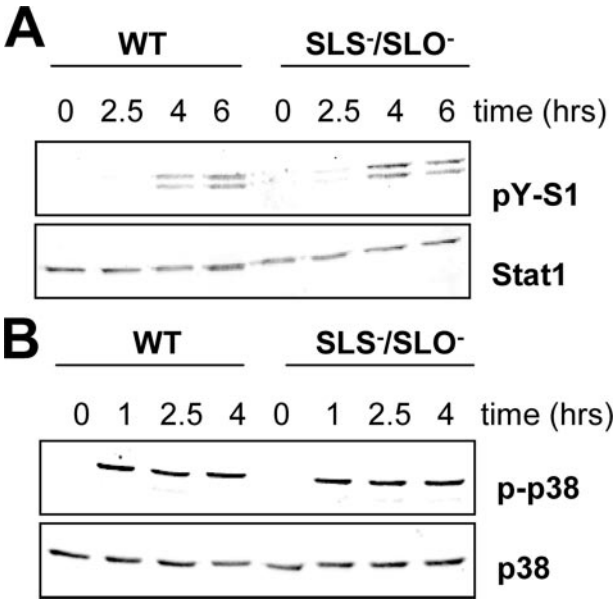


FIGURE 5. **GAS-derived cytolysins SLO and SLS are not required for induction of IFN and stress signaling.** *A* and *B*, BMDMs from wild-type mice were infected with either *S. pyogenes* WT strain or the isogenic mutant strain *sls/sagA* lacking both *sagA* (SLS) and *slo* (SLO) genes. At the indicated time points, activation of Stat1 (*A*) and p38 MAPK (*B*) was detected by Western blot analysis using antibodies to Tyr^{701}-phosphorylated Stat1 (pY-S1) and phosphorylated p38 MAPK (pp38). Equal protein loading was confirmed by reprobing the membranes with antibody to total Stat1 and p38 MAPK (p38), respectively. The data shown represent one of at least three independent infection experiments.

since our study rules out the exclusive involvement of the most prominent bacteria-recognizing receptors, TLR2, TLR4, and TLR9, or their combination. Other MyD88-dependent TLRs are the flagellin receptor TLR5 and the single-stranded RNA receptors TLR7 and TLR8 (8). Involvement of these receptors in GAS-induced MyD88-dependent signaling is not likely, since flagellin or a similar protein has not been found in GAS, and single-stranded RNA is associated with viral recognition. However, the role of these receptors cannot be entirely excluded, because PRRs are, in general, not specific for a single molecule. For example, TLR2 was reported to cooperate with dectin-1 in recognition of fungi (67). We ruled out the role of signaling through the IL-1R, which is known to be MyD88-dependent. IL-1 can be released by infected cells as a result of inflammasome-mediated caspase 1 activation, which is required for IL-1β processing (68). Although we did not address the GAS-mediated IL-1 production, a role for IL-1 as a possible mediator of the MyD88-dependent responses can be excluded. A function of IL-18 as another molecule requiring MyD88 for signaling is rather unlikely, since the IL-18 receptor is predominantly expressed on Th1 cells, whereas a prolonged treatment with *e.g.* IL-18 plus IL-12 is required for stronger expression in monocytic cells (69, 70). The ability of GAS to induce MyD88-dependent signaling independently of TLR2/4/9 may not be unique, since other Gram-positive pathogens have been reported to initiate inflammatory responses in the absence of multiple TLRs. For example, heat-inactivated group B streptococci and *Streptococcus pneumoniae* as well as spores of *Bacillus anthracis* induce MyD88-dependent responses in TLR2-, TLR4-, and TLR9-deficient macrophages or splenocytes (71–73). Despite the fact that TLR2/4/9 triple-deficient cells were not used in these studies, they support our hypothesis that some Gram-positive pathogenic bacteria are recognized by an as yet unidentified receptor upstream of MyD88.

The surprising finding that GAS is able to induce IFN-β independently of cytolysins represents another so far unique feature of GAS-induced responses in innate immune cells. In this regard, GAS-derived streptolysins are different from several other cytolysins that have been reported to induce IFN-β synthesis through activation of TLR4 (25). Another proposed mechanism of IFN-β induction by Gram-positive bacteria (*e.g. L. monocytogenes*) involves listeriolysin O-dependent liberation of bacteria and/or bacterial components from phagosomes (12, 23, 24). Although GAS is a prototype extracellular pathogen, it can be efficiently internalized by many phagocytic and nonphagocytic cells. However, GAS survives only for a short period of time in host cells due to degradation in lysosomal and autophagosomal compartments (5, 6). Interestingly, *Bacillus subtilis* engineered to express streptococcal SLO was not able to survive or multiply in infected cells, as opposed to listeriolysin O-expressing *B. subtilis* (74). This observation suggests that SLO, which displays a high degree of similarity with listeriolysin O, cannot mediate cytoplasmic escape. Thus, the reported data together with our findings support the hypothesis that GAS stimulates IFN-β synthesis by a novel mechanism that does not require cytoplasmic escape. Interestingly, the signaling events that culminate in Stat1 activation and transcription of IFN-responsive genes follow the well established pathway involving the transcription factor IRF3 and the type I IFN receptor but independent of MyD88. The increased IFN signaling in $MyD88^{-/-}$ cells may be explained by reduced expression of the inhibitor of IFN signaling SOCS1 (75). SOCS1 expression is known to be dependent on p38 MAPK (76), which we show in our study to be strongly reduced in GAS-infected $MyD88^{-/-}$ cells.

Our work demonstrates the ability of GAS to induce multiple inflammatory responses via in part novel recognition mechanisms. MyD88-dependent TLR2/TLR4/TLR9-independent signaling and cytolysin-independent IFN-β induction are so far exceptional characteristics of host cell responses to infection. Since GAS induces proinflammatory cytokine and IFN production in human macrophages (51), we assume that our findings are not restricted to the murine system, which, in the context of the whole organism, is considerably more resistant to GAS infection (66). The efficient stimulation of multiple signaling pathways may contribute to the known high capability of GAS to cause severe inflammatory diseases.

Acknowledgments—We thank Silvia Stockinger for critically reading the manuscript and Birgit Rapp for help with bacterial cultures.

REFERENCES

1. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) *Lancet* **5,** 685–694
2. Bisno, A. L., Brito, M. O., and Collins, C. M. (2003) *Lancet* **3,** 191–200
3. Chhatwal, G. S., and McMillan, D. J. (2005) *Trends Mol. Med.* **11,** 152–155
4. Staali, L., Morgelin, M., Bjorck, L., and Tapper, H. (2003) *Cell. Microbiol.* **5,** 253–265

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

5. Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S., and Yoshimori, T. (2004) *Science* **306,** 1037–1040
6. Hakansson, A., Bentley, C. C., Shakhnovic, E. A., and Wessels, M. R. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5192–5197
7. Thulin, P., Johansson, L., Low, D. E., Gan, B. S., Kotb, M., McGeer, A., and Norrby-Teglund, A. (2006) *PLoS Med.* **3,** e53
8. Akira, S., Uematsu, S., and Takeuchi, O. (2006) *Cell* **124,** 783–801
9. Lee, M. S., and Kim, Y. J. (2007) *Annu. Rev. Biochem.* **76,** 447–480
10. O'Neill, L. A., and Bowie, A. G. (2007) *Nat. Rev.* **7,** 353–364
11. Stockinger, S., Reutterer, B., Schaljo, B., Schellack, C., Brunner, S., Materna, T., Yamamoto, M., Akira, S., Taniguchi, T., Murray, P. J., Muller, M., and Decker, T. (2004) *J. Immunol.* **173,** 7416–7425
12. Stetson, D. B., and Medzhitov, R. (2006) *Immunity* **24,** 93–103
13. Takaoka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., and Taniguchi, T. (2007) *Nature* **448,** 501–505
14. Ishii, K. J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., and Akira, S. (2008) *Nature* **451,** 725–729
15. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) *Nat. Immunol.* **4,** 491–496
16. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscott, J. (2003) *Science* **300,** 1148–1151
17. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004) *J. Exp. Med.* **199,** 1641–1650
18. Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W. C., and Cheng, G. (2004) *J. Exp. Med.* **199,** 1651–1658
19. Levy, D. E. (2002) *J. Exp. Med.* **195,** F15–18
20. Taniguchi, T., and Takaoka, A. (2002) *Curr. Opin. Immunol.* **14,** 111–116
21. Strober, W., Murray, P. J., Kitani, A., and Watanabe, T. (2006) *Nat. Rev.* **6,** 9–20
22. Goldmann, O., Rohde, M., Chhatwal, G. S., and Medina, E. (2004) *Infect Immun.* **72,** 2956–2963
23. Stockinger, S., Materna, T., Stoiber, D., Bayr, L., Steinborn, R., Kolbe, T., Unger, H., Chakraborty, T., Levy, D. E., Muller, M., and Decker, T. (2002) *J. Immunol.* **169,** 6522–6529
24. O'Riordan, M., Yi, C. H., Gonzales, R., Lee, K. D., and Portnoy, D. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13861–13866
25. Park, J. M., Ng, V. H., Maeda, S., Rest, R. F., and Karin, M. (2004) *J. Exp. Med.* **200,** 1647–1655
26. Mangold, M., Siller, M., Roppenser, B., Vlaminckx, B. J., Penfound, T. A., Klein, R., Novak, R., Novick, R. P., and Charpentier, E. (2004) *Mol. Microbiol.* **53,** 1515–1527
27. Ruiz, N., Wang, B., Pentland, A., and Caparon, M. (1998) *Mol. Microbiol.* **27,** 337–346
28. Sierig, G., Cywes, C., Wessels, M. R., and Ashbaugh, C. D. (2003) *Infect. Immun.* **71,** 446–455
29. Caparon, M. G., Stephens, D. S., Olsen, A., and Scott, J. R. (1991) *Infect. Immun.* **59,** 1811–1817
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 1.74–1.84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Baccarini, M., Bistoni, F., and Lohmann-Matthes, M. L. (1985) *J. Immunol.* **134,** 2658–2665
32. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) *Immunity* **9,** 143–150
33. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752
34. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 443–451
35. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) *Nature* **408,** 740–745
36. Yasuda, K., Yu, P., Kirschning, C. J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., and Wagner, H. (2005) *J. Immunol.* **174,** 6129–6136
37. Kovarik, P., Stoiber, D., Novy, M., and Decker, T. (1998) *EMBO J.* **17,** 3660–3668
38. Alcamo, E., Mizgerd, J. P., Horwitz, B. H., Bronson, R., Beg, A. A., Scott, M., Doerschuk, C. M., Hynes, R. O., and Baltimore, D. (2001) *J. Immunol.* **167,** 1592–1600
39. Senftleben, U., Li, Z. W., Baud, V., and Karin, M. (2001) *Immunity* **14,** 217–230
40. Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W., and Ausubel, F. M. (2002) *Science* **297,** 623–626
41. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) *Nature* **416,** 750–756
42. Matsuzawa, A., Saegusa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Koyasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005) *Nat. Immunol.* **6,** 587–592
43. Abel, B., Thieblemont, N., Quesniaux, V. J., Brown, N., Mpagi, J., Miyake, K., Bihl, F., and Ryffel, B. (2002) *J. Immunol.* **169,** 3155–3162
44. Ferwerda, G., Girardin, S. E., Kullberg, B. J., Le Bourhis, L., de Jong, D. J., Langenberg, D. M., van Crevel, R., Adema, G. J., Ottenhoff, T. H., Van der Meer, J. W., and Netea, M. G. (2005) *PLoS Pathog.* **1,** 279–285
45. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., and Golenbock, D. T. (2004) *Nat. Immunol.* **5,** 190–198
46. Wei, X. Q., Leung, B. P., Niedbala, W., Piedrafita, D., Feng, G. J., Sweet, M., Dobbie, L., Smith, A. J., and Liew, F. Y. (1999) *J. Immunol.* **163,** 2821–2828
47. Gavrilin, M. A., Bouakl, I. J., Knatz, N. L., Duncan, M. D., Hall, M. W., Gunn, J. S., and Wewers, M. D. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 141–146
48. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., and Dixit, V. M. (2006) *Nature* **440,** 228–232
49. Ozoren, N., Masumoto, J., Franchi, L., Kanneganti, T. D., Body-Malapel, M., Erturk, I., Jagirdar, R., Zhu, L., Inohara, N., Bertin, J., Coyle, A., Grant, E. P., and Nunez, G. (2006) *J. Immunol.* **176,** 4337–4342
50. Glaccum, M. B., Stocking, K. L., Charrier, K., Smith, J. L., Willis, C. R., Maliszewski, C., Livingston, D. J., Peschon, J. J., and Morrissey, P. J. (1997) *J. Immunol.* **159,** 3364–3371
51. Miettinen, M., Lehtonen, A., Julkunen, I., and Matikainen, S. (2000) *J. Immunol.* **164,** 3733–3740
52. Gao, J. J., Filla, M. B., Fultz, M. J., Vogel, S. N., Russell, S. W., and Murphy, W. J. (1998) *J. Immunol.* **161,** 4803–4810
53. Toshchakov, V., Jones, B. W., Perera, P. Y., Thomas, K., Cody, M. J., Zhang, S., Williams, B. R., Major, J., Hamilton, T. A., Fenton, M. J., and Vogel, S. N. (2002) *Nat. Immunol.* **3,** 392–398
54. Decker, T., Muller, M., and Stockinger, S. (2005) *Nat. Rev.* **5,** 675–687
55. Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., Pfeffer, K., and Muller, M. (2000) *Immunity* **13,** 549–560
56. Park, C., Li, S., Cha, E., and Schindler, C. (2000) *Immunity* **13,** 795–804
57. Hug, H., Costas, M., Staeheli, P., Aebi, M., and Weissmann, C. (1988) *Mol. Cell. Biol.* **8,** 3065–3079
58. Panne, D., Maniatis, T., and Harrison, S. C. (2007) *Cell* **129,** 1111–1123
59. Alouf, J. E. (1980) *Pharmacol. Ther.* **11,** 661–717
60. Nizet, V. (2002) *Trends Microbiol.* **10,** 575–580
61. Beres, S. B., Sylva, G. L., Sturdevant, D. E., Granville, C. N., Liu, M., Ricklefs, S. M., Whitney, A. R., Parkins, L. D., Hoe, N. P., Adams, G. J., Low, D. E., DeLeo, F. R., McGeer, A., and Musser, J. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11833–11838
62. Beres, S. B., Richter, E. W., Nagiec, M. J., Sumby, P., Porcella, S. F., Deleo, F. R., and Musser, J. M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7059–7064
63. Cho, K. H., and Caparon, M. G. (2005) *Mol. Microbiol.* **57,** 1545–1556
64. Virtaneva, K., Porcella, S. F., Graham, M. R., Ireland, R. M., Johnson, C. A., Ricklefs, S. M., Babar, I., Parkins, L. D., Romero, R. A., Corn, G. J., Gardner, D. J., Bailey, J. R., Parnell, M. J., and Musser, J. M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 9014–9019

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

65. Kotb, M., Norrby-Teglund, A., McGeer, A., El-Sherbini, H., Dorak, M. T., Khurshid, A., Green, K., Peeples, J., Wade, J., Thomson, G., Schwartz, B., and Low, D. E. (2002) *Nat. Med.* **8,** 1398–1404
66. Medina, E., and Lengeling, A. (2005) *Brief. Funct. Genomics Proteomics* **4,** 248–257
67. Rogers, N. C., Slack, E. C., Edwards, A. D., Nolte, M. A., Schulz, O., Schweighoffer, E., Williams, D. L., Gordon, S., Tybulewicz, V. L., Brown, G. D., and Reis e Sousa, C. (2005) *Immunity* **22,** 507–517
68. Delbridge, L. M., and O'Riordan, M. X. (2007) *Curr. Opin. Immunol.* **19,** 10–16
69. Xu, D., Chan, W. L., Leung, B. P., Hunter, D., Schulz, K., Carter, R. W., McInnes, I. B., Robinson, J. H., and Liew, F. Y. (1998) *J. Exp. Med.* **188,** 1485–1492
70. Gerdes, N., Sukhova, G. K., Libby, P., Reynolds, R. S., Young, J. L., and Schonbeck, U. (2002) *J. Exp. Med.* **195,** 245–257
71. Henneke, P., Takeuchi, O., Malley, R., Lien, E., Ingalls, R. R., Freeman, M. W., Mayadas, T., Nizet, V., Akira, S., Kasper, D. L., and Golenbock, D. T. (2002) *J. Immunol.* **169,** 3970–3977
72. Lee, K. S., Scanga, C. A., Bachelder, E. M., Chen, Q., and Snapper, C. M. (2007) *Cell. Immunol.* **245,** 103–110
73. Glomski, I. J., Fritz, J. H., Keppler, S. J., Balloy, V., Chignard, M., Mock, M., and Goossens, P. L. (2007) *Cell. Microbiol.* **9,** 502–513
74. Portnoy, D. A., Tweten, R. K., Kehoe, M., and Bielecki, J. (1992) *Infect. Immun.* **60,** 2710–2717
75. Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Sprigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., Hertzog, P. J., Metcalf, D., and Hilton, D. J. (1999) *Cell* **98,** 597–608
76. Vazquez, N., Greenwell-Wild, T., Rekka, S., Orenstein, J. M., and Wahl, S. M. (2006) *J. Leukocyte Biol.* **80,** 1136–1144

Downloaded from www.jbc.org at MAX PERUTZ LIBRARY @IMP/VBC on July 22, 2008

5) DISCUSSION

5.1 Project 1: Lysogenic transfer of Group A streptococcus superantigen gene among streptococci

An unexplained resurgence of GAS infections observed since mid 1980s. Since then the occurrence of new and more severe infections has been observed. Particularly STSS caused by GAS was reported from Europe, USA and Japan (Cunningham, 2000) and SPEs were found to be involved in the pathogenesis of Streptococcal diseases. Furthermore GCSE and GGSE which are normally regarded as commensal organisms were found associated with streptococcal toxic shock syndrome (Hashikawa *et al.*, 2004). The question arises whether the increase in severe forms of infections by GAS is due to enhanced virulence of the pathogen itself or it is due to impairment in the host system. Many attempts to correlate a particular severe infection with a particular M serotype, could not establish a positive correlation between the two.

In GAS, bacteriophage-encoding virulence factors play a significant role in the evolution and the diversification of clones. Prophages constitute ~15% of the total GAS genome. Each of the 12 complete genome sequences of GAS contains 2 to 8 prophages per genome (Fischetti, 2007). Since many prophages contain putative or proven virulence genes on the integrated phage genome, the situation is highly conducive that GAS virulence factors can be transferred from one bacterium to others by lysogenic conversion.

Among bacterial virulence factors, superantigens are one of the most potent immunomodulatory agents. SSA is one of the widely distributed SAgs in *S. pyogenes*, which can generate symptoms like STSS. Three *ssa* alleles were found in the natural population of *S. pyogenes*, while *ssa-3* was the most predominant. SSA displays greater homology to the staphylococcal enterotoxins SEB and SEC rather than to the characterized streptococcal SAgs. A considerable number of superantigens are carried by prophages (SpeA, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM and SSA). Superantigen carrying bacteriophages have the capability to convert a non toxigenic strain into a

toxigenic strain via lysogenic conversion thus enhancing the virulence of the strains. Initial studies based on the hypothesis of intergeneric gene transfer, did not identify the *ssa* gene in *Staphylococcus aureus* clones and in Lancefield group B, C, or G streptococci (Reda *et al.*, 1996). However, later on, the identification of *ssa* in clinical isolates of GCSE and GGSE raised the possibility of interspecies gene exchange (Igwe *et al.*, 2003).

In this study, while trying to identify superantigen-encoding phages that could be released under mitomycin C treatment, we found ϕ149 from an M12 clinical isolate carrying the *ssa-3* allele. In addition to be induced by mitomycin C, ϕ149 release was also induced by hydrogen peroxide and ultraviolet radiation, which shows that ϕ149 is inducible and not a simply defective prophage like element. Sequence analysis of the att-L proximal region showed that ϕ149 is a chimeric phage as it shared one part of the phage sequence with ϕ370.2, ϕSPsP4, ϕ315.3 and ϕ370.2-like phages while the other part with ϕ315.2 and SPsP6 phages. This indicates that the recombination events have taken place in ϕ149 making it a chimeric phage. Later on, the complete genome sequence of MGAS10750 revealed ϕ10750.3 in an M4 serotype that harbours 100% att-L proximal region sequence to that of ϕ149 (Beres *et al.*, 2006). By performing lysogenization experiments we could show that ϕ149 can lysogenize seven different M serotypes (M1, M3, M5, M12, M19, M28 and M94) as well as the GCSE clinical isolates. Furthermore, ϕ149 could be transferred back from selected GAS and GCSE lysogenized strains to M12 serotype, showing that once the phage is integrated in the chromosome of the recipient strain, still the phage is active and can again infect other strains when a favorable condition is encountered. However lysogenic conversion could not be shown with GGSE. As some SAgs including the *ssa-3* allele have already been detected in GGSE (Igwe *et al.*, 2003), we think that such event can also be possible but the experimental conditions used in this study were most likely not optimal enough for their detection. This is the first direct experimental evidence of toxigenic conversion via lysogeny among several GAS serotypes and between GAS and GCSE.

In agreement to our data, recently a chimeric phage, ϕ3396, was isolated from GGSE, that not only exhibits high homology to ϕ315.1 of GAS phage but also the flanking genes of the 2 phages are the same, indicating that phage ϕ3396 has transferred to GGSE from

GAS by horizontal transfer (Davies *et al.*, 2007). Not only many GCS and GGS possess genes that encode molecules similar to SAgs but as well many GAS superantigens show sequence similarity to non-GAS superantigens indicating horizontal gene transfer (Zhao *et al.*, 2007). In addition, the globally disseminated clone M1T1 acquired several virulence factors, which also indicate the genetic exchange among streptococci (Aziz *et al.*, 2005; Aziz & Kotb, 2008).

Our data strongly support the hypothesis that the spread of superantigen genes in GCSE and GGSE have occurred by means of horizontal gene transfer from GAS using temperate phages. This event is quite likely as *S. pyogenes* and GCSE are phylogenetically closely related. Our data also question the validity of an old concept that M protein acts as a barrier for bacteriophage infection and bacteriophage transfer from one serotype to others (Cleary & Johnson, 1977; Spanier & Cleary, 1980), as our data establish the role of phage in the spread of virulence factors among the GAS population. It is quite evident now that bacteriophages are the major contributors to the genotype diversity and to the alteration of the pathogenic potential in GAS. Apart from enhancing the virulence repertoire of streptococci, acquisition of phage-encoded genes might also promote the colonization to new hosts or new ecological niches, enhanced survival, immune evasion and the emergence of fitter clones.

Given the significant role of prophages in toxigenic conversion and evolution of GAS strains, it would be important that additional information is obtained to fully understand the mechanisms of toxigenic conversion, transcriptional control of phage-encoded virulence factors, *in vivo* gene transfer, and effect of environmental signals or host signals. Simultaneously sequencing of the additional genomes would increase our knowledge about new prophage-encoded virulence factors and contribute to the advance understanding of the complex host-GAS interactions.

5.2 Project 2: A bioinformatic screen reveals novel small non-coding RNAs in *Streptococcus pyogenes*

In order to cause successful infection, bacterial pathogens need to control their virulence expression according to environmental signals. Up to recently, virulence gene expression

was thought to be mainly controlled by activator or repressor proteins. However it is quite evident now that sRNAs regulate gene expression more than previously anticipated (Johansson & Cossart, 2003; Shimizu *et al.*, 2002). In recent years, the combination of computational and molecular approaches has led to the identification of an increasing number of small, noncoding RNAs in bacteria (e.g. more than 80 small regulatory RNAs in *E. coli*). However, only a subset of these RNAs has been characterized. The most studied example of a regulatory RNA controlling bacterial virulence is the 512-nucleotide long RNAIII of *Staphylococcus aureus*, which affects both transcription and translation of virulence genes. In addition to RNAIII, the recent work by Johansson and coworkers has shown that the control of virulence gene expression in *Listeria monocytogenes* is dependent on an RNA thermosensor, the untranslated 5'-end of the *prfA* transcript. Information regarding sRNAs in the important human pathogen *S. pyogenes* is rather limited. So far only 3 putative regulatory sRNAs have been identified in *S. pyogenes*: *fasX*RNA, *pel*RNA and *riv*XRNA.

In this study we used a bioinformatic approach to identify novel sRNAs in *S. pyogenes* M1 strain SF370 as its whole genome is sequenced and it is one of the most representative serotypes. We used bioinformatic tools such as 'QRNA', 'RNAz', 'Alifoldz', 'sRNAPredict2' and the Rfam database to predict potential ncRNAs in intergenic regions. All together, 218 predictions were found at 178 loci. Candidates were chosen for Northern blot analysis on the basis of sRNAPredict2 prediction, level of sequence conservation, presence of strong RIT, presence of promoter, absence of any putative ORF and RBS. 90 of the loci were verified by Northern blot analysis and 29 sRNA (32%) were expressed under normal growth conditions. All of the selected candidates were mapped and analyzed for their stability, expression in stress conditions (pH, salt and temperature), secondary RNA structure prediction and mRNA target predictions. Overall we identified 5 riboswitches, 8 T-boxes, 5 ribosomal protein leaders, 4 functional RNAs and 5 novel sRNAs.

Comparing all the expressed RNA candidates to the Rfam database revealed 5 riboswitches, which are yybP-ykoY, Glycine, Purine, TPP and FMN. In order to validate our predictions about riboswitches, we chose FMN as an example to characterize further. Remarkably, *S. pyogenes* lacks the riboflavin synthetic genes and therefore bacterial

growth depends strictly on uptake of the metabolites from the environment, which suggests an essentiality of the riboflavin transporter gene. Northern blot and RT-PCR analyses showed that the expression of riboflavin transporter was dependent on the absence of riboflavin from the growth medium. Similarly *S. pyogenes* also lacks the terminal tryptophan biosynthetic pathway, therefore the growth of bacterium depends on tryptophan uptake from the environment. One of the unknown sRNA, candidate SpyRNA069, was found in 5' UTR region of gene Spy1016, which BLASTx analysis result indicated to be an ABC transporter substrate-binding protein, and which Panina *et al.* (2003) had predicted as tryptophan transporter. Northern blot and RT-PCR analyses indicated that indeed the Spy1016 gene has a role in tryptophan transport as this gene is expressed only in the absence of tryptophan from the growth medium. Our data shows that the metabolite transporter genes are controlled by sRNAs (residing in 5' UTR) in a way that they are expressed only when bacteria are in need of that metabolite. Our data suggest also a direct or indirect effect of these metabolites on the expression of the downstream genes via transcriptional termination or translational attenuation mechanisms involving the 5' UTR-located sRNAs. In *B. subtilis*, tryptophan biosynthesis genes are regulated either by tryptophan-activated RNA binding attenuation protein (TRAP) or by a T-box mechanism. In *S. pyogenes*, the TRAP protein-encoding gene is not present in the genome (Gutierrez-Preciado *et al.*, 2007) and the T-box consensus sequence and the structural features involved in the codon-anticodon pairing are not evident in the Spy1016 5' UTR. Thus, the expression of the *S. pyogenes* tryptophan transporter is most likely regulated by transcriptional attenuation mechanism. In gram-positive bacteria transcriptional termination is the most widely seen mode of gene regulation mechanism (Winkler & Breaker, 2003), however to our knowledge transcription attenuation mechanism operating for a tryptophan transporter has not yet been reported. Thus, taken together vital components in the regulation of expression of these important genes reside in the 5'UTRs, indicating the importance of riboswitches and leader elements in *S. pyogenes* gene regulation.

Our screen also revealed 5 novel sRNAs of unknown function. Three of these were located in 5' UTRs, one in 3' UTR while one was present in truly intergenic region. sRNA candidate SpyRNA049, which was truly present in the intergenic region, could be

a trans-acting regulatory sRNA. mRNA target analysis suggested the CAMP factor gene *cfa* as its target, as the 3' end of SpyRNA049 was base pairing with the 5' end of the *cfa* gene. As CAMP protein has been suggested to have a role in pathogenicity (Jurgens *et al.*, 1987), it was interesting to verify this target. But surprisingly, this base pairing also included the promoter region of the *cfa* gene, which either could be an artifact or a novel mechanism of regulation by sRNA binding to DNA. However, we could not observe an effect of SpyRNA049 at the level of transcription of *cfa*, but an effect at the level of translation can not be ruled out. SpyRNA049 is located near a CRISPR locus and immediately next to a CAS protein gene. As CRISPR has a role in defense against phages, it is possible that SpyRNA049 also has a role in this mechanism. But whether SpyRNA049 has a role associated with CRISPR in phage defense needs to be investigated. Interestingly, two of the novel sRNA candidates. SpyRNA014 and SpyRNA027 were found next to *scpA*, a gene encoding the virulence factor C5a peptidase and putative cardiolipin synthetase gene respectively. Thus novel sRNAs might play a role in the pathogenicity of *S. pyogenes* by regulating the expression of virulence. Therefore, it would be interesting to further characterize these candidates and explore their function in the pathogenicity, metabolism and physiology of bacteria.

Our bioinformatic screen identified eight T-boxes, which are *alaS*, *glyQS*, *ileS*, *pheST*, *thrS*, *trsA*, *valS* and *serS*. T-boxes were identified on the basis that these contained 18 nt T-box consensus sequence in the leader region and by pattern search program "fuzznuc". In gram-positive bacteria, the expression of many amino acid biosynthesis genes is regulated by T-box terminator-antiterminator mechanism (Grundy & Henkin, 1993). In *S. pyogenes* 7 out of 19 aminoacyl-tRNA synthetases (AARS) are regulated via a T-box mechanism (Steiner & Malke, 2001).

Five ribosomal protein leaders namely as L10, L13, L19, L20 and L21 were identified during the screen. In bacteria, ribosomal protein leaders are mainly regulated at the translational level by protein-mediated autoregulatory process. It is mainly described in *E coli*, where a protein released from the operon binds to its own mRNA and inhibits its translation (Haentjens-Sitri *et al.*, 2008; Zengel & Lindahl, 1994). There is limited information regarding the mechanism of ribosomal protein control in gram-positive bacteria, however it indicates rather a transcription attenuation mechanism (Choonee *et*

al., 2007). It would be interesting to investigate the mechanism of ribosomal proteins expression control in *S. pyogenes*.

This study is the first genomic search for sRNAs in the notorious human pathogen *S. pyogenes* and the first information regarding novel sRNAs, riboswitches, T-boxes and functional RNAs on a genome-wide basis. The discovery of riboswitches has helped to know the functions of many uncharacterized genes. Studies are further required as there may be a wide variety of molecular mechanisms for the control of gene regulation employed by riboswitches. As *S. pyogenes* is a poly-auxotrophic organism, it seems that it relies heavily on riboswitches for the control of important metabolite transport from the environment. Given the importance of riboswitches in bacteria, these might also serve as novel drug targets for the development of new classes of antibiotics. It would be interesting to further study novel sRNAs identified by *in silico* search to reveal their function and determine their role in pathogenicity and physiology of bacteria. As most of the sRNA candidates found in this *in silico* search were *cis*-encoded, bioinformatic programs needed to be improved so that they can allow-identifying trans-encoded RNAs in gram-positive bacteria.

5.3 Project 3: Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress and interaction with host cells

The *luxS*/AI-2 signaling pathway involved in cell-cell communication has been reported to interfere with important physiological and pathogenic functions in a variety of bacteria (Henke & Bassler, 2004; Vendeville *et al.*, 2005). Two recent studies reported a role of *luxS* in the regulation of virulence gene expression in *S. pyogenes* (Lyon *et al.*, 2001; Marouni & Sela, 2003). Here, we investigated the functional role of the *luxS*/AI-2 system in metabolism and diverse aspects of pathogenicity including the adaptation of the organism to stress conditions in two serotypes of *S. pyogenes*, M1 and M19.

In our study *luxS* expression analysis showed a monocistronic transcript of 664 bases expressed in a growth-phase dependent manner in GAS serotypes M1 and M19 whereas, a previous study based on sequence analysis had predicted *luxS* expression to be polycistronic in M6 serotype (Lyon *et al.*, 2001). Other studies showed that in

Streptococcus bovis, *luxS* is expressed in a growth phase-dependent manner but it was constitutively expressed in *Salmonella typhimurium* and V. *fischeri* (Asanuma *et al.*, 2004; Beeston & Surette, 2002; Lupp & Ruby, 2004). Similarly, in these species AI-2 like activity was observed in a temporal manner but it was not corresponding to the *luxS* expression. In our study as well, in *S. pyogenes* AI-2 like activity peaked at late-logarithmic phase, but *luxS* expression was already declining at that time point. In addition, induction experiments failed to show an auto-regulation mechanism of *luxS* expression i.e. regulation of luxS expression by AI-2, in GAS. Our results are in agreement with a previous study in *S. typhimurium*, where *luxS* expression and AI-2 like activity were not corresponding, thus suggesting that *luxS* expression was not regulated by AI-2 (Beeston & Surette, 2002).

Many reports have hypothesized the role of *luxS*/AI-2 in bacterial metabolism. Interestingly in *S. pyogenes* the AMC is incomplete as it lacks homocysteine methyltransferases (MetE or MetH) which converts SAH-derived metabolic product homocysteine to methionine. However, *S. pyogenes* contains the SAM synthetase ortholog (MetK), which converts methionine to SAM. If there is defect in *de novo* methionine synthesis, bacterial growth would depend exclusively on sulfur from external sources. Our data showed that the decrease in cysteine and methionine from media had similar effect on the growth and yield of both wild-type and *luxS* deficient strains, thus indicating that *S. pyogenes* is unable to convert homocysteine to methionine and inactivation of *luxS* did not trigger a metabolic burden on bacteria. It has also been described in *Borrelia burgdorferi* that it cannot recycle homocysteine to methionine (von Lackum *et al.*, 2006). Moreover, *luxS*-deficient strains did not have an effect on the viability and growth in various media including sulfur deficient minimal media, which suggest that *luxS* does not have an essential role in AMC-related metabolism. In *S. pyogenes*, recycling might be occurring by other types of methyltransferases.

During infection, bacteria face many different environmental challenges. Bacteria need to develop an adaptive response according to the challenging host conditions encountered. As regulatory role of *luxS* has already been shown in virulence factor expression, we investigated its role in adaptation to host-induced stress conditions. Although we could not find a role of *luxS* in adaptation to oxidative and salt stress, the *luxS*-deficient strain

survived better under acidic conditions. Accordingly, *luxS* expression and AI-2 production were also significantly lowered under low pH conditions. On the contrary, a previous study with *S. mutans* showed higher acid sensitivity (Sztajer *et al.*, 2008; Wen & Burne, 2004) and increased *luxS* expression at low pH in the *luxS* deficient strain (Hasona *et al.*, 2007; Lemos *et al.*, 2004). But the acid tolerance results need to be considered in reference to the different living habitat and pathogenesis of GAS and *S. mutans*. Furthermore, *luxS* expression and AI-2 production also decreased when cells were grown in RPMI medium supplemented with 10% serum, considered to be a host environment-mimicking medium. To further investigate that the down-regulation of *luxS*/AI-2 play a role when bacteria interact with host cells, we observed that *luxS*-deficient mutants survived 3 folds better in epithelial as well as in macrophage cells in both M1 and M19 serotypes compared to the wild-type strains. However, we could not find a significant difference in adherence rates to epithelial cells between the wild-type and *luxS*-deficient strains. Marouni *et al.* (2003) had also found that a *luxS*-deficient mutant survived better than the wild-type strain in epithelial cells at 4 hours after infection in an M3 serotype. Our results indicate that lowering the *luxS*/AI-2 response provides bacteria an advantage to survive inside host cells during infections.

After linking the role of *luxS*/AI-2 with adaptation to stress, we further wanted to investigate its role in virulence factor expression, as previous studies showed that *luxS* regulates SLS expression at transcriptional level and SpeB cysteine protease activity in an M6 serotype (Lyon *et al.*, 2001), SpeB and M protein at the transcriptional level and hyaluronic acid capsule at the post-transcriptional level (Marouni & Sela, 2003). Our data adds *fasX* and *sibA* (*psp*) as two additional targets of *luxS* to the list. *luxS* exerts a positive effect on the expression of *fasX*, a regulatory small RNA affecting virulence factor expression and suggested to have a role in local tissue destruction and bacterial aggressiveness towards host cells (Klenk *et al.*, 2005; Kreikemeyer *et al.*, 2001). *luxS* exerts a negative effect on *sibA* (*psp*), which is a virulence gene encoding a secreted immunoglobulin binding protein (Fagan *et al.*, 2001). However, *luxS* regulatory effects were seen only with M19 serotype, suggesting a strain or serotype dependent effects. *luxS*-strain specific manifestation of virulence-associated gene regulation was also observed in *Neisseria meningitides* and *Serratia marcescens* (Coulthurst *et al.*, 2004;

Dove *et al.*, 2003). Marouni *et al.* also attributed their dissimilar results with Lyon *et al.*, study about the effect of *luxS* on bacterial growth and level of SpeB regulation to a strain-specific effect (Marouni & Sela, 2003). Furthermore, differences in regulatory pathways among different GAS isolates can alter disease progression as mutations in the sensor encoding gene *covS* under selective pressure were shown to be correlated with human disease severity (Sumby *et al.*, 2006; Walker *et al.*, 2007).

Together with earlier studies demonstrating a role for *luxS* in the regulation of virulence properties in GAS, we suggest that *luxS* is not exclusively a key part of a detoxification pathway but seems to provide GAS a tool allowing to thrive under conditions often encountered during infections.

5.4 Project 4: Group A Streptococcus activates type I interferon production and MyD88-dependent signalling without involvement of TLR2, TLR4 and TLR9

GAS causes a variety of human diseases ranging from mild superficial infections like pharyngitis and impetigo to severe invasive infections like streptococcal toxic shock syndrome and necrotizing fasciitis. GAS ability to cause diversified diseases and the diversity of GAS-host interactions might be due to the fact that GAS has a number of properly regulated virulence factors, polylysogenization, GAS genome compositions and diversified transcriptome profiles. Host factors and interactions with the host cells also play major a role in the outcome of an infection. In this context, the activation of innate immune cells during GAS infections is poorly understood.

Our study showed that GAS is able to induce cytokines from macrophages by MyD88-dependent and independent pathways. Surprisingly, the induction was independent of the most commonly used receptors to recognize bacteria, TLR2, TLR4 and TLR9 and their combination. Remaining MyD88-dependent receptors flagellin recognizing TLR5 and single-stranded RNA recognizing TLR7 and TLR8 (Nakagawa *et al.*, 2004) are unlikely to be used as GAS recognizing receptors as flagellin is absent in *S. pyogenes* and single-stranded RNA are associated with viral recognition. However, roles of these receptors cannot be ruled out entirely as PRRs are not always specific for a single molecule like TLR2 also cooperate with dectin-1 to recognize fungi (Rogers *et al.*, 2005). Other gram-

positive pathogens have also been reported to induce MyD88-dependent signalling independent of multiple TLRs like heat-inactivated *S. pneumoniae* and group B streptococci as well as spores of *Bacillus anthracis*, which induce inflammatory responses in TLR2-, TLR4- and TLR9-deficient macrophages and splenocytes (Glomski *et al.*, 2007; Henneke *et al.*, 2002; Lee *et al.*, 2007) though TLR2/4/9 triple-deficient cells were not used in these studies. Moreover, recently a study in dendritic cells deficient in TLR1, TLR2, TLR4, TLR9 and TLR2/6 were not impaired in the secretion of proinflammatory cytokines against *S. pyogenes* (Loof *et al.*, 2008). All these findings are in accordance with our results that some gram-positive pathogens are recognized by a yet unidentified receptor upstream of MyD88.

Yet another interesting finding was that IFN-β synthesis was still observed when GAS deficient in SLS and SLO was used for infection thus indicating that GAS induces IFN-β independent of cytolysins. Several cytolysins have been reported to induce IFN-β synthesis through TLR4-dependent pathway, however GAS cytolysins are different from other cytolysins reported to induce IFN-β synthesis (Park *et al.*, 2004). In another IFN-β synthesis mechanism, *L. monocytogenes* induces IFN-β synthesis by involvement of escape from the phagosomes using listeriolysin O (O'Riordan *et al.*, 2002; Stetson & Medzhitov, 2006; Stockinger *et al.*, 2002). In the case of GAS, it is supposed to be an extracellular organism, however it can be internalized in many phagocytic and nonphagocytic cells (Thulin *et al.*, 2006) but degraded in lysosomal and autophagosomal compartments quickly (Hakansson *et al.*, 2005; Nakagawa *et al.*, 2004). In a study where SLO and LLO were expressed from *B. subtilis*, only LLO-expressing *B. subtilis* could multiply and survive in infected cells (Portnoy *et al.*, 1992). This indicates that although SLO shows high degree of sequence and functional similarity with LLO, it can not mediate cytoplasmic escape in GAS. All of these findings support the hypothesis that GAS induce IFN-β synthesis by a novel mechanism, which does not require cytoplasmic escape.

GAS induces multiple inflammatory responses using novel recognition mechanisms. Our data reveal some unique features of bacterial recognition like MyD88-dependent TLR2/TLR4/TLR9-independent signalling and cytolysin-independent IFN-β induction, by which GAS induces host cell responses to infection. Our findings are not only

restricted to the murine system as GAS also induces proinflammatory cytokines and IFN production in human macrophages (Medina & Lengeling, 2005). The ability of GAS to cause severe inflammatory diseases may be in part also because it can efficiently stimulate multiple signalling pathways.

6) REFERENCES

Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L. & Storz, G. (1997). A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* 90, 43-53.

Altuvia, S. & Wagner, E. G. (2000). Switching on and off with RNA. *Proc Natl Acad Sci U S A* 97, 9824-9826.

Andersen, J., Delihas, N., Ikenaka, K., Green, P. J., Pines, O., Ilercil, O. & Inouye, M. (1987). The isolation and characterization of RNA coded by the micF gene in Escherichia coli. *Nucleic Acids Res* 15, 2089-2101.

Asanuma, N., Yoshii, T. & Hino, T. (2004). Molecular characterization and transcription of the luxS gene that encodes LuxS autoinducer 2 synthase in Streptococcus bovis. *Curr Microbiol* 49, 366-371.

Ashbaugh, C. D., Moser, T. J., Shearer, M. H., White, G. L., Kennedy, R. C. & Wessels, M. R. (2000). Bacterial determinants of persistent throat colonization and the associated immune response in a primate model of human group A streptococcal pharyngeal infection. *Cell Microbiol* 2, 283-292.

Aziz, R. K., Ismail, S. A., Park, H. W. & Kotb, M. (2004). Post-proteomic identification of a novel phage-encoded streptodornase, Sda1, in invasive M1T1 Streptococcus pyogenes. *Mol Microbiol* 54, 184-197.

Aziz, R. K., Edwards, R. A., Taylor, W. W., Low, D. E., McGeer, A. & Kotb, M. (2005). Mosaic prophages with horizontally acquired genes account for the emergence and diversification of the globally disseminated M1T1 clone of Streptococcus pyogenes. *J Bacteriol* 187, 3311-3318.

Aziz, R. K. & Kotb, M. (2008). Rise and persistence of global M1T1 clone of Streptococcus pyogenes. *Emerg Infect Dis* 14, 1511-1517.

Banks, D. J., Beres, S. B. & 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., Lei, B. & Musser, J. M. (2003). Prophage induction and expression of prophage-encoded virulence factors in group A Streptococcus serotype M3 strain MGAS315. *Infect Immun* 71, 7079-7086.

Barrick, J. E. & Breaker, R. R. (2007). The distributions, mechanisms, and structures of metabolite-binding riboswitches. *Genome Biol* 8, R239.

Bassler, B. L., Greenberg, E. P. & Stevens, A. M. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* 179, 4043-4045.

Beeston, A. L. & Surette, M. G. (2002). *pfs*-dependent regulation of autoinducer 2 production in Salmonella enterica serovar Typhimurium. *J Bacteriol* 184, 3450-3456.

Beres, S. B., Sylva, G. L., Barbian, K. D. & other authors (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., Richter, E. W., Nagiec, M. J., Sumby, P., Porcella, S. F., DeLeo, F. R. & 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.

Bernheimer, A. W., Linder, R. & Avigad, L. S. (1979). Nature and mechanism of action of the CAMP protein of Group B Streptococci. *Infection and Immunity* 23, 838-844.

Bernish, B. & van de Rijn, I. (1999). Characterization of a two-component system in Streptococcus pyogenes which is involved in regulation of hyaluronic acid production. *J Biol Chem* 274, 4786-4793.

Bisno, A. L., Brito, M. O. & Collins, C. M. (2003). Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* 3, 191-200.

Biswas, I., Germon, P., McDade, K. & Scott, J. R. (2001). Generation and surface localization of intact M protein in *Streptococcus pyogenes* are dependent on *sagA*. *Infect Immun* 69, 7029-7038.

Boyd, E. F. & Brussow, H. (2002). Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends Microbiol* 10, 521-529.

Broudy, T. B. & Fischetti, V. A. (2003). In vivo lysogenic conversion of Tox(-) Streptococcus pyogenes to Tox(+) with Lysogenic Streptococci or free phage. *Infect Immun* 71, 3782-3786.

Brussow, H., Canchaya, C. & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* 68, 560-602.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A. & Brussow, H. (2003). Prophage genomics. *Microbiol Mol Biol Rev* 67, 238-276, table of contents.

Caparon, M. G. & Scott, J. R. (1991). Genetic manipulation of pathogenic streptococci. *Methods in Enzymology* 204, 556-586.

Caparon, M. G., Geist, R. T., Perez-Casal, J. & Scott, J. R. (1992). Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. *J Bacteriol* 174, 5693-5701.

Chaussee, M. A., Callegari, E. A. & 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.

Cho, K. H. & Caparon, M. G. (2008). tRNA modification by GidA/MnmE is necessary for Streptococcus pyogenes virulence: a new strategy to make live attenuated strains. *Infect Immun* 76, 3176-3186.

Choonee, N., Even, S., Zig, L. & Putzer, H. (2007). Ribosomal protein L20 controls expression of the Bacillus subtilis infC operon via a transcription attenuation mechanism. *Nucleic Acids Res* 35, 1578-1588.

Cleary, P. P. & Johnson, Z. (1977). Possible dual function of M protein: resistance to bacteriophage A25 and resistance to phagocytosis by human leukocytes. *Infect Immun* 16, 280-292.

Cleary, P. P., Kaplan, E. L., Handley, J. P., Wlazlo, A., Kim, M. H., Hauser, A. R. & Schlievert, P. M. (1992). Clonal basis for resurgence of serious *Streptococcus pyogenes* disease in the 1980s. *Lancet* 339, 518-521.

Cochrane, J. C. & Strobel, S. A. (2008). Riboswitch effectors as protein enzyme cofactors. *RNA* 14, 993-1002.

Commons, R., Rogers, S., Gooding, T., Danchin, M., Carapetis, J., Robins-Browne, R. & Curtis, N. (2008). Superantigen genes in group A streptococcal isolates and their relationship with emm types. *J Med Microbiol* 57, 1238-1246.

Coulthurst, S. J., Kurz, C. L. & Salmond, G. P. (2004). *luxS* mutants of Serratia defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. *Microbiology* 150, 1901-1910.

Cue, D., Dombek, P. E., Lam, H. & Cleary, P. P. (1998). Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells. *Infect Immun* 66, 4593-4601.

Cunningham, M. W. (2000). Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev* 13, 470-511.

Darmstadt, G. L., Mentele, L., Podbielski, A. & Rubens, C. E. (2000). Role of group A streptococcal virulence factors in adherence to keratinocytes. *Infect Immun* 68, 1215-1221.

Davies, M. R., McMillan, D. J., Van Domselaar, G. H., Jones, M. K. & Sriprakash, K. S. (2007). Phage 3396 from a Streptococcus dysgalactiae subsp. equisimilis pathovar may have its origins in streptococcus pyogenes. *J Bacteriol* 189, 2646-2652.

Delihas, N. (1995). Regulation of gene expression by trans-encoded antisense RNAs. *Mol Microbiol* 15, 411-414.

Delihas, N. & Forst, S. (2001). MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. *J Mol Biol* 313, 1-12.

Dove, J. E., Yasukawa, K., Tinsley, C. R. & Nassif, X. (2003). Production of the signalling molecule, autoinducer-2, by *Neisseria meningitidis*: lack of evidence for a concerted transcriptional response. *Microbiology* 149, 1859-1869.

Dunman, P. M., Murphy, E., Haney, S. & other authors (2001). Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. *J Bacteriol* 183, 7341-7353.

Efstratiou, A. (2000). Group A streptococci in the 1990s. *J Antimicrob Chemother* 45 Suppl, 3-12.

Ekelund, K., Skinhoj, P., Madsen, J. & Konradsen, H. B. (2005). Reemergence of emm1 and a changed superantigen profile for group A streptococci causing invasive infections: results from a nationwide study. *J Clin Microbiol* 43, 1789-1796.

Eran, Y., Getter, Y., Baruch, M., Belotserkovsky, I., Padalon, G., Mishalian, I., Podbielski, A., Kreikemeyer, B. & Hanski, E. (2007). Transcriptional regulation of the *sil* locus by the SilCR signalling peptide and its implications on group A streptococcus virulence. *Mol Microbiol* 63, 1209-1222.

Fagan, P. K., Reinscheid, D., Gottschalk, B. & Chhatwal, G. S. (2001). Identification and characterization of a novel secreted immunoglobulin binding protein from group A streptococcus. *Infect Immun* 69, 4851-4857.

Federle, M. J., McIver, K. S. & Scott, J. R. (1999). A response regulator that represses transcription of several virulence operons in the group A streptococcus. *J Bacteriol* 181, 3649-3657.

Fischetti, V. A. (2007). In vivo acquisition of prophage in Streptococcus pyogenes. *Trends Microbiol* 15, 297-300.

Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A. & Bjorck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J Biol Chem* 278, 16561-16566.

Gase, K., Ferretti, J. J., Primeaux, C. & McShan, W. M. (1999). Identification, cloning, and expression of the CAMP factor gene (cfa) of group A streptococci. *Infect Immun* 67, 4725-4731.

Glomski, I. J., Fritz, J. H., Keppler, S. J., Balloy, V., Chignard, M., Mock, M. & Goossens, P. L. (2007). Murine splenocytes produce inflammatory cytokines in a MyD88-dependent response to Bacillus anthracis spores. *Cell Microbiol* 9, 502-513.

Graham, M. R., Smoot, L. M., Migliaccio, C. A. & other authors (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. & Caparon, M. G. (2000). The RofA binding site in Streptococcus pyogenes is utilized in multiple transcriptional pathways. *J Bacteriol* 182, 1529-1540.

Grundy, F. J. & Henkin, T. M. (1993). tRNA as a positive regulator of transcription antitermination in B. subtilis. *Cell* 74, 475-482.

Gryllos, I., Levin, J. C. & Wessels, M. R. (2003). The CsrR/CsrS two-component system of group A Streptococcus responds to environmental Mg2+. *Proc Natl Acad Sci U S A* 100, 4227-4232.

Gutierrez-Preciado, A., Yanofsky, C. & Merino, E. (2007). Comparison of tryptophan biosynthetic operon regulation in different Gram-positive bacterial species. *Trends Genet* 23, 422-426.

Haentjens-Sitri, J., Allemand, F., Springer, M. & Chiaruttini, C. (2008). A competition mechanism regulates the translation of the Escherichia coli operon encoding ribosomal proteins L35 and L20. *J Mol Biol* 375, 612-625.

Hakansson, A., Bentley, C. C., Shakhnovic, E. A. & Wessels, M. R. (2005). Cytolysin-dependent evasion of lysosomal killing. *Proc Natl Acad Sci U S A* 102, 5192-5197.

Hashikawa, S., Iinuma, Y., Furushita, M., Ohkura, T., Nada, T., Torii, K., Hasegawa, T. & Ohta, M. (2004). Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. *J Clin Microbiol* 42, 186-192.

Hasona, A., Zuobi-Hasona, K., Crowley, P. J., Abranches, J., Ruelf, M. A., Bleiweis, A. S. & Brady, L. J. (2007). Membrane composition changes and physiological adaptation by Streptococcus mutans signal recognition particle pathway mutants. ***J Bacteriol*** **189, 1219-1230.**

Heath, A., DiRita, V. J., Barg, N. L. & Engleberg, N. C. (1999). A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. ***Infect Immun*** **67, 5298-5305.**

Henke, J. M. & Bassler, B. L. (2004). Bacterial social engagements. ***Trends Cell Biol*** **14, 648-656.**

Henneke, P., Takeuchi, O., Malley, R. & other authors (2002). Cellular activation, phagocytosis, and bactericidal activity against group B streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways. ***J Immunol*** **169, 3970-3977.**

Hidalgo-Grass, C., Ravins, M., Dan-Goor, M., Jaffe, J., Moses, A. E. & Hanski, E. (2002). A locus of group A Streptococcus involved in invasive disease and DNA transfer. ***Mol Microbiol*** **46, 87-99.**

Hofacker, I., Fontana, W., Stadler, P., Bonhoeffer, S., Tacker, M. & Schuster, P. (1994). Fast Folding and Comparison of RNA Secondary Structures. ***Monatshefte f Chemie*** **125, 167-188.**

Hondorp, E. R. & McIver, K. S. (2007). The Mga virulence regulon: infection where the grass is greener. ***Mol Microbiol*** **66, 1056-1065.**

Howe, R. A., Brown, N. M. & Spencer, R. C. (1996). The new threats of Gram positive pathogens: re-emergence of things past. ***J Clin Pathol*** **49, 444-449.**

Igwe, E. I., Shewmaker, P. L., Facklam, R. R., Farley, M. M., van Beneden, C. & Beall, B. (2003). Identification of superantigen genes speM, ssa, and smeZ in invasive strains of beta-hemolytic group C and G streptococci recovered from humans. ***FEMS Microbiol Lett*** **229, 259-264.**

Jaffe, J., Natanson-Yaron, S., Caparon, M. G. & Hanski, E. (1996). Protein F2, a novel fibronectin-binding protein from Streptococcus pyogenes, possesses two binding domains. ***Mol Microbiol*** **21, 373-384.**

Janzon, L. & Arvidson, S. (1990). The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in Staphylococcus aureus. ***Embo J*** **9, 1391-1399.**

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M. & Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* 110, 551-561.

Johansson, J. & Cossart, P. (2003). RNA-mediated control of virulence gene expression in bacterial pathogens. *Trends Microbiol* 11, 280-285.

Jurgens, D., Sterzik, B. & Fehrenbach, F. J. (1987). Unspecific binding of group B streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity. *J Exp Med* 165, 720-732.

Jürgens, D., Sterzik, B. & Fehrenbach, F. J. (1987). Unspecific binding of Group B Streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity. *Journal of Experimental Medicine* 165, 720-732.

Kapur, V., Nelson, K., Schlievert, P. M., Selander, R. K. & Musser, J. M. (1992). Molecular population genetic evidence of horizontal spread of two alleles of the pyrogenic exotoxin C gene (speC) among pathogenic clones of Streptococcus pyogenes. *Infect Immun* 60, 3513-3517.

Kapur, V., Reda, K. B., Li, L. L., Ho, L. J., Rich, R. R. & Musser, J. M. (1994). Characterization and distribution of insertion sequence IS1239 in Streptococcus pyogenes. *Gene* 150, 135-140.

Klenk, M., Koczan, D., Guthke, R., Nakata, M., Thiesen, H. J., Podbielski, A. & Kreikemeyer, B. (2005). Global epithelial cell transcriptional responses reveal *Streptococcus pyogenes* Fas regulator activity association with bacterial aggressiveness. *Cell Microbiol* 7, 1237-1250.

Kochiwa, H., Tomita, M. & Kanai, A. (2007). Evolution of ribonuclease H genes in prokaryotes to avoid inheritance of redundant genes. *BMC Evol Biol* 7, 128.

Kotb, M., Norrby-Teglund, A., McGeer, A. & other authors (2002). An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. *Nat Med* 8, 1398-1404.

Kreikemeyer, B., Boyle, M. D., Buttaro, B. A., Heinemann, M. & Podbielski, A. (2001). Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas) with homologies to two-component-type regulators requires a small RNA molecule. *Mol Microbiol* 39, 392-406.

Kreikemeyer, B., McIver, K. S. & 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., Nakata, M., Koller, T., Hildisch, H., Kourakos, V., Standar, K., Kawabata, S., Glocker, M. O. & Podbielski, A. (2007). The Streptococcus pyogenes serotype M49 Nra-Ralp3 transcriptional regulatory network and its control of virulence factor expression from the novel *eno ralp3 epf sagA* pathogenicity region. *Infect Immun* 75, 5698-5710.

Kwinn, L. A. & Nizet, V. (2007). How group A Streptococcus circumvents host phagocyte defenses. *Future Microbiol* 2, 75-84.

LaPenta, D., Rubens, C., Chi, E. & Cleary, P. P. (1994). Group A streptococci efficiently invade human respiratory epithelial cells. *Proc Natl Acad Sci U S A* 91, 12115-12119.

Lease, R. A. & Belfort, M. (2000). A trans-acting RNA as a control switch in Escherichia coli: DsrA modulates function by forming alternative structures. *Proc Natl Acad Sci U S A* 97, 9919-9924.

Leday, T. V., Gold, K. M., Kinkel, T. L., Roberts, S. A., Scott, J. R. & McIver, K. S. (2008). TrxR, a new CovR-repressed response regulator that activates the Mga virulence regulon in the Group A Streptococcus. *Infect Immun*.

Lee, K. S., Scanga, C. A., Bachelder, E. M., Chen, Q. & Snapper, C. M. (2007). TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Streptococcus pneumoniae. *Cell Immunol* 245, 103-110.

Lemos, J. A., Brown, T. A., Jr. & Burne, R. A. (2004). Effects of RelA on key virulence properties of planktonic and biofilm populations of Streptococcus mutans. *Infect Immun* 72, 1431-1440.

Levin, J. C. & 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.

Li, Z., Sledjeski, D. D., Kreikemeyer, B., Podbielski, A. & Boyle, M. D. (1999). Identification of *pel*, a *Streptococcus pyogenes l*ocus that affects both surface and secreted proteins. *J Bacteriol* 181, 6019-6027.

Liu, M., Hanks, T. S., Zhang, J., McClure, M. J., Siemsen, D. W., Elser, J. L., Quinn, M. T. & Lei, B. (2006). Defects in ex vivo and in vivo growth and sensitivity to osmotic stress of group A Streptococcus caused by interruption of response regulator gene *vicR*. *Microbiology* 152, 967-978.

Liu, M. Y., Gui, G., Wei, B., Preston, J. F., 3rd, Oakford, L., Yuksel, U., Giedroc, D. P. & Romeo, T. (1997). The RNA molecule CsrB binds to the global regulatory

protein CsrA and antagonizes its activity in *Escherichia coli*. *J Biol Chem* 272, 17502-17510.

Loof, T. G., Goldmann, O. & Medina, E. (2008). Immune recognition of Streptococcus pyogenes by dendritic cells. *Infect Immun* 76, 2785-2792.

Lukomski, S., Nakashima, K., Abdi, I., Cipriano, V. J., Ireland, R. M., Reid, S. D., Adams, G. G. & Musser, J. M. (2000). Identification and characterization of the scl gene encoding a group A Streptococcus extracellular protein virulence factor with similarity to human collagen. *Infect Immun* 68, 6542-6553.

Luo, F., Lizano, S. & Bessen, D. E. (2008). Heterogeneity in the polarity of Nra regulatory effects on streptococcal pilus gene transcription and virulence. *Infect Immun* 76, 2490-2497.

Lupp, C. & Ruby, E. G. (2004). Vibrio fischeri LuxS and AinS: comparative study of two signal synthases. *J Bacteriol* 186, 3873-3881.

Lyon, G. J. & Novick, R. P. (2004). Peptide signaling in Staphylococcus aureus and other Gram-positive bacteria. *Peptides* 25, 1389-1403.

Lyon, W. R., Madden, J. C., Levin, J. C., Stein, J. L. & Caparon, M. G. (2001). Mutation of luxS affects growth and virulence factor expression in Streptococcus pyogenes. *Mol Microbiol* 42, 145-157.

Malke, H., Steiner, K., McShan, W. M. & 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.

Mandal, M., Lee, M., Barrick, J. E., Weinberg, Z., Emilsson, G. M., Ruzzo, W. L. & Breaker, R. R. (2004). A glycine-dependent riboswitch that uses cooperative binding to control gene expression. *Science* 306, 275-279.

Manetti, A. G., Zingaretti, C., Falugi, F. & other authors (2007). Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation. *Mol Microbiol* 64, 968-983.

Mangold, M., Siller, M., Roppenser, B., Vlaminckx, B. J., Penfound, T. A., Klein, R., Novak, R., Novick, R. P. & Charpentier, E. (2004). Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule. *Mol Microbiol* 53, 1515-1527.

Maripuu, L., Eriksson, A. & Norgren, M. (2008). Superantigen gene profile diversity among clinical group A streptococcal isolates. *FEMS Immunol Med Microbiol*.

Marouni, M. J. & Sela, S. (2003). The luxS gene of Streptococcus pyogenes regulates expression of genes that affect internalization by epithelial cells. *Infect Immun* 71, 5633-5639.

McIver, K. S., Heath, A. S., Green, B. D. & Scott, J. R. (1995). Specific binding of the activator Mga to promoter sequences of the *emm* and *scpA* genes in the group A streptococcus. *J Bacteriol* 177, 6619-6624.

McIver, K. S. & Scott, J. R. (1997). Role of *mga* in growth phase regulation of virulence genes of the group A streptococcus. *J Bacteriol* 179, 5178-5187.

Medina, E., Goldmann, O., Toppel, A. W. & 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.

Medina, E. & Lengeling, A. (2005). Genetic regulation of host responses to group A streptococcus in mice. *Brief Funct Genomic Proteomic* 4, 248-257.

Meehl, M. A. & Caparon, M. G. (2004). Specificity of streptolysin O in cytolysin-mediated translocation. *Mol Microbiol* 52, 1665-1676.

Mitchell, T. J. (2003). The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol* 1, 219-230.

Molinari, G., Talay, S. R., Valentin-Weigand, P., Rohde, M. & 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.

Montange, R. K. & Batey, R. T. (2008). Riboswitches: emerging themes in RNA structure and function. *Annu Rev Biophys* 37, 117-133.

Morfeldt, E., Taylor, D., von Gabain, A. & Arvidson, S. (1995). Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *Embo J* 14, 4569-4577.

Musser, J. M., Nelson, K., Selander, R. K., Gerlach, D., Huang, J. C., Kapur, V. & Kanjilal, S. (1993). Temporal variation in bacterial disease frequency: molecular population genetic analysis of scarlet fever epidemics in Ottawa and in eastern Germany. *J Infect Dis* 167, 759-762.

Musser, J. M. & DeLeo, F. R. (2005). Toward a genome-wide systems biology analysis of host-pathogen interactions in group A Streptococcus. *Am J Pathol* 167, 1461-1472.

Nakagawa, I., Amano, A., Mizushima, N. & other authors (2004). Autophagy defends cells against invading group A Streptococcus. *Science* 306, 1037-1040.

Neely, M. N., Lyon, W. R., Runft, D. L. & Caparon, M. (2003). Role of RopB in growth phase expression of the SpeB cysteine protease of *Streptococcus pyogenes*. *J Bacteriol* 185, 5166-5174.

Nizet, V., Beall, B., Bast, D. J., Datta, V., Kilburn, L., Low, D. E. & De Azavedo, J. C. (2000). Genetic locus for streptolysin S production by group A streptococcus. *Infect Immun* 68, 4245-4254.

Nizet, V. (2002). Streptococcal beta-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol* 10, 575-850.

Novick, R. P. & Muir, T. W. (1999). Virulence gene regulation by peptides in staphylococci and other Gram-positive bacteria. *Curr Opin Microbiol* 2, 40-45.

Nudler, E. & Mironov, A. S. (2004). The riboswitch control of bacterial metabolism. *Trends Biochem Sci* 29, 11-17.

O'Riordan, M., Yi, C. H., Gonzales, R., Lee, K. D. & Portnoy, D. A. (2002). Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. *Proc Natl Acad Sci U S A* 99, 13861-13866.

Okada, N., Pentland, A. P., Falk, P. & Caparon, M. G. (1994). M protein and protein F act as important determinants of cell-specific tropism of Streptococcus pyogenes in skin tissue. *J Clin Invest* 94, 965-977.

Osterlund, A. & 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.

Palmer, M., Vulicevic, I., Saweljew, P., Valeva, A., Kehoe, M. & Bhakdi, S. (1998). Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* 37, 2378-2383.

Park, J. M., Ng, V. H., Maeda, S., Rest, R. F. & Karin, M. (2004). Anthrolysin O and other gram-positive cytolysins are toll-like receptor 4 agonists. *J Exp Med* 200, 1647-1655.

Petersson, K., Forsberg, G. & Walse, B. (2004). Interplay between superantigens and immunoreceptors. *Scand J Immunol* 59, 345-355.

Podbielski, A., Woischnik, M., Leonard, B. A. & Schmidt, K. H. (1999). Characterization of *nra*, a global negative regulator gene in group A streptococci. *Mol Microbiol* 31, 1051-1064.

Portnoy, D. A., Tweten, R. K., Kehoe, M. & Bielecki, J. (1992). Capacity of listeriolysin O, streptolysin O, and perfringolysin O to mediate growth of *Bacillus subtilis* within mammalian cells. *Infect Immun* 60, 2710-2717.

Rasmussen, A. A., Eriksen, M., Gilany, K., Udesen, C., Franch, T., Petersen, C. & Valentin-Hansen, P. (2005). Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* 58, 1421-1429.

Rasmussen, M., Eden, A. & Bjorck, L. (2000). SclA, a novel collagen-like surface protein of Streptococcus pyogenes. *Infect Immun* 68, 6370-6377.

Reda, K. B., Kapur, V., Goela, D., Lamphear, J. G., Musser, J. M. & Rich, R. R. (1996). Phylogenetic distribution of streptococcal superantigen SSA allelic variants provides evidence for horizontal transfer of ssa within Streptococcus pyogenes. *Infect Immun* 64, 1161-1165.

Reid, S. D., Montgomery, A. G. & 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. & 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.

Ribardo, D. A. & McIver, K. S. (2006). Defining the Mga regulon: Comparative transcriptome analysis reveals both direct and indirect regulation by Mga in the group A streptococcus. *Mol Microbiol* 62, 491-508.

Rivas, E. & Eddy, S. (2001). Noncoding RNA gene detection using comparative sequence analysis. *BMC Bioinformatics* 2, 1-19.

Roberts, S. A., Churchward, G. G. & Scott, J. R. (2007). Unraveling the regulatory network in *Streptococcus pyogenes*: the global response regulator CovR represses *rivR* directly. *J Bacteriol* 189, 1459-1463.

Roberts, S. A. & Scott, J. R. (2007). RivR and the small RNA RivX: the missing links between the CovR regulatory cascade and the Mga regulon. *Mol Microbiol* 66, 1506-1522.

Rogers, N. C., Slack, E. C., Edwards, A. D. & other authors (2005). Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22, 507-517.

Romby, P., Vandenesch, F. & Wagner, E. G. (2006). The role of RNAs in the regulation of virulence-gene expression. ***Curr Opin Microbiol*** **9, 229-236.**

Saenz, H. L., Augsburger, V., Vuong, C., Jack, R. W., Gotz, F. & Otto, M. (2000). Inducible expression and cellular location of AgrB, a protein involved in the maturation of the staphylococcal quorum-sensing pheromone. ***Arch Microbiol*** **174, 452-455.**

Sambrook, J., Fritsch, E. & Maniatis, T. (1990). ***Molecular Cloning. A Laboratory Manual,*** **2nd edn. New York: Cold Spring Harbor.**

Shelburne, S. A., 3rd, Sumby, P., Sitkiewicz, I., Granville, C., DeLeo, F. R. & Musser, J. M. (2005). Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva. ***Proc Natl Acad Sci U S A*** **102, 16037-16042.**

Shimizu, T., Yaguchi, H., Ohtani, K., Banu, S. & Hayashi, H. (2002). Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. ***Mol Microbiol*** **43, 257-265.**

Sierig, G., Cywes, C., Wessels, M. R. & 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.**

Sitkiewicz, I. & Musser, J. M. (2006). Expression microarray and mouse virulence analysis of four conserved two-component gene regulatory systems in group a streptococcus. ***Infect Immun*** **74, 1339-1351.**

Sorger-Domenigg, T., Sonnleitner, E., Kaberdin, V. R. & Blasi, U. (2007). Distinct and overlapping binding sites of Pseudomonas aeruginosa Hfq and RsmA proteins on the non-coding RNA RsmY. ***Biochem Biophys Res Commun*** **352, 769-773.**

Spanier, J. G. & Cleary, P. P. (1980). Bacteriophage control of antiphagocytic determinants in group A streptococci. ***J Exp Med*** **152, 1393-1406.**

Sriskandan, S., Unnikrishnan, M., Krausz, T. & Cohen, J. (2000). Mitogenic factor (MF) is the major DNase of serotype M89 Streptococcus pyogenes. ***Microbiology*** **146 (Pt 11), 2785-2792.**

Sriskandan, S., Faulkner, L. & Hopkins, P. (2007). Streptococcus pyogenes: Insight into the function of the streptococcal superantigens. ***Int J Biochem Cell Biol*** **39, 12-19.**

Steiner, K. & Malke, H. (2001). ***relA*****-Independent amino acid starvation response network of** ***Streptococcus pyogenes.*** ***J Bacteriol*** **183, 7354-7364.**

Stetson, D. B. & Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24, 93-103.

Stevens, D. L., Tanner, M. H., Winship, J., Swarts, R., Ries, K. M., Schlievert, P. M. & 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.

Stevens, D. L. (1992). Invasive group A streptococcus infections. *Clin Infect Dis* 14, 2-11.

Stevens, D. L. (2000). Streptococcal toxic shock syndrome associated with necrotizing fasciitis. *Annu Rev Med* 51, 271-288.

Stockinger, S., Materna, T., Stoiber, D. & other authors (2002). Production of type I IFN sensitizes macrophages to cell death induced by *Listeria monocytogenes*. *J Immunol* 169, 6522-6529.

Storz, G., Altuvia, S. & Wassarman, K. M. (2005). An abundance of RNA regulators. *Annu Rev Biochem* 74, 199-217.

Sumby, P., Whitney, A. R., Graviss, E. A., DeLeo, F. R. & 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.

Surette, M. G., Miller, M. B. & Bassler, B. L. (1999). Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A* 96, 1639-1644.

Sztajer, H., Lemme, A., Vilchez, R., Schulz, S., Geffers, R., Yip, C. Y., Levesque, C. M., Cvitkovitch, D. G. & Wagner-Dobler, I. (2008). Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effect of the *luxS* mutation. *J Bacteriol* 190, 401-415.

Terao, Y., Kawabata, S., Kunitomo, E., Murakami, J., Nakagawa, I. & Hamada, S. (2001). Fba, a novel fibronectin-binding protein from Streptococcus pyogenes, promotes bacterial entry into epithelial cells, and the fba gene is positively transcribed under the Mga regulator. *Mol Microbiol* 42, 75-86.

Thulin, P., Johansson, L., Low, D. E., Gan, B. S., Kotb, M., McGeer, A. & Norrby-Teglund, A. (2006). Viable group A streptococci in macrophages during acute soft tissue infection. *PLoS Med* 3, e53.

Tucker, B. J. & Breaker, R. R. (2005). Riboswitches as versatile gene control elements. *Curr Opin Struct Biol* 15, 342-348.

Valentin-Hansen, P., Eriksen, M. & Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. ***Mol Microbiol*** **51, 1525-1533.**

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M. & Hardie, K. R. (2005). Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. ***Nat Rev Microbiol*** **3, 383-396.**

Vitreschak, A. G., Rodionov, D. A., Mironov, A. A. & Gelfand, M. S. (2004). Riboswitches: the oldest mechanism for the regulation of gene expression? ***Trends Genet*** **20, 44-50.**

von Lackum, K., Babb, K., Riley, S. P., Wattier, R. L., Bykowski, T. & Stevenson, B. (2006). Functionality of ***Borrelia burgdorferi*** **LuxS: the Lyme disease spirochete produces and responds to the pheromone autoinducer-2 and lacks a complete activated-methyl cycle.** ***Int J Med Microbiol*** **296 Suppl. 40, 92-102.**

Voyich, J. M., Sturdevant, D. E., Braughton, K. R., Kobayashi, S. D., Lei, B., Virtaneva, K., Dorward, D. W., Musser, J. M. & DeLeo, F. R. (2003). Genome-wide protective response used by group A Streptococcus to evade destruction by human polymorphonuclear leukocytes. ***Proc Natl Acad Sci U S A*** **100, 1996-2001.**

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Vuong, C., Kobayashi, S. D., Porcella, S. F., Otto, M., Musser, J. M. & DeLeo, F. R. (2004). Engagement of the pathogen survival response used by group A ***Streptococcus*** **to avert destruction by innate host defense.** ***J Immunol*** **173, 1194-1201.**

Wagner, E. G., Altuvia, S. & Romby, P. (2002). Antisense RNAs in bacteria and their genetic elements. ***Adv Genet*** **46, 361-398.**

Walker, M. J., Hollands, A., Sanderson-Smith, M. L. & other authors (2007). DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. ***Nat Med*** **13, 981-985.**

Waters, C. M. & Bassler, B. L. (2005a). Quorum sensing: cell-to-cell communication in bacteria. ***Annu Rev Cell Dev Biol*** **21, 319-346.**

Waters, C. M. & Bassler, B. L. (2005b). Quorum sensing: cell-to-cell communication in bacteria. ***Annu Rev Cell Dev Biol*** **21, 319-346.**

Waters, C. M. & Bassler, B. L. (2006). The Vibrio harveyi quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. ***Genes Dev*** **20, 2754-2767.**

Wen, Z. T. & Burne, R. A. (2004). LuxS-mediated signaling in ***Streptococcus mutans*** **is involved in regulation of acid and oxidative stress tolerance and biofilm formation.** ***J Bacteriol*** **186, 2682-2691.**

Wessels, M. R. & Bronze, M. S. (1994). Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. ***Proc Natl Acad Sci U S A*** **91, 12238-12242.**

Wessels, M. R. (1999). Regulation of virulence factor expression in group A streptococcus. ***Trends Microbiol*** **7, 428-430.**

Winkler, W. C. & Breaker, R. R. (2003). Genetic control by metabolite-binding riboswitches. ***Chembiochem*** **4, 1024-1032.**

Winkler, W. C. & Breaker, R. R. (2005). Regulation of bacterial gene expression by riboswitches. ***Annu Rev Microbiol*** **59, 487-517.**

Wolz, C., McDevitt, D., Foster, T. J. & Cheung, A. L. (1996). Influence of agr on fibrinogen binding in Staphylococcus aureus Newman. ***Infect Immun*** **64, 3142-3147.**

Yarnell, W. S. & Roberts, J. W. (1999). Mechanism of intrinsic transcription termination and antitermination. ***Science*** **284, 611-615.**

Zengel, J. M. & Lindahl, L. (1994). Diverse mechanisms for regulating ribosomal protein synthesis in Escherichia coli. ***Prog Nucleic Acid Res Mol Biol*** **47, 331-370.**

Zhao, J., Hayashi, T., Saarinen, S. & other authors (2007). Cloning, expression, and characterization of the superantigen streptococcal pyrogenic exotoxin G from Streptococcus dysgalactiae. ***Infect Immun*** **75, 1721-1729.**

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. ***NAR*** **31, 3406-3415.**

7) ACKNOWLEDGEMENTS

I am grateful to my PhD supervisor Ass. Prof. Dr Emmanuelle Charpentier for providing me the chance to work under her leadership. My sincere thanks are for her time, training, guidance and helpful discussions during my PhD time.

I am also thankful to my PhD committee members; a.o. Prof. Dr. Rodger Novak, a.o. Prof. Dr. Angela Witte and a.o. Prof. Dr. Pavel Kovark for their precious time, valuable suggestions and support during my studies. Furthermore, my thanks to a.o. Prof. Dr. Thomas Decker, a.o. Prof. Dr. Manuela Baccarini, a.o. Prof. Dr. Pavel Kovark, a.o. Prof. Dr. Udo Blasi and a.o. Prof. Dr. Angela Witte and their students for providing me a scientific and cordial atmosphere conducive for research.

I should not forget to thank the Higher Education Commission (HEC) of Pakistan for providing me funding, which gave me education, exposure to the up-to-date techniques and training to broaden my horizons. I am also thankful to OÄD for providing me accommodation, their constant guidance, and support to solve all issues related to my stay in abroad.

My acknowledgement to my lab colleagues Rajendra Prasad Janpatla, Maria Siller, Maria Eckert, Karine Gonzales, Doris Veit and Daniela Zinkl for helping me a lot in the lab. I would like to thank as well to all my past and present lab colleagues for their cooperation.

My profound thanks go to my kind friends: Dr. Mohammd Arshad, Akhtar Banbhan, Abdul Ghafoor Khan, Asif Ali Shah, Mohammad Ali Paracha, Salim Manohara Das, Dr. Mukesh Kumar, Syam Kumar who tried their best to make my stay comfortable and gave me constant moral support. My special thanks for my brothers Amar Pirzada and Zarar Pirzada and my wife Saira Pirzada for their understanding, pains and patience.

I am deeply grateful to all the above people. Without their help, support and cooperation I would not have been able to achieve my goals.

8) CURRICULUM VITAE

PERSONAL DATA

Last name	:	**PIRZADA**
First name	:	**ZAID AHMED**
Nationality	:	Pakistan
Marital status	:	Married
Date of birth	:	October 15, 1973
Place of birth	:	Larkana, Pakistan

ACADEMIC DEGREES

2005: M.Phil. in Microbiology, University of Karachi, Pakistan
1997: M.Sc. in Clinical Microbiology, University of Karachi, Pakistan
1996: B.Sc. (Hons.) Microbiology, University of Karachi, Pakistan
1992: Intermediate in pre-medical, BISE Sukkur, Pakistan
1989: Matriculation, under BSE Karachi, Pakistan.

RESEARCH EXPERIENCE

1997: Undertook a Karachi University research project entitled "Resistance pattern of Salmonella among clinical isolates" under the supervision of Professor Shamim Ara Sidiqui.

1999 Worked as Research officer at ONR (USA) K.U. research project entitled "Marine Bacteriocins: ecological deterrence, extraction, purification and molecular characterization".

2001 Enrolled as M.Phil. student at the University of Karachi under the supervision of Dr Ajaz Rasool working on "Bacteriocins from marine environment: Screening, purification and molecular characterization".

2005 Enrolled as PhD student at the University of Vienna, Austria under the supervision of Dr Emmanuelle Charpentier on "Virulence factors in *Streptococcus pyogenes*: horizontal transfer and regulatory aspects".

TEACHING EXPERIENCE

2002-2005 Worked as Lecturer in the Department of Microbiology, University of Karachi, Pakistan.

1999-2002 Worked as full time as Cooperative teacher in the Department of Microbiology, University of Karachi, Pakistan.

1999-2001 Deputized as visiting teacher at the University of Sindh, Jamshoro, Pakistan.

FELLOWSHIP

2005-2008 Overseas Scholarship for PhD in selected fields from Higher Education Commisssion (HEC) Pakistan, ÖAD-Pakistan scholarship programme.

PUBLICATIONS

1999 **Pirzada, Z. A**., Sidiqui, S.A. and Ali, A. M. Antibiotic resistance pattern of Salmonella from clinical isolates. Pakistan Journal of Pharmacol. 16(1):13-19.

1999 Rasool, S.A., Subhan, S. A., Ali, A. M. and **Pirzada, Z. A**. Bacteriocinogenesis by marine bacteria from Arabian sea. Kar. Univ.J.Sc. 27(2):17-21.

2000 **Pirzada, Z. A.** and Rasool, S.A., Marine bacteria: Extraction, chemical properties and partial purification. Proceedings of ONR reseach projests on Arabian sea as a source of biological diversity. 166-171.

2004 Noor, N., Ajaz, M., Rasool, S.A. and **Pirzada, Z.A.** Urinary tract infections associated with multidrug resistant enteric bacilli:Characterization and genetic studies. Pak.J.Pharm.Sci. 17(2):115-123.

2004 **Pirzada, Z.A**., Ali, S.A., Khan, B.M. and Rasool, S.A. Production and physico-chemical characterization of bacteriocins-like inhibitory substances from marine bacterium ZM81. Pak.J.Biol.Sci., 7(12):2026-2030.

2008 Vojtek. I., **Pirzada, Z.A.**, Henriques-Normark, B., Mastny, M., Janapatla, R.P., Charpentier, E. Lysogenic transfer of group A Streptococcus superantigen gene among Streptococci. J. Infect. Dis. 197(2):225-234.

2008 Gratz, N, Siller, M., Schaljo, B., **Pirzada**, **Z. A.,** Gattermeier, I., Vojtek, I., Akira, S., E. Charpentier, E. and Kovarik, P. Group A type I interferon production and MyD-88 dependent signaling without involvement of TLR2, TLR4 and TLR9. J. Biol. Chem. 283(29):19879-19887.

2008 Siller, M., Janapatla, R.P., **Pirzada**, **Z. A.,** Hassler, C., Zinkl, D. and Charpentier, E. Functional analysis of the group A streptococcal *lux*S/AI-2 system in metabolism, adaptation to stress and interaction with host cells. BMC Microbiol. 8: 188.

ORAL PRESENTATION

2008 VBC student symposium II, Immunology, infection and pathogenicity, Vienna, Austria. **Pirzada, Z. A.**, Veit, D., Eckert, M. R., Gesell, T., Gonzales, K., Grabner, M., Gruber, A., Hofacker, I, Haeseler, A., Charpentier, E. A large-scale bioinformatic search for novel small, non-coding RNAs in *Streptococcus pyogenes.*

POSTER PRESENTATIONS

2006 7th ASM Conference on Streptococcal Genetics, St Malo, France. Vojtek, I., **Pirzada Z. A**., Henriques-Normark, B., Janapatla, R.P. and Charpentier, E. Transfer of a superantigen SSA-encoding gene via a novel chimeric bacteriophage among Group A streptococci.

2007 VBC student symposium I, Infection and immunity, Vienna, Austria. **Pirzada Z. A**., Vojtek, I., Henriques-Normark, B., Mastny, M., Janapatla, R.P. and Charpentier, E. Lysogenic transfer of Group A superantigen gene among streptococci.

2007 VBC student symposium I, Infection and immunity, Vienna, Austria. Gonzales, K., Siller, M., Lorenz, C., **Pirzada Z. A**., and Charpentier, E. Mode of action of *fasX* RNA on virulence factor expression in *Streptococcus pyogenes.*

2008 XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases, Porto Heli, Greece. Eckert, M., **Pirzada, Z. A.,** Veit, D. and Charpentier, E. Characterization of the flavin mononucleotide (fmn)-riboswitch and a 5' UTR regulatory element involved in tryptophan metabolism in *streptococcus pyogenes.*

2008 XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases, Porto Heli, Greece. Eckert, M., Gesell, T., **Pirzada, Z. A.,** Veit, D., Grabner, M., Gruber, A., Hofacker, I., Haeseler, v. A. and Charpentier, E. Screening for small, non-coding and functional RNAs in *Streptococcus pyogenes*: a bioinformatic approach.

2008 XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases, Porto Heli, Greece. Siller, M., Janapatla, R.P., **Pirzada**, **Z. A.,** Hassler, C., Zinkl, D. and Charpentier, E. Functions of the *luxS*/AI-2 system in M1 and M19 *Streptococcus pyogenes.*

2008 13th Annual Meeting of the RNA Society, Berlin, Germany. Eckert, M. R., Gonzales, G., **Pirzada, Z. A.,** Gesell, T., Veit, D., Grabner, M., Gruber, A., Hofacker, I., Haeseler, A. v. and Charpentier, E. Search for small, non-coding and functional RNAs in group A streptococcus.

2008 Toll 2008 Meeting, Lisbon, Portugal. Gratz, N., Siller, M., Schaljo, B., **Pirzada**, **Z. A.,** Gattermeier, I., Vojtek, I., Kirschning, C.J., Wagner, H., Akira, S., Charpentier, E. and Kovarik, P. Group A Streptococcus activates MyD88-dependent signalling and type I interferon production without involvement of TLR2, TLR4 and TLR9.

2008 SAB2008–MFPL Scientific Advisory Board Meeting Vienna, Austria. Gonzales, K., Deltcheva, E., Sharma, C., Eckert, M., Veit, D., **Pirzada, Z. A.,** Vogel, J., and Charpentier, E. Small RNAs as regulators of pathogenicity in S*treptococcus pyogenes.*