



# Development of a Strain of *Streptococcus pyogenes* that Secretes Cre Recombinase

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## Abstract

*Streptococcus pyogenes* (Group A Streptococcus, GAS) is a gram-positive, non-spore-forming, ubiquitous organism that causes diseases in humans ranging in severity from pharyngitis to necrotizing fasciitis. The mechanisms of pathogenesis by which GAS causes disease are unknown and it is debated as to whether *S. pyogenes* can exist intracellularly within the human host. In an attempt to understand colonization of hosts by *S. pyogenes* involving an intracellular phase, I want to develop a strain of GAS that will, on infection of a host transgenic mouse, enable me to track GAS infected cells and observe where they travel in the host. Therefore, the primary objective of this project is to construct a strain of GAS that secretes Cre recombinase. Upon infecting a transgenic mouse host with GAS, depending on survival of the host, the infected cells can be tracked due to expression of a beta-galactosidase gene whose expression is dependent on the presence of active Cre recombinase within the cell. The strain of GAS will express the Cre recombinase constitutively and secrete the recombinase across the cell membrane. I have attempted to construct the genetic elements necessary for this expression and secretion. Through Polymerase Chain Reaction (PCR) we have amplified a segment of DNA encoding the Cre recombinase from a plasmid DNA template. We have also amplified the secretion signal of the GAS Shr gene. Through overlapping PCR these two segments have been fused. A constitutively active transcriptional promoter, Veg, has been amplified and we have fused this upstream of the chimeric shr/cre gene. This segment is being cloned into a plasmid that can replicate in *S. pyogenes* producing a strain that constitutively synthesizes and secretes Cre recombinase.

## Aim

The goal of this project is to construct a strain of Group A *Streptococcus pyogenes* that secretes Cre recombinase.

## Introduction

*Streptococcus pyogenes* (Group A Streptococcus, GAS) is one of the most frequent pathogens of humans with an estimated 5-15% of normal individuals harboring the bacterium. The large number of unique diseases GAS causes vary from superficial and self-limiting diseases of the pharynx and skin to infections that involve increasing degrees of tissue dysfunction. The main site of infection of GAS is the human oral, nasal mucosa. However, the mechanism by which the streptococci cross mucosal or epidermal barriers is not understood, and despite extensive research efforts, we have only limited understanding of pathogenesis and the molecular bases for the diversity and severity of streptococcal disease.

*S. pyogenes* possesses multiple devices for invasion of epithelial cells. The bacteria express an array of cell surface proteins that facilitate bacterial colonization of a variety of human tissue. Changes in epidemiology and the ability of GAS to breach epithelial cells are explained by *S. pyogenes* internalization by mammalian epithelial cells at high frequencies. Evolution of GAS has allowed the alteration of inflammatory responses and impaired phagocytic clearance of the bacteria by extracellular and surface-bound factors. The more than 100 serotypes of GAS use both similar and different schemes to colonize hosts and avoid protective defenses at the biochemical level.

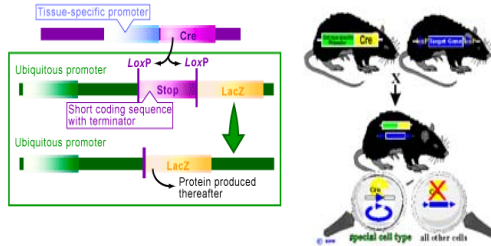
In a study of strains of *S. pyogenes* and phagocytosis by human neutrophils, it was concluded that virulent streptococci have the ability to survive and multiply in whole blood, suggesting that bacterial evasion of host defenses occurs intracellularly and survival inside human neutrophils may contribute to the pathogenesis of *S. pyogenes* and the recurrence of *S. pyogenes*. Virulence factors of GAS include M proteins which serve in resistance to phagocytosis, adherence, an intracellular invasion along with the fibronectin (FN) binding protein. The M protein is the major cause of antigenic shift and antigenic drift in GAS, and it also binds fibrinogen from serum and blocks binding of complement to underlying peptidoglycan, thereby allowing the survival of the organism by inhibiting phagocytosis.

Extracellular matrix proteins (ECM) mediate high frequency internalization targeting invasion by causing contact between the ECM binding proteins and integrin receptors on target proteins. A two receptor process is thought to occur because adherence and invasion are separate events. ECM determines the spectrum of target cells, invasion efficiencies and requirement of serum agonists.

Intracellular invasion accounts for persistence of streptococci after penicillin therapy and the recurrence of tonsillitis, and the capacity of GAS to invade epithelial cells in such a great capacity.

The aim of this project is to develop a strain of group A streptococcus that, if it invades cells in a mouse host, will leave a trace in the cells of the mouse that survive infection to find out if and where GAS colonizes mammalian hosts.

## Experimental Plan



A genetically altered mouse with a promoter that is attached to and readily regulates the gene encoding Beta-galactosidase already exists. The transgenic organism has a transcription terminator sequence between the promoter and the Beta-galactosidase gene that will terminate the expression of the lac gene. By introducing Cre into the cells of the organism, Cre-mediated recombination between flanking loxP sites deletes the terminator sequence, thereby allowing the expression of the lac gene which can be detected by histochemical staining. This will allow beta-galactosidase expression to track GAS infected cells in the mouse to better understand where the colonization takes place. The required components to reach this aim include to create a GAS strain that secretes Cre recombinase. The experimental plan calls for PCR amplification to amplify Cre recombinase from a plasmid DNA. A segment of which encodes a GAS specific protein secretion signal, the GAS Shr gene will be amplified by PCR from another plasmid template and joined in the correct reading frame to the Cre-encoding DNA segment by overlapping PCR, and the amplified new product will be cloned into a TOPO cloning vector. The Shr/Cre construct will then be amplified and fused to a constitutively active transcriptional promoter. This new DNA will then be cloned into a plasmid that can replicate in GAS. The plasmid will be introduced into a wild-type strain of GAS. This should result in the secretion of Cre recombinase from GAS.

## Materials and Methods

PCR amplification was done using Pfu error-correcting polymerase to amplify all genetic elements and all cloning was done into the Invitrogen Zero Blunt plasmid. DNA agarose gel analysis was done for verification purposes.

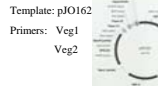
### 1. Amplification of Cre

Template: MSCVpuroCre  
Primers: Cre1  
Cre2



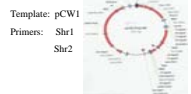
### 5. Amplification of Veg

Template: pJO162  
Primers: Veg1  
Veg2



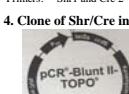
### 2. Amplification of Shr

Template: pCW1  
Primers: Shr1  
Shr2



### 3. Joining of Shr and Cre by overlapping PCR

Template: products 1 and 2  
Primers: Shr1 and Cre 2



### 4. Clone of Shr/Cre into pzeroblunt



### 6. Joining Veg Shr/Cre

### 7. Clone Veg/Shr/Cre into zeroblunt



### 8. Excise fragment from clones as EcoRI fragment.

### 9. Clone into pLZ12spc



## Results

### Amplified Cre

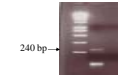


Cre was amplified from pMSCVpuro by PCR using primers Cre1 and Cre2 vector.



Amplified Cre was gel extracted and eluted resulting in a single band.

### Amplified Shr

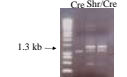


Shr was amplified from pCW1 as DNA template with 4.0mM MgCl2 added to the PCR rxn.



Amplified Shr was gel extracted and eluted resulting in a single band.

### Chimeric Shr/Cre



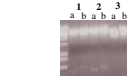
Shr/Cre was fused by overlapping PCR using the elute Cre and elute Shr as template.



Chimeric Shr/Cre was gel extracted and eluted resulting in a single band.

### Shr/Cre was cloned into Top 10 E. Coli

### Verification of Cloned DNA



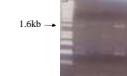
Restriction digests with Bam HI (lanes labeled "a") and EcoRI (lanes labeled "b") were done on four plasmids to verify which if any were the cloned Top 10 E. Coli transformed with Shr/Cre product into the PCR Blunt II Topo Vector. Only plasmid 4 was observed to have the Shr/Cre insert at 1.3 kb.

### Amplified Veg



Veg was amplified from CKS102 template as a single band.

### Veg was fused with Shr/Cre



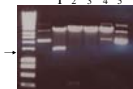
Veg/Shr/Cre was joined by overlapping PCR using Veg and Shr/Cre as template.



Veg/Shr/Cre was gel extracted and eluted resulting in a single band.

### Veg/Shr/Cre was cloned into Top 10 E. Coli

### Verification of Cloned DNA



Restriction digest with EcoRI enzyme was done on five plasmids to verify which if any of the clones contains the veg/shr/cre insert. Only Plasmid #1 was observed to have the insert at 1.6kb



Veg/Shr/Cre was gel extracted and eluted as a single band.

## Discussion

Though we did not produce a strain of GAS that secretes Cre Recombinase, we were successful in amplifying several of the genetic elements. We ligated the cloned veg/shr/cre into pLZ12 spc. Current verification attempts via restriction digestion of this clone have been inconclusive due to low plasmid concentration. It is possible that though the veg/shr/cre insert appears to be the right size it may not have the correct sequence. Further verification of this clone (e.g. restriction digestion and sequencing) is necessary before progress can be made with transforming pLZ12/S/C into GAS. We do have a scheme that appears to produce the genetic elements necessary for a strain of GAS to constitutively secrete Cre Recombinase. Initial PCR attempts were unsuccessful; however, we have learned that in order to amplify these genetic elements optimal PCR conditions must be utilized. Measures incorporated included magnesium titrations, optimizing cycling parameters, redesigning primers, and delaying primer addition. Without these conditions the desired products either failed to be amplified or generated multiple unwanted products. It was also vital to test the original templates to ensure that amplification would occur and the primers would not bind to give other products. To amplify Shr, RNase treated pCW1 prepared by phenol extraction was used as template. For amplification of Veg, the DNA template was changed from pJO162 to CKS102 (*Bacillus subtilis* genomic DNA). Even after implementation of the troubleshooting techniques other bands were sometimes detected thus the necessity for gel extraction. An error-proofing enzyme was used, but DNA sequencing is vital to the progress of the project to ensure that the clones are correct and to allow further progress.

## Future Directions

- Verify the DNA sequences of all clones
- Introduce pLZ12spc into a strain of Group A Streptococcus
- Verify that the plasmid does make and secrete Cre Recombinase
- Inject the strain of group A Streptococcus that secretes Cre Recombinase into a host transgenic mouse.
- Use histochemical staining in tissue sections to detect the group A Streptococcus

## References

- Bates, Christopher S. et al. "Identification and Characterization of a *Streptococcus pyogenes* Operon Involved in Binding of Hemoproteins and Acquisition of Iron." *Infection and Immunity*, March 2003.
- Fishetti, Vincent A. Ed. Et Al. "Intracellular Invasion by *Streptococcus pyogenes*: Invasins, Host Receptors, and Relevance to Human Disease" (Section II A Chapter 3) *Gram Positive Pathogens*, 2000.

## Acknowledgements

I would like to thank my mentor, Dr. Gordon Churchward for allowing me to work in his lab and guiding me throughout the summer. I would also like to thank Jennifer Rocco for all of her assistance with my project. Thanks to all of the members of the Churchward lab, Yi Yi Yu, Huiping Ling, Brian Dalton, and Natalie Brubaker. I would like to acknowledge Dr. Charlie Moran for providing me with pJO162 and pLZ12spc, and Dr. June Scott and Dr. Zehava Eichenbaum for pCW1. Additionally, I would like to thank the SURE program, administrators and participants, at Emory University along with the MARC program at Grambling State University. This material is based upon work supported by the Howard Hughes Medical Institute under Grant No. 52003727 and by the under National Institutes for Health Grant No. T32 AI07470 (Microbiology Department).