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Genetic Analysis of the Putative Streptolysin O Regulator from Streptococcus Pyogenes

Robert Tyrone Billingslea

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The Edwin P. McCabe Honors Program

Reference
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Senior Thesis

**“Genetic Analysis of the Putative Streptolysin O
Regulator from *Streptococcus pyogenes*”**

M. B. Tolson Black Heritage Center
Langston University
Langston, Oklahoma

Robert Tyrone Billingslea

May 2004

Langston University
Langston, Oklahoma

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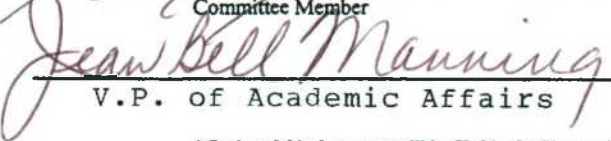
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Abstract

Group A streptococci (*Streptococcus pyogenes*) is one of the most frequent pathogens of humans. It is estimated that between 5-15% of normal individuals harbor the bacterium, usually in the upper respiratory tract, without signs of disease. *S. pyogenes* can initiate an active infection when defenses are compromised or when the organism is able to penetrate the host's defenses. When the bacteria invade vulnerable tissues, a variety of **suppurative infections** can occur. Acute *S. pyogenes* infection may be present as pharyngitis ("strep throat"), scarlet fever (rash), impetigo (infection of the superficial layers of the skin) or cellulitis (infection of the deep layers of the skin). Invasive, severe infections can result in necrotizing fasciitis ("flesh-eating bacteria"), myositis (muscle inflammation and soreness) and streptococcal toxic shock syndrome. Patients may also develop immune-mediated post-streptococcal sequelae, such as acute rheumatic fever and acute glomerulonephritis, following other infections caused by *S. pyogenes*.

Recently, the *S. pyogenes* strain SF370 genome sequencing project discovered a number of possible regulator genes. The specific regulator gene that we are observing is Spy0146 and its upstream companion gene, Spy0145. Spy0146 is homologous to the putative perfringolysin O regulator (pfoR) from *Clostridium perfringens*, while Spy0145 is a member of a family of transcriptional regulators. Our analysis of the region of the streptococcal chromosome containing gene Spy 0146 suggested that the upstream gene, Spy 0145 might be linked to Spy0146 as an operon. If Spy0145 and Spy0146 are co-transcribed, then both may play an important coordinated role in global gene regulation of *S. pyogenes*.

Previously in the laboratory, a knockout of one of these genes (Spy0146) had been demonstrated to alter the mRNA expression of many *S. pyogenes* genes, including the virulence factors streptolysin O and NADase. This mutation in Spy0146, in fact, both increased and decreased transcription activity, depending upon the particular genes analyzed.

The research being conducted here has two main goals. We would like 1) to determine whether the genes Spy0145 and Spy0146 are co-transcribed on the same polycistronic message and 2) to replace the inactivated copy of gene Spy0146 with a cloned copy in an integration vector to return the cell to wild type phenotype (natural state).

To achieve our first goal, we isolated the streptococcal RNA using the hot phenol method and synthesized cDNA. Using polymerase chain reaction (PCR), we demonstrated that both Spy0145 and Spy0146 were found on the same polycistronic message. Preliminary results further suggested that this polycistronic message is more rapidly turned over during logarithmic growth as compared to cells in stationary phase. Further, for our second goal, we isolated the gene by method of using PCR, cloned it into the *E. coli* bacteria, then transferred to a streptococcal shuttle vector (p7INT), re-introducing the gene in the mutant strain of *S. pyogenes*. This arrangement of two transcriptional factors (Spy0145 & Spy0146) on one mRNA is very unusual in bacteria. A preliminary review of the scientific literature has failed to find a similar example of regulators being co-transcribed. This unusual arrangement may suggest that these two genes may work in a coordinated fashion in the global gene regulation of *S. pyogenes*.

Introduction

In humans, diseases associated with the streptococci occur primarily in the respiratory tract, bloodstream, or as skin infections. Human diseases are most commonly associated with Group A Streptococci. Acute Group A Streptococcal disease is most often a respiratory infection that is not directly attributable to the dissemination of bacteria of group A infections. Group A Streptococcal infections remain a major worldwide health concern. Much effort is being directed toward clarifying the risk and mechanisms of these sequelae as well as identifying rheumatogenic and nephritogenic strains.

Streptococcus pyogenes has been a noteworthy pathogen (bacterium or a virus; cause of a disease) throughout history, but the complications that it causes have changed throughout time. In the early 1850's, it was identified to be the primary cause of acute childbed fever or puerperal fever (an infection of the uterus of postpartum women). Early in the 20th century, scientists understood this bacterium (a single-celled microscopic life form) to be the cause of scarlet fever (a disease that killed or debilitated many young children). For quite a long time, it was known primarily as the cause of "Strep-throat" or pharyngitis (inflammation of the pharynx; sore throat) as well as its non-suppurative sequelae (non-pus producing disease resulting from another), rheumatic fever (an acute infectious disease that causes a sore throat, fever, pain, swelling in the joints, and often damage to the heart valves), glomerulonephritis (kidney disease), and tonsillitis (inflammation of the tonsils of the mouth). In 1994, reports were released in England stating that there was a "flesh-eating bug" that killed a number of people. It was determined that a case of *Streptococcus pyogenes* caused severe cases of necrotizing

fasciitis (a severe bacterial infection that causes cell tissue to decay rapidly; flesh-eating bacteria). Although not considered part of the normal flora, it has been concluded that a small number of individuals can carry the organism asymptotically (without showing signs of infection), particularly in the nasopharynx (area between the nasal cavity and the throat), skin, vagina, and anus. The A in Group A Streptococcus refers to the Lancefield classification which is based on the antigenicity (a substance, usually a protein, on the surface of a cell or bacterium that stimulates the production of an antibody) of a carbohydrate which is soluble in dilute acid and called the C carbohydrate.

Lancefield identified 13 types of C carbohydrates in his studies, designated from A through O, which could be serologically differentiated. The organisms that are most commonly infected in human beings are found in groups A, B, D, and G. Streptococci that do not contain the C carbohydrate substance are called viridans streptococci or non-typable streptococci (not associated with a particular group). *Streptococcus pyogenes* is usually identified in the laboratory by the clear beta hemolysis (the destruction of red blood cells and the release of the hemoglobin they contain) it causes on blood agar plates and its sensitivity to bacitracin (an antibiotic that is used mainly in the treatment of skin infections caused by gram-positive bacteria). The latter is usually tested for by use of an impregnated disk placed on a particular vicinity of the organisms on a blood agar plate. Because identification is crucial so that the penicillin therapy can be instituted for the prevention of rheumatic fever, a number of commercial identification kits are available that rely on rapid antigen-antibody reactions. While most sore throats are caused by viruses, *Streptococcus pyogenes* is the most common bacterial cause, others also include: *C. diphtheriae* and the gonococcus (a sexually transmitted disease that causes

inflammation of the genital mucous membrane, burning pain when urinating, and a discharge; *Neisseria gonorrhoeae*). *Streptococcus pyogenes* is particularly dangerous because of the late non-suppurative complications that can occur subsequently to pharyngitis and impetigo (a contagious infection of the skin characterized by blisters that form yellow-brown scabs) and include rheumatic fever and glomerulonephritis. Either of these complications may occur after pharyngitis while only glomerulonephritis generally occurs only after impetigo. Rheumatic fever is hypothesized to occur due to an immunological cross reaction between the streptococcal M protein and heart muscle, and, luckily, can be prevented by a 10 day's course of penicillin. Glomerulonephritis is also thought to be immunologically-based, perhaps the result of deposition of antigen-antibody complexes on the glomerular basement membrane. Scarlet fever is not a late complication as is rheumatic fever, but rather an active infection with a strain that can produce a pyrogenic (fever causing) toxin. It may, then, be no more or less serious than any other streptococcal infection, although a rash will usually be present. This pyrogenic toxin can, however, be deadly in the presence of even insignificant amounts of endotoxin (a toxin produced within certain bacteria that is released only when the bacteria disintegrate), with which it acts synergistically (stronger when combined with another action) to produce hypotension (abnormally low blood pressure) and shock. The M protein of *Streptococcus pyogenes* is anti-phagocytic (cell that does not remove unwanted substances) and is, thus, a virulence factor (potential of being extremely poisonous, infectious, or damaging) because of its anti-phagocytic properties as well as its potential for producing cross reacting antibodies.

A prominent characteristic of the Group A Streptococcus (GAS) is the number and variety of extracellular toxins and enzymes that they release into the surrounding environment. We have identified a novel GAS gene, *sloR*, that modulates the expression in which the cytolysin streptolysin O and the co-transcribed NAD-glycohydroase with many other neighboring upstream genes. The *sloR* gene is co-transcribed with another important gene

(*yjgF*) upstream on a bioisronic message. Through previous works and research we know that both *yjgF* and *sloR* are members of putative regulators (with the former being from a family of conserved proteins from a broad range of bacteria and eukaryotes, and the latter rarely found in anything other than a select group of Gram positive species that happen to be known well as exotoxin producers such as Anthrax).

In recent years, increasing attention has been given to other streptococcal species, partly because innovations in serogrouping methods have led to advances in understanding the pathogenetic and epidemiologic significance of these species. A variety of cell-associated and extracellular products are produced by streptococci, but their cause-effect relationship with pathogenesis has not been defined. More recently, the Streptococcus pyogenes strain SF370 genome sequencing project completed at the University Of Oklahoma Health Sciences Center discovered a number of novel genes, including a few possible regulator genes. One possible regulator, gene *Spy0146*, was found in the region of the chromosome containing streptolysin O (SLO) and NAD-glycohydroase (NADase), two virulence factors. Furthermore, *Spy0146* is homologous to the putative perfringolysin O regulator (*pfoR*) from Clostridium perfringens; the

possible linkage of this putative regulator to SLO was underscored by the fact that perfringolysin O is itself a homolog of SLO. Previously in the laboratory, a successful knockout (removal) of Spy0146 had been demonstrated to alter the mRNA (messenger RNA) expression of many *Streptococcus pyogenes* genes, including SLO, NADase, and several other associated genes of uncertain function(s). This inactivation of Spy0146, in fact, both increased and decreased transcription activity, depending upon the particular genes that were analyzed.

Our analysis of the region of the streptococcal chromosome containing gene Spy 0146 suggested that the neighboring upstream gene, Spy 0145, might possibly be transcriptionally linked to Spy0146 to form an operon (a segment of a chromosome containing the genes that specify the structure of a given protein, alongside the genes that regulate its manufacture). Indeed, Spy0145 is a member of a family of transcriptional regulators, and therefore if Spy0145 and Spy0146 are co-transcribed, then both of these genes may play an important coordinated role in global gene regulation of *Streptococcus pyogenes*.

Although we ultimately hope to discover and understand everything about this bacterium, this thesis will be limited but not confined to the length of my two month summer internship assistance at Dr. J.J. Feretti's Microbiology Lab at the Oklahoma Health and Sciences Center under the supervision of Dr. William McShan.

MATERIALS AND METHODS

Bacterial Strains

Strains of NZ131 and the isogenic (having identical genes) sloR-OK85 were grown in chemically defined media. Growth was monitored by the absorbance at 600nm. Samples of the culture supernatant were removed at sequential times and prepared as described in the methods. Strains NZ131 and OK85 have similar growth characteristics in chemically defined media. Strain OK85 was delayed in the release of SLO as compared to NZ131. This delay is reproducible and also observed when using rich media. The total amount of SLO released by OK85 never achieves the levels obtained in the NZ131 wild type strain.

Streptococcus pyogenes strain NZ131 is a type 49 isolate that was originally isolated from a case of acute glomerulonephritis (kidney disease). This strain has been characterized by many studies over the last two decades, and its DNA sequence has been partially determined (unpublished results).

Strain OK85 is a derivative (hybrid) of NZ131 strain that contains an insertional inactivation of gene Spy0146 (Savic *et al.*, 2002).

Todd-Hewitt Broth supplemented with 5 % horse serum and 0.2 % yeast extract (THY-HS) was used to grow the streptococci.

Oligonucleotide primers used in these studies were:

Spy0146 L (TGAAGTATGCGGAGCAAATG),

Spy0146 R (CCAATCAAAACGGCATAACC),

Spy0145 L (ATACACCGCAGGCCAATTAC),
Spy0145 R (AACAAAAACCTGGCGACACT),
Spy0144.1 L (TCTCCTCTCACCACCTTTGC),
Spy0144.1 R (AGCGGTAAACACTTGGTTCG),
Spy0145.1 L (TGAAGAGCTACCCAGAACCAA),
Spy0145.1 R (GCGACAGCTTCGACTTCAAT),
sloR-L (AGCGGTAAACACTTGGTTCG),
sloR-R (TTACCAGCCAATAGCCAAGC),
Emm 5.F (TATTSGCTTAGAAAATTAA),
Emm5.R (GCAAGTTCTTCAGCTTGTTT),
sloR-L5 (AGCGGTAAACACTTGGTTCG), and
sloR-R5 (AGCGCTTTCAATTCTATCCAT).

Cell Culture and Growth Conditions

“Standard growth conditions” in this paper indicate growing *Streptococcus pyogenes* strain NZ131 or its derivative statically in Todd-Hewitt Broth (BBL Laboratories) supplemented with 0.2 % yeast extract (Difco) at 37° C in a 5 % CO₂ atmosphere. The medium is prepared per the manufacturer’s instructions and sterilized autoclaving. Growth of the bacteria will be monitored by absorbance at 600 nm. For some experiments, chemically defined media (CDM) will be used. CDM is prepared as published in Rijn and Kessler’s work, and strain NZ131 is capable of growth in this medium (unpublished results). One advantage of CDM is that it contains no large

macromolecules or polypeptides that could interfere with the assay of proteins released into the culture supernatant by streptococci.

Isolation of Streptococcal DNA

Bacteria were grown overnight in 10 ml THY broth at 37° C and harvested by centrifugation at $1,000 \times g$ for 15 minutes. The cells were suspended in 100 μ l of freshly prepared lysozyme (50 mg/ml in TE) + 50 Units mutanolysin (Sigma M9901). After incubation for 30 minutes at 37° C, the cells were lysed by addition of 0.5 ml GES reagent (5M guanidium thiocyanate, 100 mM EDTA, and 0.5 % sarkosyl). Cell debris was removed by the addition of 0.25 ml 7.5 M ammonium acetate and 0.5 ml chloroform-isoamyl alcohol (24:1). After centrifugation, the DNA was precipitated from the aqueous phase by the addition of 0.6 volumes of 2-propanol. The DNA pellet was washed 5 times with 70% ethanol, dried *in vacuo* for 5 minutes, and suspended in 100 μ l TE.

Isolation of Streptococcal RNA

Streptococci from an overnight culture were diluted 1:50 in fresh THY-HS. Cells were grown at 37 ° C and harvested at mid-logarithmic stage of growth ($A_{600} = .493$ and $.474$ for NZ131 & OK85, respectively) and at stationary stage (NZ131 $.953$ & OK85 $.882$) by quick freezing in a dry ice-ethanol bath. After thawing, the cells were pelleted by centrifugation for ten minutes at 4° C and washed twice, once with one volume of ice-cold 40 mM sodium acetate, pH 5.1/1m EDTA and then harvested, then repeated with 20 mM sodium acetate. The pellets were suspended in 0.7 ml of the sodium acetate solution.

SDS is added to the cell suspension to a final concentration of 1.5 %, and the cells are immediately lysed by six 1 minute cycles at 5000 RPM in a Mini-Beadbeater™ (Biospec Products). Between each cycle, the cells were rested one minute on ice.

Removal of Contaminating DNA from RNA samples.

1 µl of 50 µg/µl Proteinase K into 300 µl of Cell Lysis were diluted for each sample. Overnight cultures of E. coli were pelleted by centrifugation with the supernatant being discarded. The mix was then vortexed for ten seconds to resuspend the cell pellet. 300 µl of Cell Lysis was added and thoroughly mixed containing the Proteinase K. That collection was incubated at 65° C for fifteen minutes, while being vortexed every five minutes. Place sample on ice for three to five minutes. Add 200 µl of MPC Protein Precipitation Reagent to the lysed sample and vortex mix for ten seconds vigorously. Pellet the debris by centrifugation for ten minutes at $\geq 10,000 \times g$ in a microcentrifuge. If clear or loose, add an additional 25µl of MPC Protein Precipitation Reagent, mix and pellet debris again. Take the supernatant and transfer to a clean microcentrifuge tube and discard the pellet. Add 500µl of isopropanol to the recovered supernatant. Invert tube several (30-40) times. Pellet DNA by centrifugation at 4° C for ten minutes in a microcentrifuge. Carefully pour off the isopropanol without dislodging the RNA pellet. Rinse twice with 75% ethanol, being careful not to dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet. The recovered RNA was resuspended in TE buffer, and a sample was analyzed by agarose gel electrophoresis. The preparation was determined to be free from

contaminating DNA by the inability to amplify *Streptococcus pyogenes* specific genes by PCR.

Synthesis of Chromosomal DNA (cDNA)

Streptococcal cDNA was prepared from the isolated RNA using random hexamer oligonucleotides to prime the first strand synthesis. First strand cDNA synthesis was done using SuperScript II reverse transcriptase (Invitrogen) using the supplied reagents. Following synthesis, RNA was removed by treatment with RNase A and RNase H, and the cDNA was purified using the PCR Purification Kit from Qiagen. The quantity of cDNA was estimated by spectrophotometry (A260 and A280 nm).

Polymerase Chain Reaction (PCR)

PCR was performed using Taq polymerase using the supplier's buffers and recommended conditions (Fischer Scientific). Primers were designed using the Primer3 software package (<http://frodo.wi.mit.edu/>).

Transcript mapping

To determine the genetic composition of the mRNA containing gene Spy0146, *Streptococcus pyogenes* cDNA was used as a template for PCR amplification with the following primer pairs: Spy0145 L + Spy0145 R, Spy0146 L + Spy0146 R, and Spy0145 L + Spy0146 R. Amplification was performed using 30 cycles of 94° C for 30s, 50° C for 30s, and 72° C for 30s.

Cloning Procedures

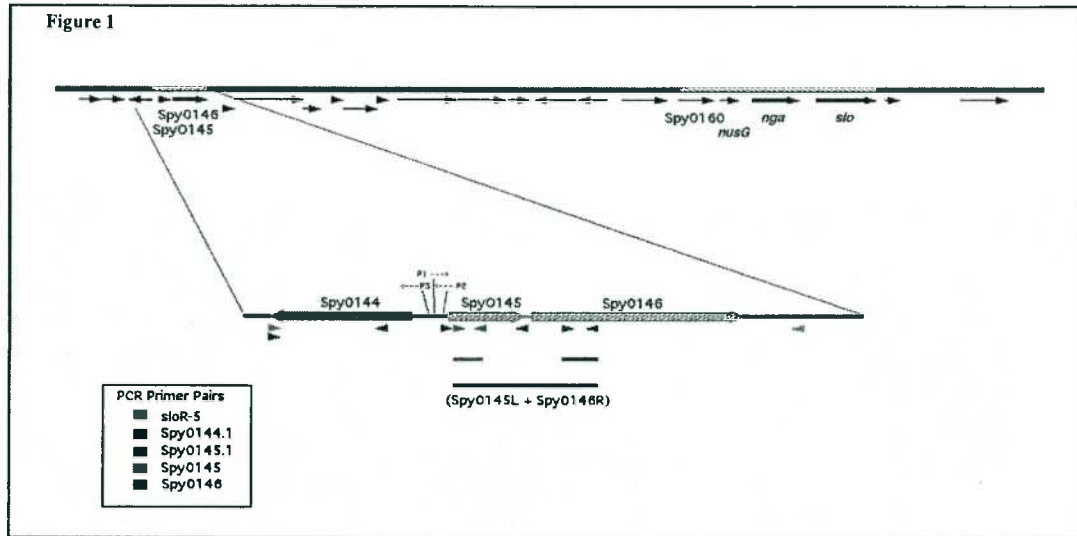
All molecular biological manipulations were done using standard protocols (Sambrook *et al.*). Regions from the NZ131 chromosome containing either internal fragments of Spy0144, Spy0145, or the entire region containing both Spy0145 and Spy0146 with the associated promoter region were amplified by PCR. Thermal cycling was performed using 30 cycles of 94° C- 30s, 50° C- 30s, 72° C- 30s for primers pairs Spy 0144.1 & Spy 0145.1, and 35 cycles of 94° C- 1 min, 50° C- 2 min, 72° C- 3 min for primer pair sloR-5. The amplified product were then ligated to PCR cloning vector pGEM T Easy (Promega) using the manufacturer's suggested protocol. *E. coli* DH5 α were transformed with the recombinant plasmids and plated on selective media containing 100 μ g/ml ampicillin. Clones were screened by PCR using the original primer pairs and conditions. The presence of an insert of correct size was confirmed by digestion restriction endonuclease pattern.

2D Electrophoresis of Streptococcal Proteins

Inoculate 20 ml of Todd-Hewitt Broth supplemented with 5 % horse serum with 0.4 ml of an overnight culture. The cells should grow at 37° C for three hours to an OD₅₆₀ of 0.25. Centrifuge 7000 \times g for five minutes at 4° C. Discard the supernatant resuspend the cells in 1 ml of ice-cold, sterile solution of 0.5 M sucrose in distilled water. Spin the cells for 15 s, discard the supernatant and resuspend the cells in the same volume of 0.5 M sucrose solution. Repeat the washing process for the cells two more times as

described. Resuspend the cells in 100 μ l of 0.5 M sucrose. To one to ten μ l of DNA solution add suspension to obtain a final volume of 100 μ l. Mix and transfer to a chilled Gene Pulser cuvette (0.2 cm electrode gap). Expose the cells to a single electric pulse. Add immediately 0.9 ml of TH-HS. Incubate at 37° C for two hours. Mix the cells with TH-HS agar containing appropriate antibiotic. Incubate at 37° C for 24 to 48 hours.

Findings and Results



The Region of the *Streptococcus pyogenes* Genome containing Spy0146.

The upper figure shows the *Streptococcus pyogenes* chromosome from genes Spy0142 through Spy0172. Previously, gene Spy0146 (in green), the perfringolysin O regulator homolog, was inactivated, and the loss of its product was shown to inhibit the expression of *slo*, *nga*, *nusG*, and Spy0160, a putative membrane lipoprotein (all shown in red). The expression of the other genes in the region was unaffected by its inactivation. Analysis of the DNA sequence surrounding Spy0146 suggested that the neighboring upstream gene, Spy0145, would be located on the same transcript as Spy0146. Further, promoter analysis using the Neural Network Promoter Prediction software (found at www.fruitfly.org/seq_tools/promoter.html) suggested that the three probable promoters (P1, P2, & P3) are found between the genes Spy0144 and Spy0145, one (P1) promoting the transcription of Spy0145 and Spy0146 while the other two (P2 and P3) potentially promoting Spy0144 (a conserved hypothetical protein). The arrangement of these

promoters may suggest some coordinated control of expression of these genes. The enlargement shows a detailed map of the Spy0146 region with the placement of the probable promoters and the location of the PCR primers used in this work.

Figure 2

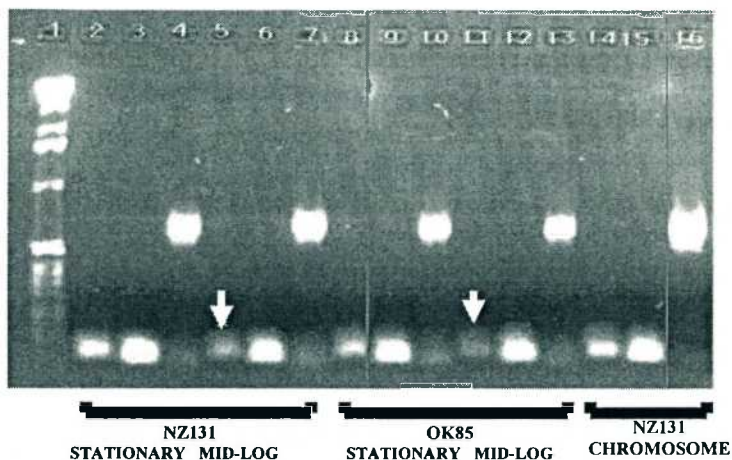
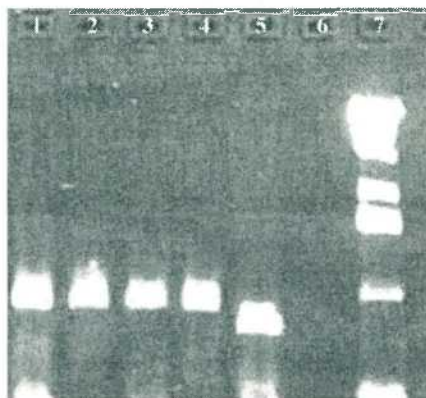


Figure 2.1



Left panel (Figure 2): Lane 1 contains the BRL Kilobase Ladder as a molecular weight marker. Lanes 2, 5, 8, 11, and 14 were amplified with Spy 0145 L & R; lanes 3, 6, 9, 12, and 15 were amplified with Spy 0146 L & R; and lanes 4, 7, 10, 13, and 16 were amplified with Spy 0145 L & Spy 0146 R.

Right panel (Figure 2.1): lane 1, NZ131 stationary; 2, NZ131 mid-log; 3, OK85 stationary, 4, OK85, mid-log; 5, chromosomal DNA; 6, NZ131 RNA; and 7, Kilobase Ladder. Lanes 1-6 were amplified with primers Emm5.F & Emm5.R).

Spy0145 and Spy0146 are contained on a Polycistronic mRNA Transcript.

cDNA isolated from either strain NZ131 or OK 85 at mid-log or stationary phase was amplified using primers specific for gene Spy0145 (Spy 0145 L & R), gene Spy0146 (0146 L & R), or both including the intergenic region (Spy 0145 L & Spy 0146 R). The results show that both Spy0145 and Spy0146 are transcribed on one mRNA molecule. Furthermore, comparing the relative amounts of the Spy0146-specific reaction between mRNA isolated from mid-log versus stationary cultures suggests that the turnover of the transcript is much faster in rapidly dividing cells (marked by arrows in lanes 5 and 11). The control reaction (lane 14) using chromosomal DNA as the PCR template confirms that this observation is not due to some variation in the PCR reaction. Amplification of the Spy0146 product in OK85 confirms that the integration of the plasmid that inactivated this gene occurred downstream of this point in the sequence. The right panel shows control reactions using primers specific for the M protein, the major surface antigen of *Streptococcus pyogenes*. This message is present in both mid-log and stationary cells under most conditions. Lane 6 in the right panel used streptococcal RNA that had not been converted into cDNA, showing that the observed reactions were not due to some kind of chromosomal DNA contamination.

Phylogenetic tree showing relationships between various species, including *Xylella fastidiosa* (labeled as *Xylella fastidiosa* 16S). The tree is rooted on the left and branches out to the right. Bootstrap values are indicated at the nodes.

Species listed (from top to bottom):

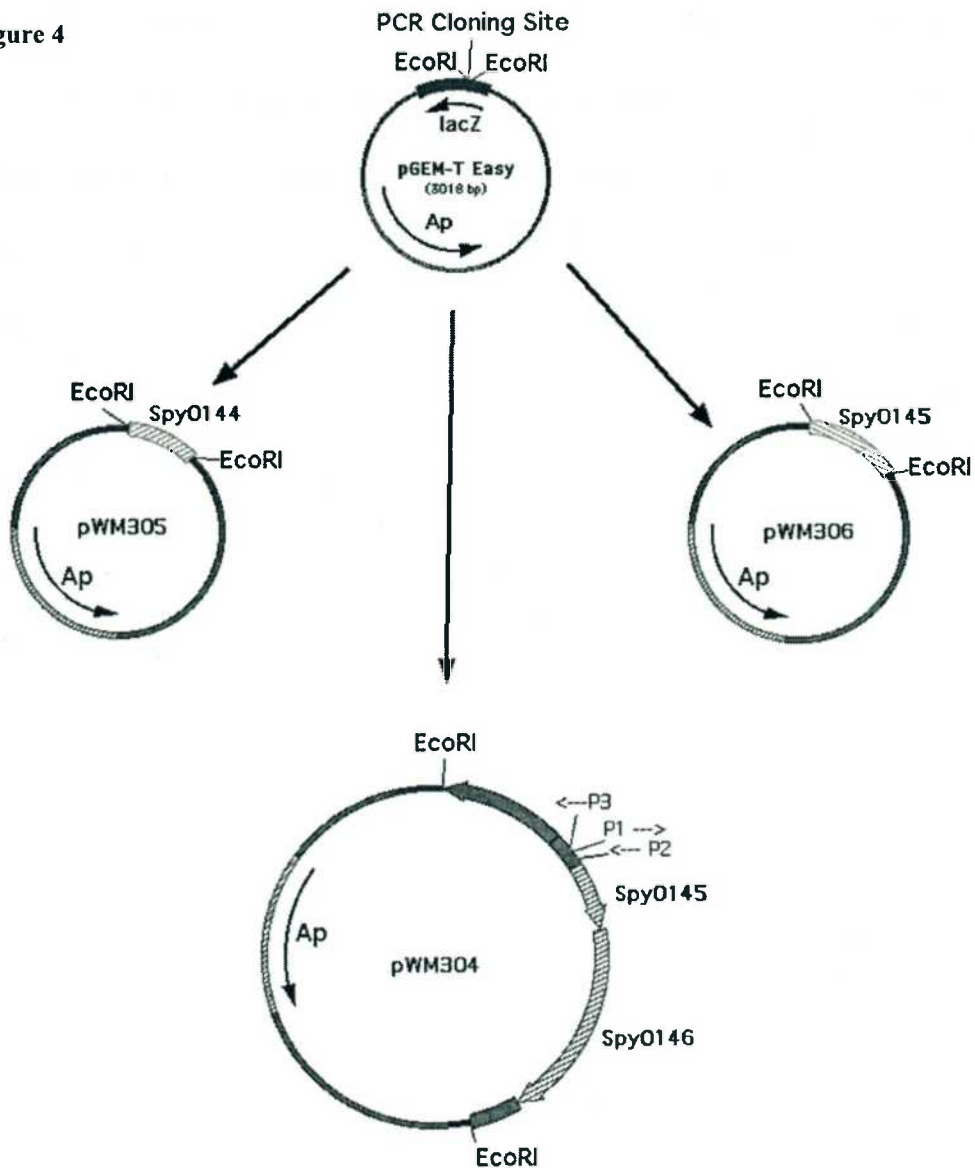
- Atoposia* (labeled as *Atoposia* 16S)
- Prochlorococcus* *erythraea*
- Acetobacter* *vinelandii*
- Metorhiza* *meningitidis*
- Xanthomonas* *campestris*
- Xanthomonas* *campestris*
- Xylella* *fastidiosa* 16S
- Agrobacterium* *petrae*
- Agrobacterium* *petrae*
- Prochlorococcus* *erythraea*
- Prochlorococcus* *erythraea*
- Prochlorococcus* *erythraea*
- Chlorobacterium* *petrae*
- Listeria* *interna*
- Listeria* *monocytogenes*
- Campylobacter* *jejuni*
- Brucella* *parvus* 16S
- Lactococcus* *lactis* 16S
- Brucella* *parvus* 16S
- Brucella* *parvus* 16S
- Thermotoga* *maritima* 16S
- Yersinia* *pestis*

Phylogenetic tree showing relationships between various bacterial strains. The tree is rooted on the left and branches to the right. The strains listed on the right are: *B. p. 145*, *B. agalactiae*, *Clostridium perfringens*, *Clostridium tetani*, *Clostridium septicum*, *Clostridium perfringens 2*, *Bacillus anthracis*, *Haemophilus influenzae*, and *Haemophilus influenzae 2*.

The genes homologous to Spy0145 (Figure 3) or Spy0146 (Figure 3.1) were identified using a BLAST search of the GenBank database. Numerous homologs of Spy0145, a putative transcription initiation inhibitor, were identified, and the most closely related proteins were selected for phylogenetic comparison. CLUSTALW was used to align the proteins and generate a dendrogram that was graphically displayed using program called TreeView X. This analysis suggests that Spy0145 is not quite that similar to the related proteins from other Gram positive organisms than to the proteins from several Gram negatives species. The same analysis was also applied to gene Spy0146 (right panel). Only a small amount of proteins were found in GenBank that were closely related to Spy0146, of which the best characterized is the PFO regulator

from *Clostridium perfringens*. One of the most noticeable observations of this list is that all of the bacterial species that contain this protein are strongly associated with exotoxin production, and other closely related species to members on this list that do not typically produce exotoxins, like *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Bacillus subtilis*, who do not contain this gene at all.

Figure 4



Cloning of the Spy0146 Region.

The PCR cloning vector pGEM T Easy was used to clone the DNA fragments generated by PCR using primers Spy0144.1 L & R (pWM305), Spy0145.1 L&R (pWM306), and sloR-5 L&R (pWM304). The cloning of these regions is the first step in the future studies of these genes. The inserts from pWM305 and pWM306 will be transferred to streptococcal suicide plasmid vectors for inactivating genes Spy0144 and Spy0145, respectively. The insert in pWM304 contains both Spy0145 and Spy0146 with their associated promoter. This region will be transferred to a vector containing a phage integrase that mediates site-specific integration into the *Streptococcus pyogenes* chromosome. This construct will then be introduced in strain OK85 in an attempt to reverse the effects seen when Spy0146 was inactivated, providing evidence that the observed effects resulted from the loss of this gene product and not from some outside factor.

CONCLUSIONS

Genes Spy0145 and Spy0146 from *S. pyogenes* are co-transcribed on the same polycistronic message and since both of these genes are potential transcriptional regulators, it further suggests that they may act in some coordinated fashion, either separately or as a complex.

This polycistronic message containing both Spy0145 and Spy0146 appears to undergo rapid turnover when the bacteria are actively dividing.

Computer analysis shows that both Spy0145 and Spy0146 are members of separate transcriptional regulator protein families. However, while the family that contains Spy0145 has many members from a wide range of bacterial species, Spy0146 belongs to a small group of proteins that appear to be only associated with bacterial species that produce exotoxins, so this co-transcription is quite an unusual combination.

Analysis of the genetic arrangement of the region of the *Streptococcus pyogenes* chromosome containing these genes suggests that the next neighboring upstream gene, Spy0144, may also somehow play a significant role in the interaction with Spy0145 and Spy0146, perhaps in some other regulatory or sensory capacity.

Future / Continuing Research

A new hypothesis has been formed to continue working with this bacterium:

“The group A streptococcus (GAS) genes Spy0144, Spy0145, and sloR form a coordinated genetic unit that influences the expression or regulation of the important virulence factors SLO, NADase, and several associated genes.”

The specific aims of this proposal are 1) to isolate and characterize the proteins encoded by Spy0144, Spy0145, and sloR to determine their cellular location and expression patterns and 2) inactivate, singly and in combination, genes Spy0144, Spy0145, and sloR to dissect the altered expression of *slo*, *nga*, and the associated regional genes between the wild-type parent and the mutant derivatives.

The proposed work is novel in that it will characterize the linkage between the putative regulator genes, Spy0144, Spy0145, and sloR, and the expression of the virulence gene cluster that includes SLO and NADase. While much is known about these proteins, very little is known concerning their regulation. Thus, not only will these studies further our understanding of the expression of an important streptococcal exotoxin, but also may help define a new class of exotoxin-associated proteins that could be ultimately targets for novel therapeutic strategies or vaccine development that could significantly reduce the incidence of serious streptococcal disease such as rheumatic fever.

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