AN ABSTRACT OF THE DISSERTATION OF

Travis K. Warren for the degree of Doctor of Philosophy in Microbiology
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Title: Advances in Expression of Heterologous Protein Products by *Streptococcus gordonii*.

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Dr. Dennis E. Hruby

Streptococcus gordonii is a bacterial species that naturally colonizes the oral cavity of most healthy humans. It resides in the mouth as an adherent to dental surfaces and, with few exceptions, does not cause disease in individuals it inhabits. It possesses qualities that encourage its use as a vector to deliver human vaccines against pathogens that enter at mucosal surfaces such as the mouth, throat, lungs, gastrointestinal or urogenital tracts. *S. gordonii* is easily engineered in the laboratory to produce foreign, or heterologous, proteins derived from pathogenic organisms. Thus, *S. gordonii* engineered to produce particular viral may stimulate protective immunity against smallpox, a potential biowarfare agent, without causing the harmful side effects associated with the current vaccine.

The research presented in this dissertation desribes new ways that *S. gordonii* can be genetically engineered for greater production of heterologous products derived from pathogens. *S. gordonii* naturally secretes two proteins, glucosyltransferase and amylase-binding protein during growth, and the genetics *S. gordonii* utilizes to produce these products were reproduced and situated on a

plasmid to facilitate production of a heterologous product. Although some plasmids constructs could not be introduced into *E. coli*, it was found that the addition of a synthetic peptide to growth medium enhanced the efficiency with which *S. gordonii* assimilated genetic material, thereby allowing the plasmid to be directly introduced into *S. gordonii*. The expression plasmid is well retained by *S. gordonii* that colonized the oral cavities of mice.

The mechanisms that *S. gordonii* uses to export products from the cell are capable of accommodating high-level expression of at least two heterologous products, smallpox vaccine candidates A27L and B5R. Further, particular viral products that had previously proven recalcitrant to expression were produced by *S. gordonii* by engineering the fusion of viral sequences with those derived from a bacterial protein.

Advances in Expression of Heterologous Protein Products by *Streptococcus gordonii*

by

Travis K. Warren

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APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Travis K. Warren, Author

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CONTRIBUTION OF AUTHORS

S. Amanda Lund assisted with data collection and performed laboratory analyses including column chromatography (Chapter 2), genetic manipulations and SDS-PAGE evaluations (Chapter 3, 4 and 5), flow cytometric evaluations (Chapter 4 and 5), and plasmid retention analyses (Chapter 4). In Chapter 3, Alex Krupkin was responsible for *E. coli* genetic manipulations and for producing and purifying *E. coli* proteins while Lindsay Brown developed the *S. gordonii* SPEX strains under the leadership of Dr. Rebecca Wilson. David King assisted with data collection presented in Chapter 3. Dr. Kevin Jones provided assistance with data interpretation, experimental design and critical review of manuscripts for Chapters 2 - 5.

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This dissertation is dedicated to my parents

Dr. Douglas D. Warren

and

Mrs. Lydia H. Warren

who gave me eyes, ears, hands, and heart.

Advances in Expression of Heterologous Protein Products by *Streptococcus gordonii*

Chapter 1

INTRODUCTION

Mucosal surfaces serve as the point of entry for many pathogens, and the oral administration of live or attenuated microorganisms has proven to be an effective approach to generating protective immunological responses against typhoid, cholera, adenovirus, and polio (32, 71). *Streptococcus gordonii*, a Gram-positive bacterial species, is currently being investigated and genetically engineered to deliver therapeutic agents and vaccines. As a vaccine-delivery vehicle, researchers are faced with the challenge of engineering *S. gordonii* strains to express foreign antigens in a manner that stimulates appropriate protective immunological responses (86). While development efforts are underway to engineer *S. gordonii* as a vaccine vector for a variety of pathogens, the investigations presented in this dissertation were conducted in support of its use primarily for vaccination against the Class A biological agent smallpox.

As a commensal organism, *S. gordonii* naturally colonizes the human oral cavity where it adheres to dental surfaces. An *S. gordonii* strain that had not been engineered to express a foreign antigen was generally well tolerated in healthy

volunteers (22) and was self-limiting, exhibiting clearance in all individuals 35 days following inoculation. Conveniently, *S. gordonii* also colonizes the mucosal surfaces of mice, which greatly facilitates laboratory immunological evaluations (37, 44).

Among the other qualities of *S. gordonii* that make it particularly attractive as a bacterial commensal vector (BCV) is its natural competence, or the ability to internalize DNA from the surrounding environment, a prevalent trait among oral-colonizing bacteria (38). The mechanism of DNA uptake has been well characterized (15) and involves secretion of a competence-stimulating peptide (CSP) by *S. gordonii*. When the peptide concentration reaches a critical threshold in the extracellular environment, peptide bound to a membrane-spanning CSP receptor on the cell surface activates cytosolic enzymes that facilitate surface expression of a DNA receptor (17, 18). These natural mechanisms can be induced *in vitro* by incubating *S. gordonii* in Brain Heart Infusion (BHI) supplemented with calf or horse serum to early log-phase growth (49). In this manner, heterologous genes derived from pathogenic organisms can be transfected for expression by *S. gordonii*.

One of the objectives in the development of *S. gordonii* expression technologies has been high-level antigen expression. One of the primary ways to achieve this has been to situate heterologous products downstream of strong native promoters. Because the genome of *S. gordonii* has only been partially sequenced and has yet to undergo annotation to identify putative genes and other genetic

features (69), identification of *S. gordonii* promoters has relied primarily on empirical techniques. Pozzi et al. (52) characterized the strength of newly identified *S. gordonii* promoters by measuring the minimum concentration of chloramphenicol that inhibits growth of clones into which a promoterless chloramphenicol acetyltransferase (*cat*) gene was randomly integrated.

This approach was used to identify a native chromosomal promoter that was utilized in development of SPEX, an expression system of *S. gordonii* in which structural heterologous genes can be expressed either as surface products or as cell-wall anchored proteins (42, 49). A second *S. gordonii* promoter was identified by Bolken et al. (7) based on homology with the lac-operon promoters of *Lactococcus* and *Streptococcus* spp. These two promoters were used to facilitate expression of two different heterologous antigens on the surface of a single *S. gordonii* clone (7).

A potential drawback to evaluating promoter strengths for purposes of expression by *S. gordonii* has been that *E. coli* – into which plasmid constructs are routinely subcloned, screened, and amplified – does not stably maintain high-copy vectors containing particular streptococcal promoters (52, 64). For this reason, it is desirable at times to transform ligated DNA containing potentially strong promoters directly into *S. gordonii* without first subcloning the DNA into *E. coli*. Even though *S. gordonii* is naturally competent, the efficiency with which DNA is introduced using traditional methodologies, i.e. by inducing competence with calf

or horse serum, has not permitted the routine introduction of small quantities of genetic material, such as the product of typical DNA ligation reactions.

In Gram-negative bacteria, the periplasm, a cellular compartment between the inner and outer membranes, provides a controlled environment for nascent proteins to interact with accessory proteins and fold into a mature conformation without exposure to the unregulated extracellular milieu. In Gram-positive bacteria, which possess only a single bilayer lipid membrane, no such compartment has been recognized and the pathways used by these bacteria to facilitate export and proper folding of products are just beginning to be understood. Recent evaluations in *S. pyogenes* (58, 59) and *Bacillus subtilis* (28, 60, 71, 89) suggest that protein export is initiated when a signal recognition particle (SRP) recognizes and binds to secretion-signal sequences positioned at the N-terminus of *de novo* translated preproteins (Fig. 1.1). A typical secretion-signal sequence, produced on most products destined for transport across the cell membrane (71, 77), consists of approximately 28 amino-acid residues (71) and facilitates the transfer of nascent unfolded preproteins to membrane-bound secretional (Sec)-pathway components for export (2). Once transported to the exterior side of the cell-membrane, the product is temporarily retained at the N-terminus by the secretion-signal sequence which remains associated with the cell membrane. Type I signal proteases liberate the protein by cleaving the secretion-signal peptide (77). The product is theorized to diffuse freely through the peptidoglycan cell wall into the extracellular milieu (11) unless retained for further

processing, which can occur by membrane-bound proteolytic enzymes that monitor for misfolded products, chaperones that help products obtain a mature confirmation, or enzymes that are required for the surface display of products (60). Recently, investigations in *S. pyogenes* have shown that Sec-pathway components, secreted products, and chaperones colocalize at a single site per cell, which has been characterized as an export organelle and given the designation ExPortal (58, 59).

S. gordonii can be engineered to produce secreted heterologous products by situating a structural gene downstream and in-frame with a prokaryotic secretion-signal sequence. This signal sequence need not be derived from a native protein, and numerous studies have utilized the secretion signal from the *S. pyogenes* M6 protein (5, 13, 48).

Schneewind et al. (74) have provided significant insights into the mechanisms used by Gram-positive bacteria to anchor exported products to the surface of the cell. Protein surface display requires translation of a COOH-terminal sorting signal, characteristically comprised of a conserved LPXTG motif, followed by a hydrophobic region and a COOH-tail of mostly positively charged residues (35, 43, 72-75). This sorting signal functions to first retain the polypeptide within the secretory system, most likely positioned at the ExPortal. In a process involving Sortase A (SrtA), the protein is cleaved between threonine (T) and glycine (G) of the LPXTG motif, and the liberated carboxyl of threonine is

covalently linked to a pentaglycyl cross-bridge of the peptidoglycan cell-wall matrix (43, 72, 73, 75).

Gram-positive bacteria abundantly secrete proteins into the extracellular milieau, making them attractive for development as BCV (86). Genomic and proteomic analyses of *B. subtilis* suggest that this Gram-positive organism secretes ~ 300 proteins, mostly via the Sec pathway (60, 70). Because products destined for export from the cell are targeted to a single location at the ExPortal, it follows that as BCV are progressively and more extensively engineered to express multiple antigens and in increasingly greater amounts, the demands for native and heterolgous protein secretion may become self-limiting if the Sec pathway cannot keep pace with production.

Numerous bacterial, viral, and eukaryotic heterologous proteins have been expressed as either secreted or anchored products from *S. gordonii* (5, 9, 37, 50, 86) by fusing gene products to native and heterologous secretion signals and, for surface display, anchoring motifs. Additionally, *S. gordonii* can be engineered to express multiple antigens from a single cell (Fig. 1.2). Nonetheless, it is important to note that particular heterologous proteins are recalcitrant to expression by *S. gordonii*. Although the *S. gordonii* expression profile capable of generating a protective immunological response against smallpox has yet to be determined, S. gordonii will likely have to be engineered to express multiple heterologous antigens in combination with immunomodulatory cytokines, ligands, or suppressors of immunological tolerance.

The purpose of the investigations reported in this dissertation was to develop the use of a multi-copy plasmid as an expression vector for high-level expression of heterologous products by *S. gordonii* in the context of BCV vaccine development, with particular emphasis on smallpox vaccine development. Additional investigations were conducted to facilitate the expression of products that were found to be difficult to express from *S. gordonii*.

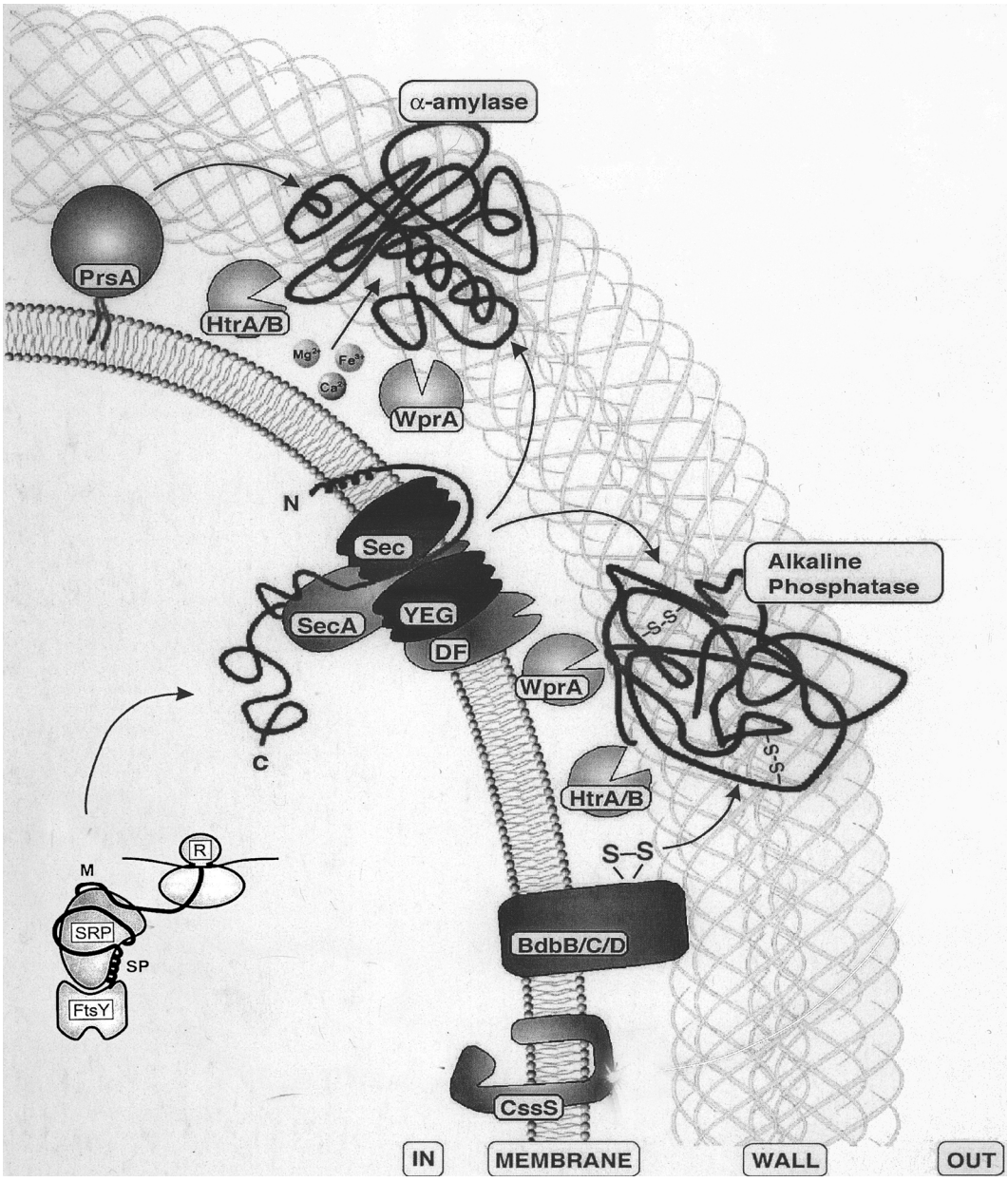

Figure 1.1. A proposed model of protein export in *S. gordonii*. This model shows the interfaces between the bacterial cell wall, plasma membrane, and cytoplasm. Illustrated components include the signal recognition particle (SRP), components of the secretory (Sec) system, and proteolytic enzymes that monitor for misfolded proteins or assist in final conformation. Adapted from Sarvas et al. (60).

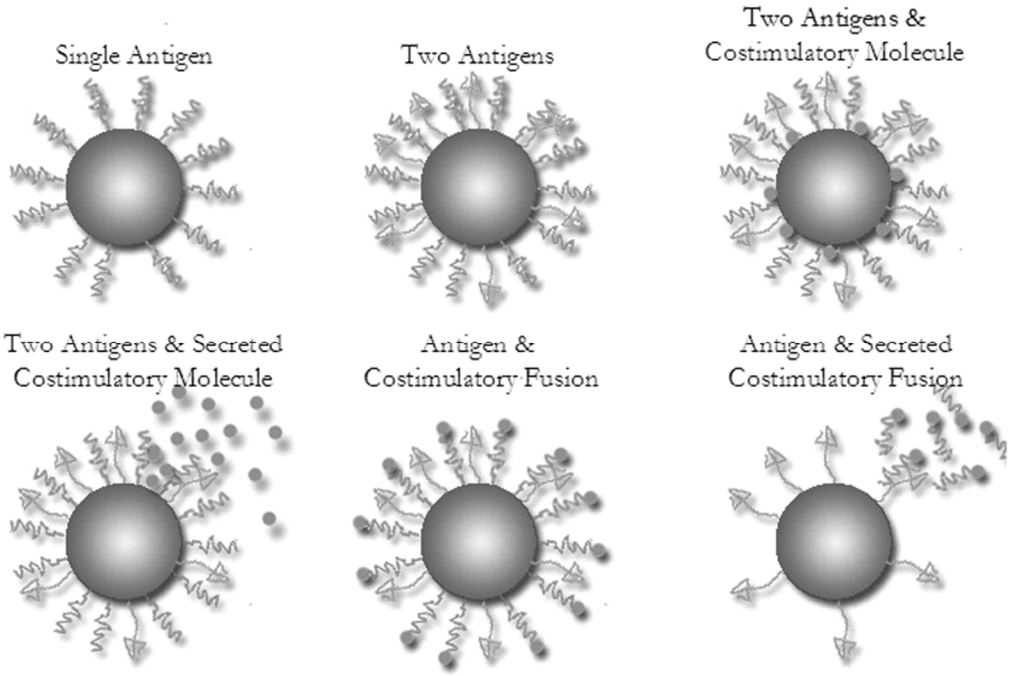

Figure 1.2. Conceptualizations of heterologous antigen expression by *S. gordonii*. Heterologous products can be displayed as a surface-anchored product or secreted into the extracellular environment. Additionally, *S. gordonii* can be engineered to express multiple products from a single cell. Costimulatory molecules can include immunomodulatory cytokines, ligands, or products that suppress immunological tolerance.

CHAPTER 2

Development of PLEX, a Plasmid-Based Expression System for Production of Heterologous Gene Products by the Gram-Positive Bacteria *Streptococcus gordonii*

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Abstract

Whereas *Escherichia coli* expression systems have been widely utilized for the production of heterologous proteins, these systems have limitations with regard to the production of particular protein products, including poor expression, expression of insoluble proteins into inclusion bodies, or expression of a truncated product. Using the Surface Protein Expression system, chromosomally integrated heterologous genes are expressed and secreted into media by the naturally competent gram-positive organism *Streptococcus gordonii.* Experiments demonstrated that *E. coli* failed to produce sufficient quantities of intact product, so we instead successfully utilized Surface Protein Expression system to produce the heterologous antigen BH4XCRR that is designed from sequences homologous to the *S. pyogenes* M-protein C-repeat region. To further enhance production of this product by *S. gordonii*, we sought to develop a novel system for the production and secretion of heterologous proteins. We observed that under various growth conditions, *S. gordonii* secreted high levels of a 172 kDa protein, which was identified by N-terminal sequence analysis as the glucosyltransferase GTF. Here we report on the development of a plasmid-based expression system, designated as PLEX, which we used to enhance production of BH4XCRR by *S. gordonii*. A region from the *S. gordonii* chromosome that contains the positive regulatory gene *rgg,* putative *gtfG* promoter, and *gtfG* secretion-signal sequence was cloned into the *E. coli/Streptococcus* shuttle plasmid pVA838. Additionally,

the *bh4xcrr* structural gene was cloned into the same plasmid downstream and in-frame with *rgg* and *gtfG*. This plasmid construct was transformed into *S. gordonii* and BH4XCRR was detected in culture supernatants from transformants at greater concentrations than in supernatants from a Surface Protein Expression strain expressing the same product. BH4XCRR was easily purified from culture supernatant using a scalable two-step purification process involving hydrophobic-interaction and gel-filtration chromatography.

Introduction

E. coli-based expression systems are widely used for the production of heterologous protein products; however, these expression systems possess limited usefulness for production of particular proteins that are sensitive to degradation by *E. coli* proteolytic enzymes, expressed in inclusion bodies, or are produced at unacceptably low levels. We have attempted to use *E. coli* expression vectors for production of a streptococcal subunit-vaccine candidate designated BH4XCRR, a circa 45 kDa antigen composed of four C-repeat regions from the *S. pyogenes* M6 protein (Fig. 2.1). These efforts have shown that BH4XCRR is susceptible to degradation when expressed in *E. coli*. Nonetheless, it can be expressed and secreted in an intact form by gram-positive *S. gordonii* using the Surface Protein Expression (SPEX) System.

Using SPEX, a heterologous structural gene, such as *bh4xcrr,* is cloned into plasmid pSMB104 so that the structural gene is flanked by regions homologous to the *S. gordonii* chromosome as well as regions corresponding to the *emm6.1* gene from *S. pyogenes* (42). The homologous flanking regions facilitate chromosomal integration of the structural gene downstream of a P2 promoter, while fusion of the heterologous gene to 16 aa of the N-terminal region of the *S. pyogenes* M6 protein provides the secretion-signal sequence. The SPEX system has been successfully used for the production of numerous heterologous proteins including derivatives of *S. pyogenes* M6-protein (40), mouse cytokines

IFN-γ and IL-2 (9), and staphylococcal nuclease A (12). However, yields of BH4XCRR from this system have been insufficient for the intended applications due to low expression and inefficient recovery methods.

During growth using typical *in vitro* culture conditions, i.e. using Todd Hewitt broth with yeast extract (THY) or Brain Heart Infusion (BHI) broth, *S. gordonii* secretes the 172 kDa enzyme glucosyltransferase (GTF) into the culture supernatant. This enzyme synthesizes glucan polymers from sucrose, which may play an important role in the *in vivo* formation of a microbial biofilm on tooth surfaces (80, 81). Mutagenesis of the gene *rgg*, located immediately upstream of *gtfG*, resulted in significantly reduced GTF activity, suggesting a possible regulatory role for *rgg* (66). Examinations into the regulatory mechanisms suggest that Rgg may act to enhance both transcription and translation of *gtfG* (80). Structural predictions of Rgg suggest the presence of a helix-turn-helix motif indicative of a DNA-binding protein (1). Interactions between Rgg and sequences located in the 3' region of *rgg* may facilitate transcriptional activity at the GTF promoter (66, 82). Rgg may be capable of enhancing translation of GTF by melting a predicted hairpin-loop secondary structure that sequesters the *gtfG* ribosomal-binding site on polycistronic *rgg/gtfG* mRNA (66). When overexpressed in *S. gordonii* using plasmid complementation, Rgg enhanced the levels of GTF activity in culture supernatants by approximately six-fold (66).

In this article, we describe the development of a new expression system, designated as PLEX, in which a resident plasmid is employed for production of a

heterologous product by *S. gordonii*. Using this system, the regulatory gene *rgg* is incorporated into the plasmid to direct expression of a structural gene, in this case BH4XCRR, from the *gtfG* promoter. Secretion of the product is facilitated by fusion of the *bh4xcrr* structural gene with the GTF secretion-signal sequence. We further report on the use of a chemically defined medium that facilitates purification.

Materials and Methods

Bacterial Strains and Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) or on LB agar (Difco). *S. gordonii* was grown in Brain-Heart Infusion (BHI) (Difco) or on BHI containing 1.5% agar. For protein production, *S. gordonii* was cultured in chemically defined medium (CDM) (JRH Biosciences, Lenexa, KS), containing 5% casamino acids (CAA)(Fisher Scientific, Pittsburgh, PA). Selection and growth for strains carrying antibiotic-resistance determinants were performed at 25 μg chloramphenicol/ml for *E. coli* strains and at 5 μg erythromycin/ml for *S. gordonii*.

DNA Isolation and Manipulation

Standard molecular biological techniques were followed in the preparation and manipulation of DNA. PCR products were subcloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA) and transformed into *E. coli* InvαF' (Invitrogen) using the manufacturer's procedure. Plasmids were isolated from *E. coli* strains using Qiagen purification kits (Valencia, CA). Following digestion with endonucleases, DNA fraqments were separated by electrophoresis and eluted from agarose gels

using QIAquick Gel Extraction Kits (Qiagen). All restriction endonucleases were obtained from New England Biolabs (Beverely, MA).

Plasmid pVA838

The *E. coli*/*Streptococcus* shuttle plasmid pVA838 has been previously characterized (30) and was isolated from *E. coli* obtained from the American Type Culture Collection (Manassas, VA). Transformed into *E. coli*, pVA838 confers resistance to chloramphenicol and erythromycin, and in *S. gordonii* it provides erythromycin resistance only.

Amplification of rgg/gtfG

Primers (forward primer TW3 5'-CCGGGATCCCGTTGACGGAGATTAG-3' and reverse primer TW4 5'-CCGGGTACCCCTGAACAGCGGACTGTTC -3')
were designed for the amplification of a 1.3 kbp product using *S. gordonii* Challis chromosome as template. These primers were designed based on the published sequence of *rgg* and the 5' region of *gtfG* (GenBank accession number M89776). TW3 introduced a *BamH1* restriction site at the 5' end of the *rgg* sequence. PCR using TW3 and the reverse primer generated a *Kpn1* restriction site at the 3'end of the *gtfG* sequence.

Design and Amplification of BH4XCRR

BH4XCRR contains four identical domains that are modeled on the conserved C-repeat region (CRR) of the M protein from *S. pyogenes* (Fig. 2.1). Each CRR seqment contains 70 amino acids. At the N terminus of the molecule and between each CRR segment are 7-amino acid spacers, each of which has a different amino acid sequence, but has the proper sequence content to maintain the alpha-helical coiled-coil nature of the molecule (48). We included a six-residue histidine tag at the C terminus with the original intent of expanding purification options. In addition, the nucleotide sequence for each CRR segment has been altered as much as possible, while keeping the amino-acid sequences of each segment identical. Last, the codon usage has been optimized for expression in *S. gordonii*. The gene was synthesized by Blue Heron Biotechnology (Bothell, WA) and was supplied as an insert in a purifed plasmid. Using this plasmid as template, we conducted PCR with forward primer TW5 (5'-CCCGGTACCTGAAACAAGAGTTAGACGAGGG-3') and reverse primer TW6 (5'-ACATGCATGCTTAATGATGGTGATGATGGTGTTG-3') to amplify the *bh4xcrr* structural gene. Amplification of *bh4xcrr* was conducted to introduce a *Kpn1* restriction site on the 5' end along with two additional nucleotides to facilitate in-frame fusion with the *gtfG* secretion-signal sequence. PCR amplification was also used to introduce an *Sph1* restriction site and a stop codon on the 3' end of *bh4xcrr*.

Cloning of pLEX1.0:bh4xcrr

For cloning, pCR2.1 containing the *rgg/gtfG* insert was sequentially digested, first with *Kpn1* and then with *BamH1*. Digestion with *Kpn1* was conducted using the manufacturer's recommended reagents for 5 h at 37^{o}C. Sodium chloride was adjusted to 50 mM and *BamH1* was added to the reaction for an additional 5 h. DNA fragments were separated using agarose-gel electrophoresis and a 1.3 kbp band was excised, extracted from the agarose matrix, and stored at -20^{o}C until further use.

The *bh4xcrr* coding sequence was excised from pCR2.1 using a simultaneous double digestion with *Sph1* and *Kpn1*, according to the manufacturer's suggested protocol, for 3 h at 37^{o}C. A 1 kbp DNA fragment was resolved using electrophoresis. This band was excised, extracted from the agarose matrix, and stored at -20^{o}C for future use.

Plasmid pVA838 was simultaneously digested using *BamH1* and *Sph1* restriction enzymes according to the manufacturer's recommended reaction conditions for 4 h at 37^{o}C. A circa 10 kbp DNA fragment was excised from the gel following electrophoresis, was extracted and stored at -20^{o}C.

The *bh4xcrr* and *rgg/gtfG* fragments were cloned into *BamH1*- and *Sph1*-digested pVA838 during a triple-ligation reaction. Digested DNA was added to the reaction at a quantity ratio of 5:5:1 *bh4xcrr:rgg/gtfG*:pVA838. Ligation was

accomplished during overnight incubation at 13°C with T4 DNA ligase (Promega, Madison, WI), using the manufacturer's suggested reaction conditions. The ligation product was immediately transformed into the InvαF' strain of *E. coli*. Transformants bearing *pVA838:rgg/gtfG/bh4xcrr* were selected from chloramphanicol-resistant colonies. Further selection was based on isolation of an appropriately sized 11.5 kb plasmid, and by PCR using isolated plasmid as template and primers TW3 and TW6. We confirmed the DNA sequence (Central Services Laboratory, Oregon State University, Corvallis, OR) of the insert using sequencing primers TW7 (5'-GCAACAGAAATATCACCTGCCG-3'), TW8 (5'-CTTGTGGCTCCAAAGGCCTTG-3'), and TW9 (5'-GGACCTTGCCAACTTGACCGC-3'). We designated this plasmid construct pLEX1.0:*bh4xcrr* (Fig. 2.2).

Transformation of Streptococcus gordonii

Purified pLEX1.0:*bh4xcrr* was introduced into *S. gordonii* GP251 using previously established transformation methods (49). Briefly, *S. gordonii* were grown in BHI in the presence of 10% fetal calf serum to induce competence and stored at –80°C in 450-µl aliquots. Plasmid pLEX1.0:*bh4xcrr* was incubated with freshly thawed competent cells for 2h at 37°C. This culture was mixed with liquified BHI agar containing 5% defibrinated sheep blood and layered onto BHI agar. After briefly allowing this layer to congeal, liquified BHI agar containing 5

μg erythromycin/ml was carefully poured onto the plates. Colonies, selected after 48 h growth at 37°C, were streaked onto BHI agar containing 5 μg erythromycin/ml to further confirm resistance and for selection of pure clones. Transformants were confirmed based on detection using Western blot of BH4XCRR in culture supernatants after overnight growth in BHI containing 5 μg erythromycin/ml at 37°C.

Electrophoresis and Western Blot

Proteins in *S. gordonii* culture supernatants were separated using SDS-PAGE. Gels were either stained using Coomassie Brilliant Blue or were transferred to PVDF membrane. Membranes were blocked with 3% BSA. Antibodies were diluted in buffer containing 0.5 % Tween 20, 0.02 % sodium azide, 0.5 M sodium chloride, and 0.01 M Tris at pH 8.2. Membranes were probed using mAb 10F5, which is specific for an epitope found in the M-protein C-repeat region of *S. pyogenes* (20), at a concentration of 2 μg/ml and goat anti-mouse IgG AP-conjugated antibody (BioRad, Hercules, CA) at a dilution of 1:1000. Blots were developed using AP-conjugate substrate kit (BioRad) diluted in buffer containing 0.06 M Tris, 0.02% sodium azide, 0.06% magnesium chloride hexahydrate at pH 9.8.

Protein Purification

A frozen stock (-80^{o}C) of SRL44 was used to inoculate 10 ml BHI containing 5 µg erythromycin/ml, and this culture was incubated at 37^{o}C overnight. It was backdiluted in 1 L filter-sterilized CDM supplemented with 5% CAA and 5 µg erythromycin/ml and was grown to OD_{650} = 0.45 (1:5 dilution in dH_2O; blanked against dH_2O) in a stationary 37^{o}C incubator. Cells were pelleted by centrifugation, and the supernatant was filtered using a filter unit with a cutoff of 0.45 µm. In preparation for hydrophobic-interaction chromatography, $(NH_4)_2SO_4$ was added to the supernatant at a concentration of 1.5 M, and the supernatant was filtered again to remove precipitates. The supernatant was loaded onto a 5-ml HiTrap Butyl FF column (Amersham BioSciences, Piscataway, NJ) that had been stripped with 5 column volumes (CV) 20mM Na_2HPO_4, pH 7.0 and equilibrated with 5 CV 50 mM Na_2HPO_4/1.5 M $(NH_4)_2SO_4$ pH 7.0. After the sample had been loaded, the column was washed with 10 CV 50 mM Na_2HPO_4/1.5 M $(NH_4)_2SO_4$ pH 7.0. Proteins were eluted using a 10-CV $(NH_4)_2SO_4$ gradient from 1.5 to 0 M, and 2-ml fractions were collected. All steps were conducted at a flow rate of 5 ml/min. Fractions containing BH4XCRR were pooled together, and proteins were precipitated by adding $(NH_4)_2SO_4$ to 60% saturation. Precipitated proteins were pelleted by centrifugation, and the pellet was resuspended in 1000 µl 50mM citric acid ($C_6H_8O_7$), pH 2.8 in preparation for gel-filtration chromatography. This solution was centrifuged to pellet undissolved proteins, and

the supernatant was loaded onto an equilibrated HiPrep16/60 Sephacryl S-300 column (Amersham). Proteins were separated at a flow rate of 1.0 ml/min using a mobile phase consisting of 50mM citric acid pH 2.8. Fractions (5 ml) were collected 30 to 120 min after the sample was loaded into the column.

Results

Secretion of BH4XCRR by S. gordonii

Following transformation of *S. gordonii*, erythromycin-resistant colonies were grown overnight at 37°C in BHI broth. All cultures were backdiluted in CDM/CAA containing erythromycin and the optical density of each culture was monitored until it reached stationary phase (OD_{650}=0.45 following 1:5 dilution in dH_2O). Culture supernatants were prepared for SDS-PAGE and BH4XCRR was visualized by Western blot (Fig. 2.3). We likewise cultured and processed samples from a BH4XCRR-secreting SPEX strain to permit comparison of production levels between these two expression systems. Intact BH4XCRR was detected in the culture supernatant of eight out of ten pLEX1.0:*bh4xcrr*-transformed *S. gordonii* and production in all transformants was enhanced relative to production in the SPEX strain (Fig. 2.3). A single pLEX1.0:*bh4xcrr*-transformed *S. gordonii* culture was retained and designated as SRL44.

While BH4XCRR could be readily detected in culture supernatants using Western blots probed with mAb 10F5, numerous approaches to quantify the BH4XCRR concentration in culture supernatants using ELISA were unsuccessful (data not shown). As an alternative, using Western blot the BH4XCRR band intensity in SRL44 culture supernatant was compared against those of serially diluted, previously purified BH4XCRR produced by the SPEX system (Fig. 2.4).

Band intensities were quantified using densitometry, and a regression line was calculated from the values. From this evaluation, we determined that BH4XCRR was secreted into medium at a concentration of 10 mg/L by SRL44.

BH4XCRR Purification

An initial cleanup step was conducted using hydrophobic interaction chromatography (Figure 2.5). BH4XCRR was extracted by passing the culture supernatant, supplemented with 1.5 M $(NH_4)_2SO_4$ over an equilibrated HiTrap Butyl FF column. The column was washed with 50mM Na_2HPO_4/1.5 M $(NH_4)_2SO_4$ and bound protein was eluted using a gradient of 1.5 M to 0 M $(NH_4)_2SO_4$. We evaluated the effectiveness of this procedure by running fractions on an SDS-PAGE gel, and proteins were visualized by staining with Coommassie Brilliant Blue (Fig. 2.5). BH4XCRR was not detected in a sample of the flowthrough taken as the sample was nearly completely loaded onto the column, suggesting that the product was efficiently extracted from the supernatant. BH4XCRR was eluted from the column in a limited number of fractions, and is represented as a single darkly stained band at 45 kDa, suggesting that it remains predominantly intact during this processing step. Additionally, BH4XCRR was separated from a number of other proteins secreted by *S. gordonii* during gradient elution.

Following this initial cleanup, BH4XCRR was further purified using gel-filtration chromatography. Fractions from the hydrophobic-interaction separation that contained BH4XCRR were pooled and proteins were precipitated using a 60% saturated solution of $(NH_4)_2SO_4$. The precipitate was pelleted, redissolved in 50mM citric acid pH 2.8, and loaded onto an equilibrated gel-filtration column. While BH4XCRR was visualized on Coomassie-stained SDS-PAGE gels in fractions that elute 40 to 75 min after sample was loaded, it was predominantly eluted in four 5-ml fractions collected between 50 and 65 min (Fig. 2.6). These fractions contained undetectable amounts of contaminating proteins. A faint banding pattern was observed below the primary BH4XCRR band in fractions eluted at 60 and 65 min. This banding pattern is indicative of minor BH4XCRR degradation, and the reactivity of these bands has been confirmed using Western blots probed with mAb 10F5.

Discussion

Because multiple heterologous products have been successfully produced using *E. coli*, we initially attempted production of the streptococcal vaccine candidate BH4XCRR using these bacteria (data not shown). While we were able to express BH4XCRR using *E. coli*, we observed substantial degradation of the product. This example highlights the limitations associated with the use of *E. coli* as an expression vector. Use of *E. coli* for production of particular proteins can result in insufficient expression, expression of insoluble proteins into inclusion bodies, or an inablility to express product in an intact form. Additional concerns arise from the use of *E. coli* as an expression vector because of the potential for traces of lipopolysaccharide (LPS), a potent toxin and immunostimulant, to be unintentionally copurified along with the desired product.

The gram-positive bacteria *S. gordonii* have been used to express numerous heterologous products, including derivatives of the *S. pyogenes* M6 protein such as BH4XCRR. Using the SPEX system, heterologous sequences are chromosomally integrated and production is driven from the native P2 promoter (10, 40, 42). Secretion is accomplished by fusion of the product with the secretion-signal sequence derived from the *emm6.1* gene from *S. pyogenes*. The SPEX system has been very successful for the production of particular antigens, and initial attempts at producing BH4XCRR were encouraging, particularly with regard to expression of an intact product. The yields of BH4XCRR, however, were not sufficient for

the intended applications. We sought, therefore, to enhance production through the development of a new expression system.

Instrumental to our approach was the observation that *S. gordonii* secretes a high-molecular weight protein under a variety of culture conditions. This protein was regularly detected as the predominant protein band in SDS-PAGE analyses of culture supernatants. N-terminal sequence analysis revealed this protein as the 172 kDa glucosyltransferase GTF (data not shown). It is positively regulated, perhaps by both transcriptional and translational mechanisms, by Rgg (66, 81), and overexpression of Rgg, using plasmid complementation, was shown to upregulate glucosyltransferase by approximately six-fold in an *rgg* deletional mutant of *S. gordonii* (66).

Several research teams have utilized plasmid-based systems for expression of heterologous products using *S. gordonii*. Shiroza et al. (63) developed a plasmid-based system for secretion of *Bacillus circulans* cycloisomaltooligosaccharide glucanotransferase using the promoter and secretion-signal sequence of the *S. sobrinus gtfI* gene. This system relies on recombination, within *S. gordonii*, between an integration plasmid and a resident plasmid for the expression of heterologous sequences. Attempts to quantify the level of expressed protein in culture supernatants were not reported. Using a derivative of pVA838 bearing the promoter and secretion-signal sequences from *S. downei* glucosyltransferase I, Kataoka et al. (21) engineered *S. gordonii* to secrete a recombinant peptide (r-pPRP-C) to inhibit *Porphyromonas gingivalis* fimbril

binding. This transformant secreted r-pPRP-C into the medium at a concentration of 8.6 μg/ml. Lee et al. (25) constructed a plasmid to express recombinant subunit S1 of pertussis toxin on the surface of *S. gordonii*. Synthesis and surface expression were accomplished using sequences from the surface protein antigen P1 gene of *S. mutans*.

The expression system we describe here was designed to drive expression of BH4XCRR from the *gtfG* promoter. Coding regions for the GTF positive regulator, Rgg, were included to enhance activity at the *gtfG* promoter and to enhance translation. Secretion of the product was accomplished by fusion of the *gtfG* secretional signal sequence to the *bh4xcrr* structural gene. We selected to express BH4XCRR, in this manner, from a plasmid with the expectation that multiple copies of these genes would facilitate enhanced production. *S. gordonii* were readily transformed with plasmid pLEX1.0:*bh4xcrr,* and the presence of intact BH4XCRR in the culture supernatants of transformants was confirmed using Western blot.

In developing a strategy to purify BH4XCRR from culture supernatant, we concentrated on techniques that could be potentially scaled to suit production-level purification practices. This approach, in combination with the relatively large volume of culture supernatant, limited the use of precipitating agents to condense supernatant proteins or for buffer exchange. Additionally, metal-affinity chromatography and ion-exchange chromatography were inefficient techniques for extracting BH4XCRR from either BHI or CDM (data not shown), perhaps because

of interference from salts contained in these formulations. However, we found that by adding moderate quantities of $(NH_4)_2SO_4$ to culture supernatants, proteins could be efficiently extracted from supernatant using hydrophobic-interaction chromatography. At a concentration of 1.5 M $(NH_4)_2SO_4$ nearly all proteins in the supernatant adsorbed to the matrix, which quickly saturated the binding capacity of the column when BHI-based supernatants were loaded. By culturing SRL44 in CDM/CAA, which contains a much lower concentration of protein products, BH4XCRR could be efficiently extracted and concentrated from a 1 L volume of supernatant using a 5-ml HiTrap Butyl FF column.

After extracting BH4XCRR from culture supernatant, we utilized size-exclusion chromatographic principles to isolate BH4XCRR from copurified proteins. Because we have found BH4XCRR to be stabilized by low pH (data not shown), we used 50mM citric acid pH 2.8 to equilibrate the column, and as a mobile phase during gel-filtration chromatography. The neutral pH of buffers used during hydrophobic-interaction chromatography apparently only minimally affected breakdown of BH4XCRR, which perhaps was stabilized by a conformation induced by $(NH_4)_2SO_4$ or by the presence of additional proteins in the supernatant.

The PLEX system has proven useful for expression of an intact protein product, BH4XCRR, that was largely degraded when expressed using *E. coli*. We are currently conducting experiments to assess the versatility of the PLEX system for the production of additional heterologous products that are not amenable to

expression by *E. coli*. The PLEX system was designed such that the expressed product retains 19 amino acids from GTF at the N-terminus. Our intent was to preserve the native GTF sequences that may be required for efficient recognition and cleavage of the GTF secretion signal by *S. gordonii* proteolytic processes. However, it may be possible to express heterologous products without the fusion of any additional sequences. Because PLEX is compatible with the SPEX expression system, it is conceivable to employ both systems within a single clone to express either individual or multiple proteins. Additionally, it may be possible to adapt the PLEX system to express proteins anchored to the *S. gordonii* cell wall. This adaptation may prove useful for efforts that are currently underway to develop *S. gordonii* as a vaccine vector. Because retention of the expression plasmid in the absence of antibiotic selective pressure is essential for use of the PLEX system in vaccine development, we are currently conducting investigations in mice regarding plasmid retention by *S. gordonii*.

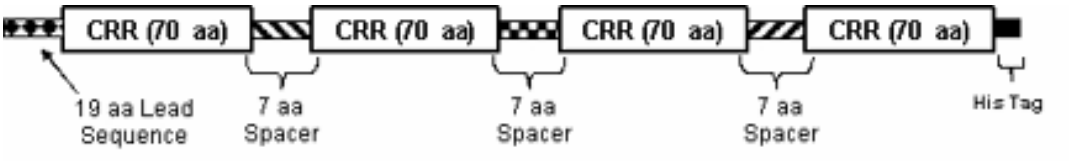

Figure 2.1. Representation of the BH4XCRR molecule showing four tandemly-repeated CRRs with intervening 7-amino acid spacers. The 19 amino acids at the N-terminus remain after the GTF signal sequence is cleaved during native processing reactions.

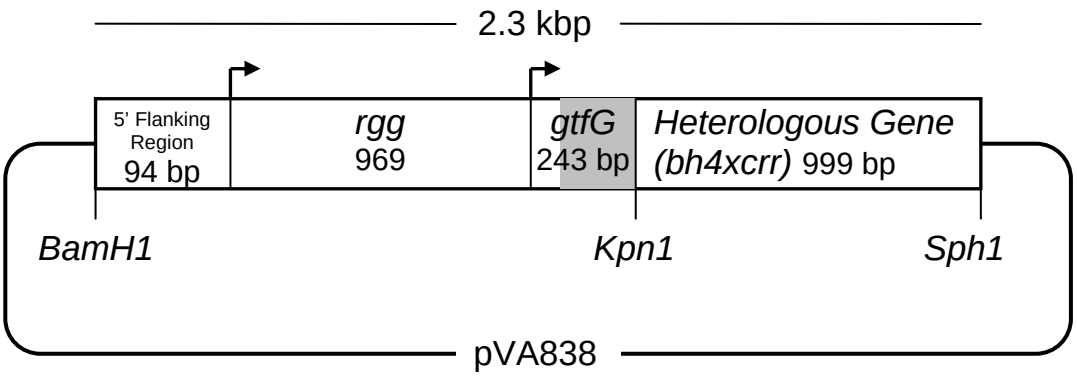

Figure 2.2. Overview of the arrangement of genetic elements in the PLEX system. A 2.3 kbp region was cloned into pVA838 using the *BamH1* and *Sph1* restriction sites on the plasmid. The putative promoters for *rgg* and *gtfG* are identified by right-angled arrows. The shaded region in GTF corresponds to the 105 bases coding for the 35-amino acid secretion signal sequence. The heterologous structural gene *bh4xcrr* is situated downstream and in-frame with the secretional signal sequence.

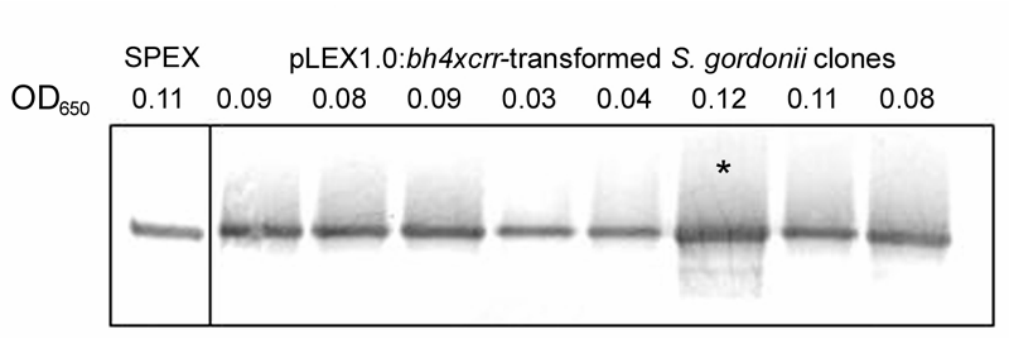

Figure 2.3. Transformation of *S. gordonii* with pLEX1.0:*bh4xcrr* enhances production relative to SPEX. Protein in culture supernatants from pLEX1.0:*bh4xcrr* -transformed *S. gordonii* and from SPEX strain SRL16 were separated using SDS-PAGE, transferred to PVDF membrane and probed with mAb 10F5. We retained the transformant indicated with the asterisk, and designated this clone as SRL44. The OD_{650} values were recorded in culture supernatants diluted 1:5 in dH_20 blanked against dH_20.

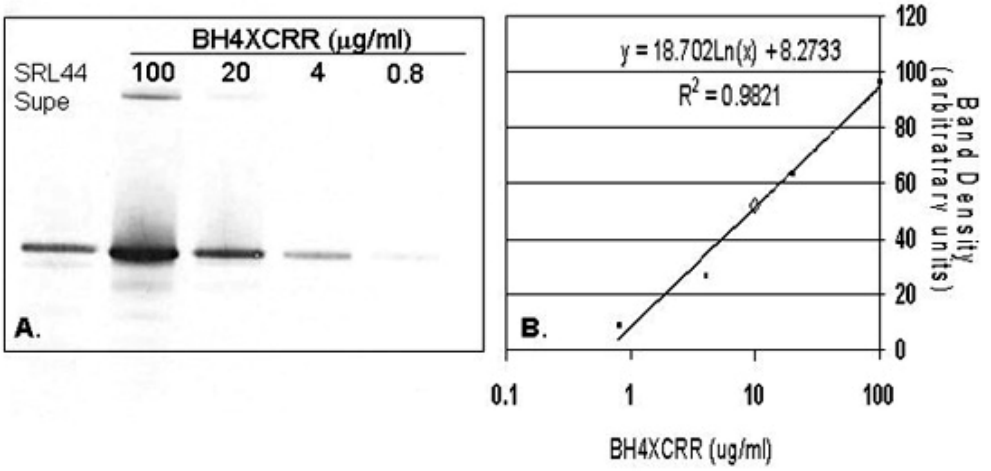

Figure 2.4. Comparison of BH4XCRR in SRL44 culture supernatants with BH4XCRR standard concentrations. A. SDS-PAGE was conducted on SRL44 culture supernatant and BH4XCRR diluted to standard concentrations. After electorphoresis proteins were transferred to PVDF membrane and probed with mAb 10F5. The stock solution of BH4XCRR used for standards had been previously produced using SPEX, purified using metal-affinity chromatography, and the protein concentration determined using a bicinchoninic acid assay (Pierce, Rockford, IL). B. Band intensities were quantified using densitometry, and a regression line was calculated from the values. The band intensity from SRL44 culture supernatant is plotted on the chart as an open diamond, and the BH4XCRR standards are represented by closed squares.

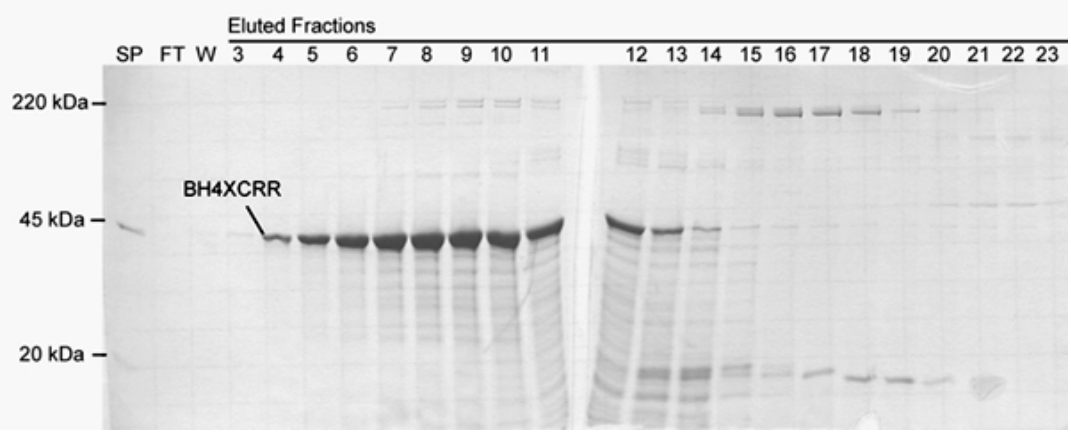

Figure 2.5. Initial cleanup and extraction of BH4XCRR from 1 L SRL44 culture supernatant (SP) is accomplished using hydrophobic-interaction chromatography. SDS-PAGE was conducted using a sample of culture supernatant, flow through (FT), wash (W) and eluted fractions (3-23). Proteins were visualized after staining with Coomassie Brilliant Blue, and BH4XCRR is represented by the predominant bands in these gels.

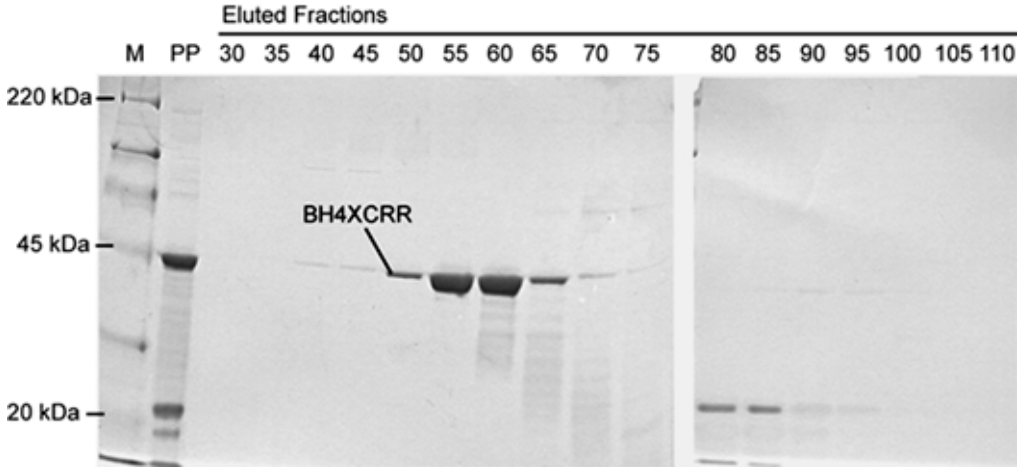

Figure 2.6. Gel filtration chromatography is used to isolate intact BH4XCRR. A partially purified product (PP) was loaded onto a citric-acid equilibrated gel-filtration column. Using a flow rate of 1 ml/min, fractions (5 ml) were collected beginning 30 min after loading the sample. Elution times (min) for each fraction are indicated above each lane. SDS-PAGE was conducted on these samples and included a molecular weight marker (M). BH4XCRR, the predominant band on these gels, was visualized after staining with Coomassie Brilliant Blue.

Chapter 3

Comparison of Heterologous Protein Expression by *Streptococcus gordonii* Native Promoters Using a Multi-Copy Plasmid

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Abstract

An active area of research in the development of *S. gordonii* for use as a bacterial commensal vector involves the identification and utilization of strong promoters for high-level expression of heterologous products. *E. coli* plasmid vectors containing different streptococcal promoters often fail to become established in *E. coli* for unknown reasons. Therefore, it is desirable at times to transform *S. gordonii*, which is naturally competent, with small quantities of nascently ligated DNA without using *E. coli* first to amplify or screen the product. By comparing the efficiency of two methods used to induce competence in *S. gordonii*, it was shown that the use of a synthetic competence stimulating peptide substantially enhanced plasmid uptake by *S. gordonii*. We isolated and characterized the amylase-binding protein (*abpA*) promoter from *S. gordonii*, and, using synthetic peptide to induce competence, introduced directly plasmid DNA containing this promoter into *S. gordonii* as an unamplified product of ligation. This plasmid facilitated abundant secretion of a heterologous product by *S. gordonii*. By assessing the levels of heterologous product secreted by two plasmid constructs, it was possible to evaluate the relative strength of two native promoters.

Introduction

Streptococcus gordonii, a human commensal bacterium, is being developed as a vaccine vector due to its ability to elicit mucosal and systemic immune responses (86). Additionally, it may be useful as a vector for delivery of therapeutic molecules (54), and has been used for protein production due to its ability to secrete soluble proteins into the culture supernatant (24, 84). These applications all require high-level expression of heterologous products, which can be facilitated through the use of native promoters and secretion-signal sequences situated on multi-copy plasmids. An active area of research involves the identification and evaluation of the strength of native promoters (52).

Under glucose-deficient culture conditions *S. gordonii* abundantly secretes a 20.5 kDa amylase-binding protein (AbpA)(56). Amylase, the most abundant enzyme in human saliva, catalyzes the hydrolysis of α-1,4-glucosidic linkages in starch (57). Based on observations that amylase-binding streptococci appear to be restricted to animal hosts that secrete salivary amylase, it has been theorized that the binding of amylase by oral streptococci may be critical for their ability to colonize and persist in the host (8, 61). Additionally, the binding of amylase may facilitate bacterial growth by providing fermentable carbohydrates from dietary starch. Analysis of the *abpA* sequence revealed a putative catabolic response element (CRE) 153 bp downstream of the start codon. This CRE may participate in transcriptional repression of *abpA* in the presence of saccharides, when starch

metabolism is not necessitated (57). Rogers and Scannapieco (2001) identified the transcriptional start site of abpA, but the genomic sequence further upstream, including the putative -35 and -10 promoter sequences, remain uncharacterized.

A potential drawback to evaluating promoter strengths for purposes of expression by *S. gordonii* is that *E. coli* – into which plasmid constructs are routinely subcloned, screened, and amplified – does not stably maintain high-copy vectors containing particular streptococcal promoters (52, 64). For this reason, it is desirable at times to be able to transform ligated DNA containing potentially strong promoters directly into *S. gordonii* without subcloning into *E. coli*. Even though *S. gordonii* is naturally competent, the efficiency with which DNA is introduced using traditional methodologies, i.e. by inducing competence with calf or horse serum, does not permit the routine introduction of small quantities of DNA, such as the product in typical ligation reactions.

In *S. gordonii,* natural competence induction is regulated by a quorum-sensing system in which the accumulation of a secreted competence stimulating peptide (CSP) induces a transient state of competence (17, 18). This CSP is synthesized by *S. gordonii* as an inactive precursor with a double glycine leader sequence. The sequence is recognized and cleaved by its cognate ABC-transporter, resulting in the secretion and activation of the peptide. When the CSP reaches a critical level in the extracellular medium, it activates a regulatory cascade that triggers the changes in gene expression necessary for competence. It has been found that strain-specific synthetic peptides can be used to induce

competence in various streptococcal strains, including *S. gordonii* (15), suggesting that the use of CSP may facilitate DNA uptake during particularly challenging molecular cloning efforts in *S. gordonii*.

Here we present a procedure for preparing frozen competent cells using a synthetic competence stimulating peptide and provide a comparison of this technique versus the traditional method. By using a CSP, competence induction and high transformation efficiencies were consistently achieved. We further report on the use of this technique to transform unamplified ligated DNA, containing a strong native streptococcal promoter, directly into *S. gordonii* thus bypassing the need for subcloning or further amplification by *E. coli*. Additionally, we report that expression of a test antigen, streptococcal vaccine candidate BH4XCRR, is expressed at enhanced levels relative to previously developed expression systems.

Methods and Materials

Bacterial Strains and Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) or on LB agar (Difco). *S. gordonii* was grown in Brain-Heart Infusion (BHI) (Difco) or on BHI containing 1.5% agar. For protein production, *S. gordonii* was cultured in chemically defined medium (CDM; JRH Biosciences, Lenexa, KS) (76), containing 5% casamino acids (CAA; Fisher Scientific, Pittsburgh, PA). Selection and growth for strains carrying antibiotic-resistance determinants were performed either at 25 μg chloramphenicol/ml or 300 μg erythromycin/ml for *E. coli* strains and at 5 μg erythromycin/ml for *S. gordonii*.

DNA Isolation and Manipulation

Standard molecular biological techniques were followed in the preparation and manipulation of DNA. PCR products were subcloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA) and transformed into *E. coli* InvαF' (Invitrogen) using the manufacturer's procedure. Plasmids were isolated from *E. coli* strains using Qiagen purification kits (Valencia, CA). Following digestion with endonucleases, DNA fraqments were separated by electrophoresis and eluted from agarose gels

using QIAquick Gel Extraction Kits (Qiagen). All restriction endonucleases were obtained from New England Biolabs (Beverely, MA).

Induction and Transformation of S. gordonii using CSP

The unmodified competence-stimulating peptide, N-DVRSNKIRLWWENIFFNKK, was custom synthesized by Sigma-Genosys (The Woodlands, TX)(17, 18). Peptide was resuspended in sterile water and the concentration was determined using a BCA™ Protein Assay kit (Pierce, Rockford, IL). Resuspended peptide was stored in convenient aliquots at -20^{o}C until further use. To prepare competent cells, an overnight culture of *S. gordonii* Challis, strain GP251 (45), was grown at 37°C in Todd-Hewitt broth with 1% yeast extract (THY) (Becton Dickinson, Sparks, MD) supplemented with chloramphenicol (5 µg/ml). The following day CSP was added to the overnight culture at a final concentration of 10 µg/ml. Cells were immediately frozen in 100 µl aliquots at -80°C following the addition of 10% glycerol.

To transform cells, an aliquot of frozen competent cells was quickly thawed and 900 µl THY and 1 µg of plasmid DNA were added. Cells were incubated at 37°C for 3 hours, and 25 µl was plated on BHI agar containing erythromycin. If the plasmid required chromosomal integration (e.g. pSMB104), 450 µl of the culture was mixed with liquified BHI agar containing 5% defibrinated sheep blood and this mixture was layered onto BHI agar without

antibiotic. Cells were incubated in this layer at 37°C for 90 min, and liquified BHI agar containing 5 μg erythromycin/ml was carefully overlayered onto the plates to select for antibiotic-resistant colonies. Plates were incubated for 48 h at 37°C.

Induction and Transformation of S. gordonii using Fetal Calf Serum

S. gordonii were grown in BHI in the presence of 10% fetal calf serum to induce competence and stored at -80°C in 450-μl aliquots (49). Plasmid (1 μg) was incubated with freshly thawed competent cells for 2h at 37°C. This culture was mixed with liquified BHI agar containing 5% defibrinated sheep blood and layered onto BHI agar. Cells were incubated at 37°C for 90 min, and liquified BHI agar containing 5 μg erythromycin/ml was carefully overlayered onto the plates. Colonies were quantified after 48 h growth at 37°C.

Plasmids

Plasmids pLEX1.1:*bh4xcrr* and pSMB104 were isolated from *E. coli* strains using QIAGEN Plasmid Midi kits (Valencia, CA). pLEX1.1:*bh4xcrr* is a derivative of the *E.coli/Streptococcus* shuttle vector, pVA838 (29). It is an 11.2 kbp plasmid in which expression of a model heterologous product, BH4XCRR, is driven by the *gtfG* promoter of *S. gordonii* (84). In *S. gordonii*, pLEX1.1: *bh4xcrr*

does not integrate into the chromosome but is replicated as a multi-copy resident plasmid, conferring resistance to erythromycin in transformed cells. Plasmid pSMB104 is a 5.6-kbp *E. coli* plasmid which integrates into the chromosome of *S. gordonii* by homologous recombination. In *S. gordonii*, replication of pSMB104 genetic material and conference of erythromycin resistance is dependent on chromosomal integration (45).

Amplification of abpA

A 300-bp region containing sequences for the *abpA* promoter, secretional signal sequence, and part of the mature protein were initially amplified using genomic DNA from *S. gordonii* Challis as template and forward primer ML4 (5'-GGATCCCTTCCCCCTGACCACAAG) and reverse primer ML9 (5'-GCATGCAAGGTAGTATGCGCCGTCG) (Fig. 3.1). Amplification in this manner introduced a *BamHI* restriction at the 5' end of the product and an *SphI* restriction site at the 3' end. This PCR product was subcloned into pCR2.1 TOPO to create pCR2.1:*abpA*. Because pCR2.1:*abpA* was poorly tolerated by *E. coli* InvαF' (demonstrated by poor plasmid yield), this plasmid was used as template to amplify *abpA* using standard primers M13F (5'-GTAAAACGACGGCCAG) and M13R (5'-CAGGAAACAGCTATGAC), which are homologous to regions of the pCR2.1 TOPO vector.

Construction of pLEX1.1:bh4xcrr

Plasmid pLEX1.1:*bh4xcrr* was derived from parental plasmid pLEX1.0:*bh4xcrr* (84), which contains two *EcoRI* restriction sites, in the 3' region of *rgg* and in the chloramphenicol acetyltransferase (*cat*) gene. Because it was desirable to use *EcoRI* to disable the *cat* gene without affecting *rgg*, site-directed mutagenesis was conducted using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturers protocol. A silent T860→C nucleotide mutation was introduced into the 3' region of *rgg* using pLEX1.0:*bh4xcrr* as template and primers EcoRI-F (5'-CAAAGAACAATTTGAGCGAATCCAACTAACAGT) and EcoRI-R (5'-TCTGCAACTACTGTTAGTTGGATTCGCTCAAAT). Parental plasmid was digested using *DpnI* and products were transformed into *E. coli* XL-10 Gold cells (Stratagene). An erythromycin-resistant clone was screened for the presence of the desired mutation by confirming that *EcoRI* was unable to excise a band during a digestion reaction. This plasmid was subsequently sequenced at Oregon State University Central Services Laboratory (OSUCSL) to further confirm the mutation. This plasmid construct was designated pLEX1.1:*bh4xcrr*.

Construction of pLEX2.0:bh4xcrr

The *bh4xcrr* gene was amplified as previously described (84) except that an *SphI* restriction site was incorporated into the 5' end of the product using primer BH4XCRR SphI (5'-GCATGCAAACAAGAGTTAGACGAGGGA) and an *EcoRI* was added to the 3' end with primer BH4XCRR EcoRI (5'-GAATTCTTAATGATGGTGATGATGGTGTTG). This PCR product was subcloned into pCR2.1 TOPO to create pCR2.1:*bh4xcrr*. pLEX1.1:*bh4xcrr* was digested using a simultaneous double digestion with *EcoRI* and *SphI*, according the manufacturer's suggested protocol. A 10-kbp fragment was resolved using electrophoresis. This band was excised and extracted from the agarose matrix, and stored at -20^{o}C until further use. pCR2.1:*bh4xcrr* was likewise digested using *EcoRI* and *SphI* to produce a 1.0 kbp fragment that was resolved by electrophoresis and extracted. EcoRI- and SphI-digested DNA fragments were ligated using 88 ng *bh4xcrr* and 36 ng digested pLEX1.1:*bh4xcrr*. Ligation was accomplished during overnight incubation at 13^{o}C with T4 DNA ligase (Promega, Madison, WI). The ligation product was immediately transformed into *E. coli* InvαF', and transformants were selected on the basis of erythromycin resistance and sensitivity to chloramphenicol. The plasmid sequence was confirmed by sequence analysis conducted by OSUCSL, and the plasmid was designated pLEX1.1:4xcrrSphIEcoRI (pML1).

PCR amplification was conducted using pCR2.1:*abpA* as template and standard primers M13F and M13R. This product and pML1 were digested using a simultaneous double digest with *BamHI* and *SphI*, according to the manufacturer's recommended reaction conditions for 2h at 37^{o}C. A 6.7 kbp plasmid fragment and a 300-bp *abpA* fragment were resolved using electorophoresis, and fragments were ligated in an overnight reaction at 13^{o}C using T4 DNA ligase with 60 ng plasmid DNA and 270 ng *abpA* insert. This plasmid was designated pLEX2.0:*bh4xcrr* (Fig. 3.2). An aliquot (5 µl) of the ligation reaction was used in attempts to transform competent InvαF', according the manufacturer's recommended protocol, and 15 µl was used to transform CSP-induced competent *S. gordonii* Challis strain GP251.

Electrophoresis and Western Blot

Proteins in *S. gordonii* culture supernatants were resolved using SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 3% BSA. Antibodies were diluted in buffer containing 0.5 % Tween 20, 0.02 % sodium azide, 0.5 M sodium chloride, and 0.01 M Tris at pH 8.2. Membranes were probed using mAb 10F5 (20), which is specific for an epitope found in the heterologous product BH4XCRR (a derivative of the M-protein C-repeat region of *S. pyogenes*), at a concentration of 2 µg/ml and goat anti-mouse IgG AP-conjugated antibody (BioRad, Hercules, CA) at a dilution of 1:1000. Blots were

developed using AP-conjugate substrate kit (BioRad) diluted in buffer containing 0.06 M Tris, 0.02% sodium azide, 0.06% magnesium chloride hexahydrate at pH 9.8.

Results

Comparison of FCS- and CSP-induced transformation efficiency

To permit comparison of transformation efficiencies between FCS- and CSP-induced techniques, *S. gordonii* was transformed with pLEX1.1:*bh4xcrr* using each procedure (49). Putative transformants were screened on the basis of erythromycin resistance and by detection of BH4XCRR on PVDF colony overlays after 48h growth. Transforming *S. gordonii* with pLEX1.1:*bh4xcrr* using CSP resulted in greater than a 1000-fold increase in the transformation efficiency versus the FCS method of transforming *S. gordonii* (Fig. 3.3A and 3.3B). Additionally, >90% of these transformants were found to secrete the heterologous protein BH4XCRR as shown using PVDF colony overlays (Fig. 3.3C), which is consistent with the expected transformed phenotype. When transforming with pSMB104, which requires integration into the chromosome, a 174-fold increase in the number of CSP- induced transformants was observed (Table 1).

Identification of S. gordonii abpA putative promoter

Using N-terminal sequence analysis of protein concentrated from *S. gordonii* culture supernatant, a 14.2 kDa protein was identified as AbpA. Although

previous research findings using primer extension had identified the transcriptional start for the amylase binding protein (57), the transcriptional promoter was not characterized. A BLAST search of the incomplete *S. gordonii* genome sequence at http://www.tigr.org/index.shtml revealed the sequence of the region upstream of the transcriptional start (69). The internet program BPROM at http://www.softberry.com/berry.phtml identified two sequences, TTGACA and ATATATAAT, which conform to the -35 and -10 consensus sequences, respectively, of bacterial σ^{70} promoters (Fig. 3.2). The -10 region of this putative promoter is precisely located at the empirically determined transcriptional start site (57).

E. coli *fails to stably maintain the* abpA *promoter*

Whereas a copy of the putative promoter for *abpA* was successfully ligated into the high-copy subcloning vector pCR2.1, yield of this plasmid from *E. coli* strain InvαF' was uncharacteristically low (data not shown) to the extent that it was impractical to use pCR2.1:*abpA* for restriction digest reactions as was originally intended. Additionally, when transformed with an aliquot of the pLEX2.0:*bh4xcrr* ligation reaction, only four erythromycin-resistant *E. coli* colonies grew, none of which developed as expected when streaked onto selective LB agar, and no plasmid DNA could be detected in plasmid preparations of cultures (data not shown). These findings are significant in the context that an

aliquot from the same reaction was used to successfully transform competent *S. gordonii*. These results are consistent with other research suggesting that plasmids bearing strong streptococcal promoters are poorly maintained by *E. coli* (52).

Transformation of S. gordonii with pLEX2.0:bh4xcrr

We sought to transform *S. gordonii* directly with the pLEX2.0:*bh4xcrr* ligation reaction because it was suspected that *E. coli* would be poorly transformed with the DNA containing the *abpA* promoter. Additionally, it was reasoned that the enhanced efficiency associated with the use of CSP might permit transformation with the minimal amount of DNA that would be would be present in the reaction. After transforming with 15 µl of the ligation product, 16 *S. gordonii* colonies developed on BHI selective agar. Five were selected for additional analysis. Colonies were grown overnight at 37^{o}C in BHI broth containing erythromycin. Cultures were backdiluted in CDM/CAA with erythromycin and grown at 37^{o}C until cultures reached stationary phase. Culture supernatants were prepared for SDS-PAGE and BH4XCRR was visualized using Western Blot. To permit the comparison of the relative amounts of BH4XCRR produced by different promoters, we likewise cultured and processed samples from *S. gordonii* strain SRL44, from which BH4XCRR is expressed from the native *gtfG* promoter (84). BH4XCRR was apparent in the supernatant of all pLEX2.0:*bh4xcrr S. gordonii* and production by all transformants was noticeably

enhanced relative to SRL44 (Fig. 3.5). A single clone was retained and designated SRL48.

It was conceivable that minor quantities of incompletely digested parental plasmid (pML1) had been carried through to the ligation reaction and subsequently transferred to *S. gordonii* during the transformation reaction. To ensure that the pLEX2.0:*abpA* expression plasmid was driving expression of BH4XCRR, DNA was isolated from SRL48 and a region was amplified from the plasmid using primers ML7 and TW6 (Fig. 3.5) that lacked homology with known *S. gordonii* genomic sequences. The sequence analysis of this product confirmed that SRL48 had been transformed by pLEX2.0:bh4xcrr (data not shown).

Discussion

The genome of *S. gordonii* has been partially sequenced and derived sequences have yet to be annotated to identify putative genes and other genetic features. Thus, identification of *S. gordonii* promoters have relied primarily on empirical techniques. Pozzi et al. (52) have successfully characterized the strength of newly identified *S. gordonii* promoters by measuring the minimum concentration of chloramphenicol that inhibits growth of clones into which a promoterless chloramphenicol acetyltransferase (*cat*) gene has been randomly integrated.

Previously, we observed that GTF was secreted by *S. gordonii* under a variety of culture conditions. By situating the genetic elements that facilitate GTF production and secretion on a multi-copy plasmid, a heterologous gene product, BH4XCRR, could be expressed at high levels. We retrospectively designate this expression system as $PLEX_{1.0}$. Using SPEX, heterolgous genes can be expressed at high levels using a single-copy genomic P2 promoter (5, 9, 40, 42). However, because the gene copy number differs between the SPEX and $PLEX_{1.0}$ expression systems, evaluation of the relative strength of the promoters associated with each system is not straightforward.

We observed that under culture conditions used for protein production using $PLEX_{1.0}$, AbpA protein was abundantly secreted into the culture medium. Having identified putative genetic elements that facilitate expression and secretion

of AbpA, it was possible to create a multi-copy expression plasmid construct that permitted production of the BH4XCRR heterologous product using *abpA* genetics, which we designated as $PLEX_{2.0}$. The amino acid sequences of BH4XCRR produced by $PLEX_{1.0}$ versus $PLEX_{2.0}$ differ only by 16 aa, positioned at the N-terminus following cleavage of the secretion signal by native processes. BH4XCRR production was found to be enhanced using the $PLEX_{2.0}$ expression plasmid, suggesting that the AbpA promoter may more efficiently initiate transcription than the GTF transcriptional elements of $PLEX_{1.0}$. However, further research is required to evaluate the influence that the different secretion-signal sequences exert on expression levels.

The amplified region of *abpA* omitted the putative CRE 153 bp downstream of the translational start codon. The CRE has been used to facilitate transcriptional suppression of genes involved in the utilization of starch such as *abpA*. Findings in support of this theory show that when *S. gordonii* is cultured in the presence of glucose, lactose, or maltose, AbpA secretion is suppressed (57). Interestingly, when SRL48 was cultured in BHI supplemented with glucose at various concentrations from zero to 5%, no differences could be detected using Western blot in the amount of BH4XCRR in culture supernatants (data not shown). These results suggest that the promoter, as cloned on pLEX2.0:*bh4xcrr,* is not subject to the carbohydrate-induced suppression that has been observed for the same promoter in the context of the genome, perhaps because deletion of the CRE enables uninhibited transcription from the *abpA* promoter.

The research findings presented in this manuscript suggest that the *abpA* promoter may be a useful genetic component for the production of heterologous proteins using *S. gordonii*. Its use may enhance the *in vivo* delivery of vaccine antigens, costimulatory molecules, or therapeutic agents using *S. gordonii* as a bacterial commensal vector (BCV) (86). While it has been suggested that the potential for plasmid loss in the absence of antibiotic selective pressure makes the use of plasmid-based expression systems undesirable in BCV systems (52), the *in vivo* retention of plasmids by *S. gordonii* BCV may well depend on the plasmid vector used. We are currently conducting evaluations in mice regarding retention of pLEX plasmids by *S. gordonii*.

By utilizing a synthetic CSP to induce competence, *S. gordonii* transformation efficiency was greatly enhanced, making this method ideal for routine transformations, and for times in which quantities of DNA and time are at a premium. Here we have demonstrated that, when necessary, CSP can be used to bypass intermediate cloning steps in *E. coli*, by transforming ligation products directly into *S. gordonii*. In addition, we have successfully used CSP to transform *S. gordonii* with the *E. coli/S. faecalis* shuttle vector pAM401 (87) and the temperature sensitive plasmid pGhost6 (33), which could not be transformed in FCS-induced *S. gordonii* (data not shown). While the traditional use of FCS has been widely utilized, this technique commonly requires multiple transformation attempts to obtain antibiotic-resistant transformants that display the full array of phenotypic characteristics consistent with transformation, e.g. erythromycin

resistance, chloramphenicol sensitivity, and heterologous protein expression. In our laboratory, where we frequently transform *S. gordonii* for protein production purposes and for development of vaccines, the use of a synthetic CSP to induce competence has replaced serum-based methods.

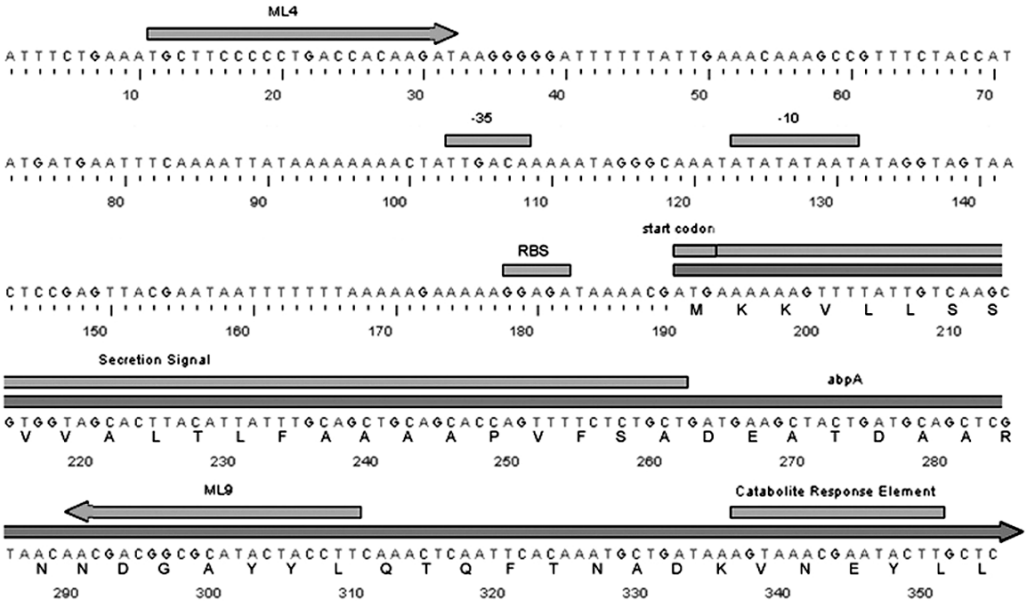

Fig. 3.1. Arrangement of putative *abpA* genetic elements as arranged on the *S. gordonii* Challis genome. The promoter for *abpA* is indicated by -35 and -10 notations above the DNA sequence, as is notation for the ribosomal binding site (RBS). The binding regions are indicated for primers ML4 and ML9, which were used for amplification of the intervening sequence. Additionally, a putative catabolite repressive element is located 153 bp downstream of the AbpA translational start codon.

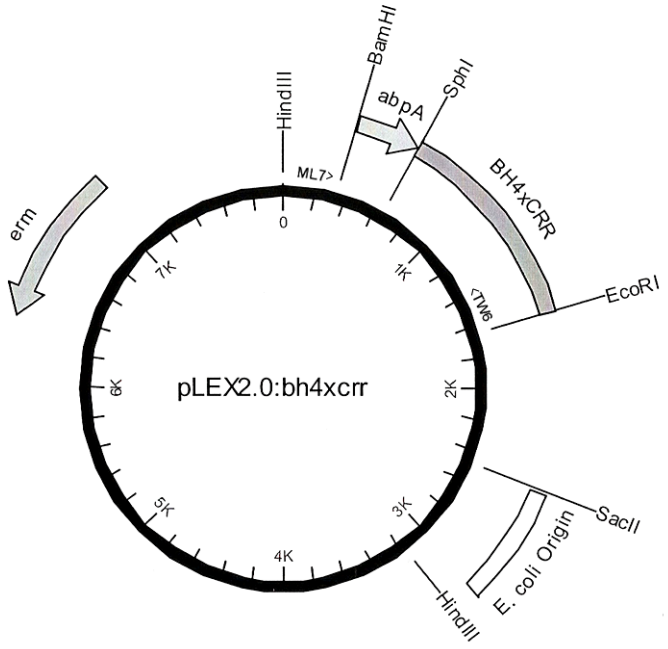

Fig. 3.2. Overview of the arrangement of genetic elements in pLEX2.0:*bh4xcrr*. A 2.3 kbp region was cloned into pVA838 using the *BamH1* and *Sph1* restriction sites on the plasmid. The heterologous structural gene *bh4xcrr* is situated downstream and in-frame with the promoter and secretion signal sequence of AbpA. Cloning of *bh4xcrr* using *SphI* and *EcoRI* deactivated a *cat* gene present on the parent plasmid. The pACYC184-derived *E. coli* origin is indicated, but the Gram-positive replication origin remains unidentified on the plasmid. *HindIII* sites used in construction of parent plasmid pVA838 are provided for reference. Following transformation into *S. gordonii*, primers ML7 and TW6, which lack homology with *S. gordonii* genomic sequences, were used to confirm transformation with pLEX2.0:*bh4xcrr*.

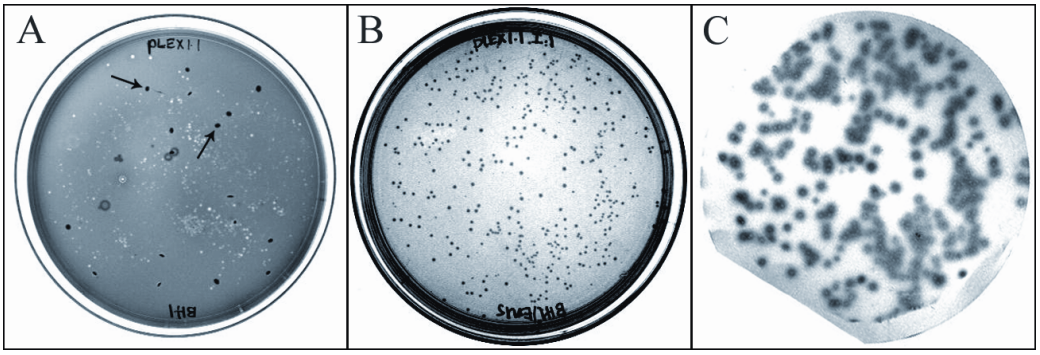

Figure 3.3. Induction of competence using strain-specific CSP enhances the ability to transform *S. gordonii*. A. Natural competence was induced using FCS and *S. gordonii was* transformed with 1 µg pLEX1.1:*bh4xcrr*. A 450-µl aliquot of the transformation reaction was mixed with melted agar and added to the plate. An additional layer of melted agar containing erthromycin was added and plates were incubated for 48h. The arrows indicate examples of imbedded colonies. B. Competence was stimulated using CSP and *S. gordonii* was transformed with 1 µg pLEX1.1:bh4xcrr. A 25-µl aliquot of the transformation reaction was added directly to BHI agar containing erythromycin, and plates were incubated for 48h. C. Putative CSP tranformants (B) were screened by colony blot for production of BH4XCRR using mAb 10F5. Greater than 90% of all putative transformants were confirmed as positive on the basis of BH4XCRR expression.

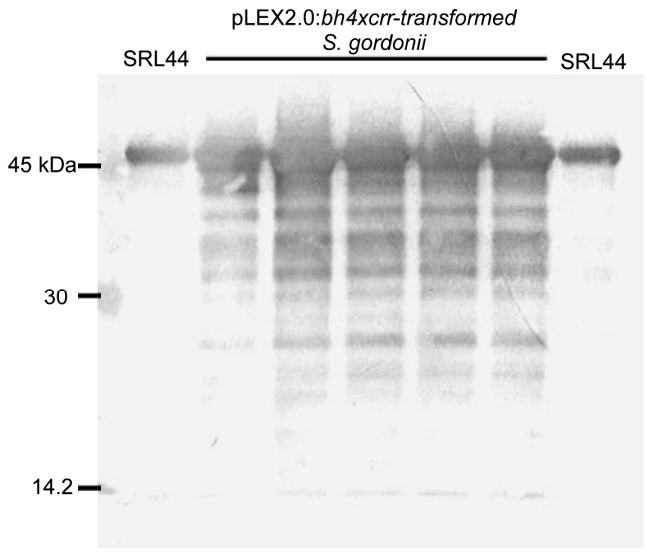

Fig. 3.4. Transformation of *S. gordonii* with pLEX2.0:*bh4xcrr* enhances production relative to pLEX1.0:*bh4xcrr* (SRL44). Cultures were grown overnight in CDM/CAA with erythromycin. Protein in culture supernatants from pLEX2.0:*bh4xcrr* -transformed *S. gordonii* and from SRL44 were separated using SDS-PAGE, transferred to PVDF membrane and probed with mAb 10F5, which reacts specifically with epitopes of the heterologous product BH4XCRR.

Table 3.1. Comparison of the transformation efficiencies of the CSP and FCS transformation induction methods.

Plasmid	Transformation efficiency[a] with indicated method	
	CSP	Traditional
pLEX1.1:*bh4xcrr*	1.4×10^4	12
pSMB104	87	0.5
None	0	0

a. Transformation efficiency = transformants per μg of DNA. All transformation experiments were performed with 1 μg of plasmid DNA, except for the negative controls which received no DNA.

Chapter 4

Streptococcus gordonii protein secretion system effectively accommodates high-level expression of multiple heterologous products

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Abstract

Streptococcus gordonii is currently being developed as a bacterial commensal vector to provide a rapid and flexible response against smallpox, a Class A warfare pathogen. To promote effective protective immune responses, *S. gordonii* will likely have to be engineered to express multiple antigens possibly in combination with immunomodulatory cytokines, ligands, or other molecules. However, mechanisms of protein export across the Gram-positive cell membrane are poorly understood and the capacity for *S. gordonii* to secrete numerous products may become limited by the demand associated with protein export in commensal vectors. In this report, a strain of *S. gordonii* that expresses vaccinia virus antigen B5R was engineered, using a multi-copy resident plasmid, to express an additional viral antigen, A27L. Demands for the dual antigen expression did not diminish expression of either antigen, relative to strains expressing individual antigen alone. Additionally, the expression plasmid was found to be well retained for ≥ 2 wk in the absence of antibiotic selective pressure by *S. gordonii* colonizing the oral cavities of BALB/c mice.

Introduction

Streptococcus gordonii is a Gram-positive commensal organism and is a normal component of human oral flora. Investigations are currenly underway to adapt this bacterium to deliver vaccine antigens (86) and therapeutic agents (54). This approach offers several advantages over traditional methods of vaccine delivery including cost-effectiveness, ease of delivery, and tolerability by humans (22).

While it has been shown that delivery of *S. gordonii* expressing a heterologous antigen can induce both a local and systemic immune response (5, 36), we have also shown that co-expression of mouse IFN-γ or IL-2 in combination with a heterologous antigen (M6 protein from *S. pyogenes*) can alter the expression profile of immunological genes following inoculation of mice with *S. gordonii* recombinants (9). Oggioni et al. (31) demonstrated that the humoral immune response in mice treated with *S. gordonii* expressing two products, heat-labile toxin B and the V3 antigen of HIV-1 gp 120, was greater than when two strains of *S. gordonii*, expressing either LTB or V3 antigen, were delivered together. Other studies demonstrate that co-delivery of ligands or cytokines in combination with an antigen can substantially augment the cellular and humoral immune responses or skew humoral responses toward TH1 or TH2 bias (88). Thus, it may be necessary to engineer *S. gordonii* to export several antigens or co-stimulatory molecules such as cytokines or particular ligands, e.g. CD40L (39), to

modulate the intensity of the immunogenic response and to activate particular immune components required for long-term protection (Wortham 1998).

Mechanisms associated with the export of proteins from the cytosol across the Gram-positive bacterial cell membrane are poorly understood, but studies primarily involving *Bacillus subtilis* (89), *Streptococcus pyogenes* (59), and *Staphylococcus aureus* (26, 62) suggest that export occurs through activities associated with the highly conserved components of the Sec pathway (2). Recently, it has been shown in *Streptococcus pyogenes* that Sec-substrate proteins are secreted from the cell exclusively through the ExPortal (58, 59). This unique microdomain contains enzymes of the Sec transport system, accessory proteins involved in protein folding and anchorage to the cell surface, and products destined for secretion. Each streptococcal cell possesses a single ExPortal (58). While the mechanisms by which proteins are transported through this site are unknown, it has been theorized that the ExPortal architecture is conserved throughout Gram-positive bacteria as an organelle for secreting proteins. Because the use of *S. gordonii* as a vaccine-delivery vector may require the secretion of multiple heterologous antigens or cytokines by bacterial cells, it was of interest to evaluate whether expression of one heterologous antigen is diminished, perhaps by secretion bottlenecks created at the ExPortal, when *S. gordonii* is engineered to express an additional product at high level.

Using the single-copy chromosomally integrated genetics associated with the SPEX (Surface Protein Expression) system, heterologous gene products have

been successfully displayed on the surface of *S. gordonii* by fusing a structural heterologous gene with the COOH-terminal sorting signal derived from the *S. pyogenes* M6 protein (42, 51). Previously we reported on the development of a plasmid-based expression system (PLEX), by which native promoters situated on a resident plasmid were used to drive the synthesis and high-level expression of secreted heterolgous products by *S. gordonii* (84). Here we evaluate the use of this system for surface anchoring a heterolgous product, VV A27L, using the COOH-terminal sorting signal of M6 protein. Further, we evaluate whether engineering a strain of VV B5R-producing *S. gordonii* to express an additional anchored viral antigen, A27L, affects the production and surface display of B5R. Additionally, we report on the *in situ* retention of the PLEX expression plasmid by *S. gordonii* in the absence of antibiotic selective pressure, following colonization in the oropharyngeal cavities of mice.

Methods and Materials

Bacterial Strains and Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) or on LB agar (Difco). *S. gordonii* was grown in Brain-Heart Infusion (BHI) (Difco) or on BHI containing 1.5% agar. Selection and growth for strains carrying antibiotic-resistance determinants were performed at 300 μg erythromycin/ml for *E. coli* strains and at 5 μg erythromycin/ml for *S. gordonii*. Selection for chloramphenicol resistant colonies were performed using 25 μg chloramphenicol/ml for *E. coli* and 5 μg chloramphenicol/ml for *S. gordonii.*

DNA Isolation and Manipulation

Standard molecular biological techniques were followed in the preparation and manipulation of DNA. PCR products were subcloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA) and transformed into *E. coli* InvαF' or Top10F' (Invitrogen) using the manufacturer's procedure. PCR-derived DNA was transformed into *E.coli* XL-10 Gold (Stratagene, La Jolla, CA) for site-directed mutagenesis. Plasmids were isolated from *E. coli* strains using Qiagen purification kits (Valencia, CA). Following digestion with endonucleases, DNA fraqments were separated by electrophoresis and eluted from agarose gels using QIAquick

Gel Extraction Kits (Qiagen). All restriction endonucleases were obtained from New England Biolabs (Beverely, MA). Sequence analyses were performed on all final plasmid constructs by CGRB Core Laboratory at Oregon State University (Corvallis, OR).

Amplification and Subcloning of VV Antigens

The genes encoding viral products have been described elsewhere (85). Briefly, the gene encoding the A27L (p14) protein was generated using VV Copenhagen DNA as a template by PCR using forward primer 5'-CGGGGTACCGACGGAACTCTTTTCCCC-3' and reverse primer 5'-CCGGAATTCCTCATATGGATCTGAAC-3', which produce 5' *KpnI* and 3' *EcoRI* restriction sites flanking the amplified gene. The PCR product was subcloned into pCR2.1 TOPO forming plasmid pCR2.1:*a27L*. Using forward primer LB7 (5'-CCGGGTACCATGACTGTACCCACTATGAATAAC), reverse primer LB9 (5'-CCGGTCGACTGCTTCTAACGATTCTATTTC), and VV Copenhagen DNA as template, the coding sequence for B5R amino acids 22-276 was amplified. The product containing a 5' *KpnI* and 3' *SalI* site was subcloned into pCR2.1 to generate pCR2.1:*b5r*Δ. Following digestion of pCR2.1:b5rΔ with *KpnI* and *SalI*, a 768-bp fragment was isolated and ligated into *KpnI*- and *SalI*-digested pSMB104 to generate pSRL39.

Construction of pLEX1.1:bh4xcrr

Plasmid pLEX1.1:*bh4xcrr* was derived from parental plasmid pLEX1.0:*bh4xcrr* (84), which contains two *EcoRI* restriction sites, in the 3' region of *rgg* and in the chloramphenicol acetyltransferase (*cat*) gene. Because it was desirable to use *EcoRI* to disable the *cat* gene without affecting *rgg*, site-directed mutagenesis was conducted using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers protocol. A silent T860→C nucleotide mutation was introduced into the 3' region of *rgg* using pLEX1.0:*bh4xcrr* as template and primers EcoRI-F (5'-CAAAGAACAATTTGAGCGAATCCAACTAACAGT) and EcoRI-R (5'-TCTGCAACTACTGTTAGTTGGATTCGCTCAAAT). Parental plasmid was digested using *DpnI* and products were transformed into *E. coli* XL-10 Gold cells. An erythromycin-resistant clone was screened for the presence of the desired mutation by confirming that *EcoRI* was unable to excise a band during a digestion reaction. This plasmid construct was designated pLEX1.1:*bh4xcrr*.

Construction of pLEX1.1:a27l(a)

Plasmid pCR2.1:*a27l* was digested using *EcoRI* and *KpnI* to produce a 286-bp fragment that was ligated into *EcoRI*- and *KpnI* -digested SPEX cloning vector pSMB104 (42). Ligation in this manner situated the *a27l* structural gene,

lacking a stop codon, immediately upstream and in frame with the anchoring region from the *S. pyogenes* M6 protein (40-42). We designated this anchored version of the *a27l* gene *a27l(a)*. Using pSMB104:*a27l(a)* as template, PCR was used to amplify *a27l(a)* flanked by *KpnI* and *NruI* restriction sites using forward primer LB37 (5' CCGGGTACCTGGACGGAACTCTTTTC) and reverse primer ML11 (5' CGGCCGTCGCGATTAGTTTTCTTCTTTGCGTTA). This product was sequentially digested with *KpnI* and *NruI*, and a circa 1.0 kbp band was isolated. pLEX1.1:*bh4xcrr* was digested with *KpnI* and *NruI* to remove the *bh4xcrr* insert and a 9.8 kbp digested plasmid band was resolved using electrophoresis and purified. The *KpnI*- and *NruI*-digested *a27l(a)* product was ligated into the pLEX1.1 plasmid using T4 DNA ligase in an overnight reaction at 13^{o}C. This product was transformed into *E. coli* InvαF', and tranformants were selected on the basis of erythromycin resistance. This plasmid construct was designated pLEX1.1:*a27l(a)*.

Construction of pLEX1.3:a27l(a)

Because pLEX1.1:*a27l(a)* confers erythromycin resistance in *S. gordonii*, this plasmid cannot be used to introduce genetic material into strains that already possess an erythromycin resistant phenotype, such as those transformed by pSMB104 derivatives. pLEX1.3:*a27l(a)* was developed to allow screening of *S. gordonii* transformants using chloramphenicol. The *cat* gene was PCR amplified using plasmid pAM401 (ATCC # 37429 as template and primers ML16 (5'-GCTAAAAATTTGTAATTAAGAAGGAGTGATTACCTCGAGATGACTTTTAATATTATTGAATTAGAAAATTGG) and ML17 (5'-CCAAATTTACAAAAGCGACTCATAGAACATATGCTAAATCCAATCATCTACCCTATG). The 3' region of each primer is homologous with the *cat* gene of pAM401 while the 5' sequences are homologous to plasmid sequences of pLEX1.1:*a27l(a)* that flank the erythromycin-resistance conferring gene (Fig. 4.1A). Use of these primers introduced *XhoI* and *NdeI* restriction sites at positions flanking the *cat* structural gene during PCR amplification. The PCR product from this reaction was used to prime a second PCR reaction using pLEX1.1:*a27l(a)* as template (Fig. 4.1B). Product from this reaction was digested with *DpnI* to remove parental plasmid and was transformed into *E. coli* strain XL-10 Gold. Transformants were screened on the basis of chloramphenicol resistance and susceptibility to erythromycin. This plasmid construct was designated pLEX1.3:*a27l(a)* (Fig. 4.1C).

Transformation of S. gordonii

When *S. gordonii* was transformed with plasmid derived from pSMB104, the following transformation method was used (49). Briefly, *S. gordonii* was grown in BHI in the presence of 10% fetal calf serum to induce competence and stored at –80^{o}C in 450-µl aliquots. Plasmid (500 ng) was incubated with freshly thawed competent cells for 2h at 37^{o}C. This culture was mixed with liquified BHI agar containing 5% defibrinated sheep blood and layered onto BHI agar. After briefly allowing this layer to congeal, liquified BHI agar containing 5 µg erythromycin/ml was carefully poured onto the plates. Colonies, selected after 48 h growth at 37^{o}C, were streaked onto BHI agar containing 5 µg erythromycin/ml to further confirm resistance and for selection of pure clones.

When transforming *S. gordonii* with any of the pLEX plasmids, a synthetic competence-stimulating peptide (CSP), N-DVRSNKIRLWWENIFFNKK (Sigma-Genosys, The Woodlands, Texas) was used (15). Briefly, *S. gordonii* were grown overnight in BHI containing appropriate selective antibiotic. To prepare cells for transformation, CSP (10 µg/ml final concentration) and glycerol (10% final concentration) were added to the culure, and cells were frozen at -80^{o}C in 100 µl aliquots. To transform *S. gordonii*, cells were quickly thawed and 1 µg DNA and 900 µl THY were added. Cells were incubated at 37^{o}C for three hours and were plated out on BHI agar containing selective antibiotic.

E. coli expression and purification of VV A27L and B5R proteins

Expression of viral products and methods used for purification have been described by Wilson et al. (85). Briefly, a PCR product encoding A27L was generated using VV Copenhagen DNA as template. Products were cloned into the *KpnI-EcoRI* restriction sites of the IPTG-inducible-expression plasmid pET41b (Novagen, Schwalbach, Germany), forming plasmid pET41b:*a27l*. This plasmid was subsequently introduced into the protein expression strain *E. coli* Rosetta (Invitrogen). The *KpnI-SalI* digested DNA fragment from plasmid pSMB104:*b5r*Δ was ligated into the *KpnI-SalI* restriction sites of pET41b forming plasmid pET41b: *b5r*Δ. Plasmid pET41b: *b5r*Δ was transformed into strain BL21. Plasmid pET41b expresses products as GST fusions (Fig. 4.2), so A27L- and B5RΔ-GST fusions were purified using column chromatography with Ni-NTA Agarose (Qiagen, Valencia, CA), and Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to manufacturer's protocol.

Generation of antisera

Rabbits were immunized intramuscularly with *E. coli*-expressed A27L protein to raise antibodies for use as an immunological reagent. Briefly, two New Zealand white rabbits were immunized with 200 μg of purified protein in Titermax

Gold adjuvant (Sigma), at week 0, then boosted with 100 μg of protein in adjuvant at weeks 3 and 7. Rabbits were bled prior to each immunization and, finally, exanguinated at the termination of the immunization protocol.

To generate mouse antisera reactive with VV antigens B5R and A27L, these proteins were produced using *E. coli* as GST fusions. Purified products were diluted in PBS to a concentration of 250 μg/ml. MPL+TDM (Sigma) was mixed with the antigen at a ratio of 1:1, and 200 μl (25 μg) was delivered to 5 BALB/c mice per treatment when mice were five weeks of age. Treatments were delivered again three weeks later and once again two weeks after that. Whole blood (~120 μl) was collected from the saphenous vein 3 weeks after the final inoculation. Serum was separated by centrifugation and sera from animals in like treatments were pooled and stored at -80^{o}C.

Flow cytometric evaluations

Rabbit anti-VV Lister was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). FITC-conjugated goat anti-rabbit IgG was obtained from Sigma (St. Louis, MO) and PE-conjugated goat anti-mouse IgG was obtained from Invitrogen-Molecular Probes (Eugene, OR).

Overnight cultures were backdiluted 1:20 in BHI containing an appropriate selective antibiotic and incubated without agitation at 37^{o}C until OD_{650} = 0.5. Cells were washed twice in FACS buffer (PBS supplemented with 1% FCS and

0.1% sodium azide) to remove the growth media and were resuspended in FACS buffer at one-third the original culture volume. A 50-µl aliquot was transferred to a 96-well plate and cells were pelletted by centrifugation. Cells were resuspended in FACS buffer containing a 1:50 dilution of antisera and were incubated on ice for 30 min. If appropriate to the analysis, cells were washed and again incubated with a second antisera in like manner. After washing cells twice with FACS buffer, fluorochrome-labeled secondary antibody was applied at a 1:50 dilution in 50 µl FACS buffer. After incubating on ice for 30 min, cells were washed and resuspended in 300 µl FACS buffer for analysis. Fluorescence intensities were quantified for 10000 particles using a Beckman Coulter FC500.

Inoculation of mice with plasmid-bearing S. gordonii

Retention of the PLEX expression plasmid by *S. gordonii* in the absence of selective pressure was assessed in 10 four-wk old BALB/c mice. Two days prior to inoculation, streptomycin was added to the drinking water of mice at a concentration of 5 g/L to clear the oral flora. Strain SRL44 (pLEX1.0:*bh4xcrr*-transformed *S. gordonii*) (84) was grown in BHI broth containing 1000 µg streptomycin/ml to OD_{650}=0.85. Bacteria were washed with PBS to remove the culture supernatant, resuspended in one-sixth of the original culture volume, and 50 µl was delivered in two 25-µl aliquots to the mouth and nares of lightly isoflurane-anesthetized mice. The growth of bacteria and inoculation of mice was

repeated 3 d later, at which time drinking water was replaced with tap water containing no antibiotics. Mice received 3 x 10^7 CFU and 3.5 x 10^8 CFU during the first and second inoculations, respectively.

Samples of the oral flora of mice were collected using calcium alginate swabs (Fisher Scientific) at 1, 2, 3, and 5 wk intervals. At each of these timepoints, swabs were streaked on BHI agar containing 5% defibrinated sheep blood and 500 μg streptomycin/ml. Colonies exhibiting α-hemolytic phenotypes (indicative of *S. gordonii*) were transferred to BHI agar containing 5 μg erythromycin/ml, and colonies were screened for BH4XCRR production by probing PVDF colony overlays with mAb 10F5, which is specific for an epitope of BH4XCRR (20). Colonies were scored as positive for plasmid retention if they grew on erythromycin and exhibited reactivity with 10F5.

Results

Surface display of VV A27L by S. gordonii

A single clone of SRL45 (pLEX1.1:*a27l*(a)-transformed *S. gordonii*) was isolated and cultured in BHI. This culture was backdiluted in BHI containing antibiotic and was grown to mid-log phase (OD_{650}=0.5). Cells were prepared for flow cytometric analysis of surface proteins. We likewise cultured and processed *S. gordonii* strains GP251 (negative control) and SRL21, which expresses A27L on the bacterial surface using SPEX genetics, to permit comparison of the relative levels of A27L expressed by the different expression systems. Parental *S. gordonii* strain GP251 exhibited a low background level of fluorescence [mean fluorescence channel (MFC) = 2.38] (Fig. 4.3). We observed substantial shifts to greater fluorescence channels relative to GP251 for both A27L(A) producing strains, suggesting that both PLEX and SPEX-based expression systems capably facilitate surface expression of the heterologous VV antigen. SRL45 demonstrated slightly greater fluorescence (MFC=16.3) compared with SRL21 (MFC=13.3). Cells incubated with FITC-labeled secondary antibody alone exhibited a MFC less than that of GP251 (data not shown), suggesting that fluorescence resulted from specific binding of the anti-VV antisera.

Surface expression of VV B5R by S. gordonii

S. gordonii strain GP251 was transformed with pSRL39. Transformants were screened on the basis of erythromycin resistance, and PCR was conducted to confirm the presence of the B5RΔ genetic construct. A single clone was retained and designated as SRL39. To assess surface expression of B5R, GP251 and SRL39 were incubated with rabbit anti-VV serum. Using flow cytometry, we observed a substantial shift to higher fluorescence channels for the SRL39 sample compared to GP251 (similar results confirmed in Fig. 4.4D). These findings were consistent with our expectations and suggest that B5R is expressed on the surface of *S. gordonii* strain SRL39.

Dual Expression of VV B5R and A27L surface antigens

After confirming B5R expression in *S. gordonii* strain SRL39, we sought to express an additional vaccinia viral antigen, A27L, by transforming this strain with pLEX1.3:*a27l(a)*. Using CSP to facilitate plasmid uptake, we introduced pLEX1.3:*a27l(a)* into SRL39 and screened transformants for chloramphenicol resistance. Putative transformed clones were isolated and incubated in BHI to mid-log phase. Strains GP251, SRL39, and SRL45 were likewise cultured and cells were prepared for flow cytometric analysis of surface proteins. Cells were cultured with both mouse antisera developed against *E. coli*-derived B5R, and rabbit antisera from an animal immunized with *E. coli*-produced A27L. GP251

exhibited a low background level of both FITC (MFC=0.794) and PE (MFC=1.43) fluorescence, and transformation of this strain with the PLEX parent plasmid pVA838 produced no substantial shift in fluorescence values (Fig. 4.4Aand 4.4B). As expected, SRL45 (pLEX1.1:*a27l*(a)-transformed *S. gordonii*) exhibited a shift in A27L-FITC fluorescence (MFC=21.3) relative to GP251 but did not show a PE (B5R) shift (Fig. 4.4C). Conversely, but also consistent with expectations, SRL39 exhibited enhanced fluorescence for B5R-PE (MFC=5.3) but not FITC (Fig. 4.4D). A pLEX1.3:*a27l(a)*-transformed SRL39 clone exhibited both A27L-FITC (MFC=19.5) and B5R-PE (MFC=9.57) fluorescence at levels comparable to that observed in SRL45 and SRL39 (Fig. 4.4E), respectively, suggesting that this clone expressed both A27L and B5R at levels equivalent to strains that express only one of these products. This clone was retained and designated SRL48.

Retention of the pLEX plasmid by S. gordonii in mice

One week after inoculating mice with *S. gordonii* strain SRL44, the PLEX plasmid had been retained in all evaluated colonies from 7 of the 10 mice (Fig. 4.5 and Table 2). Of the remaining three mice, ≥ 70 percent of the colony isolates exhibited phenotypes consistent with retention of the expression vector. The mean percentage of plasmid retention fell slightly from 95 percent after one week of colonization to 88 percent following two weeks. At the wk-2 timepoint, retention

ranged from 70 to 100 percent, with colonies from four of the mice showing full retention of the plasmid. By wk 5 post-inoculation, the average percentage of plasmid-positive colonies had decreased to 33 percent, with individual values ranging from 0 to 60 percent.

Discussion

Gram-positive bacteria abundantly secrete proteins into the extracellular milieu, making them attractive for development as BCV (86). In streptococcal bacteria, recent reports suggest that secreted products, including those targeted for surface display, are exported from the cell at a single site, at which enzymes of the Sec pathway, chaperones, and other proteins are localized (58, 59). Based on these findings it follows that as BCV are progressively and more extensively engineered to express multiple antigens and in increasingly greater amounts, the demands for native and heterolgous protein secretion may become self-limiting if the Sec pathway cannot keep pace with production.

Using flow cytometry, we have presented findings showing that the surface expression of the heterologous antigen B5R shows no diminishment when an additional demand for A27L production is placed on *S. gordonii*. When inoculating rabbits and mice to generate antisera for A27L and B5R detection, we were mindful that antibodies would likely be generated against protein products, such as GST, that were fused to the VV antigens. However, the non-viral antigens present on the *E.coli*-expressed products contained no homology with the non-viral antigens fused to A27L and B5R expressed on the surface of *S. gordonii* (Fig. 4.2). Thus we are confident that fluorescence associated with cells resulted from antisera reacting directly with viral epitopes, and not from antibodies binding non-viral regions. Findings from these analyses suggest that *S. gordonii* secretory and

attachment mechanisms are capable of meeting the demand imposed by genetic transformation of at least two, and possibly more, high-level expression systems.

Schneewind et al. (74) have provided significant insights into the mechanisms used by Gram-positive bacteria for the surface display of proteins through the study of *Staphylococcus aureus*. It has been shown that protein surface display requires translation of a COOH-terminal sorting signal, characteristically comprised of a conserved LPXTG motif, followed by a hydrophobic region and a COOH-tail of mostly positively charged residues (35, 43, 72-75). This sorting signal functions to first retain the polypeptide within the secretory system, most likely positioned at the ExPortal. In a process involving Sortase A (SrtA), the protein is cleaved between threonine (T) and glycine (G) of the LPXTG motif, and the liberated carboxyl of threonine is covalently linked to a pentaglycyl cross-bridge of the peptidoglycan cell-wall matrix (43, 72, 73, 75).

The COOH-terminal sorting signal from the *S. pyogenes* M6 protein has previously been used in association with various expression systems to facilitate the surface display of heterologous products by *S. gordonii* (5, 9, 31, 42, 51). Through the evaluations presented here, we demonstrated that the COOH-terminal sorting signal could also be effectively utilized in the $PLEX_{1.0}$ expression system. Previously, we have shown that plasmid-borne genetics of both the $PLEX_{1.0}$ (84) and $PLEX_{2.0}$ expression systems can facilitate high-level expression of secreted forms of heterologous products, and these expression systems are currently being evaluated as part of the overall development of *S. gordonii* as a BCV.

Nonetheless, these expression plasmids must be retained by the bacterium in order for the heterologous products they encode to be expressed. Selective antibiotic is added to *in vitro* cultures to ensure plasmid retention, but it is neither feasible nor prudent to administer antibiotics to animals, including humans, for the timecourse required to generate long-term protective immune responses. It has been theorized that without antibiotics to select for plasmid retention, plasmids will not be replicated as cells in the initial inoculum divide to colonize the oral cavity (52).

We evaluated whether the pLEX1.0:*bh4xcrr* expression plasmid was retained in the absence of antibiotic selective pressure by screening cultures obtained from the oral cavity after inoculating BALB/c mice. Our findings demonstrate that the expression plasmid is well retained by bacteria for up to two weeks following inoculation. Because mice are typically given a booster of BCV at two-week intervals, we propose that the PLEX expression plasmids are retained to a satisfactory extent for BCV application. To acertain this, we are currently conducting comparisons of the immune responses to antigens delivered by *S. gordonii* using PLEX plasmids versus the chromosomal-based SPEX system. Taken together the findings presented here further support the development and use of *S. gordonii* as a BCV.

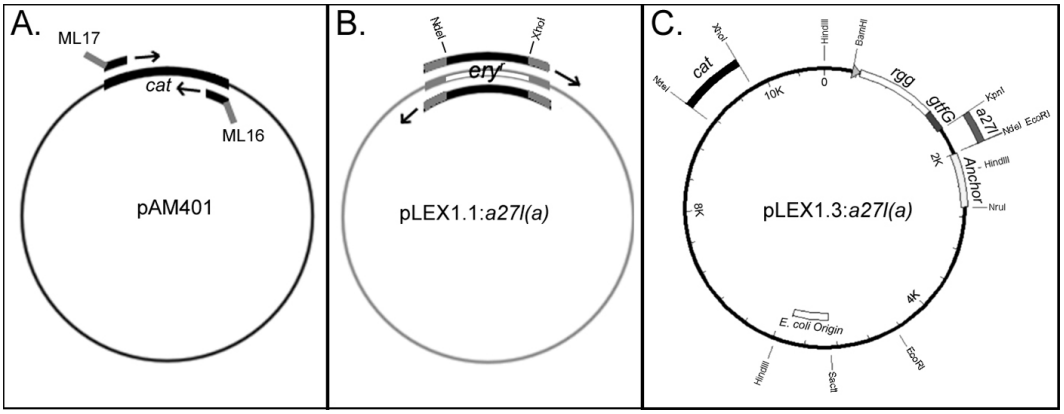

Figure 4.1. Construction of pLEX1.3:*a27l(a).* A. Plasmids ML16 and ML17 were used to amplify a promoterless *cat* gene using pAM401 as template. B. Product from this reaction (A) was used to prime the PCR amplification of pLEX1.1:*a27l(a)*. C. *DpnI*-digested PCR product (B) was transformed into *E. coli* and pLEX1.3:*a27l(a)* was isolated. The pLEX1.3:*a2l(a)* plasmid map indicates incorporation of the *cat* gene into the plasmid, and the *rgg*, *gtfG*, and anchoring elements responsible for the expression and surface display of VV A27L.

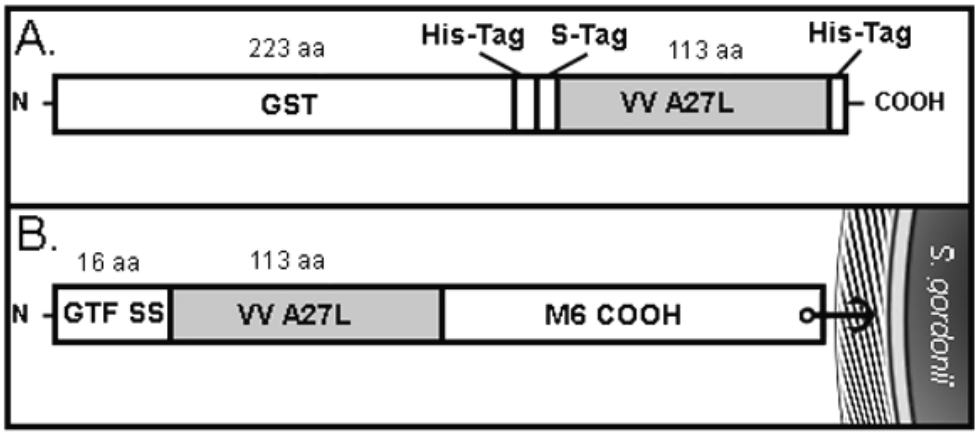

Figure 4.2. Comparison of mature A27L products as expressed by *E. coli* and *S. gordonii*. A. A27L was expressed by cloning the VV Copenhagen *a27l* gene into the pET41b vector for expression by *E. coli* strain BL21. Product was purified using Glutathione Sepharose 4B and the purified product was delivered to mice and rabbits to generate A27L-reactive antisera. B. The VV Copenhagen *a27l* gene was cloned into the *S. gordonii* expression plasmid pLEX1.1, which facilitates export of the product using the native glucosyltransferase secretion signal (GTF SS). This product was expressed as a fusion with the COOH-terminal anchoring signal of the *S. pyogenes* M6 protein.

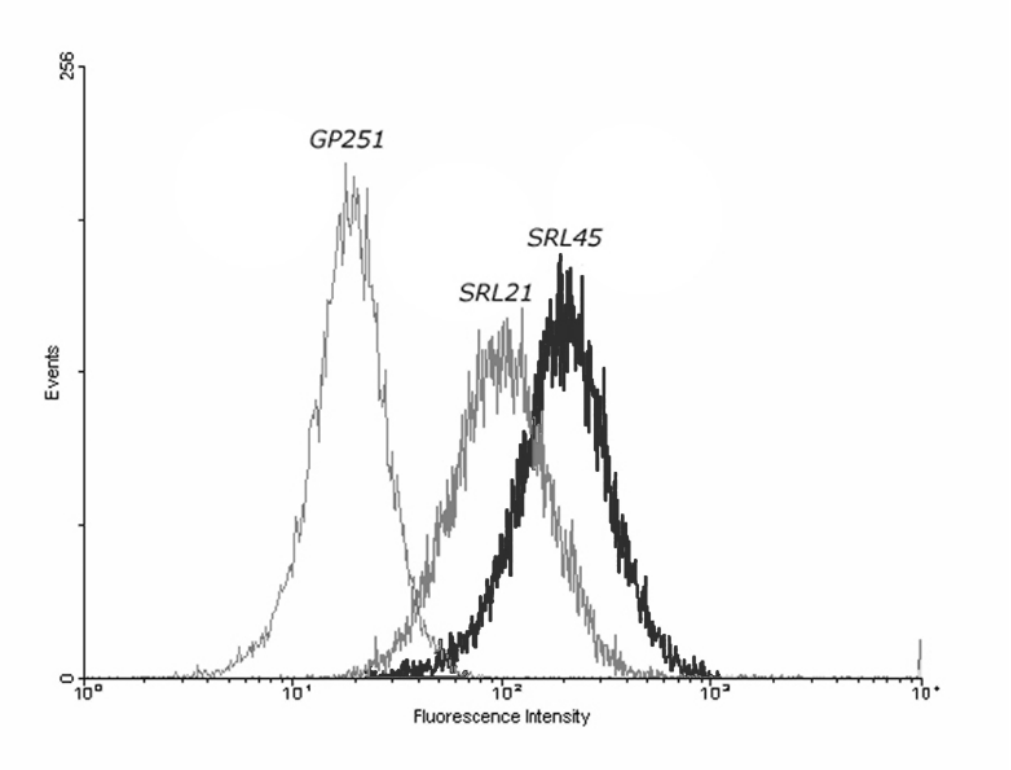

Figure 4.3. Flow cytometric evaluation of VV A27L surface display by SPEX- and PLEX- transformed *S. gordonii*. Overlaid histograms are shown that were generated using rabbit anti-VV and FITC-conjugated anti rabbit-IgG to detect A27L on the surface of *S. gordonii* strains GP251, SRL21, and SRL45.

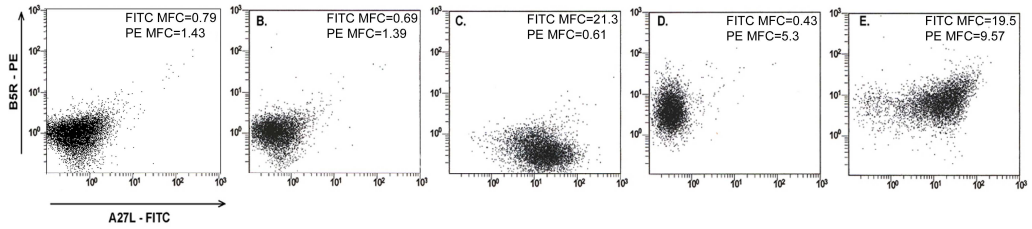

Figure 4.4. B5R and A27L are expressed at comparable levels in *S. gordonii* strains expressing one versus both antigens. Surface expression of B5R and A27L was evaluated using flow cytometry with antisera generated in mice and rabbits immunized with *E. coli* recombinant proteins. Rabbit antisera specific for A27L was detected using FITC-conjugated antibodies, and B5R was detected using PE-conjugated anti-mouse IgG. Antigen display was evaluated in parent strain GP251 (A), GP251 transformed with pLEX parent plasmid pVA838 (B), SRL45 expressing A27L alone (C), SRL39 expressing B5R alone (D), and SRL48 expressing A27L and B5R (E). All strains were incubated with both primary antibodies prior to evaluation.

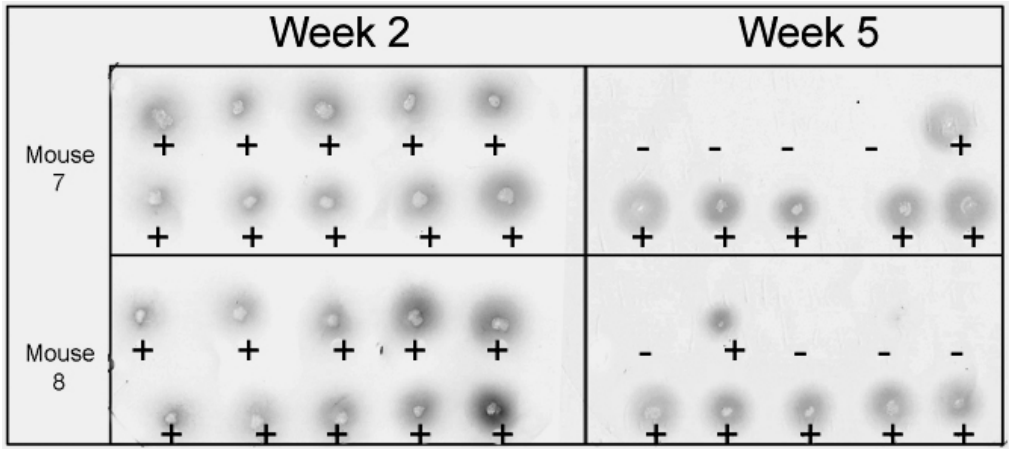

Figure 4.5. Evaluation of pLEX plasmid retention by *S. gordonii.* Ten 5-wk old BALB/c mice were inoculated with *S. gordonii* strain SRL44, which bears plasmid pLEX1.0: *bh4xcrr*. At weeks 1, 2, and 5 following inoculation, 10 streptomycin-resistant colonies from oral swabs were transferred to BHI agar containing erythromycin. After 48 h of growth, colonies were overlaid with PVDF and membranes were probed with mAb 10F5, which is specific for the heterologous product BH4XCRR. Retention of the plasmid by *S. gord*onii colonies was scored as positive if colonies grew on erythromycin plates and demonstrated obvious 10F5 reactivity. Representative PVDF colony overlays are presented for mouse 7 and 8 at two timepoints (Week 2 and Week 5).

Table 4.1 Bacterial Strains and Plasmids

Strain or Plasmid	Relevant Markers or Characteristics	Reference or Source
E. coli		
InvαF'	F′ *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 Φ80*lac*ZΔM15 Δ*lac*ZYA-*arg*F)*U169*	Invitrogen
Top10F'	F′{*lacI*^{q}, Tn*10*(Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
XL-10 Gold	Tet^R Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI*^{q}*ZΔM15 tn10(*Tet^R) Amy Cam^R]^a	Stratagene
BL21	F$^-$ *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)	Invitrogen
Rosetta	F$^-$ *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3) pRARE	Novagen
S. gordonii		
GP251	pSMB104 recipient strain carrying cat gene: Cm^5_r	G. Pozzi
SRL21	GP251 transformed with pSMB104:*a27l(a)*: Ery_5^r	(85)
SRL39	GP251 transformed with pSMB104:*b5rΔ*: Ery_5^r	(85)
SRL44	GP251 tranformed with pLEX1.0:*bh4xcrr*, Sm_{500}r, Ery_5r	(84)
SRL45	GP251 transformed with pLEX1.1:*a27l(a)* : Ery_5^r	This work
SRL48	SRL39 transformed with pLEX1.3:*a27l(a)*: Cm_5^r, Ery_5^r, Sm_{500}^r	This work
Plasmids		
pCR2.1 TOPO	High copy *E. coli* subcloning vector: *E. coli* Amp_{50}^r	Invitrogen
pSMB104	Recombination plasmid carrying M protein C-terminus fused to *ermC: E. coli* Amp_{50}^r, *S. gordonii* Ery_5^r	G. Pozzi
pSMB104:*b5r*Δ	VV Copenhagen b5rΔ cloned into pSMB104	This work
pSMB104:*a27l(a)*	VVCopenhagen A27L cloned into pSMB104	This work
pLEX1.0:*bh4xcrr*	*E. coli/Streptococcus* shuttle plasmid; *S. gordonii* resident BH4XCRR expression vector: *E. coli* Ery_{300}^r, Cm_{25}^r; *S. gordonii* Ery_5^r	(84)
pLEX1.1:*bh4xcrr*	*E. coli/Streptococcus* shuttle plasmid; *S. gordonii* resident BH4XCRR expression vector: *E. coli* Ery_{300}^r, Cm_{25}^r; *S. gordonii* Ery_5^r: *EcoRI* restriction site available for cloning	This work
pLEX1.1:*a27l(a)*	*E. coli/Streptococcus* shuttle plasmid; *S. gordonii* resident A27L(A) expression vector: *E. coli* Ery_{300}^r, Cm_{25}^r; *S. gordonii* Ery_5^r	This work
pLEX1.3:*a27l(a)*	*E. coli/Streptococcus* shuttle plasmid; *S. gordonii* resident A27L expression vector: *E. coli* Cm_{25}^r; *S. gordonii* Cm_5^r	This work
pAM401	E. coli/Enterococcus shuttle plasmid	ATCC 37429
pET41b	*E. coli* High level expression of cloned genes fused with GST, 6xHis, and S-Tag	Invitrogen
pET41b:*b5r*Δ	Expression of VV Copenhagen B5R from pET41b	(85)
pET41b:*a27l(a)*	Expression of VV Copenhagen A27L from pET41b	(85)

Table 4.2.
Retention of the PLEX plasmid by *S. gordonii* in BALB/c mice.

	Percent Plasmid Retention		
Mouse	Week 1	Week 2	Week 5
1	100	100	50
2	90	70	10
3	100	100	50
4	90	100	0
5	100	100	40
6	70	90	40
7	100	80	60
8	100	80	60
9	100	80	20
10	100	80	0
Average ± Std. Dev.	95 ± 9.7	88 ± 11.4	33 ± 23.6

Chapter 5

COOH-terminal requirements for secretion of heterologous products by *S. gordonii*

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Abstract

The Gram-positive organism *Streptococcus gordonii* is currently being developed as a bacterial commensal vector to provide a rapid and flexible response against smallpox, a Class A warfare pathogen. To promote effective protective immune responses, *S. gordonii* will likely have to be engineered to express multiple antigens possibly in combination with immunomodulatory cytokines, ligands, or other molecules. A number of expression systems have been developed to facilitate export of heterologous products across the plasma membrane by fusing secretion-signal sequences to the N-terminus of heterologous products. *S. gordonii* was successfully engineered to express smallpox-vaccine antigen A27L as a surface protein by fusing the anchoring motif from *S. pyogenes* M6 protein to the A27L COOH-terminus. However, when *S. gordonii* was engineered to secrete A27L by deleting the M6 residues, no product could be detected in culture supernatants. However, abundant A27L secretion was observed when the D-repeat region of M6 was fused to the A27L COOH-terminus. Viral products H3L and G1L, which had previously proven recalcitrant to expression by *S. gordonii* were likewise successfully secreted by fusing the M6 D-repeat region to the respective COOH-terminal ends of these proteins. Additionally, we found that deletion of putative hydrophobic regions from H3L and G1L further enhanced secretion of these products from *S. gordonii*. These results indicate that inclusion of the M6 D-repeat region in chimeric proteins will enhance their secretion, and suggests it may play a similar role in its native context.

Introduction

The Gram-positive *Streptococcus gordonii* is regarded as a promising bacterium for use as a bacterial commensal vector (BCV) because of its tolerability in humans, cost-efficiency, susceptibility to genetic manipulation, and ability to deliver therapeutics and stimulate immune responses at the mucosal sites where pathogens first enter the body. In our laboratory, *S. gordonii* has been genetically engineered to display vaccinia viral (VV) antigens on the bacterial surface as part of overall developments efforts of a fifth-generation vaccine against smallpox, a Class A biowarfare pathogen (85). Additional manipulations may include the introduction of genetic alterations to facilitate secretion of immunomodulatory cytokines, receptors, or other molecules.

Protein export from the Gram-positive bacterial cytosol across the plasma membrane is poorly understood, but recent evaluations in *S. pyogenes* (58, 59) and *Bacillus subtilis* (28, 60, 71, 89) suggest that protein export is initiated when a signal recognition particle (SRP) recognizes and binds to secretion-signal sequences positioned at the N-terminus of *de novo* translated preproteins. A typical secretion-signal sequence, produced on most products destined for transport across the cell membrane (71, 77), consists of approximately 28 amino-acid residues (71) and facilitates the transfer of nascent unfolded preproteins to membrane-bound Sec-pathway components (2) for export. Once transported across the plasma membrane, preproteins may be processed by quality-control

proteases, folding factors, and chaperones. Proteins destined for surface display, such as *S. pyogenes* M6 protein, possess a COOH-terminal sorting motif, which is processed by sortase (SrtA) resulting in covalent attachment to the peptidoglycan matrix of the cell wall (6, 74).

Numerous bacterial, viral, and eukaryotic heterologous proteins have been expressed as either secreted or anchored products from *S. gordonii* (5, 9, 37, 50, 86) by fusing gene products either to native or heterologous secretion signals and, for surface display, anchoring signals. However, we have found that particular heterologous proteins are recalcitrant to expression by *S. gordonii* using these techniques. Here we present methodologies used to secrete products for which no expression had been previously detected or which had proven difficult to secrete. These results suggest that attributes of the COOH-terminus of certain proteins may have a larger role in protein export from Gram-positive organisms than has been previously recognized.

Methods and Materials

Bacterial Strains and Growth Conditions

E. coli strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) or on LB agar (Difco). *S. gordonii* was grown in brain-heart infusion (BHI) (Difco) or on BHI containing 1.5% agar. Selection and growth for strains carrying antibiotic-resistance determinants were performed at 300 μg erythromycin/ml for *E. coli* strains and at 5 μg erythromycin/ml for *S. gordonii*.

DNA Isolation and Manipulation

Standard molecular biological techniques were followed in the preparation and manipulation of DNA. Ligation products were subcloned, screened, and amplified in *E. coli* InvαF' or Top10F' (Invitrogen, Carlsbad, CA) using the manufacturer's procedure. Plasmids were isolated from *E. coli* strains using Qiagen purification kits (Valencia, CA). Following digestion with endonucleases, DNA fraqments were separated by electrophoresis and eluted from agarose gels using QIAquick Gel Extraction Kits (Qiagen). All restriction endonucleases were obtained from New England Biolabs (Beverely, MA). Sequence analyses were performed on all final plasmid constructs by CGRB Core Laboratory at Oregon State University (Corvallis, OR).

Amplification and Cloning of VV A27L, B5R, and G1L

Vaccinia virus Copenhagen DNA was used as template to amplify viral genes, and all primers were designed to encode 5' *KpnI* and 3' *EcoRI* restriction sites flanking the amplified gene, unless otherwise indicated (Table 1). The gene encoding the A27L protein was amplified by PCR using forward primer 5'-CGGGGTACCGACGGAACTCTTTTCCCC and reverse primer 5'-CCGGAATTCCTCATATGGATCTGAAC. Two truncated forms of VV H3L encoding amimo acids 2-283 and 21-170 were amplified and designated H3LΔ and H3LΔΔ, respectively. H3LΔ was amplified using forward primer LB13 (5'-CCGGGTACCGCGGCGGTGAAAAC) and reverse primer LB14 5'-CCGGAATTCAAAAAATGAAATCAGTGGAGTAGTAAACGCG), and H3LΔΔ was amplified using forward primer ML30 (5'-GGGGTACCACATTTCCTAATGTTCATGAGC) and reverse primer ML34 (5'-CGGAATTCTTTGTCCATTACAAGCTCGG). The full-length G1L gene (1822 bp) and truncated forms of the gene were amplified using primers as indicated in Table 1. COOH-terminal regions of *S. pyogenes* M6 protein (GenBank accession #P08089) were amplified using pSMB104:A27L(A) as template. For simplification, we have used the term CRR to designate the C-repeat region of M6 protein (residues 264 -346, relative to the native N-terminus) and DRR to designate the region from the DRR start to the residue immediately prior to leucine

(L) of the LPSTG anchor motif (amino acids 347-448). (For a complete review of the M6 protein, refer to Fischetti (13)).

Genes were cloned into the pLEX expression plasmid such that heterologous genes were situated downstream and in-frame with the secretion-signal sequence of glucosyltransferase (84). To create COOH-terminal fusions, stopless structural heterologous genes were cloned into the expression plasmid to facilitate translational read through to sequences downstream of the heterologous gene.

Transformation of S. gordonii

When transforming *S. gordonii* with any of the pLEX plasmids, a synthetic competence-stimulating peptide (CSP), N-DVRSNKIRLWWENIFFNKK (Sigma-Genosys, The Woodlands, Texas) was used (15). Briefly, *S. gordonii* were grown overnight in BHI containing appropriate selective antibiotic. To prepare cells for transformation, CSP (10 µg/ml final concentration) and glycerol (10% final concentration) were added to the culture, and cells were frozen at -80^{o}C in 100 µl aliquots. To transform *S. gordonii*, cells were quickly thawed and 1 µg DNA and 900 µl Todd-Hewitt broth with 1% yeast extract (THY) were added. Cells were incubated at 37^{o}C for three hours and were plated out on BHI agar containing selective antibiotic.

SDS-PAGE and Western Blot

Proteins in *S. gordonii* culture supernatants were resolved using SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 3% BSA. Antibodies were diluted in buffer containing 0.5 % Tween 20, 0.02 % sodium azide, 0.5 M sodium chloride, and 0.01 M Tris at pH 8.2. Membranes were probed using mAb 10F5 (20), which is specific for an epitope found in the *S. pyogenes* M6 CRR fusion partner, anti-FLAG mAb, or rabbit A27L antisera, developed using *E. coli*-expressed protein. Goat anti-mouse IgG or goat anti-rabbit IgG AP-conjugated antibodies (BioRad, Hercules, CA) were used for detection. Blots were developed using AP-conjugate substrate kit (BioRad) diluted in buffer containing 0.06 M Tris, 0.02% sodium azide, 0.06% magnesium chloride hexahydrate at pH 9.8.

Flow Cytometric Evaluations

Overnight bacterial cultures were backdiluted 1:20 in BHI containing an appropriate selective antibiotic and incubated without agitation at 37^{o}C until OD_{650} = 0.5. Cells were washed twice in FACS buffer (PBS supplemented with 1% FCS and 0.1% sodium azide) to remove the growth media and were resuspended in FACS buffer at one-third the original culture volume. A 50-µl aliquot was transferred to a 96-well plate and cells were pelletted by centrifugation. Cells

were resuspended in FACS buffer containing a 1:50 dilution of rabbit anti-VV Lister (Accurate Chemical and Scientific Corp., Westbury, NY) and were incubated on ice for 30 min. After washing cells twice with FACS buffer, FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) was applied at a 1:50 dilution in 50 µl FACS buffer. After incubating on ice for 30 min, cells were washed and resuspended in 300 µl FACS buffer for analysis. Fluorescence intensities were quantified for 10,000 particles using a Beckman Coulter FC500.

Results

Expression of VV A27L Fusion Products by S. gordonii

As we have previously reported (85), *S. gordonii* can be engineered to facilitate expression of the vaccinia virus A27L protein as an anchored product, designated A27L(A), by fusing the *S. pyogenes* M6 anchor sorting signal (representing amino acids 264-483 relative to the M6 start codon) to the COOH-terminus of A27L (Fig. 5.1). Initially, we attempted to engineer *S. gordonii* to secrete A27L according to previously established genetic manipulations for heterologous product secretion (9), which entails encoding a translational stop codon immediately downstream of the structural gene on the expression plasmid. This putative product was designated as A27Lstop (Fig. 5.1). The A27Lstop expression plasmid was introduced into *S. gordonii* and multiple transformed clones grew on BHI selective agar (containing erythromycin). Initial screening of the transformants, conducted by probing a PVDF colony overlay with anti-A27L antibodies, revealed limited anti-A27L reactivity associated with colonies (data not shown), suggesting poor A27Lstop production by the transformants. To more stringently evaluate production, a transformed clone was isolated and grown to stationary phase in BHI. Cells were separated from the culture supernatant, and the supernatant was subjected to SDS-PAGE and Western transfer. The faint protein band detected in the culture supernatant (Fig 5.2, Lane E) provided further

confirmation that A27Lstop secretion by this clone was minimal. These results were unexpected. Using flow cytometric analysis, we had already observed that A27L could be successfully transported from *S. gordonii* and anchored as a surface antigen. In an attempt to facilitate secretion we had altered only COOH-terminal residues, which would not be predicted to influence secretion according to current models of Gram-positive protein export (19).

COOH-terminal residues facilitate VV A27L secretion

We investigated in greater detail the influence of COOH-terminal residues on A27L secretion by engineering a number of constructs differing only by residues fused to the A27L COOH terminus (Fig. 5.1). Three variations of A27L-M6 fusions were constructed: A27L fused to CRR and DRR together (A27L-CRRDRR) and A27L fused to each of these regions separately (A27L-CRR and A27L-DRR) (Fig. 5.1).

Plasmid-transformed *S. gordonii* were cultured overnight in BHI. Cultures were centrifuged and culture supernatants were subjected to SDS-PAGE and Western Blot analysis. *S. gordonii* most abundantly secreted A27L as a fusion product with residues derived from either the *S. pyogenes* CRRDRR or DRR (Fig. 5.2, Lanes B and D). A27L was expressed at a substantially lesser level using the CRR fusion partner (Fig. 5.2, Lane C). These results suggest that the M6 DRR

domain situated at the A27L COOH terminus contributed substantially to A27L-CRRDRR secretion by *S. gordonii*.

Secretion of VV H3L by S. gordonii

During protein transport in Gram-positive bacteria, it is unclear how and to what extent proteins are exposed to the hydrophobic lipid bilayer of the plasma membrane. Thus it is conceivable that product hydrophobicity could significantly affect secretion from the cell. While VV A27L possesses relatively few hydrophobic domains (Fig. 5.1), VV H3L N-terminal (amino acids 2-20) and COOH terminal (171-283) hydrophobic domains were identified (Fig. 5.3) according to a Kyte-Doolittle hydrophobicity analysis (23). An H3L construct encoding a domain of lesser hydrophobicity (amino acids 21-170, designated H3LΔΔstop) was PCR amplified to encode a stop codon immediately downstream of the product (Fig. 5.3). This construct was cloned into the *S. gordonii* pLEX expression plasmid, and the plasmid was introduced into *S. gordonii.* A transformed erythromycin-resistant clone was isolated for analysis. Following overnight growth in BHI, culture supernatant was processed and subjected to SDS-PAGE and Western transfer to PVDF. However, products in culture supernatant exhibited negligible reactivity with anti-H3L rabbit antisera (data not shown), suggesting that H3LΔΔstop was ineffectively secreted by *S. gordonii*. No reactivity was observed in supernatant probed with mAb 10F5 (Fig. 5.4, Lane D).

Based on our findings that COOH-terminal sequences facilitated A27L product secretion, we engineered the H3LΔΔstop pLEX expression plasmid to express H3LΔΔ product as fusion with CRR/DRR (H3LΔΔ-CRRDRR) (Fig 5.3). As before, the plasmid was introduced into *S. gordonii* to obtain a transformed clone, and the culture supernatant was screened for the presence of H3LΔΔ-CRRDRR. A circa 52-kDA protein band was observed to react strongly with mAb 10F5 (Fig. 5.4, lane C). These findings provide additional support to the theory that amino acid residues positioned at the COOH terminus of certain secreted products facilitate protein secretion.

We further examined whether inclusion of a putative hydrophobic domain of H3L (i.e. amino acids 171-283) could be secreted from *S. gordonii*. DNA encoding amino acids 21-283 (H3LΔ) was amplified and cloned into pLEX upstream and inframe with CRR/DRR (H3LΔ-CRRDRR). A transformed *S. gordonii* clone was isolated and grown in BHI overnight. Culture supernatants were prepared for SDS-PAGE and H3L was visualized using Western Blot. To permit the comparison of the relative amounts of H3L produced by different H3L constructs, we likewise cultured and processed samples from other pertinent *S. gordonii* strains. H3LΔ-CRRDRR was detected in culture supernatant but at a substantially lower level than for H3LΔΔ-CRRDRR, suggesting that perhaps the hydrophobic domains of H3LΔ-CRRDRR inhibit secretion of the product from *S. gordonii*.

Secretion of VV G1L by S. gordonii

A Kyte-Doolittle hydrophobicity plot was generated using native G1L amino acids 2-389 (relative to the viral product N-terminus) fused to CRR/DRR from *S. pyogenes* M6 protein (Fig. 5.5). From this analysis, it was apparent that G1L residues were consistently more hydrophobic than those of CRR/DRR. While the 200 N-terminal residues in particular generated numerous hydrophobic crests (hydrophobicity score > 1.0), G1L residues at the COOH-terminus exhibited somewhat more limited hydrophobicity.

Because previous attempts to express a full-length version of the 66 kDA G1L protein had proven unsuccessful (data not shown), we evaluated expression of truncated forms representing amino acids 2-389 G1Lt, 188-389 G1Lt1(s), 253-389 G1Lt2(s), 354-389 G1Lt3(s) and 188-335 G1Lt4(s) (Fig. 5.5). G1Lt was engineered with and without fusion to CRRDRR-- designated G1Lt and G1Lt(S), respectively -- but the remaining truncated forms of G1L were expressed only as fusions with CRRDRR. Supernatants from each strain were prepared from overnight cultures for SDS-PAGE and Western blot. PVDF blots were probed with either anti-FLAG or mAb 10F5, which is specific to a CRR epitope (20). FLAG-tagged G1Lt, which lacks the CRRDRR fusion, could not be detected in culture supernatant using anti-FLAG mAb (Fig 5.6B). However, protein bands representing G1L truncated proteins were observed in all culture supernatants from *S. gordonii* strains transformed to express G1L residues as CRR/DRR fusions (Fig

5.6A). G1Lt(s), the fusion possessing the greatest number of hydrophobic domains, was found to be secreted at lower levels compared to the other G1L truncates. *S. gordonii* engineered to express G1Lt1(S) expressed the greatest amount of product. G1Lt2(s), G1Lt3(S), and G1Lt4(s) were secreted at comparable levels. These findings provide further comfirmation that COOH-signals facilitate protein secretion in *S. gordonii*, but also suggest that removal of hydrophobic regions may not necessarily boost secretion.

Discussion

Current models of Gram-positive protein transport suggest that one of the first steps involves recognition of the N-terminal secretional-signal peptide by a signal recognition particle (SRP), which shuttles the product to translocases associated with an SRP receptor at the cell membrane (28, 46). Highly conserved components of the Sec pathway facilitate transport across the membrane (2, 65, 78, 83), where the preprotein is subject to the activity of accessory proteins, including Type I signal peptidases that cleave the membrane-bound secretion signal (77). Once the secretion signal has been cleaved, the liberated mature product is postulated to freely diffuse through the peptidoglycan cell-wall matrix into the extracellular milieu (11) unless other enzymes, such as sortase, retain the product for further processing. In Gram-positive bacteria, sortase processes COOH-terminal sequences and catalyzes the covalent attachment of the product to the cell-wall peptidoglycan (6, 74).

The findings presented in this manuscript suggest that COOH-terminal sequences may have a larger role in protein export than has been previously recognized. Secretion of three heterologous viral products – A27L, H3L, and G1L – were secreted into culture supernatant by *S. gordonii* at substantially greater levels, when the products were translated as fusions with derivatives of the *S. pyogenes* M6 protein situated at the COOH terminus than when they were expressed without a fusion partner. By deleting the M6 CRR region from the

A27L-CRRDRR fusion, we demonstrated that amino acids contained in the M6 DRR promote product secretion by *S. gordonii*.

Based on current understandings of protein export, it is enigmatic why fusion of the viral products with M6 domains would so dramatically affect their secretion. It is possible that the addition of M6 protein residues to the COOH terminus of viral products may have provided negative (i.e. inhibitory) signaling to proteolytic enzymes that monitor exported products for misfolding, thus allowing diffusion of the fusion product from the cell. This theory is supported by our inability to detect G1L-FLAG in culture supernatants (Fig. 5.6B), cytoplasmic preparations, or on the surface of *S. gordonii* following transformation with pLEX:G1L-FLAG (data not shown). Investigations involving *B. subtilis* suggest that preproteins are transported across the membrane in an unfolded state and must fold into a mature confirmation quickly to avoid degradation by proteolytic enzymes that maintain the quality of secreted proteins (60). As viral proteins, A27L, H3L, and G1L, expressed without fusion partners may have lacked the conformation necessary to avoid degradation by proteolytic enzymes, such as HtrA, which has been shown to colocalize with enzymes of the Sec pathway in *S. pyogenes* (58). We are currently conducting investigations regarding whether these viral products can be secreted from an *S. gordonii htrA* mutant.

Another possibility is that residues present in the M6 DRR interact with enzymes that facilitate protein export. While the identity of an enzyme(s) that responds both to COOH-terminal signaling and provides a role in protein export

has not been identified in Gram-positive bacteria, SrtA may be involved. In *S. gordonii*, this enzyme has been shown to recognize and process *S. pyogenes* M6 COOH-terminal sequences to facilitate protein surface display (6). Because an *S. gordonii* mutant possessing a disabled *srtA* gene has already been developed (6), it would be straightforward to evaluate whether SrtA is involved in secretion of the viral fusions.

While the fusion products engineered in these investigations were designed with N-terminal secretion signals derived from native glucosyltransferase (GTF) (66, 84), it is not possible to dismiss the possibility that by fusing the M6 COOH to heterologous products, these products were routed to Sec independent export mechanisms. Recent evaluations in *B. subtilis* (70) and *Listeria monocytogenes* (27) suggest that Sec-independent protein secretion is more common than previously thought. Indeed, it has been shown that *S. gordonii* possesses, in addition to canonical Sec enzymes similar to those of *E. coli*, non-canonical accessory Sec enzymes that may mediate export of particular products (2, 3, 67, 68). While protein export via the Tat pathway may be common in Gram-positive bacteria (4, 55), the GTF secretional signal used in these experiments lacks the twin-arginine signal motif characteristic of Tat signaling, suggesting that Tat transport is not involved.

Results from our investigations of the influence of hydrophobicity on secretion suggest that, in some cases (e.g. H3L), deletion of putative hydrophobic residues can markedly enhance secretion of the product by *S. gordonii*. It may be

possible that when these regions are present, they exhibit an extracellular conformation that targets them for degradation by the quality-control proteolytic enzymes. Alternatively, these regions may be difficult for the Sec pathway enzymes to accommodate. In *E. coli*, the first 20 amino acids of the mature protein have been shown to affect N-terminal loop formation important for the inititiation of translocation (83). Thus, in *S. gordonii*, hydrophobic residues situated near the N-terminus of the mature product may interfere with effective processing of the secretion signal by the Sec translocase.

Regardless of the mechanisms at work in *S. gordonii*, the results provided by the investigations reported here illuminate techniques that may be useful for enhancing production of recalcitrant proteins. We have used the M6 CRRDRR fusion partner to secrete additional viral products A33R, D8L, and B5R from *S. gordonii*, and we are currently evaluating whether production of murine cytokines IL-2 and IFN-γ can be enhanced by fusion with bacterial sequences.

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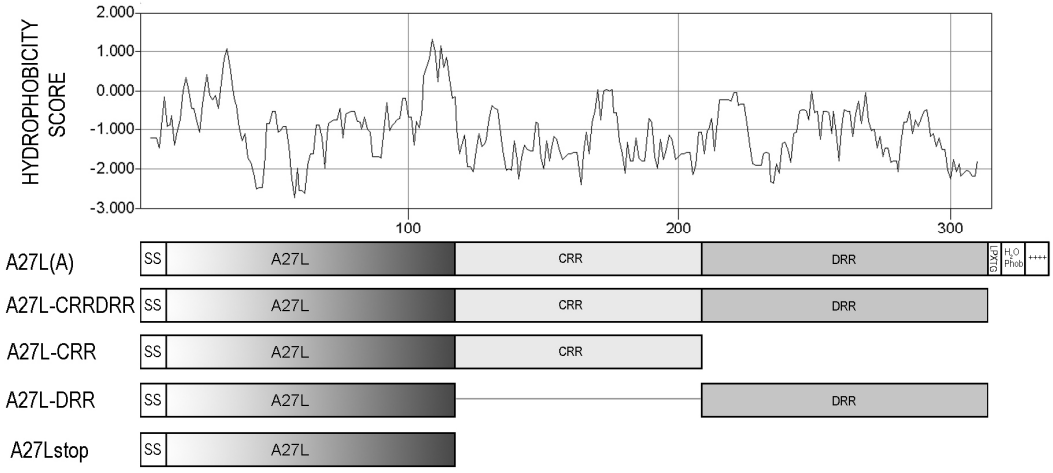

Figure 5.1. Hydrophobicity and nomenclature of A27L products. VV A27L was cloned into the *S. gordonii* pLEX expression vector. To promote anchoring of the product, A27L was fused at the COOH-terminus to domains derived from the *S. pyogenes* M6 protein, including the C-repeat region (CRR), D-repeat region (DRR), and anchor sorting signals comprised of LPXTG, a hydrophobic region, and positively charged tail (++++). Other A27L constructs were engineered as indicated. The thin gray line indicates deleted domains. The domains are relatively scaled according to their size and are positioned directly beneath a Kyte-Doolittle hydrophobicity analysis of their amino-acid residues.

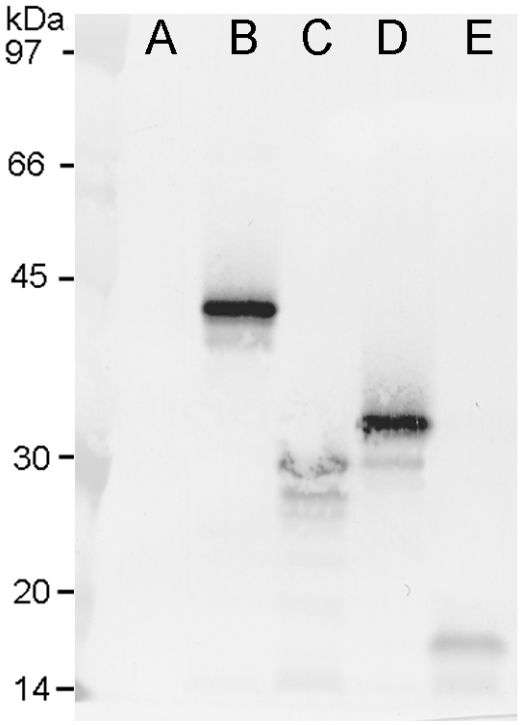

Figure 5.2. Fusion of VV A27L to *S. pyogenes* M6 protein domains facilitates secretion in *S. gordonii*. Supernatants from S. gordonii were subjected to SDS-PAGE and Western transfer to PVDF. The membrane was probed with rabbit A27L anti-sera. *S. gordonii* was transformed with the pLEX expression plasmid encoding the CRR-DRR domains (A), A27L-CRRDRR (B), A27L-CRR (C), A27L-DRR (D), or A27Lstop (E).

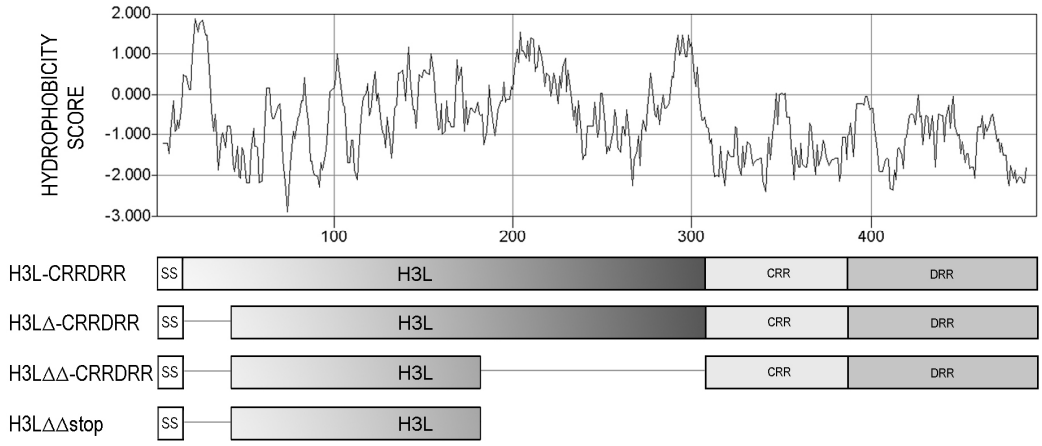

Figure 5.3. Hydrophobicity and nomenclature of H3L products. VV H3L was cloned into the *S. gordonii* pLEX expression vector. H3L was fused at the COOH-terminus to domains derived from the *S. pyogenes* M6 protein, including the C-repeat region (CRR), D-repeat region (DRR). Other H3L constructs were engineered as indicated. The thin gray line indicates deleted domains. The domains are relatively scaled according to their size and are positioned directly beneath a Kyte-Doolittle hydrophobicity analysis of their amino-acid residues.

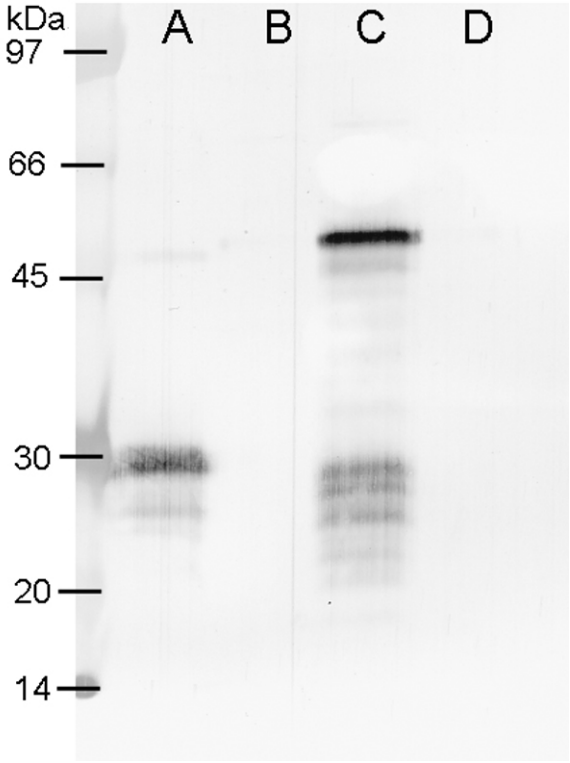

Figure 5.4. Fusion of VV H3L to *S. pyogenes* M6 protein domains facilitates secretion by *S. gordonii*. *S. gordonii* supernatants were subjected to SDS-PAGE and Western transfer to PVDF. The membrane was probed with mAb 10F5 specific for the M6 CRR domain. *S. gordonii* was transformed with the pLEX expression plasmid encoding the CRR-DRR domains (A), H3LΔ-CRRDRR (B), H3LΔΔ-CRRDRR (C), or H3LΔΔ (D).

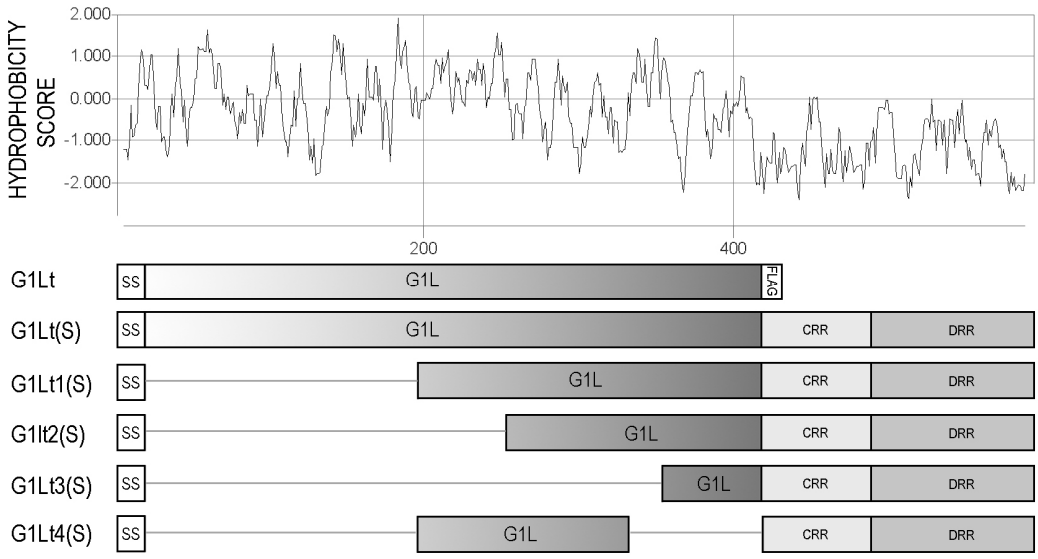

Figure 5.5. Hydrophobicity and nomenclature of G1L products. Truncated versions of VV G1L was cloned into the *S. gordonii* pLEX expression vector. G1L was fused at the COOH-terminus to the FLAG tag (DYKDDDDK) or domains derived from the *S. pyogenes* M6 protein, including the C-repeat region (CRR), D-repeat region (DRR), as indicated. Thin gray lines indicate deleted G1L domains. Domains are relatively scaled according to their size and are positioned directly beneath a Kyte-Doolittle hydrophobicity analysis of their amino-acid residues.

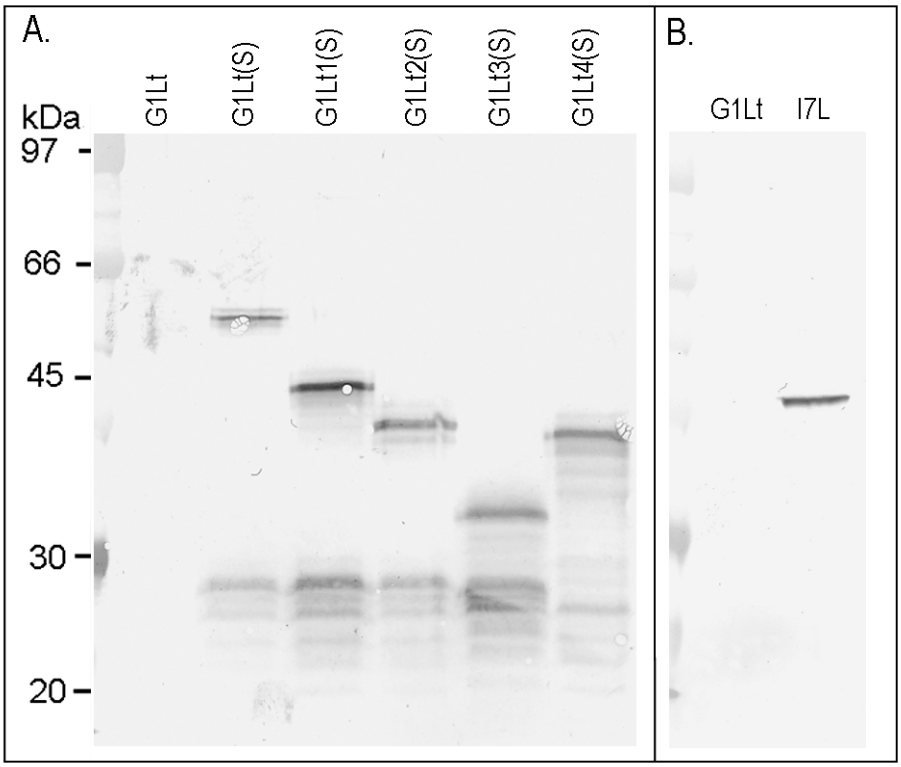

Figure 5.6. Fusion of VV G1L truncates to *S. pyogenes* M6 protein domains facilitates secretion in *S. gordonii*. *S. gordonii* supernatants were subjected to SDS-PAGE and Western transfer to PVDF. A. The membrane was probed with mAb 10F5 specific for the M6 CRR domain. *S. gordonii* was transformed with the pLEX expression plasmid encoding G1Lt, G1Lt(S), G1Lt1(S), G1Lt2(S), G1Lt3(S), or G1Lt4(S). B. Supernatant from *S. gordonii* transformed to express FLAG-labeled G1Lt was probed with anti-FLAG mAb, along with VV I7L-FLAG, included as a positive control.

Table 5.1.
Plasmids used for amplification of products for cloning into pLEX.

Gene Product (Encoded Amino Acids Relative to Start Codon)	Primer Names	PCR Primers (5'-3')	Encoded Features
A27L (2-110)	RW 5' A27L	CGGGGTACCGACGGAACTCTTTTCCCC	KpnI
	RW 3' A27L	CCGGAATTCCTCATATGGATCTGAAC	EcoRI
H3LΔ (21-283)	LB13	CCGGGTACCGCGGCGGTGAAAAC	KpnI
	LB14	CCGGAATTCAAAAAATGAAATCAGTGGAGTAGTAAACGCG	EcoRI
H3LΔΔ (21-170)	ML30	GGGGTACCACATTTCCTAATGTTCATGAGC	KpnI
	ML34	CGGAATTCTTTGTCCATTACAAGCTCGG	EcoRI
G1L (2-607)	GILFLAG PLEX5'	CGTAGGGGTACCTGATTGTCTTACCGAATAAAGTTCG	KpnI
	GILFLAG PLEX3'	GTCTAGCATGCTCACTTGTCGTCATCGTCTTTATAATC	FLAG Stop SphI
G1Lt (2-389)	GILFLAG PLEX5'	CGTAGGGGTACCTGATTGTCTTACCGAATAAAGTTCG	KpnI
	TW17	GGAATTCTCACTTGTCGTCATCGTCTTTATAATCTCGTATA AATA	FLAG Stop EcoRI
GILt(s) (2-389)	TW16	GGGGTACCATTGTCTTACCGAATAAAGTTCGTATTTTC	KpnI
	TW21	GCGAATTCTCGTATAAATATTTCATTAGATGTTAGACGCTT CA	EcoRI
G1Lt1(s) (188-389)	TW39	GGGGTACCGTTAAGAGATTAGGACCTGG	KpnI
	TW22	TCCCCGCGGCCGCTCTTACTGTCTCTTAGTTTCC	BsrBI/SacII
G1Lt2(s)(253-389)	TW40	GGGGTACCTACGAAACTTACCACTTAATAGACTATGAG	KpnI
	TW22	TCCCCGCGGCCGCTCTTACTGTCTCTTAGTTTCC	BsrBI/SacII
G1Lt3(s)(354-389)	TW41	GGGGTACCGTTATTTACCCAAACTTTAGTAAGGCC	KpnI
	TW22	TCCCCGCGGCCGCTCTTACTGTCTCTTAGTTTCC	BsrBI/SacII
G1Lt4(s)(188-335)	TW39	GGGGTACCGTTAAGAGATTAGGACCTGG	KpnI
	TW42	CGGAATTCCGACTTGCTATCGTCATTAATACGTGTAATG	EcoRI
A27LM6Anchor (M6 264-483)	RW5' A27L	CGGGGTACCGACGGAACTCTTTTCCCC	KpnI
	ML11	CGGCCGTCGCGATTAGTTTTCTTCTTGCGTTTTA	NruI Stop
A27LCRRDRR (M6 264 -448)	RW5'A27L	CGGGGTACCGACGGAACTCTTTTCCCC	KpnI
	ML25	CGAAGATCTTTACTGTCTCTTAGTTTCCTTCATTGG	BglII Stop
A27LCRR (M6 264 -346)	RW5'A27L	CGGGGTACCGACGGAACTCTTTTCCCC	KpnI
	ML27	CGGCCGTCGCGATTAAGCTTTTTCAACTTGTTTCTTAG	Nru I Stop
M6DRR (347-448)	ML28	GGAATTCTTAGAAGAAGCAAACAGCAATTAGC	EcoRI
	ML24	GGAATTCTTACTGTCTCTTAGTTTCCTTCATTGG	EcoRI Stop

Chapter 6

Conclusions

The investigations presented in this dissertation serve to reinforce the use of *S. gordonii* as a bacterial commensal vector (BCV), and suggest that the promoters of AbpA and GTF as well as the pLEX expression vector may be useful genetic tools for *S. gordonii* manipulations. The multi-copy pLEX plasmid represents a powerful tool to rapidly introduce DNA into *S. gordonii* and it facilitates high expression levels through multiple gene copies. Use of the $pLEX_{2.0}$ expression plasmid facilitates even greater expression using the strong *apbA* promoter. Thus, heterologous antigen expression can be achieved from single-copy genes, using any of a number of native chromosomal promoters, or from multiple copies using pLEX.

The pLEX plasmids additionally represent a flexible and powerful tool for evaluating *S. gordonii* promoter strength, as described in Chapter 3. In the future, it would be of interest, and a relatively straightforward analysis, to evaluate the strength of the inducible *lacG* promoter, by incorporating this element into the

pLEX plasmid for production of BH4XCRR. Evaluation of the strength of this promoter could be evaluated by comparing BH4XCRR production with that facilitated by the $pLEX_{1.0}$ and $pLEX_{2.0}$ plasmids already developed (Chapters 2 and 3).

Other variations on the pLEX technology would be interesting to evaluate as well. For example, it has been suggested that the *E. coli* T7 promoter may function as a strong transcriptional promoter in *S. gordonii,* and could be easily cloned into pLEX. Full functionality of the T7 promoter would require that *S. gordonii* be engineered to express the single-unit T7 polymerase, possibly from the same plasmid bearing the T7 promoter.

While the position of the streptococcal origin of replication has not been identified on the plasmid, the origin sequences can be narrowed to a relatively small region, deduced from the manner in which the original parent plasmid, pVA838, was created (30). It would be interesting to replace this region with that from a plasmid that has been shown to replicate at higher copies in *S. gordonii* or other Gram-positive bacteria such as *B. subtilis* or *Lactococcus lactis*.

It is of interest to evaluate whether the pLEX plasmids and the genetic elements they bear are replicated and function effectively in *L. lactis*, as this bacteria has also been shown to have promise as a live bacterial vector vaccine delivery (16, 34, 53). *L. lactis* is generally recognized as safe by the US Food and Drug Administration (USFDA) and, like *S. gordonii*, has been shown to induce

both systemic and oral humoral responses to heterologous surface antigens following oral delivery.

One of the larger remaining obstacles to developing *S. gordonii* as a vaccine-delivery vector involves the use of antibiotic-resistance genes for genetic manipulations. While these genes have been convenient for screening purposes the USFDA discourages inclusion of antibiotic-resistance markers in clinical applications. Several clonal-selection strategies have been identified to overcome the challenges of genetically manipulating bacteria without introducing antibiotic resistance in the final constructs. The temperature-sensitive pGHOST plasmid is being utilized by personnel at Siga to facilitate chromosomal integration of A27L at the Lac operon, using a technique first developed in *Listeria monocytogenes (14)*. To apply this technique, the plasmid is first engineered such that the heterologous gene is flanked by native sequences to facilitate a single-crossover recombinational event with chromosomal sequences. Once the plasmid has been integrated into the chromosome, plasmid replication is induced at the permissive temperature, and spontaneous recombination removes antibiotic resistance elements from the chromosome. Excised plasmids are cured by incubation at non-permissive temperature, and transformants are selected on the basis of the loss of antibiotic resistance. Another approach to introducing heterologous genes without conferring antibiotic resistance was also developed in *L. monocytogenes* and involves transforming a D-alanine racemase-deficient strain with a resident plasmid that complements the deficiency (79). Because the plasmid complements

mutations in essential genes, the plasmid must be retained for cell survival. The approach requires inactivation of two well conserved chromosomal genes involved in D-alanine metabolism in the plasmid-recipient strain.

It is important to recognize that the evaluations of *S. gordonii* promoter strength described in this dissertation have been conducted *in vitro*. Whether promoter activity *in vitro* correlates with activity during oral colonization is a matter for speculation. Evaluating promoter activity *in vivo* is not straightforward; however, four approaches may be useful to identify expression of heterologous products by *S. gordonii* during oral colonization. One of the most obvious approaches is to assess development of the immunological response to heterologous antigens. It would be important to evaluate expression of a heterologous product that possessed a high-degree of antigencity. While this dissertation describes studies in which mice were inoculated with *S. gordonii* expressing BH4XCRR, this molecule is poorly immunogenic and is not an ideal heterologous product to use in immunogenic evaluations. Moderate to high-level humoral immunogenic responses to viral antigens presented on the surface of *S. gordonii* have been documented (85); however, a comprehensive evaluation of the humoral response generated by individual BCV strains has yet to be conducted.

A second possible approach to assessing *in vivo* expression during oral colonization involves engineering the *S. gordonii* promoters of interest to transcribe a luminescent product. Advances in imaging technology have permitted the use of highly photosensitive digitial equipment to detect the internal

distribution of bioluminescent-tagged *S. pyogenes* in anesthetized mice (47). A similar approach would presumably allow *S. gordonii* to be detected in the oral cavity of mice, where the luminescent intensity of bacteria would provide an indication of the activity at the promoter responsible for expression of the bioluminescent product. The high cost of the imaging equipment is an obvious drawback associated with this assessment.

A third, low-cost approach to evaluating *in vivo* expression involves engineering *S. gordonii* to express β-galactosidase as a surface product. Following oral colonization, tissue sampled from the oral cavity, or perhaps from draining lymph nodes, could be briefly incubated in the presence of X-gal, which is converted into a readily visualized blue precipitate upon hydrolysis by β-galactosidase. Tissue that develops greater blue intensities would suggest enhanced β-galactosidase surface expression at the time of sample collection. The final approach to *in vivo* analysis of expression involves characterizing the RNA transcripts from oral bacteria. This approach has been successfully used to identify active promoters in *S. pyogenes* during murine challenge.

The findings presented in Chapter 5 demonstrate that COOH-terminal fusion of M6 domains to viral products facilitate secretion by *S. gordonii*. These findings may provide the background for more extensive investigations into the mechanisms used by *S. gordonii* to secrete proteins. One potential target for further study is whether HtrA (a DegP homologue) is involved in proteolytic degradation of viral products. This enzyme possesses an established role in

monitoring the extracellular space in Gram-positive organisms for misfolded products; however, the mechanisms and substrate attributes that activate proteolysis are not known in *S. gordonii*. If HtrA was responsible for degrading viral products, it would be expected that transformation of an *htrA* deletional mutant with the pLEX plasmid would promote secretion of viral products by *S. gordonii* without the use of a fusion partner. Other questions stimulated by these findings include whether the M6 DRR could facilitate equivalent levels of secretion if positioned at the N-terminus of fusion products. Would domains from native anchored products facilitate secretion if fused to viral products?

While removal of putative hydrophobic domains from products was found to influence secretion, it is not known in what manner the deleted regions exert this influence. Hydrophobic residues may promote protein conformations that are inconsistent with native secreted products and subsequently provide the necessary signaling for degradation by quality-control enzymes. Alternatively, the Sec-pathway components may be poorly capable of accommodating hydrophobic products. It would be interesting to evaluate amino-acid sequences of the numerous products secreted by *B. subtilis* (70, 71) to assess the extent to which hydrophobic domains occur in proteins secreted by this bacterial species.

While the investigations presented in this dissertation may facilitate the development of *S. gordonii* as BCV, it is apparent that the scientific community will have to perform numerous investigations before the mechanisms and processes involved in product export by *S. gordonii* are fully understood.

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