Thermoregulation of Capsule Production of Streptococcus pyogenes Strain HSC5

Trilce Michelle Galeas

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THERMOREGULATION OF CAPSULE PRODUCTION IN *Streptococcus pyogenes* STRAIN HSC5

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MD, Universidad Autónoma de Santo Domingo, Santo Domingo. Dominican Republic. 2005

A Thesis Submitted in Partial Fulfillment of the Requirements
For the Master of Science Degree

Department of Microbiology
Molecular Biology, Microbiology, and Biochemistry Graduate Program
Southern Illinois University, Carbondale, IL 62901
December, 2009.
THESIS APPROVAL

THERMOREGULATION OF CAPSULE PRODUCTION IN *Streptococcus pyogenes* STRAIN HSC5

By

Trilce Michelle Galeas Peña

A Thesis Submitted in Partial Fulfillments of the Requirements for the Degree of Master of Science in the field of Microbiology, Molecular Biology and Biochemistry

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Group A Streptococcus (GAS) is responsible for mild and common infections like tonsillitis and pharyngitis, and more serious invasive disorders like necrotizing fasciitis and glomerulonephritis. The ability to invade tissues is closely linked to the virulence factors expressed by the bacterium. Hyaluronic acid capsule expression is variable among all the strains in *S. pyogenes* and confers the capacity to evade the immune response. In a previous study, it was found that capsule production in CovR mutants was temperature-regulated, showing no capsule production at 37°C but increased production was observed at 25°C. In this study, the objective is to find the elements involved in the thermoregulation using a genetic approach. First, mutants were created by knocking-out CovR, the response regulator of the CovRS two-component system that controls about 15% of GAS genome. Transposon mutants were screened to find changes in capsular phenotype. Colonies expressing capsule at 37°C were selected for sequencing. The sequencing revealed three different events in different mutants. Two of them pointed at hypothetical proteins, one of them,
SpyM3_1255, was phage associated protein with a DnaD domain and the other one, SpyM3_1377, encoded cvfA. A third over-producer mutant showed an insertion in the promoter area of the has operon, the operon that encodes for hyaluronan synthase production, upstream from other disruptions in the promoter area that generated non-producing mutants. This suggest that there is more than one factor involved in thermoregulation of capsule production.
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INTRODUCTION

*Streptococcus pyogenes* is a Gram positive human pathogen that grows in chains (Figure 1) and is the causative agent of relatively mild diseases such as tonsillitis, pharyngitis, pyoderma and impetigo or serious invasive infections like necrotizing fasciitis and streptococcal toxic shock syndrome. In some cases, after infection with Group A Streptococcus (GAS) post-streptococcal systemic diseases like rheumatic fever, glomerulonephritis, endocarditis (91) and Sydenham’s chorea (56) could develop. *S. pyogenes* can be found naturally in the human skin and throat. It is an aerotolerant anaerobe called β-hemolytic because of the complete hemolysis pattern formed in blood agar (β-hemolysis) (67).

In the United States, each year there are over 10,000 cases of invasive streptococcus infections such as necrotizing fasciitis, bacteremia and toxic shock syndrome, resulting in 1,000 – 1,800 deaths (76).

Global epidemiology is unknown due to poor report and surveillance systems in most developing countries. However, it is estimated that there are at least 517,000 deaths each year linked to severe streptococcal infections and post-streptococcal diseases. The prevalence of GAS is at least 18.1 million cases with 1.78 million new cases each year (13) and there are at least 663,000 new cases with 163,000 deaths each year from invasive GAS infections. That added to the fact that there are more than 111 million cases of pyoderma and 616
million cases of pharyngitis per year, it is easy to conclude that there is a high prevalence of streptococcal diseases in the clinical practice.

Rebecca Lancefield, in 1928, published a method to serotype streptococcus based on its M protein, resulting in over 100 serotypes (59). Later, in 1946, she published another serotyping method based on the T protein, 20 serotypes are known in this system (60), and 4 of the antigens related to T protein are pili, a structure relevant to attachment to host cells (75).

Pathogenesis and virulence factors of S. pyogenes

There are several virulence factors in S. pyogenes that enable the bacterium to attach to host cells, invade deep tissues favoring spreading and avoid immune responses. Among those is the hyaluronic acid capsule which is the subject of this research and will be discussed in detail later.

M protein, which is a major factor in phagocytosis evasion uses its ability to bind two different components of the immune response (C4BP and IgA) inhibiting opsonization and phagocytosis (14). It is also related to the autoimmune response in rheumatic fever and rheumatic heart disease derived from post-streptococcal infection by eliciting a molecular mimicry mechanism and activating opsonic antibodies and lymphocytes (42). Another relevant characteristic of the well studied M protein is its interaction with peptides derived from the fifth domain of beta-2 glycoprotein I (beta-2-GPI), a human heparin binding plasma protein, which has antibacterial activities against Gram-positive and Gram-negative bacteria. Also, protein H and M1 protein, released from the bacterial cell wall by
FIGURE 1: Photomicrograph of Streptococcus bacteria, 900x magnification, showing its typical spherical shape and chain growth. (CDC Public Health Image Library). http://phil.cdc.gov/phil/details.asp
polymorphonuclear-derived proteases, bind to, and inhibit the activity of, beta(2)GPI-derived antibacterial peptides. Taken together, it is suggested that the interaction between the streptococcal proteins and beta(2)GPI or beta(2)GPI-derived peptides presents a novel mechanism to resist an antibacterial attack by beta(2)GPI-cleavage products (77).

Another virulence factor is F protein, which facilitates adherence to host cells (9), and is linked to severe soft tissue infections (80). It also mediates invasion in HeLa cells and enables the bacteria to be internalized, proving that M protein is essential for adhesion, but F protein is essential for internalization (82). F protein shall not be confused with streptococcal pyrogenic exotoxin SpeF, previously referred as mitogenic factor, which, in Toxic Shock-like Syndrome caused by Streptococcus, causes permeabilization of lung vessels, leading to Acute Respiration Distress Syndrome (70). Moreover, F protein has superantigenic properties. Fibronectin-binding properties of S. pyogenes are also mediated by protein F (50). While M protein of S. pyogenes mediates the binding of the bacterium to keratinocytes, protein F directs the adherence of the organism to Langerhans' cells (81).

Many of the virulence factors of GAS are not present in all clinical isolates, however, Streptolysin O (SLO), an oxygen-labile (thiol-activated) cytolysin, is present in most and is a powerful toxin that is partially responsible for hemolytic pattern and is highly cardiotoxic (49, 46). When absent, S. pyogenes shows a significant decrease in lethality and ability to cause disease (63).
A recent study found this pore-forming cytolytic streptolysin O as responsible and sufficient for apoptosis induction of macrophages and neutrophils involving caspase-1. This requires internalization of the bacterium by the phagocyte, contributing to GAS virulence and immune evasion (102). This toxin has the potential to establish a novel class of suicide gene therapeutic reagents (114). Membrane damage by SLO is analogous to that mediated by previously studied channel formers, namely, the C5b-9 complement complex and staphylococcal alpha-toxin (8). On capsule production, capsular and acapsular M3 strains were tested and the result was that the cytotoxic effects of streptolysin O protect GAS from phagocytic killing and enhance bacterial virulence, particularly of strains that may be relatively deficient in hyaluronic acid capsule (96).

Another key virulence factor is streptolysin S (SLS), that is partially responsible, along with SLO, for the beta-hemolytic phenotype. It is encoded by the sagA gene (28). Despite a long time since its discovery, its chemical structure remains unknown (74). SLS is not immunogenic; thus, no neutralizing antibodies are evoked during the course of natural infection, but recently, a synthetic peptide containing aminoacids 10 to 30 from the sagA gene coupled with keyhole limpet hemocyanin elicited an antibody response in a rabbit and reversed the hemolytic phenotype, giving cues to develop a new vaccine (28).

GAS also secretes streptokinase which binds and activates plasminogen (7), the enzyme that destroys fibrin clots. Interactions between bacteria, fibrinogen, streptokinase and plasminogen resulted in acquisition of cell-associated enzymatic activity that can lyse fibrin clots despite the presence of the
major physiological plasmin inhibitor, alpha 2-antiplasmin (107), and this interaction promotes bacterial invasion of tissues (101). It is strongly believed that streptokinase plays a mayor role in acute post-streptococcal glomerulonephritis by initiating the nephritis process by glomerular deposition, which leads to local activation of the complement cascade. Detection of streptokinase in kidney tissue increased with the degree of glomerular hypercellularity. Thus, the severity of the pathological process may be a reflection of the degree of streptokinase deposition (78, 79).

Virtually all strains of the human bacterial pathogen *Streptococcus pyogenes* express a highly conserved extracellular cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB) (65) that is used by GAS to kill phagocytes, as demonstrated by Lukomski et al, where a mutant lacking expression of SpeB was unable to resist phagocytosis and subsequent dissemination (66). SpeB also affects directly the production of hyaluronic acid capsule, when inactivated, extracellular hyaluronic acid levels decreased (112). It also degrades IgG, IgM, IgA, IgD and IgE (23). The mechanism to inactivate IgG is by cleaving IgG into Fc and Fab fragments, therefore, increasing bacterial survival (24, 37). Another proposed mechanism of phagocytic evasion by SpeB is by mitochondrial damage to the polymorphonuclear cells at an early infectious stage (18). As reviewed so far, SpeB cleaves or degrades host serum proteins such as human extracellular matrix, immunoglobulins, complement components, and even GAS surface and secreted proteins. Destruction of both host and
bacterial proteins makes SpeB the key virulence factor in GAS pathogenesis (19).

To invade host tissues, *S. pyogenes* uses hyaluronidase as one of the many virulence factors involved in invasion and dissemination. This chromosomal and phage-associated enzyme degrades hyaluronic acid from host tissues and increase adherence to epithelial host cells (6). A recent study found that hyaluronidase digests tissue hyaluronic acid and facilitates spread of large molecules but is not sufficient to cause subcutaneous diffusion of bacteria or to affect lesion size (98). Along with hyaluronidase, GAS also produces hyaluronic acid for its capsule, and another interesting finding was that hyaluronidase is not sufficiently active to remove capsule during peak synthesis (98).

**Purpose of this Study**

Human pathogens are exposed to changes in the environmental conditions, such as temperature, osmolarity and pH. In order to survive, bacteria have to regulate their gene expression according to environmental factors and, thus, ensure a successful colonization that allows them to thrive in the highly hostile human body.

Temperature is one of the most relevant environmental factors. Several thermoregulation studies have been done in other bacteria, demonstrating how differential gene expression may be temperature-dependent, especially in pathogenesis, where body temperature is a significant cue for the bacterium to start the virulence machinery.
In the specific case of Group A Streptococcus, where colonization of both skin surface and blood is possible, there must be a thermoregulation in the capsule production that allows the bacteria to switch from one place to another while evading phagocytosis and internalization.

Also, the products of temperature-regulated genes with central roles in physiology and virulence could be targets for novel therapeutics or mutation to generate defined live attenuated vaccines.

**Hyaluronic acid capsule structure and synthesis**

The most relevant virulence factor for this study is the hyaluronic acid (HA) capsule produced in the surface of the bacterial cell that plays a key role in virulence and invasiveness in mucoid strains (109). The capsule is also a mechanism to avoid oxygen damage by isolating the cell from the environment, making oxygen diffusion through the capsule much more slow, providing the bacterium with a shield from oxygen metabolites (22). The virulence of HA capsule is derived from its capacity to confer phagocytosis resistance to the bacterium (110) and is not necessary to be heavily expressed to confer full virulence, as demonstrated by Ashbaugh, where moderately encapsulated strains and heavily encapsulated strains posed the same invasiveness and virulence (4). The capsule in *Streptococcus pyogenes* is also required for biofilm formation by helping in the process of maturation into a tridimensional structure (20).
The composition of hyaluronic acid is shared by bacteria and mammalians alike, therefore the immune system is unable to recognize encapsulated bacteria leading to failed opsonization and phagocytosis (27). Encapsulated strains are very invasive in soft tissue infections, as oppose to uncapsulated strains that are internalized by keratinocytes (93) resulting in a noninvasive disease, and evidencing the fact that in order to spread, GAS needs to avoid phagocytosis and internalization and this is accomplished by capsule production (30). Introduction of GAS into the pharynx or deep tissues activates capsule production in capsulated strains (44), however, the underlying mechanism remains unknown, although several putative environmental triggers have been studied with little success. In this regard, a recent publication tried to identify the trigger, after noticing how mucoid strains on primary culture lost their encapsulated phenotype after laboratory passage, suggesting that the trigger must be present in the host environment and absent in most laboratory culture media. They found that cathelicidin LL-37, a human antimicrobial, had the ability to stimulate CovRS-regulated virulence factors (45).

HA is synthesized at the protoplast and its structure is a polymer of disaccharides, themselves composed of D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds. Hyaluronic acid can be 25,000 disaccharide repeats in length. Polymers of hyaluronan can range in size from 5,000 to 20,000,000 Da \textit{in vivo} (99, 73, 100) (Figure 2). The average molecular weight of extracellular HA is $2 \times 10^6$, and its production is only effective until the cell reach stationary phase, where production
is halted, possibly because of changes in membrane conformation (106). HA release after formation is mediated by an ABC transport system, coded adjacent to the HA synthase gene with a reading frame in opposite direction (84).

Synthesis of hyaluronic acid requires much energy and takes a high percentage of sugar, therefore, the obvious advantages of its synthesis is not widespread among pathogenic bacteria. The enzyme responsible for hyaluronic acid synthesis is Hyaluronic Acid Synthase. The gene encoding this enzyme was first discovered in 1993 from GAS (31) and after that, it has been described in different species in bacteria and mammals as well. These various hyaluronic acid synthases share many common features and aminoacid sequence regions identity or similarity (108). In Streptococcus pyogenes, the HA synthase is known as HAs Class I.

The first work identifying the HA synthase was in 1950 by Dorfman. In his paper, he described the enzyme as membrane-bound that needed Mg$^{++}$ and used two sugar-nucleotide substrates, uridine diphospho-glucuronic acid (UDP-GlcA) and uridine diphospho-H-acetylglucosamine (UDP-GlcNAc) to polymerize a hyaluronic acid chain (69) adding one after another at the reducing end (103). Different from proteoglycans, hyaluronic acid chain initiation does not need a protein backbone, the sole presence of the nucleotide sugar precursors are enough to elicit initiation and elongation (84).
FIGURE 2: Structure of hyaluronic acid. Hyaluronic acid consists of repeating disaccharides composed of $N$-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA).
HA synthase is very unique; it has two different functions as glycosyltransferase. The product of the first sugar addition is the substrate for the next addition (108). The overall reaction for the synthesis of one hyaluronan disaccharide unit is:

\[
\text{UDP-GlcA} + \text{UDP-GlcNAc} + (\text{HA})_n \xrightarrow{\text{hyaluronan synthase}} (\text{HA})_{n+1} + 2 \text{UDP}
\]

Although this reaction seems simple, it is believed that at least six different reactions take place to complete the cycle. Before it is released, the chain can grow to more than 40,000 monosaccharides, corresponding to a mass of more than 8 million Da. The sugar-nucleotide substrates are produced and used by the synthase inside the cell, and the hyaluronan chain is continuously transferred (translocated) so that it is extruded into the exterior of the cell to form the capsule (Figure 3). It was also believed that in absence of precursors, capsule would not be able to be synthesized and that hyaluronidase may play a role in solving this problem. To demonstrate a possible nutritional role for hyaluronidase, GAS was shown to grow with N-acetylglucosamine but not d-glucuronic acid (both components of HA) as a sole carbon source. However, only hyaluronidase (+) strains could grow utilizing HA as a sole carbon source, suggesting that hyaluronidase may permit the organism to utilize host HA or its own capsule as an energy source (98).

As bacterial hyaluronic acid is very similar to human and other vertebrates molecule, different strains of Streptococcus is being use for production and later use for purpose of human health and cosmetic industry (113). Also recombinant
FIGURE 3: Enzyme functions needed for hyaluronic acid biosynthesis. The diagram shows the membrane-bound hyaluronan synthase and the six independent activities required for the enzyme to make a disaccharide unit and extend the growing hyaluronic acid chain. (Image used with permission of Glycoforum. www.glycoforum.gr.jp).
HA from *E. coli* (115), *B. subtilis* (111) and Agrobacterium sp. (68) is used for commercial purposes.

**The hyaluronic acid synthesis (has) operon in Streptococcus species.**

Hyaluronic acid production is coded in an operon containing three genes: *hasA*, *hasB*, and *hasC*, which encode hyaluronan synthase, UDP-Glc dehydrogenase and UDP-Glc pyrophosphorylase, respectively (Figure 4). *hasA* codes for the enzyme responsible for the overall hyaluronic acid synthesis reaction (35), *hasB* is necessary to make UDP-GlcA, one of the substrates needed for HA production, from UDP-Glc in an oxidation that requires 2 NAD\(^+\) (34), and *hasC* codes for the enzyme that creates UDP-Glc from UTP and Glc-1-phosphate (25). Deletion analysis of the operon by Ashbaugh demonstrated that *hasC* gene, although it is part of the transcript, is not necessary for hyaluronic acid production, therefore *hasAB* are the only genes necessary for capsule production (5) and probably the *hasC* function is shared by other metabolic pathways in the cell. This operon is quite unique for Streptococcal species and recent phylogenetic studies show that there is no evidence of detectable inter-species lateral gene transfer suggesting intra-species genetic rearrangement as the origin of *has* operon (34). It also shows that the *hasA* is highly conserved among streptococcal strains, different from the M protein that is highly variable (32). Even when all streptococcal strains posses the operon, not every strain have the capsular phenotype. In acapsular phenotype, no operon mRNA
HAS OPERON: 3606 base pairs (bp)

P                        T
↓                        ↓

\[
\text{hasA (1259 bp)} \quad \text{hasB (1208 bp)} \quad \text{hasC (915 bp)} \quad \text{spyM3_1854} \quad \text{recF}
\]

FIGURE 4: General representation of has operon and its genomic context. The P represents the promoter area and the T is the terminator.
transcript is expressed (26). Further characterization of the operon showed a promoter consensus sequence upstream the hasA gene, and no terminator sequence was found on hasA, indicating that the has operon promoter could regulate transcription of the entire operon, thus producing a polycistronic mRNA (26), therefore, mutations in the has operon promoter or in the hasA gene lead to a polar effect.

The level of capsule production sometimes differs among individual strains of the same M-type. It is likely that strain-specific factors also affect capsule production along with the intrinsic strength of the has promoter conferred by the serotype-specific has promoter sequence (1).

**Streptococcus pyogenes gene regulation**

Gene regulation in *Streptococcus pyogenes* is mediated by several regulators that either activate or repress certain genes in response to growth phase or environmental triggers this is achieved by transcriptional regulator proteins, like Mga (12), a regulator of gene expression, which is responsible for the activation of expression during exponential growth of genes involved in adhesion, internalization and immune evasion and influences the expression of over 10% of all GAS genome, primarily genes and operons involved in metabolism and sugar utilization (53). Mga functions as a DNA-binding protein that interacts with sites both proximal and distal to the start of transcription for the
genes that it regulates (2). Ribardo et al. (89) described an open reading frame named amrA that is required for maximal activation of the Mga regulon during exponential phase and is strongly conserved across different streptococcal M types. Inactivation of amrA in an M3 serotype also resulted in reduction of the gene coding M protein, emm, transcripts, therefore, the role of amrA does not appear to be serotype specific. Although the specific function of AmrA is unknown, its putative membrane localization and homology to transporters involved in cell wall synthesis suggest a link between growth and Mga (89).

Many virulence regulators in other bacteria respond to carbon catabolite repression, a potential catabolite-responsive element important for binding by the catabolite control protein A (CcpA) was found in the promoter area of Mga upstream of its distal P1 start of transcription, supporting the role for CcpA in the early activation of the Mga promoter and establishing a link between carbon catabolite repression and Mga regulation in GAS (3). A link in a regulatory pathway has been found between CovRS and Mga, this is mediated by a novel two-component system regulator, which is CovR repressed, named TrxR that regulates the expression of Mga regulon genes, therefore, connecting CovR expression with Mga, affecting pathogenesis in GAS (61).

Gene regulation in S. pyogenes is also achieved by two-component systems like CovRS, the global response regulator that mediates expression of about 15% of all GAS genome (43).

Two-component regulatory systems constitute an extensive superfamily of pairs of functionally linked proteins that modulate the activity of biochemical
pathways and regulate the expression of virulence factors in many bacteria. The sensor component typically consists of a membrane-bound protein that responds to an environmental signal by phosphorylating a conserved histidine residue in a cytoplasmic transmitter domain of the sensor protein. The phosphate group is subsequently transferred from the sensor protein to a conserved aspartic acid residue in the receiver domain of the response regulator, a cytoplasmic protein. Regulator proteins of this type influence expression of target genes by acting as transcriptional activators or repressors, and their activity at binding sites upstream of target genes depends upon the phosphorylation state of the protein. Phosphorylation alters the DNA binding properties of the response protein regarding either affinity or target specificity.

Levin and Wessels described the effect of the system on hyaluronic acid capsule production and named the system csrRS for “capsule synthesis regulator” and found that csrR was the negative response regulator and csrS was the sensor component and are very conserved among Streptococcus (62). Federle found five different sites of CovR binding to the has operon promoter area and concluded that, except for the almost overlapping binding site pair CB-1 and -2, binding of CovR to each of the five sites surrounding the hasA promoter is independent of binding at the other sites. However, binding at all of these sites is needed for complete repression of this promoter in vivo (38).

CovRS protein products are members of the OmpR/EnvZ two-component signal transduction family (29). It is widely accepted that CovS mediates CovR phosphorylation, however, further studies gave evidence that in some
exceptional cases CovS may not be needed for CovR phosphorylation and consequent activation, even when these two proteins are cotranscribed, there could be another element in the cell able to phosphorylate CovR in absence of CovS (29). Phosphorylation of CovR by acetyl phosphate results in dimerization (48) significantly enhancing repression of one group of genes (e.g. speA, hasA, ska) while reducing repression of a second group (e.g. speB, grab, spd3) (105).

CovS is required for growth under stress conditions like high temperature, high salt concentration and low pH (29). CovRS has been described as a double-negative regulator, where CovR represses transcription of the covRS operon by promoter occlusion (47), so as long as CovR is active, very little new CovR protein is made.

The consensus region of CovR binding at the promoter area of both covR and has operons is ATTARA, where R could be any purine. In this sequence the T residues key in CovR binding (40). Another consequence of stress-induced inactivation of CovR is derepression of CovR synthesis. It can be expected that under stress conditions, more inactive CovR accumulates so that when stress is relieved and CovR becomes active, there is a rapid repression response. The design of this system thus makes it very sensitive to environmental change, like temperature. Since CovR-regulated promoters may vary in their degrees of sensitivity to CovR phosphorylation, CovR-regulated genes may exhibit differential expression under both normal and stress conditions and may respond to various degrees when the GAS encounters stress conditions (29).
CovR is also regulated by RocA, named for “regulator of Cov”, which plays an important role in regulation of the operon by increasing its expression and activation of covR transcription is increased about threefold. As expected, a rocA mutant is mucoid and produces more transcript from the has promoter since this promoter is repressed by CovR. This regulator is present in GAS but not in other species like Group B Streptococcus or Group G Streptococcus (10).

There are other regulators than the previously described CovRS and Mga. For instance, the RofA-like proteins (RALP) including RofA and Nra (39), regulate genes involved in persistence; FasA, which is involved in growth phase regulation of virulence gene expression (58) and Rgg (RopB), which activates expression of extracellular proteins such as SpeB (16). Rgg has also been shown to play a role in regulating the metabolic activities of GAS (17).

**Thermoregulation in bacterial gene expression**

Temperature is a relevant stress factor sensed by bacteria and used to regulate gene expression. Depending on the induction mechanism, two different pathways have to be distinguished, namely the heat shock response and the high temperature response. While the heat shock response is induced by temperature increments and is transient, the high temperature response needs a specific temperature to become induced and proceeds as long as cells are exposed to that temperature. The heat shock response is induced by denatured proteins and aimed to prevent definitive denaturation and formation of protein aggregates by refolding, and the high temperature response is mainly used by
pathogenic bacteria to detect entry into a mammalian host followed by induction of their virulence genes (94). Thus, thermoregulation in gene expression has been described in several bacterial species. Several pathogenic bacteria undergo temperature shift when going from one host to another or from one environment to another, e.g. skin to deep tissues. Genes are up or down-regulated depending on different temperatures; this difference, in some cases, has been linked to promoter sequence, as for maRAB and acrAB in *Salmonella thyphimurium* (51). Another report on Salmonella found a strong thermosensing for virulence phenotype where bacteria grown at 30°C or lower were unable to activate the intracellular type III secretion system even under strong inducing signals such as synthetic medium, contact with macrophages, and exposure to the murine gut (36).

Three different macromolecules have been identified being able to sense temperature: DNA, proteins and mRNA. For example, DNA supercoiling can control gene expression of pVY virulence plasmid in *Yersinia enterocolítica* (90) as well as *virB* expression in *Shigella* (87). Protein regulation that are temperature-dependent are found in the expression of flagella gene in Listeria species (95) and the two-component signal transduction BvgAS in *Bordetella pertussis* (55). RNA regulation is conferred by riboswitches and an example is the LcrF production in *Yersinia pestis* (52) that induces expression of several virulence factors. An example of how temperature regulates the expression of a two-component signal transduction is BvgSR in *Bordetella pertussis*, the causative agent of whooping cough (57). This system is transcribed and up-
regulated at 37°C and down-regulated at 25°C (88) and the mechanism is very similar to CovR activation by phosphorylation (55). Microarray studies with *Francisella tularensis* showed that after shifting temperature from 26°C to 37°C about 11% of the bacterium’s genes were differentially regulated and 40% of those genes are linked to pathogenesis (54). In group B streptococcus (GBS) changes in the genome occurred after incubation in blood at a temperature similar to those in patients with infection (about 40°C) (72), proving adaptative changes mediated by temperature in response to invasive infection. In a recent study in *E. coli* the effect of temperature on colicin K production was investigated and the results showed that expression was higher at 37°C than at 22°C and is hypothesized that SOS response indirectly regulates thermoregulation of colicin K (11). Another example of bacteria that uses temperature as a regulatory cue for pathogenicity is *Borrelia burgdorferi*, the causative agent of Lyme disease. It regulates expression of RevA in response to temperature, with the protein being synthesized by bacteria cultivated at 34°C but not by those grown at 23°C (15).

In some cases, a change in DNA topology, instead of promoter sequence, can lead to thermoregulation, as reported for virB transcription in *Shigella flexneri*, where a reduction in negative superhelicity at the promoter area was identified (104).

As for *Streptococcus pyogenes*, previous studies have related some gene expression and temperature. At least 9% of the genes are expressed differentially at 29°C relative to 37°C, as shown in microarray analysis. These genes include transporter proteins, proteins involved in iron homeostasis,
transcriptional regulators, phage-associated proteins, and proteins with no known homologue. Relatively few known virulence genes were differentially expressed at this threshold (97). However, at 40°C more gene expression was shifted, showing that there is thermoregulation in gene expression in GAS.

**Research Aims**

The aim of this study was to identify the gene(s) or protein(s) responsible for thermoregulation and which areas of the *has* operon promoter are involved. For this purpose transposon mutagenesis was performed.
MATERIALS AND METHODS

Media, Solutions and Standard Protocols

All the recipes for media, buffers and solutions used in this research are provided in Appendix A. The protocols are described in Appendix B.

Bacterial Strains and Plasmids

The bacterial strains used in this research and their relevant characteristics are described in Table 1. Plasmid vectors are listed in Table 2. Molecular cloning experiments were performed with Top10 E. coli cells (64) which were cultured in Luria-Bertani broth at 37°C with agitation at 250 rpm. For routine culture of S. pyogenes, Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium) was employed. For growth in liquid media, S. pyogenes was cultured at 37°C in sealed tubes without agitation. To produce solid media, Bacto agar (Difco) was added to a final concentration of 1.4% (wt/vol). When appropriate, antibiotics were added to the media (see Appendix A).

Plasmid construction

The plasmids used in this work are listed in Table 2. Gene manipulation and cloning were carried out according to standard protocols (Sambrook et al, 1989). Plasmid constructs were verified by PCR and gel electrophoresis.
Creation of CovR mutants

CovR is the response regulator in the CovRS two-component system that regulates capsule expression. CovR mutants were created by two methods. One was an insertional disruption on the covR gene that was achieved by using pSpc18 vector that confers spectinomycin resistance. The plasmid is a 2.94 kb suicide vector with a multicloning site and an origin of replication for E. coli. A fragment of the covR gene was inserted to create the disruption in the target loci of the chromosome. Also, an in-frame deletion mutant was created using the temperature induced plasmid pJRS233; no resistance is conferred after plasmid excision. Both transformations were performed as described in Appendix B.

Transposon mutagenesis

Electroporation was performed to introduce the transposon into competent S. pyogenes cells prepared as described in Appendix B. Mutants were plated in THY plates with the appropriate antibiotic and incubated at 37°C as described in standard methods (21). Transformants with insertions of pMGC8, a plasmid carrying a transposon, were then recovered following overnight incubation at 37°C on THY medium plates supplemented with kanamycin. Insertion was confirmed with PCR. For mutants displaying the desired phenotypes (overproducers or non-producers) the transposon insertion site was identified by chromosomal DNA sequencing. The strain used for transposon mutagenesis was CovRIFD which does not produce capsule at 37°C but produces it at 25°C.
**Capsule quantitation assay**

As different strains expressed capsule differently, a method to measure the amount of capsule was developed and optimized using Stains-all reagent; the complete protocol can be found in Appendix B. Stains-all is known to bind to acid polysaccharides. As hyaluronic acid is a highly acidic polysaccharide, Stains-all was used to quantitate the capsule amount produced by cells. The spectrophotometric readings were done using a 640 nm wavelength. The first method included a resuspension of THY medium pellet along with the colony to be measured. This method was discontinued because the media caused interference with the spectrophotometer readings. The new method included only the colony in the resuspension. Another problem was the continuous growth of cells when incubating at room temperature for 24 hrs after the initial 37°C incubation. To solve this problem, readings were done the same day using different sets of cells for each temperature to be measured.

A standard curve was created by measuring the absorbance of hyaluronic acid concentrations from 1 to 5 µL at 640 nm wavelength. The values obtained were plotted. The X axis represents optical density and Y axis the hyaluronic acid concentration. This generated an Y/X slope that was used to multiply the OD of the samples, the resulting number was then multiplied considering the dilution factor, which in this case was 2. The subsequent value was divided between the total cell number of the colonies and the result was converted from µg/cfu to fg/cfu. All samples were duplicated and the values were averaged.
## TABLE 1: Bacterial Strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong> Top10</td>
<td><em>hsdR, mcrA, lacZΔM15, endA1, recA1</em></td>
<td>(64)</td>
</tr>
<tr>
<td><strong>S. pyogenes strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC5</td>
<td>Wild type, M14 serotype</td>
<td>(50)</td>
</tr>
<tr>
<td>OPTM18</td>
<td>Transposon generated mutant with a disruption in <em>has</em> operon promoter area created with pMGC8. Overproducer phenotype</td>
<td>This study</td>
</tr>
<tr>
<td>OPTM 2</td>
<td>Transposon mutant with a disruption in a hypothetical protein created by pMGC8. Over-producer phenotype</td>
<td>This study</td>
</tr>
<tr>
<td>OPTM7</td>
<td>Transposon mutant with a disruption in a hypothetical protein created by pMGC8. Overproducer phenotype.</td>
<td>This study</td>
</tr>
<tr>
<td>ΩCovR</td>
<td>HSC5 with a mutation in the <em>covR</em> gene created with pSPC18</td>
<td>(84)</td>
</tr>
<tr>
<td>CovRIFD</td>
<td>HSC5 with an in-frame deletion in the <em>covR</em> gene created with pJRS233</td>
<td>This study</td>
</tr>
<tr>
<td>MGAS315</td>
<td>Wild type</td>
<td>(65, 85)</td>
</tr>
<tr>
<td>MGAS315ΩCovR</td>
<td>MGAS315 with an in-frame deletion in the <em>covR</em> gene created with pSPC18</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Relevant characteristics</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>pMGC8</td>
<td>Transposon containing <em>km</em> gene that confers kanamycin resistance.</td>
<td>(41)</td>
</tr>
<tr>
<td>pJRS233</td>
<td>Temperature-sensitive shuttle vector. Confers erythromycin resistance.</td>
<td>(86)</td>
</tr>
<tr>
<td>pSPC18</td>
<td>pUC18-based suicide vector containing <em>aad9</em> (spectinomycin resistance gene from <em>Enterococcus faecalis</em>).</td>
<td>(20)</td>
</tr>
<tr>
<td>pCIV2</td>
<td>pUC18-based streptococcal integration vector; contains <em>aphA3</em> conferring kanamycin resistance</td>
<td>(21)</td>
</tr>
<tr>
<td>pLZ12</td>
<td>Self replicating plasmid. Confers chloramphenicol and kanamycin resistance.</td>
<td>(29)</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Wild types and the generation of CovR mutants

One of the wild type strains used in this study was the non-mucoid HSC5, which shows no capsule production at any temperature. To study the thermoregulation of capsule production, CovR, the response regulator of the CovRS two-component system was mutated, conferring a less regulated capsule expression with an obvious temperature-dependent production. The CovR mutants presented a clear temperature-dependent phenotype where no capsule was produced at 37°C and there was capsule production after incubation at 25°C (Figure 5). Because HSC5 is not fully sequenced, MGAS315 was used for genomic and phenotypical comparison. It showed the same non-mucoid phenotype as HSC5.

To generate ΩCovR, the wild type was transformed with pSPC18, a plasmid carrying spectinomycin resistance that creates a disruption and partial duplication.

An in-frame deletion CovR mutant was also created using the temperature regulated plasmid pJRS233 carrying erythromycin resistance. The resulting phenotype was similar to the ΩCovR mutant. For transposon mutagenesis experiments, this was the strain used because the in-frame deletion generates no partial duplicates, as opposed to insertional disruption (Figures 25 and 26). And because no double antibiotic resistance genes are carried (for each plasmid insertion).
HSC5, MGAS315 and CovR mutant strains showed a low capsule reading at 37°C, around 40 - 130 fg/μL. The difference in capsule production between 37°C and 25°C was not significant, showing always low values. However, MGAS315 wild type usually had a higher values in capsule quantitation, compared to HSC5, but macroscopically, it showed no characteristic coat of hyaluronic acid, but only slightly larger colonies. For CovR mutants, the difference was significant (Tables 3-5) showing an increased production of hyaluronic acid capsule at 25°C (Figures 6-9). Only in one measurement, HSC5 showed capsule production (Tables 6-8, Figures 10-13) but further attempts to obtain the same phenotype failed, suggesting that the capsule production observed in HSC5 was a consequence of a technical or human error.

Measurements at 30°C were performed to determine if there was an intermediate phenotype at an intermediate temperature. The results for this reading showed that CovR mutants start their capsule production slowly after removal from the 37°C environment.

**CovRS complementation**

The phenotype of CovR mutants shows a temperature dependent capsule expression. To rule out a possible secondary mutation causing the phenotype a complementation experiment was performed. The plasmid used was pLZ12 containing a fragment of the CovRS two-component system. After electroporation, revertants were obtained, showing the same phenotype
observed in the wild type. Capsule quantitation assay demonstrated a lack of capsule production in the complementation mutants (Tables 9-11, Figures 14-17).

**Temperature dependence of capsule expression**

The mutant strains ΩCovR and CovRIFD showed a temperature-dependent expression of capsule. At 37°C, the strain showed no hyaluronic acid production, but at 25°C an evident coat of hyaluronic acid capsule was produced (Figure 5). In order to study the temperature dependence in HSC5, capsule measurements at 30°C were performed. It was observed that the amount of capsule produced at this intermediate temperature was lower than the amount produced at 25°C, but higher than the amount at 37°C (Tables 4, 7 and 10. Figures 7, 11 and 15).

**Transformation of MGAS315**

The strain used in this study, HSC5, is not fully sequenced and in order to have genomic profiles of the disrupted genes we used the sequence of MGAS315, a strain with the closest similarity to HSC5. The capsular phenotype of MGAS315 was the same as HSC5. An ΩCovR mutant using MGAS315 was created and the phenotype corresponded to that of the ΩCovR mutant with HSC5 background. Capsule quantitation assays showed only a slight increment in capsule amount (Tables 3-5), but the thermoregulated pattern of the MGAS315 CovR mutant was maintained (Figures 6-9).
FIGURE 5: Capsule phenotype. a) no capsule production. b) and c) capsule production.
**TABLE 3: Capsule quantitation of wild types and CovR mutants at 37°C**

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.017</td>
<td>720,000</td>
<td>140.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC5-2</td>
<td>0.018</td>
<td>730,000</td>
<td>147.14</td>
<td>144.02</td>
<td>4.41</td>
</tr>
<tr>
<td>MGAS315-1</td>
<td>0.020</td>
<td>700,000</td>
<td>170.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGAS315-2</td>
<td>0.020</td>
<td>780,000</td>
<td>153.01</td>
<td>161.75</td>
<td>12.36</td>
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<tr>
<td>ΩCovR-1</td>
<td>0.023</td>
<td>396,000</td>
<td>301.38</td>
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<tr>
<td>ΩCovR-2</td>
<td>0.021</td>
<td>394,000</td>
<td>257.48</td>
<td>279.43</td>
<td>31.04</td>
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<tr>
<td>MGAS315ΩCovR-1</td>
<td>0.020</td>
<td>480,000</td>
<td>285.94</td>
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<tr>
<td>MGAS315ΩCovR-2</td>
<td>0.017</td>
<td>500,000</td>
<td>250.63</td>
<td>268.28</td>
<td>24.96</td>
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</table>
TABLE 4: Capsule quantitation of wild types and CovR mutants at 30°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.020</td>
<td>440,000</td>
<td>264.51</td>
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<tr>
<td>HSC5-2</td>
<td>0.020</td>
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<tr>
<td>MGAS315-1</td>
<td>0.030</td>
<td>480,000</td>
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<tr>
<td>MGAS315-2</td>
<td>0.030</td>
<td>510,000</td>
<td>342.31</td>
<td>353.00</td>
<td>15.12</td>
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<tr>
<td>ΩCovR-1</td>
<td>0.100</td>
<td>244,000</td>
<td>2384.92</td>
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<tr>
<td>ΩCovR-2</td>
<td>0.090</td>
<td>236,000</td>
<td>2219.19</td>
<td>2302.05</td>
<td>117.18</td>
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<tr>
<td>MGAS315ΩCovR-1</td>
<td>0.120</td>
<td>370,000</td>
<td>1887.31</td>
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<tr>
<td>MGAS315ΩCovR-2</td>
<td>0.110</td>
<td>330,000</td>
<td>1939.73</td>
<td>1913.52</td>
<td>37.07</td>
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</table>
## TABLE 5: Capsule quantitation of wild types and CovR mutants at 25°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.05</td>
<td>3,600,000</td>
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<tr>
<td>HSC5-2</td>
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<td>3,700,000</td>
<td>78.64</td>
<td>79.73</td>
<td>1.54</td>
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<tr>
<td>MGAS315-1</td>
<td>0.04</td>
<td>3,300,000</td>
<td>70.54</td>
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<td></td>
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<tr>
<td>MGAS315-2</td>
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<td>3,200,000</td>
<td>72.74</td>
<td>71.64</td>
<td>1.55</td>
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<tr>
<td>ΩCovR-1</td>
<td>0.15</td>
<td>350,000</td>
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<tr>
<td>ΩCovR-2</td>
<td>0.13</td>
<td>33,000</td>
<td>2,292.41</td>
<td>2,393.18</td>
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<tr>
<td>MGAS315ΩCovR-1</td>
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<td>180,000</td>
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<tr>
<td>MGAS315ΩCovR-2</td>
<td>0.09</td>
<td>190,000</td>
<td>2,756.46</td>
<td>2,833.03</td>
<td>108.28</td>
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</tbody>
</table>
FIGURE 6: Capsule quantitation of wild types and CovR mutants at 37°C.

FIGURE 7: Capsule quantitation of wild types and CovR mutants at 30°C. Note the different capsule amount in the CovR mutants, showing that in those strains the capsule expression starts after leaving the 37°C incubation.
FIGURE 8: Capsule quantitation of wild types and CovR mutants at 25°C.

FIGURE 9: Effect of temperature in wild type and CovR mutants. Note how CovR mutants present a thermoregulated capsule phenotype, as opposed to wild types HSC5 and MGAS315.
<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.004</td>
<td>4,000,000</td>
<td>58.09</td>
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</tr>
<tr>
<td>HSC5-2</td>
<td>0.004</td>
<td>4,500,000</td>
<td>51.63</td>
<td>54.86</td>
<td>4.56</td>
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<tr>
<td>CovRIFD-1</td>
<td>0.004</td>
<td>6,500,000</td>
<td>35.75</td>
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<tr>
<td>CovRIFD-2</td>
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<td>6,600,000</td>
<td>44.00</td>
<td>39.87</td>
<td>5.84</td>
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<tr>
<td>ΩCovR-1</td>
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<td>7,800,000</td>
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<td>7,900,000</td>
<td>36.768</td>
<td>37.00</td>
<td>0.33</td>
</tr>
<tr>
<td>Strain (duplicated)</td>
<td>OD640</td>
<td>Colony number (cfu/mL)</td>
<td>Capsule conc (fg/cfu)</td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>------------------------</td>
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<td>---------</td>
<td>--------------------</td>
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<td>321.30</td>
<td>353.63</td>
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<tr>
<td>ΩCovR-1</td>
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<td>283.83</td>
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<td>ΩCovR-2</td>
<td>0.09</td>
<td>1,970,000</td>
<td>268.41</td>
<td>276.12</td>
<td>10.90</td>
</tr>
</tbody>
</table>
TABLE 8: Capsule quantitation of HSC5 and CovR mutants at 25°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.17</td>
<td>1,270,000</td>
<td>786.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC5-2</td>
<td>0.18</td>
<td>1,350,000</td>
<td>783.36</td>
<td>784.90</td>
<td>2.18</td>
</tr>
<tr>
<td>CovRIFD-1</td>
<td>0.18</td>
<td>1,590,000</td>
<td>665.12</td>
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<td></td>
</tr>
<tr>
<td>CovRIFD-2</td>
<td>0.17</td>
<td>1,470,000</td>
<td>679.44</td>
<td>672.28</td>
<td>10.13</td>
</tr>
<tr>
<td>ΩCovR-1</td>
<td>0.20</td>
<td>1,670,000</td>
<td>703.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR-2</td>
<td>0.22</td>
<td>1,600,000</td>
<td>807.84</td>
<td>755.73</td>
<td>73.69</td>
</tr>
</tbody>
</table>
FIGURE 10: Capsule quantitation of HSC5 and CovR mutants at 37°C.

FIGURE 11: Capsule quantitation of HSC5 and CovR mutants at 30°C.
FIGURE 12: Capsule quantitation of HSC5 and CovR mutants at 25°C.

FIGURE 13: Effect of temperature on HSC5 and CovR mutants. Note that HSC5 wild type showed increased capsule production at 30 and 25°C, which is not the usual behavior.
TABLE 9: Results of capsule quantitation of complementation strain and controls at 37°C.

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.003</td>
<td>422,000</td>
<td>45.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC5-2</td>
<td>0.003</td>
<td>427,000</td>
<td>45.43</td>
<td>45.70</td>
<td>0.38</td>
</tr>
<tr>
<td>ΩCovR-1</td>
<td>0.006</td>
<td>820,000</td>
<td>47.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR-2</td>
<td>0.007</td>
<td>1,030,000</td>
<td>43.94</td>
<td>45.63</td>
<td>2.38</td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-1</td>
<td>0.005</td>
<td>820,000</td>
<td>39.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-2</td>
<td>0.006</td>
<td>860,000</td>
<td>45.11</td>
<td>42.27</td>
<td>4.01</td>
</tr>
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</table>
TABLE 10: Capsule quantitation for complementation strain and controls at 30°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.006</td>
<td>730,000</td>
<td>47.05</td>
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</tr>
<tr>
<td>HSC5-2</td>
<td>0.006</td>
<td>760,000</td>
<td>45.19</td>
<td>46.12</td>
<td>1.31</td>
</tr>
<tr>
<td>ΩCovR-1</td>
<td>0.177</td>
<td>1,790,000</td>
<td>566.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR-2</td>
<td>0.171</td>
<td>1,680,000</td>
<td>582.68</td>
<td>574.37</td>
<td>11.75</td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-1</td>
<td>0.009</td>
<td>1,040,000</td>
<td>49.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-2</td>
<td>0.009</td>
<td>1,030,000</td>
<td>50.02</td>
<td>49.78</td>
<td>0.34</td>
</tr>
</tbody>
</table>
TABLE 11: Capsule quantitation for complementation strain and controls at 25°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5-1</td>
<td>0.008</td>
<td>1,030,000</td>
<td>44.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC5-2</td>
<td>0.007</td>
<td>1,060,000</td>
<td>37.80</td>
<td>41.13</td>
<td>4.71</td>
</tr>
<tr>
<td>ΩCovR-1</td>
<td>0.240</td>
<td>2,070,000</td>
<td>663.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR-2</td>
<td>0.243</td>
<td>1,980,000</td>
<td>693.89</td>
<td>678.81</td>
<td>21.33</td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-1</td>
<td>0.010</td>
<td>1,240,000</td>
<td>46.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΩCovR(pCovRS)-2</td>
<td>0.009</td>
<td>1,360,000</td>
<td>37.88</td>
<td>42.02</td>
<td>5.86</td>
</tr>
</tbody>
</table>
FIGURE 14: Capsule quantitation for CovRS complementation strain and controls at 37°C.

FIGURE 15: Capsule quantitation for CovRS complementation strain and controls at 30°C.
FIGURE 16: Capsule quantitation for CovRS complementation strain and controls at 25°C.

FIGURE 17: Effect of temperature in complementation strain and controls.
Transposon mutagenesis

The efficiency is expected to be high in this kind of experiments because the insertion is random and the mobile element is very stable. After electroporation and incubation, several mutants were screened and selected mutants showing anomalous capsule expression were tested for proper insertion and sequencing. The strain used in these experiments was CovRIFD. The capsular phenotypes observed in the transposon mutants were a) non-producer, which didn’t produce capsule at room temperature, and b) overproducer that produced capsule at 37°C, as opposed to the background strain CovRIFD. Approximately 20 non-producer colonies were examined and all of them showed insertions in either hasA gene or hasA promoter area. Of particular interest were the overproducer phenotype, and those were sequenced to identify the disrupted area. The mutants were named OPTM for “overproducing transposon mutant”.

The data obtained from the transposon mutagenesis experiments revealed more than one chromosomal location involved in the thermoregulation of capsule production and that a mutation in this system confers an increased capsule production at 37°C, thus, giving the potential of more virulence and invasiveness, adding more evidence to the fact that virulence regulation is complex in S. pyogenes and that it could respond to more than one molecular trigger.

Four mutants with three different insertion sites that resulted in massive capsule expression at 37°C, were analyzed. The strains were called OPTM2,
OPTM7, OPTM17 and OPTM18. All were sequenced to obtain the insertion location.

**OPTM2**

The first mutant sequenced was named OPTM2. Blasting the sequence against an MGAS315 genomic database showed an insertion in the gene *spyM3_1254* (Figure 18). There was a possibility that this disruption caused polarity effect on downstream genes and gave an overproducer phenotype. There are about 14 genes downstream of *spyM3_1254* that are also hypothetical proteins encoding phage associated proteins whose products have not been well characterized. Inactivation in any of these genes by polar effect may lead to an overproducing phenotype from a gene different from the one disrupted.

**OPTM7 and OPTM16**

Two other overproducers, OPTM7 and OPTM16 showed an insertion in the *cvfA* (*spyM3_1377*) gene (Figure 19). Downstream of *cvfA* there is a gene encoding a guanylate kinase. CvfA is a predicted HD superfamily hydrolase that in previous studies it has been found to be linked to virulence in *S. pyogenes*, as well as virulence in *B. subtilis* and *S. aureus* (71). As part of the continued research, protein work is being performed in order to elucidate the possible interaction between *cvfA* and thermoregulation of capsule production.
OPTM18

Another over-producer mutant, OPTM18, showed a different insertion site. Identification of the transposon insertion loci revealed that this mutant had an insertion in the intergenic area, upstream from the insertion site at the promoter area of hasA, found in non-producers (Figure 20).

There are five binding sites for CovR at the promoter area; none of these was affected by the transposon insertion, but this particular finding may imply that part of the sequence upstream from the promoter region of the has operon is involved in the thermoregulation in GAS, along with other elements found in the other mutants obtained in this study.

Capsule was measured in mutants and wild type strains. OPTM18 showed an increment in capsule production at 37°C compared with the wild type, reaching values higher than 900 fg/μL at 37°C (Figures 21 and 22).

However, insertional inactivation of the same promoter area region by site directed mutagenesis did not lead to any observable defect on capsule production.
**spyM3_1254:**

ATGATGAAGAAGAAAGGAAGAAGCATTTGGACAAAAAAGAAGGTTGGATAATTT
CCAAATAATTTGGATGCGTCTCTTATCACCATTGGAGATTGAGACATTAATAAG
▼
TGGATTTGACGAAGAGAAGATGCCTGGTGAAGTTGGTTAAGCGAGGCACTAAAAT
CGACAATTCTATAACGCACACAAAACCTTAGATACTTAAAACAGAGTCTTTGAAC
AACTGGAAGCGACAAGGGATTTGATACAGTCGAGAAAGTCGAATTTGCTAGGT
TGCAATTTGAAAATAAAAAGCTCAGTCAAAATAAAATCATCAATCCAACGTCC
CAAGCTGGTCGAATCCGACTACAAAAAGAACAGATTAAAAGAATTTTGACTA
stop
GGAAGCATAGACGGTATAGAAGATGGATCAGGAGATTTT
TAA

**FIGURE 18:** Complete sequence of *spyM3_1254*. The insertion site of the transposon is shown with a vertical arrow. The gene is 414 nucleotides long. This insertion generated OPTM2.
**spyM3_1376:**

\[
\begin{align*}
\text{ATG} & \quad \text{GTTAATATTATTATTAATTGTTTCTGCCCCTCATTGGTTTAATATTAGTTAGTTTGCAC} \\
& \quad \text{TATTCCGATTAGCTCAAGTCGTCGAAAGGAAGCTGCGAGTTGACTCTTTTAACG} \\
& \quad \text{CTGAACAAGAAGCTTGGATATCGTGAGCAAGAATGTAGTATGTGCGACACATCA} \\
& \quad \text{AAAAAACAGCTAAACGGAAAAGTTGCTTGCTCGAGTGACAGAAAGCGCCGTTGAAGT} \\
& \quad \text{GATTGTTAATGGAGACGGGAAACAAAACCTGAGCCATGAAATGCGACGCCGATTGCGA} \\
& \quad \text{TGCAGTGACATCAAGGAGCCACAGTTAAGAGCCGCAAGACTGGTTAGGAGCG} \\
& \quad \text{AAAGAGTGCACGCGCTCTTTGTGTGAGATGTAGTATGCTGAGCTGACACATGA} \\
& \quad \text{GTATTTAGAGATGAGGCAAGGCGCTTCCAGGCGCTCGTACTCGTGAGTCCACACCTTGAG} \\
& \quad \text{TGCAGAACGACAGCAACATGAAGGCGCAATTAGGGACGCGTAATATCC} \\
& \quad \text{GTACTTTAGAGAGCTTGGACTGCGCATTAGGCCTTCAAGTACTCTCCTAAGTTTTG} \\
& \quad \text{TATTTATCCAGGAGTTGGATCTTATCCGACTGAAATTTGCAGCTGACTTTTGAGAT} \\
& \quad \text{TCTCTGCTGGGATGTGCACAGCTAGGTCGAGAGTGAGTATACGTCTCAATGAC} \\
& \quad \text{TATTTAGTGAGTTAGGGTAGGAAATGTTTGCTCTTGCCCGCCGCTGCTGTGTTTCTTGCAT} \\
& \quad \text{GATATGGAAGTAAAGCTTACAGGCTTTGTGTAGGTAGGTTGAAAGTTGATGCTGTCGAG} \\
& \quad \text{CAGGTGGTCCAGGAAGGAAGCATCAGGTTTGTTGCAACACTATTGCTGACCCAC} \\
& \quad \text{ACGGGAGATTGAGGACCCAGTTGTGATTGCTGACTTGGATGCTGACTGACGACGCC} \\
& \quad \text{CTCAGTTCGGCTCGTCCAGGGCCTCGTAATGAGTCAATGGGAAGATTACATCAAGCGT} \\
& \quad \text{CTTCGTTATTTAGAGAAGAAATCGGCAGAAGTTGATTGGTGCCAAAATGTTTTGCTCTC} \\
& \quad \text{TACAAAGCCTGGGAGATTTAGGTTGTTCAACCTGAAAATTTACAGATGATCA} \\
& \quad \text{GGTTGTCAATTTTGTCGCATAAGAGAAGAAAATTTGAAAAACAATCTGATTACCAC} \\
& \quad \text{CGAAAATATTTAACACTGTTATTCCGTGAGATGAGAGCGGTTGGATTATGCCAAAGTGA}
\end{align*}
\]

**FIGURE 19:** Partial sequence of *spyM3_1376*, the *cvfA* gene. The gene is 1608 nucleotides long. Here only the first 259 nucleotides are shown. The vertical arrows represent the insertion site of the transposon. Arrow “a” points the insertion site found in OPTM16, arrow “b” shows insertion site found in OPTM7.
5' - spym3_2199

TCTTAATCAGATGAAAGTTGCTACTCCCTGAACAAATTTTTCAATAATGTTTCCTTAATA
AATAGGTGATCCCTACGATTATAACCATTTTTATTCAGGTAGGTGAGTGATAA
CTTTTTTTGACTTAAATTGTCGGAATTCTAGTTAGTTAATATAGTTTTCTACA
ATATATCCTTTACCAGTTATCATATTTCTTGATATTTTTTAAAAATCAAATATATC
▼a
TATTTTTTCATAAATTAGATAAAAACACTTTGGAAAAAGTTTTACTAAAATAATTTATA
ACAATTCAATTATCCTGATTTTTTCGNNNGGAATTITTTTTATTGAACA
-35
CAATTTTATATAATATCCTATGCTTAGTTGACAATTATTTCTTAGTTATTAC
-10 +1 ▼b RBS
AAATAATTGAGCTCCTTTCTTTCAAGGAAATTAAAAAGAAAGAGGTGTAATT

5' - hasA

GTGCCTATTTTTTTTTTTTTTTTTTTTTATTGAAACA

FIGURE 20: The hasA promoter region. The start of hasA transcription is shown as +1. Promoter elements (-10 and -35 region) and the ribosome-binding site (RBS) are indicated in bold. 5'-ends of hasA and spy_2199 are indicated by horizontal arrows. Five CovR binding sites are underlined. The two vertical arrows indicate transposon insertion sites showing opposite phenotypes. The transposon insertion close to the transcription start site (vertical arrow b) generated a capsule non-producer. The transposon insertion 178 bases upstream of the start codon (vertical arrow a) generated a capsule overproducer at 37˚C (OPTM18).
### TABLE 12: Capsule quantitation of transposon mutants at 37°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGAS315-1</td>
<td>0.004</td>
<td>340,000</td>
<td>85.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGAS315-2</td>
<td>0.005</td>
<td>420,000</td>
<td>86.97</td>
<td>86.46</td>
<td>0.72</td>
</tr>
<tr>
<td>MGAS315ΩCovR-1</td>
<td>0.006</td>
<td>470,000</td>
<td>93.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGAS315ΩCovR-2</td>
<td>0.006</td>
<td>440,000</td>
<td>99.62</td>
<td>96.45</td>
<td>4.50</td>
</tr>
<tr>
<td>OPTM2-1</td>
<td>0.648</td>
<td>4,600,000</td>
<td>1,029.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPTM2-2</td>
<td>0.618</td>
<td>4,100,000</td>
<td>1,101.22</td>
<td>1,065.19</td>
<td>50.95</td>
</tr>
<tr>
<td>OPTM7-1</td>
<td>0.547</td>
<td>4,800,000</td>
<td>832.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPTM7-2</td>
<td>0.532</td>
<td>4,300,000</td>
<td>903.88</td>
<td>868.22</td>
<td>50.43</td>
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<td>OPTM18-1</td>
<td>0.597</td>
<td>4,600,000</td>
<td>948.17</td>
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<tr>
<td>OPTM18-2</td>
<td>0.666</td>
<td>4,200,000</td>
<td>1,158.49</td>
<td>1,053.33</td>
<td>148.72</td>
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</tbody>
</table>
TABLE 13: Capsule quantitation of transposon mutants at 25°C

<table>
<thead>
<tr>
<th>Strain (duplicated)</th>
<th>OD640</th>
<th>Colony number (cfu/mL)</th>
<th>Capsule conc (fg/cfu)</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGAS315-1</td>
<td>0.007</td>
<td>620,000</td>
<td>82.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGAS315-2</td>
<td>0.008</td>
<td>680,000</td>
<td>85.95</td>
<td>84.22</td>
<td>2.45</td>
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<tr>
<td>MGAS315ΩCovR-1</td>
<td>0.191</td>
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<td>MGAS315ΩCovR-2</td>
<td>0.193</td>
<td>1650000</td>
<td>807.48</td>
<td>803.82</td>
<td>5.18</td>
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<tr>
<td>OPTM2-1</td>
<td>0.735</td>
<td>5,500,000</td>
<td>976.32</td>
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<td>OPTM2-2</td>
<td>0.771</td>
<td>5,900,000</td>
<td>954.71</td>
<td>965.51</td>
<td>15.28</td>
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<td>OPTM7-1</td>
<td>0.697</td>
<td>6,200,000</td>
<td>821.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPTM7-2</td>
<td>0.713</td>
<td>6,400,000</td>
<td>813.91</td>
<td>817.61</td>
<td>5.23</td>
</tr>
<tr>
<td>OPTM18-1</td>
<td>0.803</td>
<td>6,100,000</td>
<td>961.73</td>
<td></td>
<td></td>
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<tr>
<td>OPTM18-2</td>
<td>0.796</td>
<td>5,900,000</td>
<td>985.66</td>
<td>973.70</td>
<td>16.92</td>
</tr>
</tbody>
</table>
FIGURE 21: Capsule production of transposon mutants at 37°C. Note the increased amount of capsule in transposon mutants.

FIGURE 22: Capsule production of transposon mutants at 25°C. Note how wild type MGAS315 does not change the capsule amount, contrasting to the other strains.
Knock-out experiment

Knock-out experiments were done for phenotype confirmation of OPTM18. The target promoter area was amplified by PCR (Table 14) and cloned into PCIV2, a 4.195 kb plasmid harboring kanamycin resistance. After transformation, the phenotype of the transformant was similar to the background strain, no capsule production at 37°C and capsule production at 25°C. The insertion mechanism of insertional inactivation is different from transposon mutagenesis and this could explain the difference in phenotype. While PMGC8 was inserted into the target using transposase (Figure 25), PCIV2 was integrated causing an insertional disruption with a fragment of the target gene, generating partial duplicates of certain target sequences that would be inactive (Figure 26) but this could generate a partial binding site or recognition sequence.

The knock-out was confirmed using a confirmation primer that contains a sequence in the target gene area, OPhasAP-confirm, and a set of primers that anneal to the vector in two different sites in a reverse and forward direction, M13-f and M13-r, respectively (Table 15). The insertion is confirmed when an amplicon of 782 bp is present in a PCR reaction with the confirmation primer and one of the M13 primers. In this experiment, bands were present with the M13-r set, showing a reverse direction insertion (Figures 23 and 24).

However, knock-out experiments failed to mimic the same overproducer phenotype. This may be partially explained by the different insertion mechanisms for both, plasmid and transposon. While in transposon mutagenesis the continuity of the DNA sequence is disrupted by the insertion of the mobile
element, in insertional disruption a partial duplication is created (figures 25 and 26).

The influence of temperature in pathogen gene expression has been widely studied, and many thermoregulated genes are known. Several mechanisms are described in different species and specific genomic changes have been determined by microarray analysis in GAS, however, most of the differential expression revealed was in hypothetical proteins (97).

The results presented in this study could throw some light upon the hypothetical function of uncharacterized proteins and help to develop a model of thermoregulation in *S. pyogenes* capsule production, leading to better understanding of virulence and physiology in this species.
### TABLE 14: PCR primers used for *hasA* promoter area amplification

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5OPhasASph</td>
<td>5' - AAAAGCATGCTCTGTCTAGATTCAATGTC C - 3'</td>
</tr>
<tr>
<td>3OPhasASph</td>
<td>5' - AAAAGCATGCTCTGTCTAGATTCAATGTC C - 3'</td>
</tr>
</tbody>
</table>

The first 4 As of the sequence are overhangs, GCATGCT is the recognition site of the restriction enzyme SphI. The optimum PCR condition for amplification of the *hasA* promoter area was 55°C for annealing temperature and one minute of extension.

### TABLE 15: Primers used for *hasA* promoter area knock-out confirmation

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPhasAP-confirm</td>
<td>5' - TACTCCCTGAACAATTTTCATAAT - 3'</td>
</tr>
<tr>
<td>M13 - f</td>
<td>5' - CGCCAGGGTTTCCAGTCAGGAC - 3'</td>
</tr>
<tr>
<td>M13 - r</td>
<td>5' - AGCGGATAAACATTTCCACACAGGA - 3'</td>
</tr>
</tbody>
</table>

The optimum PCR condition to check the right insertion was 52°C of annealing temperature and one minute of extension.
FIGURE 23: Gel electrophoresis of nucleic acids for knockout experiments. a) amplification of the promoter area of *has* operon. b) Bands confirming the insertion of PCIV2 in a reverse direction and consequent disruption of a region of the *has* operon promoter area. Lanes 1-5 are transformation products. Lane 6 is a positive control and lane 7 is a negative control.
FIGURE 24: Primer annealing for knock-out confirmation. The black bold lines represent the chromosome region of hasA. The grey line in between represents the inserted plasmid. The fine line below the chromosome with plasmid insertion is the extension obtained in this experiment, confirming a reverse insertion. The green rectangles are the primers in their annealing site.
FIGURE 25: Graphic representation of transposon insertion. The green circles are transposase. The transposon is inserted randomly into the genome, causing a wide variety of mutations on each transformation, derived from gene disruption at the insertion site.
FIGURE 26: Graphic representation of insertional inactivation. The plasmid is inserted into the chromosome by homologous recombination between a fragment of the target gene and the chromosomal target gene loci. The insertion creates a disrupted gene and a partial duplication of the target sequence.
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APPENDICES
I. Media

1) Luria-Bertani Broth (per liter):

10 grams NaCl

10 grams Tryptone (Fisher Scientific)

5 grams Yeast extract (Difco)

Adjust pH to 7.6 with NaOH solution. Autoclave at 20 psi for 30 minutes.

2) THY broth (per liter):

30 grams Todd Hewitt (BBL)

2 grams Yeast extract (Difco)

Autoclave at 20 psi for 30 minutes.

3) TP broth (per liter):

30 grams Todd Hewitt (BBL)

2 grams Yeast extract (Difco)

20 grams Peptone (Bacto brand)

Autoclave at 20 psi for 30 minutes
4) THY agar plates (per liter, approximately 40 plates):

30 grams Todd Hewitt

2 grams Yeast extract

14 grams Agar (Bacto brand)

Autoclave at 20 psi for 30 minutes. Let cool down to 55°C. Add appropriate antibiotic if needed before pouring plates.

5) LB agar plates (per liter, approximately 40 plates):

10 grams NaCl

10 grams Tryptone

5 grams Yeast extract

14 grams Agar (Bacto brand)

Autoclave at 20 psi for 30 minutes. Let cool down to 55°C. Add appropriate antibiotic if needed before pouring plates. Add X-Gal if needed.
## II. Antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Abbreviation</th>
<th>Solvent</th>
<th>Concentration of stock solution</th>
<th>Storage</th>
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<tr>
<td>Chloramphenicol</td>
<td>Cam, Cm</td>
<td>50% EtOH</td>
<td>10 mg/ml</td>
<td>4°C</td>
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<td>Erythromycin for</td>
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<td>Erm, Em</td>
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<td>1 mg/ml</td>
<td>4°C</td>
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<tr>
<td><em>S. pyogenes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Kan, Kn</td>
<td>ddH₂O, filter</td>
<td>100 mg/ml</td>
<td>4°C</td>
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<tr>
<td>Spectinomycin</td>
<td>Spc</td>
<td>ddH₂O, filter</td>
<td>100 mg/ml</td>
<td>4°C</td>
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For *E. coli*:

<table>
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<th>Antibiotic</th>
<th>Final concentration (µg/ml)</th>
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<td>Erythromycin</td>
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<tr>
<td>Kanamycin</td>
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<td>5</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100</td>
<td>10</td>
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For *S. pyogenes*:

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<th>Final concentration (µg/ml)</th>
<th>Volume of stock solution (µL)</th>
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<td>Erythromycin</td>
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<tr>
<td>Kanamycin</td>
<td>700</td>
<td>70</td>
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<tr>
<td>Spectinomycin</td>
<td>150</td>
<td>15</td>
</tr>
</tbody>
</table>
III. Buffers and solutions:

1) PBS buffer (10X)

80 grams  NaCl

2 grams  KCl

2 grams  K phosphate monobasic

11.5 grams  Na phosphate dibasic heptahydrate

pH 7.2

2) TAE Buffer (10X)

48.8 grams  Tris Base

11.42 mL  Glacial acetic acid

2 mL  0.5M EDTA pH 8.0

q.s. to 1 Liter with ddH₂O
APPENDIX B: Protocols

I. Generation of In-Frame Deletion Strain

1. Once Erm resistant colonies are obtained (which have been grown at 30°C) pick colonies and grow them in 10 ml of THY+Erm1 at 30°C.

2. Next morning, make freezer stocks of the overnight cultures and inoculate 10 µL of overnight cultures into 10 ml of THY+Erm1 and incubate at 39°C. This shift of temperature will select for the integration of the plasmid into the chromosome, as pJRS233 cannot replicate at 39°C.

3. Next morning, inoculate 10 µL of overnight culture into 10 ml of THY+Erm1 and incubate at 39°C. Passing twice at 39°C in presence of Erm1 ensures integration of the plasmid.

4. Next morning, inoculate 10 µL of overnight culture into 10 ml THY without Erm and incubate at 30°C. This temperature shift selects for the excision of the plasmid, which could leave either the wild type or deletion allele of the gene.

5. Repeat step 4) twice. Three times passing of the cultures at 30°C without Erm is necessary to get sufficient percentage of Erm sensitive cells in the culture.
6. After the final passage, make freezer stock of 2 cultures and plate, and dilutions on THY agar in the absence of Erm, and incubate overnight at 30°C in an anaerobic conditions.

7. Pick colonies and replica-plate (by patching with sterile wooden sticks) on THY+ Erm1 and THY plates. Patch 100 colonies. Incubate overnight in anaerobic conditions at 30°C.

8. Grow up those patches which were Erm sensitive at 37°C and make freezer stocks and confirm chromosomal structure by PCR after chromosomal extraction, as these strains could have either wild type or deleted chromosomal structure. Test 10 colonies. If all the 10 colonies have the wild type genotype, try again from the beginning.

9. Make freezer stock of wild type revertant as a control and another stock of settled down strains.

II. Isopropanol precipitation

1. Transfer the sample to an Eppendorf tube. Sample volume in a tube should be less than 800 µL. if sample volume is over 800 µL split in two tubes.

2. Add 1/10 volume of 3M Sodium Acetate solution (pH5.2) to the sample.
3. Add 70% of the final volume of room temperature isopropanol to the DNA solution and mix well.

4. Centrifuge the sample immediately at 10,000 – 15,000 x g for 1 hour at 4°C.

5. Carefully decant the supernatant without disturbing the pellet.

6. Add 1 ml of 70% EtOH to a tube gently.

7. Centrifuge at 10,000 – 15,000 xg for 15 minutes at 4°C.

8. Carefully decant the supernatant without disturbing the pellet.

9. Remove the residual ethanol solution with a pipette very carefully.

10. Dry the pellet at 50°C for 5 – 20 minutes (depending on the size of the pellet).

11. Add DW. Added amount of DDW depends on the final DNA concentration you want to get. Usually 50 µL is enough.

12. Tap the tubes vigorously to dissolve DNA for about 5 minutes per tube.

13. Examine DNA concentration by gel electrophoresis or nanodrop.

14. Store sample at -20°C.
III. Electroporation of *S. pyogenes*

1. Prepare DNA

2. Prepare THY plates containing appropriate antibiotics.

3. Add 100 µL of filter sterilized 2M glycine solution to 10 ml TP medium and add appropriate antibiotic.

4. Inoculate *S. pyogenes* and incubate them overnight at 37°C.

5. Next morning, add 1 ml of filter sterilized 2M glycine solution to 200 ml of TP medium plus appropriate antibiotic.

6. Take out 2 ml for blanks (1 ml per cuvette) for spectrophotometry and add 2 ml of overnight culture to 98 ml of TP+ glycine and add appropriate antibiotic.

7. Incubate the culture at 37°C until the culture reaches OD600=0.24.

8. Distribute cultures into 4 tubes of 50 ml falcon tubes.

9. Harvest cells by centrifugation (7,000 x g for 10 minutes at room temperature). Resuspend total cell pellets in 4 ml of the supernatant.

10. Incubate cells at 43°C for 9 minutes.

11. Add 6 ml of 15% glycerol solution and centrifuge the cells (7,000 x g) for 10 minutes at room temperature.
12. Discard the supernatant and wash the cells three times with 10 ml of 15% glycerol solution.

13. Remove the supernatant as much as possible.

14. Resuspend the cell pellet in 800 µL of 15% glycerol solution.

15. Keep competent cells at room temperature until electroporation.

16. Place tubes of 10 ml of THY media and electroporator cuvettes on ice.


18. Place DNA in an ice-cold 2 mm electroporator cuvette:

   Suicide vectors: 10 – 20 µL

   Shuttle vectors: 0.5 – 2 µL

   Transposon vectors: 10 – 20 µL

   pJRS233: 10 – 20 µL

19. Add 200 µL of competent cells into the cuvettes ad pipette up and down to mix well.

20. Give electric shock.

21. Immediately, remove cells from the cuvette and add the cells into the cold 10 ml THY media.
22. Record the time constant.

23. Incubate cells for 30 minutes on ice, then 1 hour and 30 minutes at 37°C. Dry the plates during this time.

24. Harvest cells by centrifugation (7,000 x g) for 10 minutes at room temperature and resuspend cells in 500 µL of THY medium.

25. Plate 100 µL of cells on each plate.

26. Incubate overnight at 37°C in anaerobic conditions.

IV. Electroporation of *S. pyogenes* with pJRS233 derivatives

1. Prepare pJRS233 using a Maxi prep kit (initial volume: 300 ml of overnight culture, final volume: 50-100 µL in DDW).

2. Prepare THY agar plates with no antibiotic (24 ml THY agar/plate)

3. Add 100 µL of filter sterilized 2M glycine solution to 10 mL of TP medium.

4. Inoculate *S. pyogenes* and grow overnight at 37°C.

5. Next morning, add 1 ml of filter sterilized 2M glycine solution to 100 mL of TP medium.

6. Take blanks for spectrophotometry (2 mL) and add 2 mL of overnight culture to 98 mL of TP+Glycine medium containing proper antibiotic.
7. Incubate the culture at 37°C until the culture reaches OD= \( \approx 0.24 \).
   Usually it takes 2 hours for wild type.

8. Turn on the heat block containing a 15 ml tube block and set temperature at 43°C.

9. When the culture reaches OD= \( \approx 0.24 \), distribute cultures into two 50 mL falcon tubes and harvest cells by centrifugation (7,000 x g for 10 minutes at 14°C).

10. Resuspend total cell pellets in 2 mL of the supernatant and transfer to a 15 mL falcon tube.

11. Incubate cells at 43°C for 9 minutes. Use the heat block.

12. Add 8 mL of 15% glycerol solution and centrifuge the cells (7,000 x g for 10 minutes at 14°C).

13. Discard the supernatant and wash the cells twice with 10 mL of 15% glycerol solution (centrifugation at 14°C).

14. During the final wash, place tubes of 10 mL of THY media and electroporator cuvettes on ice. The tube number are transformation number plus one (for negative control). The cuvette number is the same as the transformation number.

15. Remove supernatant as much as possible after the final wash.

16. Resuspend the cell pellet in 600 µL of 15% glycerol solution.
17. Keep the competent cells at room temperature, not on ice. Perform a
Electroporation as soon as possible.

18. Set the electroporator (voltage: 2100V, Capacitor: 25µF, Resistance:
200 Ω, electroporator cuvette: 2 mm).

19. Place DNA in an ice-cold 2 mm electroporator cuvette.

   a. pJRS233 derivative: 10-20 µg

   b. DNA concentration after Maxi-prep using 300 mL of 20-24 hrs
culture: 3-4 µg/µL

20. Add 200 µL of competent cells into the cuvettes and pipette up and
down to mix well.

21. Press the red button on the electroporator.

22. Immediately, remove cells from the cuvette and add the cells into the
cold 10 mL THY media.

23. Record the time constant.

24. Incubate cells for 30 minutes on ice, then 1 hour in the 30°C incubator.
   You can dry the agar plates during this time if plates are not dry.

25. Harvest cells by centrifugation (7,000 x g for 10 minutes at room
temperature) and resuspend cells in 300 µL of THY medium.
26. Add 300 µL of cells to 15 mL of THY soft agar (cooled down to 43°C), mix gently and pour 3 mL of cells-soft agar mix onto a THY + Erm1 plate.

27. Incubate the plates in an anaerobic jar at 30°C at least 2 hours and no more than 3 hours.

28. Remove the plates from the anaerobic jar. Overlay with an additional 3 mL THY soft agar containing erythromycin (4 µg/mL)

29. Incubate plates at 30°C for 48 hours in an anaerobic jar.

V. *E. coli* transformation:

1. Thaw frozen Top10 competent cells on ice.

2. To each Top10 cell aliquot add 1 – 200 ng of transforming DNA in a volume of 10 µL or less. For a ligate, use 10 µL (maximum amount). Include a positive control (the undigested vector for the cloning) and no DNA control.

3. Place cells on ice for 30 minutes.

4. Heat shock cells for 30 seconds at 43°C.

5. Add 1 ml of LB broth to each tube and incubate at 37°C for one hour, shaking intermittently or in a 37°C shaker incubator.
6. Dry agar plates.

7. Plate 200 µL of cells on selective agar plates (6 plates for transformation and 1 plate for control cells).

8. Incubate plates overnight at 37°C.

**VI. Quantitation of *S. pyogenes* capsule:**

1. Grow *S. pyogenes* overnight in 10 ml THY media with appropriate antibiotic.

2. Next morning, dilute cells to $10^{-5}$, $10^{-6}$, and $10^{-7}$ with sterile PBS buffer and plate 100 µL of cells on THY agar plates. Incubate the plates at 37°C in anaerobic conditions. Make triplicate dilutions and place each set in a different jar (or as many as the temperature that will be tested).

3. 24 hours later, take out the jars and place one at 25°C (if more temperatures will be tested, then move each jar to each temperature) and save one jar for measurement at 37°C. Measure the capsule amount produced by the 37°C jar.

4. 4-5 hours later, measure the capsule amount produced by colonies on the plates incubated at 25°C.
Capsule Quantitation using Stains-All reagent solution

Analyze in duplicate. Do not use PBS buffer in the Stains-All assay because it inhibits the reaction

5. 2X Stains-All reagent: 20 mg of Stains-All and 60 μL of glacial acetic acid in 50 mL of 50% formamide. Wrap the reagent bottle with foil because it’s degraded by light. Store at 4°C. The reagent is effective within a month.

6. Take colonies carefully using a spatula and place this colony into a tube with 10 ml of DDW. Duplicate the samples.

7. Vortex the tubes about 1 minute to resuspend cells and capsule.

8. Take 100 μL of the resuspension and dilute it in 900 μL of PBS buffer. Save for later serial dilution and plating. This will be the first tube of the serial dilution.

9. Centrifuge the 10 ml tubes to precipitate cells (6,000 x g) for 10 minutes at room temperature.

10. Take 500 μL of supernatant into a spectrophotometry cuvette.

11. Mix with 500 μL of 2X Stains-all reagent.

12. Prepare hyaluronic acid solutions for a standard curve. Add 1 μL, 2 μL, 3 μL, 4 μL and 5 μL of 1 mg/ml hyaluronic acid in 500 μL of DDW in a spectrophotometer cuvette. Mix the solution very well with a P1000
pippetman. This mixing step is very important because hyaluronic acid does not dissolve in the Stains-all reagent. Add 500 µL of the Stains-all reagent to each cuvette and mix well.

13. Prepare blanks with 500 µL of DDW mixed with 500 µL of Stains-all.

14. Measure OD640

15. Dilute resuspended cells to $10^{-3}$, $10^{-4}$ and $10^{-5}$ with PBS buffer and plate 100 µL of cells on THY agar plates. Make the dilution and plating in duplicate. Next morning count appropriately spread colonies and calculate cell concentration (cell number/ml).

16. Using the standard curve and cell concentration, calculate the amount of capsule produced per CFU.

17. 5 hours later, measure the capsule amount produced by colonies on the plates at room temperature the same way it was done before, make dilutions, plate and then count colonies to calculate cell concentration and capsule production.

\section*{VII. Storing cells at -80°C freezer}

1. Grow cells overnight in THY media with antibiotics if needed.

2. Transfer 0.7 mL of culture into a cryogenic tube and add 0.3 mL of 80% glycerol solution. Mix well.
3. Label the tube and store at -80°C.

VIII. Plasmid extraction

All plasmids were propagated in *E. coli* Top10 cells and extracted using Biotech Gerard Maxi Prep Kit according to manufacturer’s protocol, except that final volume was 2 mL per sample. Initial volume: 300 mL of LB broth.

IX. DNA Purification for PCR products

PCR products were purified using Qiagen PCR Purification Kit following the protocol provided by the manufacturer.

X. Preparation of Chromosome of *S. pyogenes* with Sigma column

1. Grow 10 ml of overnight culture in THY broth with 100 µL of 20mM glycine and add appropriate antibiotic if necessary.

2. Harvest cells by centrifugation at 7,000 rpm for 10 minutes.

3. Wash twice with 3 ml of TE buffer.

4. Add 200 µL of lysozyme (100 mg/µL), 2 µL of mutanolysin (100 µL/µL), 80 µL of RNAse and 520 µL of Gram positive solution.

5. Incubate 1 hour at 37°C with intermittent shaking.
6. Follow the remaining steps provided by the manufacturer.

**XI. Preparation of *E. coli* competent cells**

1. Grow Top10 cells in 10 mL of LB broth at 37°C overnight.

2. Dilute 1 mL of cells into 200 mL of LBB in a 500 mL flask.

3. Make solutions and keep them on ice.

4. Grow cells with vigorous shaking until they reach $10^8$ cells/mL. It takes approximately 2 hours.

5. Divide 200 mL culture into four 50 mL conical tubes.

6. Harvest cells at 5,000 x g for 5 minutes at 4°C. From this point on, cells must be kept ice cold.

7. Gently resuspend each pellet in 10 mL ice cold, sterile solution I.

8. Recover cells by centrifugation at 5,000 x g, 5 minutes, 4°C.

9. Discard supernatant. Gently resuspend each pellet in 10 mL of solution II. Pipette up and down. Do not vortex.

10. Place tubes on ice for 15 minutes.

11. Recover cells by centrifugation, 5,000 x g, 5 minutes, 4°C. gently resuspend all pellets in 10 mL of solution II containing 10% (v/v) glycerol.
12. Divide cells in 200 µL aliquots in eppendorf tubes (approximately 50 tubes) and freeze at -80°C.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
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| Solution I | 10 mM MOPS (KOH) pH 7.0  
10 mM RbCl |
| Solution II | 100 mM MOPS (KOH) pH 6.5  
50 mM CaCl₂  
10 mM RbCl |
| Stock solutions | .1 M MOPS pH 7.0  
.1 M RbCl  
1.0 M MOPS pH 6.5  
0.5 M CaCl₂ |
| Solution I | 32 mL ddH₂O  
4 mL 0.1M MOPS pH 7.0  
4 mL 0.1 RbCl |
| Solution II | 35 mL ddH₂O  
5 mL 1.0 M MOPS pH 6.5  
5 mL 0.1 M RbCl  
5 mL 0.5 M CaCl₂ |
| Solution II containing 10% glycerol | 10 mL solution II  
1.43 mL of 80% glycerol |
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Thermoregulation of Capsule Production in *Streptococcus pyogenes* strain HSC5.

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