Development of An Antibiotic Marker-Free Gene Delivery System in *Streptococcus gordonii*

by

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Abstract

Delivery of vaccine antigens by live bacterial carriers has the potential to elicit both mucosal and systemic immune responses. *Streptococcus gordonii,* a commensal oral bacterium, is considered a good candidate to function as a live oral vaccine vector. The introduction of vaccine antigen genes into *S. gordonii* relies on the use of antibiotic resistance genes as selectable markers, which is undesirable. In this study, we used auxotrophic complementation (deletion of an essential gene from the chromosome and insertion into a plasmid) as a means to create an antibiotic marker-free gene delivery system in *S. gordonii.* The dependence of *S. gordonii* on thymidylate synthase for growth was exploited for the construction of the auxotrophic complementation system. The *thyA* gene encodes thymidylate synthase, an essential gene for the synthesis of DNA precursors. *S. gordonii ΔthyA* was created by inserting the *ermAM* gene into *thyA*, and *ermAM* was subsequently deleted using the *cre/loxP* system. *S. gordonii ΔthyA* is a thymidine auxotroph and only grew in media supplemented with thymidine. The *ermAM* cassette was successfully deleted from the *thyA* mutant, which showed sensitivity to erythromycin. The *thyA* mutation was complemented by cloning *thyA* onto pDL276. The kanamycin resistance gene was then removed from pDL276/*thyA* in order to obtain an antibiotic marker-free expression plasmid, pDL276/*thyA*del*kan*. The resulting marker-free *thyA* mutant, when transformed with pDL276/*thyA*del*kan*, gave an unexpected 100-fold increase in transformation efficiency as compared to pDL276. The pDL276/*thyA*del*kan* transformants arose from both single and double crossing over. The increase in transformation efficiency suggests that a highly efficient antibiotic marker-free system to deliver genes to the chromosome has been created using *thyA* complementation. To test the efficiency of the new system, the vaccine antigen gene of pertussis, *fha/cr-1*, was cloned onto the antibiotic marker-free expression system, pDL276/*thyA*del*kan*FHA/CR-1, and transformed into the antibiotic marker-free *thyA* mutant. Although the transformation efficiency of pDL276/*thyA*del*kan*FHA/CR-1 was similar to that of pDL276/*thyA*del*kan*, the frequency of pDL276/*thyA*del*kan*FHA/CR-1 transformants with single crossing over was very low. This was investigated following the transformation of pDL276/*thyA*del*kan*FHA/CR-1 into the erythromycin-resistant *thyA* mutant and transformants were selected based on an erythromycin-resistant phenotype. Erythromycin-resistant transformants (*erm*^{r}), with the plasmid integrated into the chromosome by single crossing over, were screened for protein production by Western blot and found able to produce the vaccine protein successfully. In addition, successful PCR amplification was done in order to identify the precise location where the plasmid was integrated in the chromosome. The results showed that the single crossing over of *thyA* gene occurred equally at both the upstream and downstream portions of *thyA* in chromosomal *thyA::ermAM*. Although our investigation of the successful delivery of the vaccine gene into the chromosome has not been carried in the antibiotic marker-free system, it did allow us to determine that single-copy integration of the plasmid had occurred at low frequency with pDL276/*thyA*del*kan*FHA/CR-1. Future work can be done to increase the probability of transformants with single crossing over using a different strategy in the construction of the *thyA* mutant. In conclusion, an antibiotic marker-free delivery system has been successfully established in this study for testing vaccine antigen gene delivery and expression in *S. gordonii*.

List of Abbreviations Used

ASL- adenylosuccinate lyase

BCG- Bacille Calmette-Guerin

BCIP- 5-bromo-4-chloro-3-indolyl phosphate

BHI- brain heart infusion

BHICS- brain heart infusion calf serum

bp- base pair

cfu- colony forming unit

DAP- Diaminopimelic acid

dH_2O- deionized water

dNTP- deoxynucleotide triphosphate

dTMP- deoxythymidine monophosphate

dUMP- deoxyuradine monophosphate

EDTA- ethylenediamine tetracetic acid

FHA- filamentous hemagglutinin

GI- gastrointestinal

GTE- glucose-Tris-EDTA

IgA- immunoglobulin A

IgG- immunoglobulin G

KAc- potassium acetate

kb- kilobase

KDa- kiloDalton

LAB- lactic acid bacteria

LB- Luria-Bertani

LLO- *Listeria* listeriolysin O

LPS- lipopolysaccharide

LT-B- heat labile enterotoxin B subunit of *E. coli*

M- Molar

mg- milligram

min- minutes

ml- milliliter

mM- milli Molar

NBT- nitro blue tetrazolium

OD- optical density

ORT- Operator-repressor titration

PBS- phosphate-buffered-saline

PBS- phosphate-buffered saline with Tween 20

PCR- polymerase chain reaction

PT- pertussis toxin

SB- super broth

SDS-PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis

scFv- single-chain variable fragment antibody

TAE- Tris acetate-EDTA

TE- Tris- EDTA

TTFC- tetanus toxin fragment C

TYG- tryptone yeast extracts glucose

UDP- uridine diphosphate

UV- ultraviolet light

x g- times gravity

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Chapter1. Introduction

1.1. Mucosal Vaccines

The first vaccine was developed by Edward Jenner in 1796 in order to protect against deadly smallpox. Since then, many vaccines have been developed that play important roles in the fight against microbial diseases. Most current vaccines rely upon parenteral administration of antigens to stimulate a systemic immune response. However, most pathogenic microorganisms invade via the mucosal surfaces (Medina and Guzman, 2000). Vaccines administered by a mucosal route mimic the immune response elicited by natural infections (McGhee *et al,* 1992). Therefore, mucosally-delivered vaccines have the potential to induce both systemic and mucosal immunity (Medina and Guzman, 2000). As a result, parenterally-administered vaccines, which may be limited in their capacity to induce mucosal immune responses, are less potent in preventing infection by mucosal pathogens (McNeilly *et al,* 2008).

The administration of vaccines by a mucosal route has other non-immune related advantages over parenteral vaccinations. For example, the mucosal administration route eliminates the use of needles, as well as the need for trained personnel. Vaccination with needles produces dangerous infectious waste materials that may pose serious health threats to both patients and health care professionals. In addition, needle-free vaccine delivery can make vaccination in less developed countries cheaper and more accessible. Moreover, mucosal vaccination lowers the risk of localized site-specific complications such as pain, erythema and swelling at the vaccination site.

Various strategies have been used to deliver vaccine antigens by the mucosal route. Among the live vectors studied, bacteria have the advantage that their genome is able to carry a number of foreign genes. As a result, recombinant bacteria have the potential to produce various foreign antigens. However, the use of engineered bacterial vaccine strains in humans requires a balance between the level of attenuation and the stimulation of protective immune responses. Two different approaches have been taken to meet these requirements: the development of vaccine strains through attenuation of bacterial pathogens and the development of vaccine vehicles from commensal microorganisms.

1.1.1. Attenuated mucosal pathogens as live vaccine vectors

Live attenuated mutants of several pathogenic bacteria have been exploited as potential live vaccine vectors for heterologous antigen delivery by the mucosal route. The advantage is that pathogenic bacteria, being more immunogenic than commensal microorganisms, are able to stimulate protective immune responses. The use of live attenuated bacteria to deliver vaccine antigens safely to the mucosal surfaces for the stimulation of protective immune responses has been attempted in several different ways. While some of these approaches have been applied, others are still not considered to be safe and acceptable ways to develop and deliver this kind of vaccine. Those most studied organisms include attenuated strains of Bacille Calmette-Guerin (BCG), *Salmonella spp.*, *Shigella flexneri*, and *Vibrio cholera* (Mahairas *et al.*, 1998; Levine et al., 1989; Tacket et al., 1999).

Repetitive culture passage of pathogenic bacteria leads to a number of genetic changes that may give rise to attenuation of some pathogenic bacteria. Bacillus Calmette-Guerin (BCG), a vaccine consisting of *Mycobacterium bovis* developed to prevent *Mycobacterium tuberculosis* infection, is an attenuated mutant vaccine that was produced by this technique. The original BCG Pasteur strain was developed by 230 repetitive serial passages and has never reverted to the virulent strain (Mahairas *et al.*, 1998). BCG is the most widely administered vaccine in the world today. The vaccine reduces the risk of tuberculosis by 50% and reduces the complications associated with the disease by up to 80% (Colditz et al., 1994). Moreover, BCG vaccine is capable of inducing a protective immune response against tuberculosis for prolonged periods of time (Husain et al., 2010). Although BCG was originally administered orally, intradermal injection is the most common method because it delivers a more precise dose of vaccine.

Another successful example of a live attenuated bacterial vaccine derived by laboratory passaging is the vaccine strain *Salmonella enterica* serovar Typhi Ty21a. Ty21a is an attenuated mutant strain of *S. typhi* Ty2 that was isolated by random chemical mutagenesis using nitrosoguanidine. Ty21a has prominent GalE and virulence (Vi)-negative phenotypes (Germanier and Furer, 1975). Ty21a is deficient in the enzyme uridine diphosphate (UDP)-galactose-4-epimerase, which is responsible for the conversion of UDP-glucose into UDP-galactose. UDP-galactose is subsequently used in the biosynthesis of the lipopolysacharide (LPS) O chain. In addition to the mutation in galactose metabolism, synthesis of the virulence associated (Vi) polysaccharide capsule is mutated in strain Ty21a, leading to a further attenuation. Since the LPS O antigen is the main antigenic determinant on the cell surface, the formation of LPS O antigens is

important for the Ty21a vaccine's induction of the immune response. Thus, Ty21a was supplied with a limiting source of exogenous galactose during the production of the vaccine because of the defect in *galE* gene. The Ty21a vaccine has been shown to be safe and protective as a live oral vaccine, and currently is the only licensed live oral vaccine for protection against typhoid fever (Levine et al., 1989).

The Ty21a vaccine has been used to express antigens from other closely related enteric pathogens, such as the B subunit of the heat labile enterotoxin (LT-B) of *E. coli* (Clements and El-Morshidy, 1984). Tests in mice and guinea pigs showed that Ty21a vaccine carrying the LT-B on a plasmid, SE12, was safe when given orally and was capable of inducing a significant antibody response to LT-B when injected parenterally (Clements and El-Morshidy, 1984). In addition to the induction of an immune response, SE12 was rapidly cleared after intraperitoneal injection in mice. In agreement with the previous study, Formal *et al.* (1981) confirmed that the oral vaccine strain of *S. typhi* Ty21a serves as a useful carrier for other antigenic determinants to protect against different intestinal infections. Thus, Ty21a is considered a successful live attenuated vaccine vector for both homologous and heterologous antigens. Although those previous studies claimed that this vaccine strain has good stability, Germanier and Furer (1971) commented that some strains might be subjected to a secondary mutation that leads to its persistence inside the human body, thus causing some concerns regarding its safety.

The technology of attenuating pathogenic bacteria by laboratory culture passage is no longer considered optimal for the development of live attenuated vaccines. Low expression of certain antigens by the bacterial strain, which might be important for protective immunity, probably occurs. Therefore, finding another way to attenuate

pathogenic bacteria for use as a live vaccine is necessary. Repetitive culture passage may also create deletions or mutations in genes other than those related to virulence, including genes involved with eliciting immune responses, thus reducing the overall immunogenicity of vaccine strain. It is preferable to develop better-defined attenuated bacterial vaccines rather than facing the uncertain consequences of random mutations within the virulence stain.

One of the most widely studied classes of attenuated bacteria is auxotrophies in the aromatic amino acid (*aro*) biosynthetic pathway genes. This pathway is the only way by which bacteria can synthesize important aromatic compounds responsible for folate and nucleotide synthesis. Because mammalian cells do not possess this pathway, these important aromatic compounds are not available in the host tissues. Mutations in these genes render the bacteria unable to replicate unless cultivated on special growth medium under *in vitro* conditions. Thus, pathogenic bacteria carrying mutations on *aro* genes cannot grow *in vivo* despite having other virulence factors. A study by Dougan *et al.* (1987) clearly demonstrated that attenuated *Salmonella typhimurium*, which has a mutation in the *aroA* gene, can colonize the reticuloendothelial system (RES) in mice at a much slower rate and persist at lower levels than the parent strain. These mutants are usually completely cleared after a few weeks but confer a strong protection against virulent challenge. The *aroA* mutant of *S. typhimurium* has good potential as a live attenuated vaccine against typhoid infection.

Deletion of virulence genes in pathogenic bacteria reduces their pathogenicity. In many cases, deletion or inactivation of a single virulence gene—often the gene encoding the main toxin—results in non-pathogenic organisms. For example, CVD 103- HgR, a

live attenuated *Vibrio cholerae* O1 Inaba strain vaccine, was produced by deleting 94% of the gene encoding the cholera toxin A subunit (*ctxA*) gene while keeping the expression of the non-toxic but immunogenic B subunit (*ctxB*) gene, leading to intermediary strain CVD 103. Subsequently the gene encoding resistance to mercury was inserted into the hemolysin *hlyA* gene to provide a marker to differentiate the vaccine strain from wild type *V. cholerae* classical Inaba strains (Ketley et al., 1993).

The safety and immunogenicity of CVD 103- HgR vaccine were evaluated in human volunteers using a challenge model. Excellent efficacy of CVD 103-HgR against wild type *V. cholerae* challenge infection was demonstrated, in which 91% of vaccine recipients developed significant increases in serum vibriocidal antibody titers following oral immunization (Tacket et al., 1999). In addition, no significant adverse reactions related to the vaccine were observed (Richie et al., 2000)

The most important safety feature of CVD 103-HgR vaccine is that the vaccine strain is unable to revert to a virulent phenotype, as shown by Kaper et al. (1994). The study examined the potential conjugal transfer of *ctxA* genes from wild type *V. cholerae* O1 to CVD 103-HgR vaccine strain by performing a series of mating experiments. The study reported the failure to demonstrate transfer of *ctxA* genes to *V. cholerae* CVD 103-HgR, with no evidence that CVD 103-HgR could reacquire *ctxA* genes from wild-type *V. cholerae* O1 strains (Kaper et al., 1994). However, if the donor *V. cholerae* O1 strains were genetically manipulated to add genes that allow chromosomal gene transfer, then *ctxA* sequences could be acquired by CVD 103-HgR (Kaper et al., 1994).

Despite the safety of some attenuated pathogenic bacteria, deletion of an important active-site residue, glutamic acid-148, from the diphtheria toxin of the strain *Corynebacterium diphteriae* did not prevent reversion. Secondary site mutations in sequences adjacent to the deletion in the active site of diphtheria toxin has occurred (Killeen et al., 1992). This suggests that partial deletion of virulence genes from pathogenic bacteria with the aim of developing live bacterial vaccines may not work in all cases.

One way to circumvent potential pathogenic reversion is to use the live commensal bacteria as vectors to deliver antigens from pathogens.

1.1.2. Commensal bacteria as live vaccine vectors

Commensal bacteria can provide a safe substitute to live attenuated bacteria by eliminating the possibility of a live attenuated organism reverting to a pathogenic state and causing disease. These recombinant commensal microorganisms can establish a niche in the host and stimulate an immune response (Fischetti et.al., 1993). The native organisms are non-invasive and are typically well tolerated, and can colonize and persist on mucosal surfaces and display immunogenic antigens for long periods of time. The tolerance of the host immune system to non-pathogenic commensal bacteria could be considered as an advantage in relation to mucosal delivery as it reduces the risk of provoking immune responses to the bacterial strain from repeated administration. However, this could also represent a limitation to their potential as a vaccine vector if they are unable to elicit effective immune responses to the vaccine antigens. Engineering commensal bacteria in order to be more visible to the immune system and induce an

immune response can be achieved. Examples of commensal bacteria that have been used as vaccine carriers are lactic acid bacteria and *Streptococcus gordonii.*

1.1.2.1. Lactic acid bacteria as a commensal mucosal vaccines vectors

Over the past decade, there has been increasing interest in the use of lactic acid bacteria (LAB) such as *Lactococcus spp.* and *Lactobacillus spp.*, which are widely used in the food industry, as mucosal delivery vehicles. A number of LAB have been used, including *Lactobacillus plantarum* (Corthesy et al., 2005; Grangette et al. 2001; Oliveira et al., 2006), *Lactococcus lactis* (Bahey-El-Din et al., 2008 ; Hanniffy et al., 2007; Lee et al., 2001; Mannam et al., 2004; Perez et al., 2005), *Lactobacillus acidophilus* (Chu et al., 2005), *Lactobacillus casei* (Lee et al., 2006; Poo et al., 2006), and *Lactobacillus helveticus* (Oliveira et al., 2006). Antigens expressed in LAB include *Helicobacter pylori* urease B subunit gene (Corthesy et al., 2005; Lee et al., 2001), tetanus toxin fragment C (TTFC) (Grangette et al. 2001), *Streptococcus pneumoniae* antigens (Hanniffy et al., 2007; Oliveira et al., 2006), *Streptococcus pyogenes* M6 antigen (Mannam et al., 2004), enterotoxigenic *Escherichia coli* K99 fimbriae (Chu et al., 2005), SARS-associated coronavirus spike antigen segments (Lee et al., 2006), VP7 protein of rotavirus (Perez et al., 2005), listeriolysin O antigen of *Listeria monocytogenes* (Bahey-El-Din et al., 2008) and E7 protein of Human Papilloma Virus 16 (Poo et al., 2006). Immune responses against all these antigens were elicited in animal models when vaccinated with the recombinant bacteria.

There are several factors that could affect the immunogenicity of LAB vaccines. The choice of bacterial species for vaccine delivery is important and dependent on the intrinsic antigenicity of the organism. This was investigated by using a single model antigen, *H. pylori* urease B subunit, which has been expressed in two different bacterial hosts, *L. lactis* and *L. plantarum* (Lee et al., 2001 & Corthesy et al., 2005). Using the same mouse strain as a recipient, the recombinant *L. lactis* and *L. plantarum* strains elicited different protective immune responses to challenge with *H. pylori.* Oral immunization of mice with *L. plantarum* expressing *H. pylori* urease B subunit exhibited a better protection against *Helicobacter* infection than the recombinant *L. lactis* (Lee et al., 2001 & Corthesy et al., 2005). Recombinant *L. lactis* induced a very weak antibody response (Lee et al., 2001). The weak immune responses observed might be a reason for the poor persistence of the strain in the gastrointestinal (GI) tract since *L. lactis* has a lower capacity to colonize the digestive tract than *L. plantarum* (Klijn et al., 1995 & Pavan et al., 2003).

The immunogenicity of the expressed antigen has an effect on the stimulation of efficient immune responses in LAB vaccines. For example, *L. lactis* expressing tetanus toxin fragment C (TTFC) has successfully elicited local immunoglobulin A (IgA) responses in gut secretions following oral immunization in the mouse model (Robinson et al., 1997). However, when recombinant *L. lactis* expressing *H. pylori* urease B subunit was used to immunize mice orally, low levels of antibody responses were obtained (Lee et al., 2001). The difference between these two experiments suggests that efficient immune response induction depends upon the immunogenicity of the expressed antigen.

It is known that TTFC is very immunogenic and could be responsible for the high immune response (Norton et al., 1996).

1.1.2.2. *Streptococcus gordonii* as a commensal mucosal vaccine vector

S. gordonii is a commensal organism that inhabits the oral cavity of humans. This organism is generally considered non-pathogenic in healthy children and adults, and is most abundantly found in the oral cavity. *S. gordonii* colonizes the oral cavity at an early age and remains as a normal inhabitant of the oral mucosa in adults (Zou et al., 2004). *S. gordonii* plays an integral role in initiating colonization of other organisms by creating surfaces for other colonizers to adhere to. The efficiency of *S. gordonii* in colonizing the oral cavity suggests its theoretical potential for the stimulation of the mucosal immune system and providing life-long protection following a single oral inoculation.

In addition, like other members of the *Streptococcus* genus, *S. gordonii* possesses mechanisms to uptake genetic material from its environment. This feature of natural competence can be exploited in the laboratory for genetic manipulation of *S. gordonii*. The strain "challis" of *S. gordonii*, which is naturally competent, is the most common strain used for the expression of heterologous antigens (Macrina *et.al*., 1980).

As a commensal, different characteristics make *S. gordonii* an attractive choice for a live vaccine vehicle for immunization against the mucosal pathogens. Its ability to colonize, its persistence on the mucosa, and the natural elicitation of immune responses are all advantages that make *S. gordonii* a good candidate to be a live mucosal vaccine

vector. Furthermore, intranasal administration of *S. gordonii* has been recently shown to be safe in human volunteers (Kotloff et al., 2005).

S. gordonii has been previously used as a recombinant commensal live bacterial vaccine to express a variety of different antigens. As an example, pertussis antigens have been successfully expressed in *S. gordonii*, either as surface-expressed pertussis toxin S1 fragment (Lee et al., 1999) or as a secreted fusion protein consisting of PT S1S3 fragments with filamentous hemagglutinin (FHA) (Lee et al., 2002a). The S1 subunit has been surface-expressed in *S. gordonii* by genetic fusion to the surface protein gene *spaP* from *S. mutans* (Lee et al., 2002a). The *spaP* promoter and surface localization domain were shown to function in *S. gordonii* (Homonylo-McGavin and Lee, 1996).

Examples of other antigens expressed in *S. gordonii* include diphtheria toxin fragment A (Lee et al., 2004), human papillomavirus E7 protein (Medaglini et al., 1997), *S. pyogenes* M6 protein (Oggioni et al., 1995), and hornet venom allergen (Medaglini et al., 1995). Immune responses against all these antigens were tested in mice by oral or intranasal route of inoculations with recombinant *S. gordonii*. Furthermore, these recombinant *S. gordonii*, after a single immunizing dose, were able to colonize the oral cavity of mice for a period of time that lasted up to 19 weeks (Lee et al., 2002b; Medaglini et al., 1995). This stable colonization may represent an effective approach of overcoming the requirement for frequent doses of antigens. Despite the persistent colonization and the induction of mucosal immunoglobulin at the mucosal sites, protective immunity was not readily achieved. It has been suggested that the lack of

induction of a protective antibody immune response may have been due to low levels of antigen expression in *S. gordonii* (Mallaley et al., 2006).

1.2. Heterologous protein expression in *S. gordonii*

The reason behind the low quantities of heterologous protein expression in *S. gordonii* is not known. However, many attempts have been made to investigate whether an increase in antigen production by the *Streptococcus* vaccine strain results in a better antibody response. For example, the use of an inducible promoter to increase the S1 antigen production in *S. gordonii* and *S. mutans* has been investigated (Mallaley et al., 2006). The promoter tested, the *B. subtilis*/*E. coli* chimeric tetracycline-inducible *xyl/tetO* promoter, was cloned upstream of the SpaP/S1 fusion protein into pDL276, an *E. coli*-streptococci shuttle vector and introduced into both *S. gordonii* and *S. mutans* by natural transformation. The inducibility of the *xyl/tetO* promoter by tetracycline has been shown in both bacteria, with the highest expression levels in *S. mutans*. However, intranasal immunization of mice with *S. mutans* (*Pxyl/tetO*-SpaP/S1) resulted in a very weak IgA immune response against S1, and no serum IgG antibody was observed. Mallaley et al. (2006) suggested that the reason for the low level of immune response observed in the recombinant *S. gordonii* may relate to the nature of the SpaP/S1 recombinant protein.

Inefficient protein translocation and folding may limit heterologous protein production. Failure to fold into a proper configuration generally produces proteins that are rapidly degraded by protease. A study by Davis *et al.* (2011) investigated the role of the cell wall in modulating heterologous protein production in *S. gordonii*, examining

whether the charge density in the cell wall of *S. gordonii* has an influence on the expression of SpaP/S1. Their results suggest that a negatively charged cell wall can increase SpaP/S1 production. The increase in expression level of SpaP/S1 was due to an increase in PrsA levels, a peptidyl-propyl *cis/trans* isomerase whose expression is regulated by the cell envelope stress two-component regulatory system (LiaSR). It was suggested that LiaSR senses and responds to cell wall charges and regulates the expression of PrsA in *S. gordonii* (Davis et al., 2011). In *B. subtilis,* the PrsA protein is an essential membrane-bound lipoprotein that is assumed to assist post-translocational folding of exported proteins and stabilize them in the compartment between the cytoplasmic membrane and cell wall (Vitikainen *et al.,* 2004). A number of other studies found that increasing PrsA production results in increased expression of heterologous proteins in *B. subtilis* and *L. lactis* (Kontinen & Sarvas, 1993; Marugg *et al.*, 1996; Williams *et al.,* 2003).

In support of a role for PrsA in SpaP/S1 production from *S. gordonii*, Davis et al. (2011) showed that cloning the *prsA* gene into the plasmid carrying the *spaP/s1* fusion gene resulted in a PrsA and SpaP/S1 overproducing strain. The new strain produced 2.6-fold more PrsA and 3.5-fold more SpaP/S1. Thus, the proper folding of secreted proteins has an important role in regulating protein production.

One other reason that might limit heterologous protein expression is codon-usage bias. In bacteria, the highly expressed genes have biases toward using certain codons that are recognized by the most abandunt tRNA molecules. As a result, tRNA that recognizes a rarely used codon exists in low amounts. Heterologous genes containing clusters of rare

codons stand a higher chance to reduce the expression level of the synthesized protein. A study by Lee et al. (2009) investigated whether the problem of codon-usage bias exists and causes the limitation of heterologous protein expression in *S. gordonii*. The study identified 12 codons deemed as rare codons in *S. gordonii* and seven other streptococcal species, such as *S. mutans*. Transformation of the plasmid carrying 10 of the 12 tRNA genes, encoding rare codones, into strains of *S. gordonii* expressing the fusion protein SpaP/S1, the anti-complement receptor 1 (CR1) single-chain variable fragment (scFv) antibody, or the *Toxoplasma gondii* cyclophilin C18 protein resulted in an increased production of the three heterologous proteins. These heterologous proteins contained high percentages of amino acids encoded by rare codons (Lee at al., 2009). In addition, the study reported the increase production of anti-CR1 scFv in *S. mutans* following tRNA gene supplementation. The results of this study demonstrated that codon-usage bias exists in *Streptococcus spp*. and it is a reason for the limitation of heterologous protein expression in *S. gordonii* (Lee at al., 2009).

1.3. Approaches for introduction of vaccine genes into live bacterial vectors

Live bacterial vectors can be constructed to produce proteins from any number of pathogens with the antigen-encoding gene being located on a plasmid or integrated within the chromosome. The major advantage of using the plasmid expression system is the ability to produce high levels of antigen from the multicopy expression plasmids. In addition, using plasmid vectors in vaccine development offers greater flexibility such that several different antigens can be introduced or cloned rapidly (Verch et al., 2004).

However, multicopy plasmids are usually unstable *in vivo*. Plasmid retention is a prerequisite for the expression of heterologous antigens in live bacterial vectors.

The problems resulting from plasmid instability can be avoided by integrating antigen-encoding genes into the bacterial chromosome via recombination. Chromosomal integration of a heterologous gene can ensure stable gene expression. However, the antigen will only be expressed from a single copy of the foreign gene, compared with multiple copies of the gene present in a multicopy expression plasmid. This may result in low expression of the antigen (Spreng and Viret, 2005) and, as a result, low immunogenicity (Cardenas and Clements, 1993). Moreover, the requirement of finding a suitable gene into which to insert the vaccine antigen gene without causing an undesirable polar effect is another disadvantage.

1.4. Use of antibiotic resistance as a selectable marker

The development of recombinant bacterial vaccines usually requires the use of selectable markers, often conferring antibiotic resistance. For example, the integration of the SpaP/S1 fusion gene into the chromosome of *S. gordonii* by homologous recombination depends on the use of kanamycin resistance for the *in vitro* selection (Lee et al., 2002b). Bacterial delivery systems carrying plasmids also rely on antibiotic resistance markers as selection (Dunstan et al., 2003; Pilgrim et al., 2003). The use of antibiotic resistance markers in live bacterial vaccine carriers raises several safety concerns and, as a result, the use of this kind of vaccine is not recommended by regulatory agencies. The European pharmacopoeia 7.0 states that, “Unless otherwise

justified and authorized, antibiotic-resistance genes used as selectable genetic markers, particularly for clinically useful antibiotics, are not included in the vector construct. Other selection techniques for the recombinant plasmid are preferred" (European Pharmacopeia 7.0, 2011).

The main reason for avoiding the use of antibiotic resistance genes in the medical field is the risk of spreading antibiotic resistance genes to pathogenic organisms in the environment, possibly rendering them resistant to antibiotic treatment. In addition, horizontal gene transfer of the antibiotic resistance gene to the host's normal flora may lead to the spread of multidrug resistance microorganisms (Lemaitre et al., 1998; NARMS, 2001). A second reason is the possibility of allergies or other serious reactions to particular types of antibiotics. The US Food and Drug Administration recommends that, "penicillin and other β-lactam antibiotics be avoided during production, due to the risk of serious hypersensitivity reactions in the patients. If antibiotic selection is used during production, it is preferable not to use selection markers which confer resistance to antibiotics in significant clinical use, in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes" (US Food and Drug Administration, 1998). Thus, the use of plasmids carrying antibiotic-resistance genes for human use is not ideal and bacterial vaccine systems that do not contain antibiotic-resistance markers are needed. Finding a way to circumvent the need for an antibiotic resistance gene to protect plasmid stability is required.

1.5 Alternative strategies to antibiotic resistance genes

1.5.1 Toxin-antitoxin-based systems

Toxin-antitoxin-based systems, also called post-segregational killing systems, mediate plasmid stabilization via the killing of plasmid-free segregants. This system is made of two key components: a biologically active protein molecule (toxin) and the corresponding inhibitor (antitoxin). When these two components are contained on a plasmid, only the daughter cells having the plasmid will survive after cell division. After the loss of the plasmid, the unstable antitoxin is degraded and the stable toxic protein kills the new cell.

The well-characterized toxin-antitoxin module of *E. coli* plasmid R1 is the *hok-sok* system (Gerdes et al., 1997). The *hok* gene encodes for a lethal pore-forming Hok protein, and *sok* encodes a small antisense RNA that blocks Hok translation. Due to the high susceptibility of *sok* RNA to nuclease degradation, the absence of *sok* antitoxin RNA in bacteria results in the accumulation of stable *hok* mRNA, leading to sustained synthesis of Hok protein and cell death (Gerdes et al., 1997). This method presents several advantages, such as no involvement of chromosomal mutagenesis of the live bacterial vectors and the easy introduction of such a stabilized plasmid into any live vector strain. The post-segregational killing function of the *hok-sok* module revealed enhanced inheritance of plasmids in attenuated strains of *S. typhi* (Galen et al., 1999) and *S. flexineri* (Altboum et al., 2003); however, this system proved to be ineffective during

prolonged culture, as plasmid-free segregants that escaped the lethal effects of the toxin rapidly proliferated in the culture (Galen et al., 1999).

1.5.2. Operator-repressor titration

Operator-repressor titration systems can be used to control gene expression. In this situation, introducing an operator sequence upstream of a specific gene on the chromosome will allow the expression of that gene only in the presence of a multicopy plasmid containing the same operator sequence. One or several operators on the plasmid will facilitate the titration of the repressor, leading to expression of the chromosomal gene. In the absence of the plasmid, gene expression is repressed and the cell dies.

Operator-repressor titration (ORT) technology has been applied to the live attenuated vaccine *S. typhimurium* to express protective antigens against *Yersinia pestis* (Garmory et al., 2004) and *Bacillus anthracis* (Leckenby et al., 2009). To generate the ORT strain, the essential gene *dapD*, which encodes tetrahydrodipicolinate *N*-succinyl-transferase (catalyst for a step in the lysine/diaminopimolic acid pathway), was replaced with a cassette containing *dapD* under the control of the *lac* operator/promoter and *lacI* repressor. Diaminopimelic acid (DAP) is an essential component of the bacterial cell wall. When the LacI repressor binds to the chromosomal *lac* operator, cell growth is prevented unless an inducer is present. However, the LacI repressor protein can be titrated away from the *lacO* if a multi-copy plasmid encoding the *lacO* sequence was transformed into the ORT strain, leading to successful chromosomal gene expression, allowing cell growth and providing a mechanism for plasmid selection and maintenance.

Plasmid maintenance was investigated in ORT strains of *S. typhimurium* containing pORT plasmids, and the results showed that there was no plasmid loss over the 5 days of subculture (Garmory et al., 2004; Leckenby et al., 2009). Subsequently, plasmid maintenance was evaluated *in vivo* following oral immunization of mice with recombinant bacteria; bacteria were detected in the spleen and Peyer's patches of the mice, indicating that the plasmid was maintained (Garmory et al., 2004; Leckenby et al., 2009). Moreover, ORT *Salmonella* vaccine strains expressing antigens of *Y. pestis* or *B. anthracis* conferred excellent protection against bacterial pathogens in a mouse model (Garmory et al., 2004; Leckenby et al., 2009). These results indicate that utilization of ORT technology can provide an antibiotic-free plasmid maintenance system efficient for genetic stability in live bacterial vector vaccines.

1.5.3 Auxotrophic complementation of bacterial strains

Auxotrophic complementation employs an essential gene deleted from the bacterial chromosome to enforce an obligate requirement for the gene product. A plasmid vector possessing the wild type gene, called "balanced lethal vector", is used to complement the lethal effects of the chromosomal mutation. During cultivation, selection based on antibiotics is not necessary. Only plasmid-carrying cells can grow, so that the strain is totally dependent on the maintenance of the plasmid. To date, a number of balanced lethal vectors have been developed based on the complementation of genetic defects in biosynthetic pathways of cell wall, DNA precursor, and amino acids.

1.5.3.1. Plasmid maintenance system based on auxotrophies in cell wall synthesis

A balanced lethal host-vector system was designed in the early 1990s by Curtiss et al. (1990) for *Salmonella spp*., which involved auxotrophic complementation of *asd* mutants. The *asd* gene encodes for aspartate-β-semialdehyde dehydrogenase, which is involved in the biosynthesis of diaminopimelic acid (DAP) from aspartate. DAP is an essential constituent of the peptidoglycan layer of the cell wall of Gram-negative bacteria and *asd* mutants cannot grow in medium without supplementation of DAP. However, plasmids carrying the wild type allele of the *asd* gene allow the *asd* mutant to grow. Since DAP is not found in the animal host, essentially 100% of the surviving avirulent *Salmonella* recovered from an immunized animal host still contain the recombinant plasmid and express the foreign antigen (Curtiss et al., 1990). Asd-balanced lethal vectors carrying heterologous genes such as the pneumococcal surface protein A gene (PspA) (Kang et al., 2002) and the *Campylobacter jejuni A* gene (*CjaA*) (Wyszynska et al., 2004) have been evaluated in the *S. typhimurium asd* mutant and were shown to confer stable gene expression and elicit significant immune responses to both *Salmonella* and heterologous antigens. In addition, studies have shown that immunization with the attenuated *S. typhi asd* mutant strain encoding hepatitis B core-pre-S antigens on an Asd^+ plasmid vector is safe in humans (Nardelli-Haefliger et al., 1996; Tacket et al., 1997).

Alanine racemases, enzymes that are ubiquitous among bacteria and absent in humans (Strych et al., 2007), catalyze the reversible racemization of L- and D-alanine. D-alanine is an essential component in the biosynthesis of the bacterial peptidoglycan of

both Gram-positive and Gram-negative bacteria. To date, all investigated bacteria have been found to have either one or two distinct alanine racemase genes.

In *E. coli* and *L. monocytogenes*, two genes that control the synthesis of D-alanine, *alr/dadX* (for *E. coli*) and *dal/dat* (for *L. monocytogenes*), had to be inactivated in order to achieve complete D-alanine auxotrophy (Wild et al., 1985; Verch et al., 2004). Inactivation of the *alr* and *dal* genes in *S. pneumoniae* and *B. subtilis*, respectively, resulted in D-alanine auxotrophic phenotype (Strych et al., 2007; Xia et al., 2007).

D-alanine racemase-deficient mutant strains complemented by a gene located on a high copy plasmid have been applied to *B. subtilis* and *L. monocytogenes* to express different heterologous antigens, such as *Bacillus stearothermophilus* β-galactosidase reporter gene *bgaB* and human papillomavirus E7 fused to *Listeria* listeriolysin (LLO), respectively (Xia et al., 2007; Verch et al., 2004). Because D-alanine mutants cannot grow in growth medium without supplementation of D-alanine, this specificity was used as pressure for selection of recombinant strains. Both strains achieved good maintenance of the recombinant plasmids with a high expression level of BgaB reporter and LLO-E7 (Xia et al., 2007; Verch et al., 2004).

1.5.3.2. Plasmid maintenance system based on auxotrophies in DNA synthesis

Thymidylate synthase (*thyA*) is essential for DNA synthesis. It catalyzes the conversion of dUMP and 5, 10-methylenetetrahydrofolate to dTMP and 7, 8-dihydrofolate. A *thyA* mutant strain is virtually incapable of growing in media lacking thymidine or thymine and undergo so-called “thymidine-less death” (Ahmad et al., 1998).

A *thyA* balanced lethal plasmid system has been developed for *S. typhi* Ty21a for the heterologous expression of *Vibrio cholerae* O1 Inaba lipopolysaccharide O antigen (Tacket et al., 1990; Attridge et al., 1991) and *H. pylori* urease subunits A and B antigens (Bumann et al., 2002). This system confers plasmid stability in *S. typhi* Ty21a, and most of the Ty21a clones elicited significant immune responses against the heterologous antigens. Moreover, the vaccine was well tolerated in human volunteers and no significant adverse reactions were observed.

Auxotrophic complementation system has been applied to *Lactobacillus acidophilus* (Fu and Xu, 2000) and to a *V. cholerae* vaccine candidate (Liang et al., 2003). The cloned *thyA* gene on the plasmid of *L. acidophilus thyA* mutant was taken from *L. casei*, since the sequence of the *thyA* gene of *L. acidophilus* was not available. The *thyA* gene of *L. casei* complemented the mutation of *thyA* gene in the *L. acidophilus* strain, and no plasmid loss was observed after 40 generations.

The *purB* gene encodes for adenylosuccinate lyase (ASL), an enzyme that catalyzes an essential step in the pathway for *de novo* synthesis of adenosine monophosphate in the purine biosynthetic pathway. Attenuated *Salmonella spp.*, *S. typhi* and *S. typhimurium* deleted for *phoP/phoQ* virulence regulon were engineered to express immuno-reactive urease A and B subunits from a balanced lethal plasmid expression system based upon chromosomal deletion and plasmid complementation of the *purB* gene (DiPetrillo et al., 1999; Angelakopoulos and Hohmann, 2000). In order to enhance recovery of the deletion mutants (*phoP/phoQ/purB*), adenine was included in all growth media. Previously, evaluation of *phoP/phoQ*-deleted *S. typhi* demonstrated that this strain was safe and immunogenic in adult volunteers (Hohmann et al., 1995). This led to an

evaluation of *phoP/phoQ*-deleted *S. typhi* expressing urease in human volunteers. All volunteers had strong mucosal immune responses to the bacterial vector; however, there were no detectable humoral or mucosal responses to urease antigen (DiPetrillo et al., 1999). In contrast, a single oral dose of attenuated *S. typhimurium* expressing the *H. pylori* urease strain resulted in detectable immune responses to both vector and urease antigens (Angelakopoulos and Hohmann, 2000). These two studies, taken together, suggest that attenuated *S. typhimurium* is more effective than *S. typhi* in induction of immune responses against the urease A/B antigens.

In *S. typhimurium* and *S. typhi*, plasmid stability *in vivo* is important for the expression of foreign antigens that lead to induction of adequate immune responses. Western blot analysis of immuno-reactive urease proteins in attenuated S*almonella* vectors demonstrated that the two strains produced the same amount of urease (Angelakopoulos and Hohmann, 2000). However, the stabilities of the urease encoding plasmids within the carrier strains were markedly different *in vitro*. For both strains, more than 90% of the colonies retained the plasmid within 12 or 14 h of cultivation. At 16 h and later, the percentage of colonies having the urease plasmid declined rapidly to 20% in *S. typhi* culture, whereas more than 90% of *S. typhimurium* colonies retained the plasmid up to 36 h (Angelakopoulos and Hohmann, 2000). These findings provided an explanation for the observation that the *S. typhimurium* strain induced a *H. pylori*-specific immune response, whereas the *S. typhi* failed to do so.

1.5.3.3. Plasmid maintenance system based on auxotrophies in amino acid synthesis

Balanced lethal plasmid stabilization systems have been employed in a number of bacteria based on auxotrophies in genes involved in the biosynthesis of peptidoglycan and nucleic acid building blocks. Genes involved in amino acid and protein synthesis have also been targeted in this system.

Glutamate and glutamine serve as the primary nitrogen donors for all cellular metabolites in most enteric bacteria (Klose and Mekalanos, 1997). Glutamine synthetase (encoded by the *glnA* gene) is an enzyme responsible for the synthesis of glutamine and ammonia assimilation under conditions of low extracellular nitrogen concentrations. *S. typhimurium glnA* mutant is a glutamine auxotroph that cannot grow on media unless supplemented with glutamine (Klose et al., 1997).

Complementation of the chromosomal deletion of *glnA* with a plasmid expressing *glnA* was used as a balanced lethal system for *in vivo* expression of an antigen in *V. cholerae* vaccine strain (Peru2) (Ryan et al., 2000). A $glnA^{+}$ plasmid was maintained in *V. cholerae* Peru2Δ*glnA* in medium deficient of glutamine without the need for antibiotic selection pressure. Oral inoculation of germ-free mice with *V. cholerae* Peru2Δ*glnA* complemented with the *glnA*-encoding plasmid resulted in successful intestinal colonization in which *V. cholerae* Peru2Δ*glnA* was recoverable from the stool for 10 days. Despite complete auxotrophy in minimal medium, plasmid free *V. cholerae* was recovered on day 8 after inoculation, suggesting that glutamine is present in sufficient quantities in the intestinal lumen of mice (Ryan et al., 2000). However, this antigen

expression system induced prominent specific systemic and mucosal immune responses compared to those elicited by a vaccine strain expressing the same antigen from a single copy gene on chromosome (Ryan et al., 2000).

The live attenuated *Mycobacterium bovis* BCG has characteristics that make it one of the most attractive live vectors for multivalent vaccines. Auxotrophic complementation has been used as an effective selectable marker for the construction of potential recombinant BCG. Genes coding for the biosynthesis of leucine and lysine amino acids, *leuD* and *lysA*, were targeted in BCG strain to construct the auxotrophic complementation (Borsuk et al., 2007; Saubi et al., 2012). A BCG Δ*leuD* was obtained by gene knockout, and a plasmid encoded *leuD* gene was used as a selectable marker (Borsuk et al., 2007). BCG Δ*lysA* complemented with $lysA^+$ plasmid was used to express HIV-1 clade A immunogene (HIVA) in addition to the vector antigens (Saubi et al., 2012).

The BCG Δ*leuD* cultured on medium not supplemented with leucine maintained the vector for over 20 subcultures (Borsuk et al., 2007). In both mutant strains, BCG Δ*leuD* and BCG Δ*lysA*, the complemented plasmids were stable *in vivo* for more than 20 weeks after mouse immunization (Borsuk et al., 2007; Saubi et al., 2012). In addition, BCG Δ*lysA* complemented with $lysA^+$ plasmid expressing HIVA was well tolerated in newborn mice and induced HIV1 and *Mycobacterium tuberculosis* specific immune responses in both adult and newborn mice (Saubi et al., 2012).

Another plasmid stabilization system has been constructed by Hagg et al. (2004) for *E. coli* based on the auxotrophic complementation of the *infA* gene. The *infA* gene codes

for translation initiation factor 1 (IF1), a small intracellular protein essential for cell viability. The vector was stable for at least 120 generations of growth in the absence of antibiotics. Such a stabilization system can be used in attenuated live bacterial vaccine in order to avoid antibiotics and antibiotic resistance genes during vaccine development.

1.6. Objectives of study

S. gordonii is a promising live oral vaccine vector. The non-pathogenic nature of this bacterium and its ability to colonize the oral and nasal cavities make it an attractive candidate as a live bacterial vaccine vector. Since an antibiotic marker-free system has not been developed in *S. gordonii*, the purpose of this study was to construct an antibiotic resistance marker-free expression system in *S. gordonii*. To circumvent the use of antibiotics, which is often required for the recombinant plasmid stability, an essential gene was deleted from the chromosome and inserted on a plasmid. This auxotrophic complementation strategy enables the stable maintenance of plasmids and has been successfully implemented in a number of bacteria. This was accomplished by taking advantage of *S. gordonii*'s dependence for growth on the enzyme thymidylate synthase, which is encoded by the *thyA* gene and essential for DNA synthesis. A *S. gordonii thyA* mutant would be auxotroph and only able to grow in media supplemented with thymidine. When the *thyA* gene is placed on a plasmid and introduced into the mutants, the mutants are forced to maintain the plasmid. The objective of this work was to use the antibiotic marker-free system to test the efficiency of the vaccine antigen delivery in *S. gordonii.* The results obtained demonstrated that a system such as auxotrophic complementation is effective for the delivery of vaccine antigen in *S. gordonii* based on

thyA complementation. The results suggest that the system can be used *in vivo* and bring it a step closer to practical use in humans.

Chapter 2: Materials and Methods

2.1. Bacteria and growth conditions

Bacterial strains, characteristics, and sources are listed in Table 1.

2.1.1. *Streptococcus gordonii*

S. gordonii was cultured in tryptone yeast extract glucose (TYG) broth (1% tryptone, 0.25% yeast extract, 1% glucose and 1% K_2HPO_4, w/v), or Brain Heart Infusion broth (BD BBL Microbiology Systems) at 37°C and 5% CO_2 without agitation. *S. gordonii* DL1 was grown without antibiotics. *S. gordonii thyA::ermAM* mutant was grown in the presence of 20 µg/ml of thymidine (BioShop, Burlington, ON) and 10 µg/ml of erythromycin. *S. gordonii* Δ*thyA*Δ*erm* was cultured in the presence of 20 µg/ml of thymidine. *S. gordonii thyA::ermAM* carrying pDL276/*thyA* was cultured with 250 µg/ml kanamycin. The expression of the *cre* gene from pTV1-OKCre was induced by adding 10 ng/ml tetracycline to the growth media. Cultures at late exponential growth were harvested and stored frozen at -80˚C in 25% glycerol in 0.5 ml aliquots.

2.1.2. *Escherichia coli*

E. coli were cultured aerobically with agitation at 200 rpm and 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, w/v). *E. coli* Δ*thyA*Δ*dap* was grown in the presence of 25 µg/ml of thymidine and 100 µg/ml of 2, 6-diaminopimelic acid (Sigma-Aldrich, Oakville, ON, Canada).

Table 1. Bacterial strains used in this study.

Bacterial strain	Characteristics	Source
S. gordonii		
S. gordonii DL1	Parent strain	(LeBlanc & Hassell, 1976)
S. gordonii thyA::ermAM	*thyA* gene interrupted by *ermAM* cassette	This study
S. gordonii Δ*thyA*Δ*erm*	*ermAM* gene deleted from the mutant with a retention of *thyA* mutation	This study
S. gordonii Δ*thyA*Δ*erm* (pTV1-OKCre)	DL1 *thyA* mutant carrying pTV1-OKCre	This study
S. gordonii thyA::ermAM (pDL276/*thyA*)	DL1 *thyA* mutant complemented with pDL276/*thyA*	This study
E. coli		
E. coli XL1-blue	Alpha complementation	(Stratagene, LaJolla, CA,USA)
E. coli Δ*thyA*Δ*dap*	χ 2338 strain of *E. coli* has a mutation in *thyA57* and *dapD8*	*E. coli* Genetic stock center

All antibiotics were purchased from Sigma-Aldrich Chemical Co. (Oakville, Ontario, Canada). Stock concentrations of kanamycin and ampicillin were prepared in deionized water (dH_2O) (Millipore, Billerica, USA), filter sterilized with a 0.2 μm filter and then aliquoted into 1.5 ml tubes. Tetracycline was prepared in 95% ethanol and erythromycin was prepared in methanol. All antibiotics were stored at -20˚C.

For agar plates, both TYG and LB agars were prepared by adding 1.5 % (w/v) agar to the media prior to sterilization. Following sterilization, the agar was cooled in a 55˚C water bath for approximately 15 minutes. If necessary, antibiotics were added to the agar at this point. The agar was poured into sterile petri dishes, allowed to solidify at room temperature and then dried at 37˚C for 10 minutes.

2.2. Chromosomal DNA extraction

To extract the genomic DNA from *S. gordonii*, 0.5 ml of overnight bacterial culture was centrifuged (5 min, 10,000 x g, 4˚C) and the supernatant was discarded. Two hundred microliters each of TE buffer (10 mM Tris buffer, pH 8.0, and 1 mM EDTA) and chloroform, as well as 70 mg of 400 μm-glass beads, was added to the pelleted cells. The tube was vortexed vigorously for 1 minute and centrifuged for 5 minutes (10,000 x g, 4 ˚C). The resulting aqueous layer was collected into a new Eppendorf tube and the DNA was precipitated in 1 ml of cold 95% ethanol containing 2.5 % potassium acetate via incubation at -80˚C for 30 minutes. Following incubation, the samples were centrifuged

(10 min, 14,000 x g, 4 ˚C) and the pellet was washed with 70% ethanol. The DNA was vacuum-dried and then dissolved in 10 µL of dH_2O.

2.3. Agarose gel electrophoresis

The quality of the DNA was assessed by agarose gel electrophoresis, as described by Sugden *et al.* (1975). A gel consisting of 0.8 % agarose was prepared by adding 0.8 g of agarose into 100 ml of 1X TAE buffer (1M Tris base, 50 mM EDTA, and 0.057% glacial acetic acid). The solution was heated until the agarose dissolved, poured into a gel tray supplied with the electrophoresis tank, and allowed to solidify at room temperature. The gel was then placed in an electrophoresis tank filled with 1X TAE buffer containing 0.5 µg/ml of ethidium bromide. Ten microliters of sample was mixed with 2 µL of 6X loading buffer (0.25% bromophenol blue, 30% glycerol in water) and then loaded into the wells of the gel. Ten microliters of 1 kb DNA ladder (New England Biolabs, ON, Canada) was also loaded into a well. The gel was run at 115 volts for 40 minutes and then examined under UV light using the UVP BioDoc-It Imaging System (Upland, CA, USA).

2.4. Construction of *alr*-knockout gene

To make an *alr* gene inactivation construct, polymerase chain reaction (PCR) was used to amplify two 500 bp fragments (upstream and downstream) of the *alr* gene from the genomic DNA of *S. gordonii*. The forward and reverse primers SL797/SL798 (upstream fragment) and SL799/SL800 (downstream) were used in this reaction (Table

2). The erythromycin resistance cassette (*ermAM* gene, 800 bp) was also amplified from a synthetic construct template (Tremblay et al., 2009) using SL609/SL729 primers. The PCR reactions consisted of 100 µL PCR reaction mixtures containing 2 mM $MgCl_2$, 0.2 mM dNTPs, 1X PCR reaction buffer, 100 pmoles of each forward and reverse primers, 0.5 µL of *Taq* DNA polymerase and 1 µL of template DNA dissolved in dH_2O. The PCR program consisted of 30 cycles each of denaturing (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 30 s) followed by a final extension at 72°C for 2 minutes.

The PCR products were extracted twice with chloroform. To construct the *alr::ermAM* knockout, the upstream fragment of the *alr* gene and the 0.8 kb *ermAM* gene were first digested with EcoRV for 1 hour at 37˚C. The digested DNA was then electrophoresed on a 0.8% agarose gel. The DNA bands were recovered from the gel pieces by electro-elution at 100 V for 30 minutes and purified using a DNA affinity column, Elutip column-D (Schleicher & Schuell, Keene, NH). The DNA was precipitated by 95% ethanol, washed with 70% ethanol and ligated using 0.5 µL of T4 DNA ligase. The ligation was carried out overnight at room temperature.

The following day, 1 µL of the ligated DNA was subjected to PCR using SL797 and SL 729 primers under the PCR conditions described above. The 1.4 kb band of the *alr up-ermAM* PCR product was then extracted with chloroform, digested with BamHI for 1 hour at 37˚C and then ligated to the BamHI-cut downstream fragment of the *alr* gene. After ligation, 1 µL of the product was PCR amplified using SL841 and SL844 primers to obtain the 2 kb *alr::ermAM* construct.

Table 2. Primers used in this study. All primers were synthesized by Alpha DNA (Montréal, Québec). The underline sequences notify the restriction sites, while the bold sequences are *loxP*.

primer	Nucleotide sequence	Description
SL106	GTT TTC CCA GTC ACG AC	Reverse primer of a sequence on pDL276
SL609	TGA GAT ATC CCG GGC CCA AAA TTT GTT TGA T	Forward primer for *ermAM*, EcoRV
SL666	ACG CCA AGC TTG CAT GCC TGC	Forward primer of a sequence on pDL276
SL729	TAC GGA TCC AGC GAC TCA TAG AAT TAT TT	Reverse primer for *ermAM*, BamHI
SL797	CTA GTA TAC ATA GGC CAA G	Forward primer for up-fragment of *alr* gene
SL798	TGA GAT ATC **ATA ACT TTG TAT AGC ATA CAT TAT ACG AAG TTA T**GC AAA GTG AGT AAA GAT TC	Reverse primer for up-fragment of *alr* gene, **loxP** and EcoRV
SL799	TAC GGA TCC **ATA ACT TCG TAT AAT GTA TGC TAT AGC AAG TTA T**TC GTT TGG GAG ATA TTA TTT	Forward primer for down-fragment for *alr* gene, **loxP** and BamHI
SL800	TAC GAA TTC TTA ATT CTT GTA AAT TCT TGG	Reverse primer for down-fragment of *alr* gene, EcoRI

primer	Nucleotide sequence	Description
SL841	CAG GCA TGC GAA TAT TGG ATC AAT CCT T	Forward primer for up-fragment of *thyA* gene, SphI
SL844	CTC GGT ACC CCG AAA AAC TTC GGT AG	Reverse primer for down-fragment of *thyA* gene, KpnI
SL849	TAC GGT ACC ATA GGG GTT ATC GGG TGA	Forward primer for *cre* gene, KpnI
SL850	TAC GAA TTC CCG CGT TAA TGG CTA ATC	Reverse primer of *cre* gene, EcoRI
SL912	TGA GAT ATC **ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA** TAT TCC AGG CTA TTG GAC	Reverse primer for up-fragment of *thyA* gene, **loxP** and EcoRV
SL913	TAC GGA TCC **ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA** TGG AAG TTG GCG ATA CG	Forward primer for down-fragment of *thyA* gene, **loxP** and BamHI
SL985	ATC GCG GCC GCC AAG CTT GCA TGC CTG C	Forward primer for FHA/CR-1 gene, NotI
SL986	ATA CGA TCG CGT TAA GAA GCG TAG TCC GGA ACG TC	Reverse primer for FHA/CR-1 gene, PvuI

primer	Nucleotide sequence	Description
SL1016	GTT AAC AGA AGC AGT CTC	Reverse primer, 100 bp downstream the reverse primer of *thyA* gene
SL1017	ACT CAT CGA TAA TGG AGAA	Forward primer, 100 bp upstream the forward primer of *thyA* gene

To obtain an *alr*-null mutant of *S. gordonii*, 30 µL the *alr::ermAM* construct's PCR product was transformed into *S. gordonii* DL1 competent cells (see Section 2.6). Transformants were selected on BHI agar plates containing 50 µM D-alanine (Sigma) and 10 µg/ml erythromycin. The transformants were screened for the *S. gordonii alr* mutant by replica plating colonies onto two BHI agar plates containing 10µg/ml erythromycin, with or without D-alanine.

2.5. Construction of *thyA*-knockout gene

The strategy used for the construction of the *thyA*-knockout gene was similar to that used used for the construction of the *alr*-knockout. Two 550 bp fragments of the *thyA* gene were amplified from the chromosomal DNA of *S. gordonii* via PCR using the primers SL841/SL912 (upstream) and SL913/SL844 (downstream). The *ermAM* cassette was also amplified using SL609/SL729 primers. The three fragments, *up-ermAM-down*, were ligated together following two rounds of digestion and ligation in order to obtain the final construct of the *thyA::ermAM* gene. The upstream *thyA* fragment and *ermAM* gene were digested with EcoRV and then ligated overnight using T4 DNA ligase. The following day, 1 µl of the ligated product was PCR amplified using *Taq* polymerase with the primers SL841/SL729 to give 1.4 kb fragment. Ten microliters of chloroform-extracted PCR product was then digested with BamHI and ligated with the similarly restricted *thyA* downstream fragment. One microliter of the overnight ligation product was PCR amplified in a 100 µL PCR reaction mixture containing 1 mM $MgCl_2$, 0.2 mM

dNTPs, 1X PCR reaction buffer, 100 pmoles of each primer SL841/SL844, 0.5 µL of *Tag* DNA polymerase, and 1 µL of template DNA dissolved in dH_2O.

Thirty microliters of the PCR product was introduced into *S. gordonii* DL1 competent cells by natural transformation in order to construct the *S. gordonii thyA* mutant strain. The transformants were selected on TYG agar plates containing 20 µg/mL thymidine and 10 µg/mL erythromycin.

2.6. Natural transformation of *S. gordonii*

The protocol used for transformation of *S. gordonii* cells was developed by Perry and Kuramitsu (1981). To prepare *S. gordonii* competent cells, 4 mL of Brain Heart Infusion (BHI) broth containing 200 µL heat-inactivated calf serum (BHICS) were inoculated with 30 µl of *S. gordonii* frozen culture. For *S. gordonii thyA::ermAM* and *S. gordonii* Δ*thyA*Δ*ermAM* competent cells, 80 µl of thymidine (10 mg/ml) were added to the cultures. The cultures were grown at 37˚C in a CO_2 incubator without agitation for 16 hours. One hundred microliters of the overnight culture were used to inoculate 4 mL of pre-warmed BHICS and incubated at 37˚C for 90 minutes. Seven hundred and fifty microliters of culture were transferred to an Eppendorf tube and 30 µL of PCR product of the *alr*-knockout or *thyA*-knockout genes, or 10 µL of plasmids were added. The culture was incubated for 30 min at 37 ˚C and then incubated for another 90 minutes at 37˚C following the addition of 750 µL of pre-warmed BHICS. The culture was then centrifuged and 1 mL of supernatant was discarded. The remaining volume (about 500 µL) was used to re-suspend the pelleted cells, and 250 µL of the suspension were plated

onto each of two BHI or TYG agar plates containing the appropriate antibiotics or supplements. For the transformation of pDL276/*thyA*del*kan* into the *S. gordonii* Δ*thyA*Δ*ermAM*, the culture was diluted to 10^{-8} before plating onto the agar plates in order to obtain well isolated colonies.

2.7. Preparation of electro-competent *E. coli*

Electro-competent *E. coli* cells were prepared using a protocol established by Barbas et al. (2001). Twenty microliters of frozen culture of *E. coli* XL1-blue or *E. coli* Δ*thyA*Δ*dap* were added to 5 mL of super broth (SB) (3.5% tryptone; 2% yeast extract; 0.5% NaCl; 0.25% 2N NaOH) containing 10 µg/ml tetracycline for *E. coli* XL1-blue or 100 µg/ml 2, 6-diaminopimelic acid and 20 µg/ml thymidine for *E. coli* Δ*thyA*Δ*dap*, and incubated overnight at 37˚C in a shaker incubator at 200 rpm. Two and a half milliliters of overnight culture were added to each of two 500 ml of fresh SB broth in 2 Liter flasks. For each flask, 10 mL of 20% (w/v) glucose and 5 mL of 1 M $MgCl_2$ were added and incubated at 37˚C with shaking until OD_{600}= 0.7. The cultures were chilled on ice for 15 minutes and then centrifuged for 20 minutes (3000 x g, 4˚C). The supernatants were discarded, and pelleted cells were re-suspended in 25 mL of pre-chilled (on ice) 10% (v/v) glycerol. The two re-suspended pellets were combined in one pre-chilled 500 mL centrifuge bottle. Pre-chilled 10% (v/v) glycerol was added to the bottle up to about 500 mL and the contents were centrifuged for 20 minutes (3000 x g, 4˚C). The pelleted cells were re-suspended again in 500 mL of pre-chilled 10% glycerol and centrifuged for a third time, followed by the re-suspension in 25 mL of pre-chilled 10% glycerol. The

suspension was centrifuged for 15 minutes (2500 x g, 4˚C), and the supernatant was carefully poured off. The pellet were re-suspended with the remaining volume of glycerol (about 5 mL), divided into 300 µL aliquots and stored at -80˚C.

2.8. Electroporation of temperature sensitive plasmid

Electroporation of pTV1-OK was done using the protocol described by Barbas et al. (2001). In a chilled 1.5 mL Eppendorf tube, 50 µL of thawed electro-competent XL1-blue cells was added, mixed with 1 µL of plasmid (pTV1-OK), and transferred into a 0.2 cm electroporation cuvette. The cuvette was incubated for 1 minute on ice and electroporated at 2.5 kV and 25 µF capacitance for 3.9 milliseconds in a Gene Pulser II apparatus (BioRad Laboratories). Immediately after, 1 mL of SB broth was added to the cuvette and the cell suspension was transferred to a polypropylene tube containing 2 mL of SB broth and incubated for 3 hours at 30˚C. After 3 hours incubation, the culture was serially diluted and plated on LB agar plates containing 300 µg/ml erythromycin and 10 µg/ml kanamycin, and then incubated for 2 days at 30˚C.

E. coli transformants were screened for the presence of pTV1-OK by plasmid isolation (see Section 2.11). The plasmid was used to express the *cre* gene.

2.9. Preparation of $CaCl_2$ – induced competent *E. coli* cells

Competent *E. coli* cells were prepared using $CaCl_2$ induction as described by Sambrook et al. (1989). One milliliter of overnight culture of *E. coli* Δ*thyA*Δ*dap* was

added to 45 ml of LB broth containing 25 µg/ml thymidine and 100 µg/ml 2, 6-diaminopimelic acid, and incubated for 2-3 hours at 37˚C in a shake incubator until it reached an OD_{600} of 0.35. The cells were harvested by centrifugation at 10 000 x g for 10 minutes. The pelleted cells were washed with 50 mL of cold transformation buffer #1 (Tfm1) (10 mM Tris, 150 mM NaCl, pH 7.5) and re-centrifuged. After that, the cells were re-suspended in 50 mL of cold transformation buffer #2 (Tfm2) (50 mM $CaCl_2$), mixed gently, and kept on ice for 45 min. The suspension was then centrifuged and the cells re-suspended in 3 ml of Tfm2. Following re-suspension, 2 mL of 50% glycerol were added; the result was divided into 250 µL aliquots and stored at -80 ˚C.

2.10. Transformation of $CaCl_2$ – induced competent *E. coli*

E. coli cells were transformed using a procedure described by Sambrook et al. (1989). Following the addition of 200 µL of *E. coli* competent cells and 100 µL of cold transformation buffer 3 (Tfm3) (10 mM Tris, 50 mM $CaCl_2$, 10 mM $MgSO_4$, pH 7.5), the tube of ligated plasmid incubated on ice for 45 minutes. The cells were heat shocked at 37˚C for exactly 2 minutes and then incubated for 10 minutes at room temperature. Five hundred microliters of LB broth was added and incubated for 1 hour at 37˚C. The transformed cells were inoculated onto four LB agar plates containing the appropriate antibiotics. The plates were incubated overnight at 37˚C.

2.11. Plasmid DNA isolation from *E. coli* using alkali lysis

The plasmids were isolated from *E. coli* strains using alkali lysis, as described by Birnboim and Doly (1997). One thousand and five hundred microliters of bacterial cultures grown overnight in a shaker incubator were centrifuged (5 minutes, 10 000 x *g*, 4 ˚C) and the resulting pellets were re-suspended in 100 µl of GTE solution (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA). One hundred eighty six microliters of dH_2O with 1 µL of RNase (10 µg/µL) were added to the tube and vortexed. Fourteen microliters of lysis solution (10 µL 20% (w/v) SDS, 4 µL 10 M NaOH) were added and mixed gently followed by 5 minutes incubation at RT. After that, 150 µL of cold potassium acetate (KAc) solution (60% of 5 M KAc, 11.5% of glacial acetic acid, and 28.5 % of dH_2O) were added and mixed by inversion. The samples were then incubated on ice for 10 minutes and centrifuged for 5 minutes at 14,000 x g, and the resulting supernatant was transferred into a new eppendorf tube. The DNA was extracted twice with chloroform and centrifuged (5 min, 10 000 x *g*). The top layer was collected into a new tube and the DNA was precipitated by adding 1 ml of cold 95% ethanol containing 2.5% KAc. The samples were incubated at -80˚C for 30 minutes and centrifuged (10 min, 14,000 x *g*). The pellets were washed with 70% ethanol. The plasmid was vacuum-dried and dissolved in 20 µL of dH_2O with 0.1 µL of RNase.

2.12. Cloning methodology

Plasmid DNA, characteristics, and sources are listed in Table 3

2.12.1. Cloning of *cre* gene into pTV1-OK

To clone the *cre* gene into pTV1-OK, the 1.3 kb PCR product of the *cre* gene was PCR amplified from pLEW100Cre-del-tetO using *Taq* DNA polymerase and the primers SL849/SL850. The PCR product was extracted twice with chloroform and restricted with KpnI. A previously constructed plasmid, pTET6, was used to clone the *cre* gene behind the *xyl/tetO* promoter (Mallaley *et al.,* 2003). The KpnI restricted plasmid and *cre* gene were run on a 0.8% agarose gel and recovered from the gel by electro-elution. The DNA was purified with Elutip column-D (Schleicher & Schuell, Keene, NH, USA), ethanol-precipitated and washed. The DNA of the *cre* gene and pTET6 was ligated overnight at room temperature using T4 DNA ligase. Following ligation, 1 µL of ligated product was PCR amplified using the primers SL666/SL850 to obtain the resulting 2.2 kb of $P_{xyl/tetO}$ *cre* gene.

The PCR product and pTV1-OK were double digested with EcoRI and PstI for 1 hour at 37˚C. Following the digestion, the samples were run on a 0.8% agarose gel and recovered from the gel by electro-elution. The DNA was then purified and ligated together. The ligated product (pTV1-OKCre) was transformed into electro-competent *E. coli* XL1-blue cells by electroporation as described above.

Table 3. Plasmid DNA used in this study.

Plasmids	Characteristics	Source
pTV1-OK	Plasmid that has a temperature-sensitive replicon from pWVO1, the plasmid is 11 Kb, kan^R & erm^R	(Gutierrez *et al.,* 1996)
pLEW100Cre-del-tetO	pLEW100Cre with the *tet* operator deleted expressing Cre recombinase, pLEW100Cre backbone (6.6 kb), Amp^R	Addgene (non-profit plasmid repository)
pTET6	*xyl/tetO* promoter in pDL276	(Mallaley *et al*., 2003)
pTV1-OKCre	*Cre* gene from pLEW100Cre fused to *xyl/tetO* promoter from pTET6 and cloned into pTV1-OK, the plasmid is 5.7 kb and kan^R	This study
pDL276	*E. coli- Streptococcus* shuttle vector, the plasmid has a streptococcal replication origin and *E. coli* replication origin, kan^R (7 kb).	(Dunny *et al*., 1991)
pDL276*/thyA*	*thyA* gene cloned into pDL276, the plasmid is 8.2 kb and kan^R	This study

pDL276/*thyA*del*Kan*	Kanamycin resistance gene deleted from pDL276*/thyA*, the plasmid is 7 kb	This study
pSecFHA/CR-1 #12	*xyl/tetO* promoter, P1 signal sequence, *fha/cr-1* fusion gene in pDL276 backbone (10 kb), kan^R	(Lee *et al*., Unpublished)
pDL276/*thyA*del*Kan*FHA/CR-1	*fha/cr-1* from pSecFHA/CR-1 cloned to pDL276/*thyA*del*Kan,* pDL276 backbone (9.5 Kb)	This study

2.12.2. Cloning of *thyA* gene into pDL276

The *S. gordonii thyA* gene, along with 200 bp DNA containing the predicted promoter, was PCR amplified from the *S. gordonii* chromosomal DNA. The gene was amplified by using *Taq* DNA polymerase and primers SL841/SL844. The PCR reaction mixture (100 µL) contained 1 µL of chromosomal DNA, 100 pmoles each of SL841 and SL844 primers, 2 mM $MgCl_2$, and 0.5 µL *Taq* DNA polymerase (New England Biolabs, ON). The PCR program consisted of 30 cycles each of denaturing (94°C, 30 s), annealing (50°C, 30 s), extension (72°C, 30 s) and final extension at 72°C for 2 minutes. The PCR product was double restricted with SphI and KpnI for 1 hour at 37˚C and ligated into the SphI and KpnI sites on the *E. coli-Streptococcus* shuttle vector, pDL276. The ligated DNA was transformed into *E. coli* Δ*thyA*Δ*dap* competent cells. Transformants were selected on four LB agars containing 100 µg/ml of 2,6-diaminopimelic acid and 50 µg/ml of kanamycin.

2.12.2.1. Removal of kanamycin resistance gene from pDL276/*thyA*

To remove the kanamycin resistance gene from pDL276/*thyA*, 5 µL of pDL276/*thyA* was digested with BssHII (New England Bio-labs) and treated with 0.5 µL Klenow fragment (Invitrogen) and 0.5 µL of 10 mM dNTPs for 15 minutes at room temperature. The Klenow enzyme was heat inactivated at 75˚C for 20 minutes and the plasmid was then digested with ScaI (New England Bio-labs). The digested plasmid was

electrophoresed on a 0.8% agarose gel and visualized under UV light. The 1.5 kb drop off band of kanamycin from pDL276/*thyA* was seen under UV light. The 7 kb band of pDL276/*thyA*del*kan* was recovered from the gel by electro-elution and purified by Elutip column-D. The plasmid was self-ligated with T4 DNA ligase, incubated overnight at room temperature and transformed into *E. coli* Δ*thyA*Δ*dap* competent cells. The transformed cells were inoculated onto four LB agar plates containing 100 μg/ml 2,6-diaminopimelic acid and incubated overnight at 37˚C.

2.12.3. Cloning of *fha/cr-1* into pDL276/*thyA*del*kan*

To construct pDL276/*thyA*del*kan*FHA/CR-1, the 3-kb DNA fragment carrying the TetR repressor gene, the tetracycline-inducible *xyl/tetO* promoter, the ribosomal binding site and signal sequence of *spaP* originating from *Streptococcus mutans* with the DNA coding for the type 1 domain of filamentous hemagglutinin (FHA) and anti-CR1 scFv and the C-terminal histidine and hemagglutinin (HA) tags was amplified from the previously constructed plasmid, pSecFHA-CR1#12 (Lee et al. Unpublished). The 3 kb DNA fragment was amplified using Phusion DNA polymerase (Finnzymes, ON) and primers SL985/SL986. The PCR reaction mixture (50 μL) contained 0.25 μL of pSecFHA/CR-1 #12, 50 pmoles of each primer, and 0.5 μL of Phusion DNA polymerase. The PCR program consisted of 30 cycles of denaturing (98°C, 5 s), annealing (50°C, 10 s), extension (72°C, 45 s) and final extension at 72°C for 10 minutes. The PCR product was analyzed by electrophoresis on a 0.8% agarose gel for FHA/CR-1. Twenty microliters of the chloroform extracted PCR product was digested with PvuI for 2 hours

at 37˚C. The antibiotic marker-free pDL276/*thyA*del*kan* was first restricted with KpnI, blunt ended with Klenow, and then restricted with PvuI. The digested PCR product and plasmid were ligated together overnight at room temperature with T4 DNA ligase. The ligated product was then transformed into the electro-competent *E. coli* Δ*thyA*Δ*dap* cells by electroporation.

The next day, seventy isolated colonies of these cells were grown individually in LB broth containing 100 µg/ml 2, 6-diaminopimelic acid. Following incubation, the seventy cultures were divided into seven pools, each consisting of ten individual cultures. The plasmid DNA was extracted from each pool using an alkali lysis procedure (see Section 2.11) and then screened for presence of the correct plasmid via PCR amplification for *fha/cr-1* using the primer pair SL985/SL986. The individual cultures from the positive pool (i.e., the one with the correct plasmid) were grown in fresh LB broth containing 100 µg/ml 2, 6-diaminopimelic acid. The following day, the plasmid from each culture was isolated and restricted with EcoRI. The expected fragments from the restriction of pDL276/*thyA*del*kan*FHA/CR-1 were 9.1 kp and 400 bp.

2.13. SDS-PAGE

Protein production of FHA/CR-1 from bacterial cultures of *E.coli* Δ*thyA*Δ*dap* or *S. gordonii thyA::ermAM* following the transformation of pDL276/*thyA*del*kan*FHA/CR-1 was examined using sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis. One milliliter of overnight bacterial cultures was centrifuged (5 min, 10 000 x g) and the bacterial pellets were re-suspended in 50 µL of 1X sample buffer (25 % (w/v) β-mercaptoethanol, 25% (w/v) 0.5 M Tris-HCl buffer (pH 8.0), 0.347 M SDS, 50%

glycerol, and 3 mM bromophenol blue). The samples were boiled for 5 minutes and then centrifuged again for 5 min at 10 000 x g. Fifteen microliters of both prestained marker (New England Biolabs Ltd; Pickering, ON) and the supernatants were loaded onto the stacking gel and 7.5 % separating acrylamide gel. SDS-PAGE was conducted at 200 V for 30 minutes in a mini Trans-Blot cell (BioRad; Mississauga, ON) using a Power Pac 300 (BioRad Laboratories Inc).

2.14. Western Immunoblotting

Following electrophoresis, the gel was placed on a nitrocellulose membrane (BioRad Laboratories Inc) that was pre-wet with 1X Transfer buffer (0.025 M Tris base, 20% methanol, 0.192 M glycine) and then sandwiched between two pieces of filter paper. The proteins were transferred at 200 mA for 1 hour and then the nitrocellulose membrane was blocked with 1% (w/v) gelatin in phosphate-buffered saline with tween (PBST) (8.7 mM (w/v) Na_2HPO_4, 1.5 mM (w/v) $NaH_2PO_4 \cdot H_2O$, 145 mM (w/v) NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 3.07 mM NaN_3, and 0.1% (v/v) Tween 20). The blocking solution was discarded and the membrane incubated overnight at 4˚C with the mouse anti- FHA monoclonal antibody 5E at a concentration of 1:4000 in PBST. The immunoblots were washed four times with PBST and then incubated for 1 hour at room temperature (RT) with the goat anti-mouse IgG-alkaline phosphatase conjugates (Sigma); 1:8000 in PBST. The blots were then washed four times with PBST and developed in 10 ml of alkaline phosphatase buffer (0.01 mM $MgCl_2 \cdot 6H_2O$, 0.1 M NaCl, and 0.2 M Tris-HCl, pH 9.8) with 33 µl of 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (50% BCIP (w/v) in dH_2O)

and 66 μl of nitroblue tetrazolium (NBT) (50% NBT (w/v), 70% dimethyl-formamide). The immunoblot were covered with aluminum foil and developed at RT on a rocker until the desired color intensity was obtained.

Chapter 3. Results

3.1. Attempts at constructing *alr*-null mutant of *Streptococcus gordonii*

3.1.1. Construction of *alr*-knockout gene

I attempted to create a mutant of *S. gordonii* defective in the alanine racemase gene (*alr*). Once constructed, that would be followed by the complementation of the mutant by cloning the *alr* gene into a plasmid. The proposed strategy to create a *S. gordonii alr*-null mutant is outlined in Figure 1**.** For the construction of the *alr*-knockout gene, an erythromycin resistance gene was used as a selectable marker in generating the deletion mutant. Two 500 bp fragments of the *alr* gene, upstream and downstream, were each amplified from the genome of *S. gordonii* using PCR. The 0.8 kb erythromycin resistance gene (*ermAM*) was amplified from a synthetic construct template (Tremblay et al., 2009) (Figure 2A). The *ermAM* gene was ligated to the two *alr* fragments facilitated by the restriction sites (EcoRV and BamHI) engineered in the primers to construct the 1.8 kb *alr::ermAM* gene. The ligated product was amplified by PCR using the primers SL797, which binds to the upstream fragment of the *alr* gene, and SL800, which binds to the downstream fragment (Figure 2B). The PCR product of the *alr*-knockout gene was then introduced into *S. gordonii* DL1 by natural transformation to obtain the chromosomal mutation of the *alr* gene via homologous recombination.

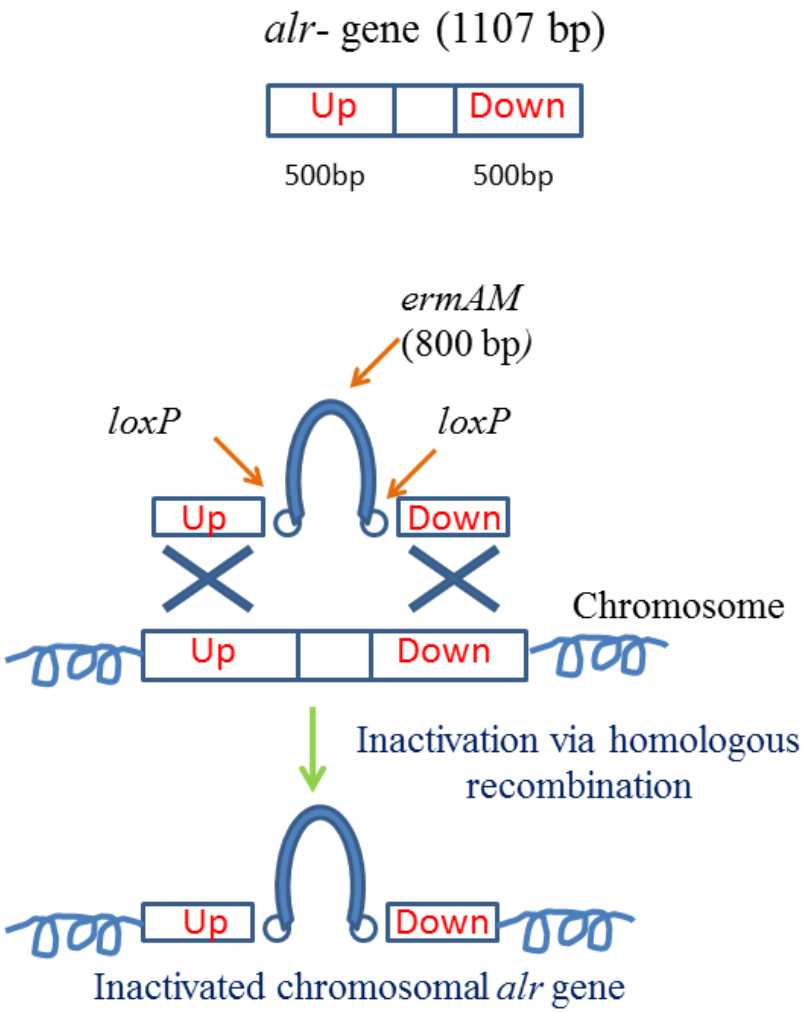


Figure 1. Strategy for the construction of *alr*-null mutant of *S. gordonii* by double cross-over homologous recombination.

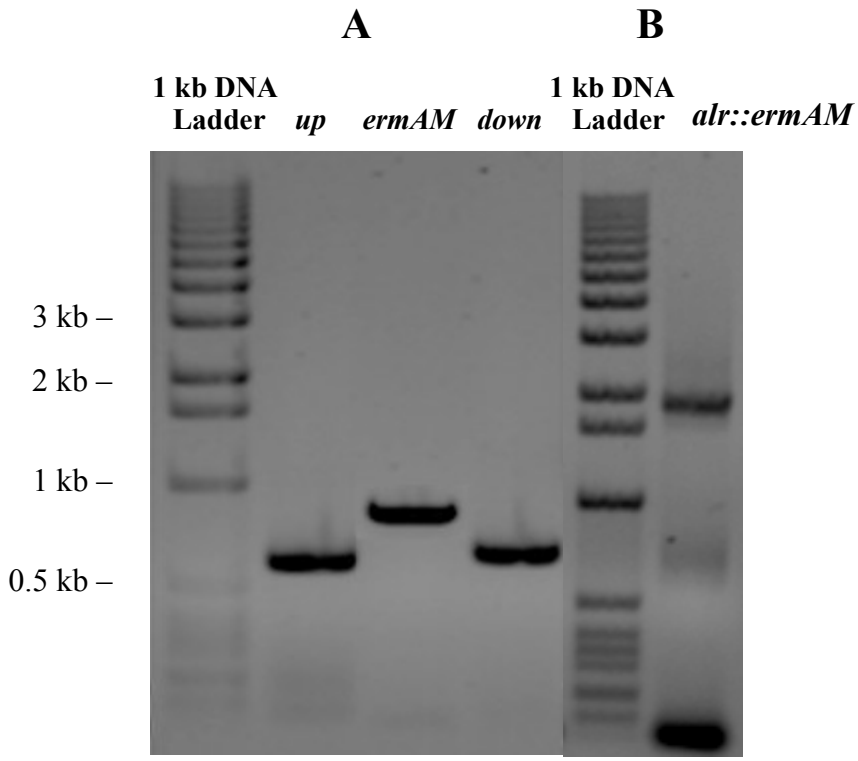


Figure 2. Agarose gel electrophoresis of the PCR amplification of the alanine racemase gene (*alr*) fragments for an attempt to construct an inactive *alr* gene. (A) The upstream (*up*) and downstream (*down*) portions of the *alr* gene were amplified by PCR from *S. gordonii* genomic DNA, in addition to an erythromycin resistance cassette (*ermAM*). (B) The fragments were ligated together and amplified by PCR with the primers SL797/SL800 to generate a 1.8 kb construct that was used to transform *S. gordonii*.

3.1.2. *alr*-null mutant of *S. gordonii* could not be obtained

Transformants were expected to carry the erythromycin resistance gene and to be auxotrophic for D-alanine. The transformants were resistant to erythromycin but not auxotrophic for D-alanine when replica-plated on BHI agar containing 10μg/ml of erythromycin (data not shown). All thirty-three of the transformants analyzed by PCR still carried the intact *alr* gene (Figure 3). The results indicate not only that the transformants maintained a functional *alr* gene but also that the *ermAM* gene integrated elsewhere on the chromosome. This deduction was consistent with the phenotypic observation that all the transformants grew in media without D-alanine (data not shown).

3.2. Construction of *thyA*-null mutant of *S. gordonii*

3.2.1. Construction of *thyA*-knockout gene

From the above results, I determined that obtaining an *alr* mutant of *S. gordonii* would be difficult. Thus, I selected another gene, *thyA,* to target. The *thyA* gene encodes thymidylate synthase, which is an essential gene for the synthesis of DNA precursors. *S. gordonii thyA* mutants would be auxotrophic for thymidine. I applied the same strategy used to generate the *alr* gene knockout for constructing the *thyA* mutation (Figure 4). As the objective of the project was to establish an antibiotic marker-free system for *S. gordonii*, and the *ermAM* cassette was used to select for *thyA* mutants, the strategy was to delete the erythromycin marker subsequently via the *cre/loxP* system. The primers designed for the

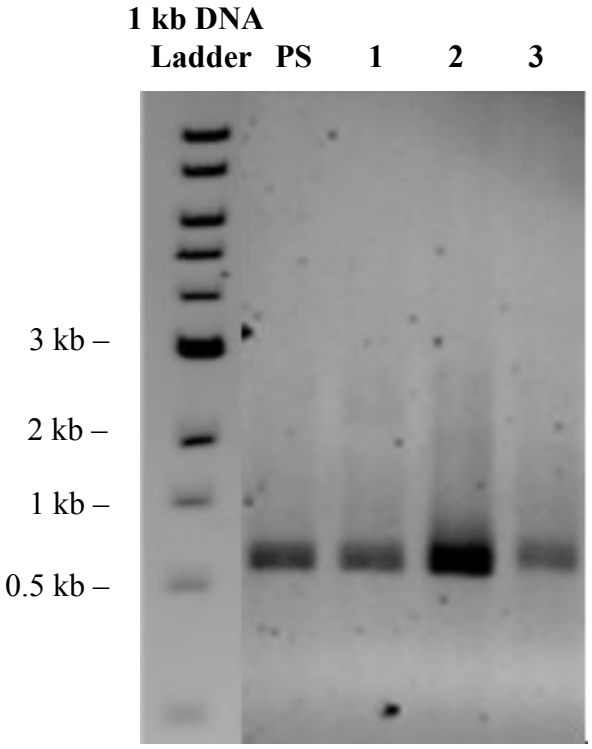


Figure 3. Erythromycin-resistant transformants maintain an intact *alr* gene. PCR analysis of the *alr* gene from the parent strain (PS) and representative erythromycin resistant mutants (Lanes 1-3). Genomic DNA prepared from *S. gordonii* cultures were used as a template with the primer part SL797/SL800.

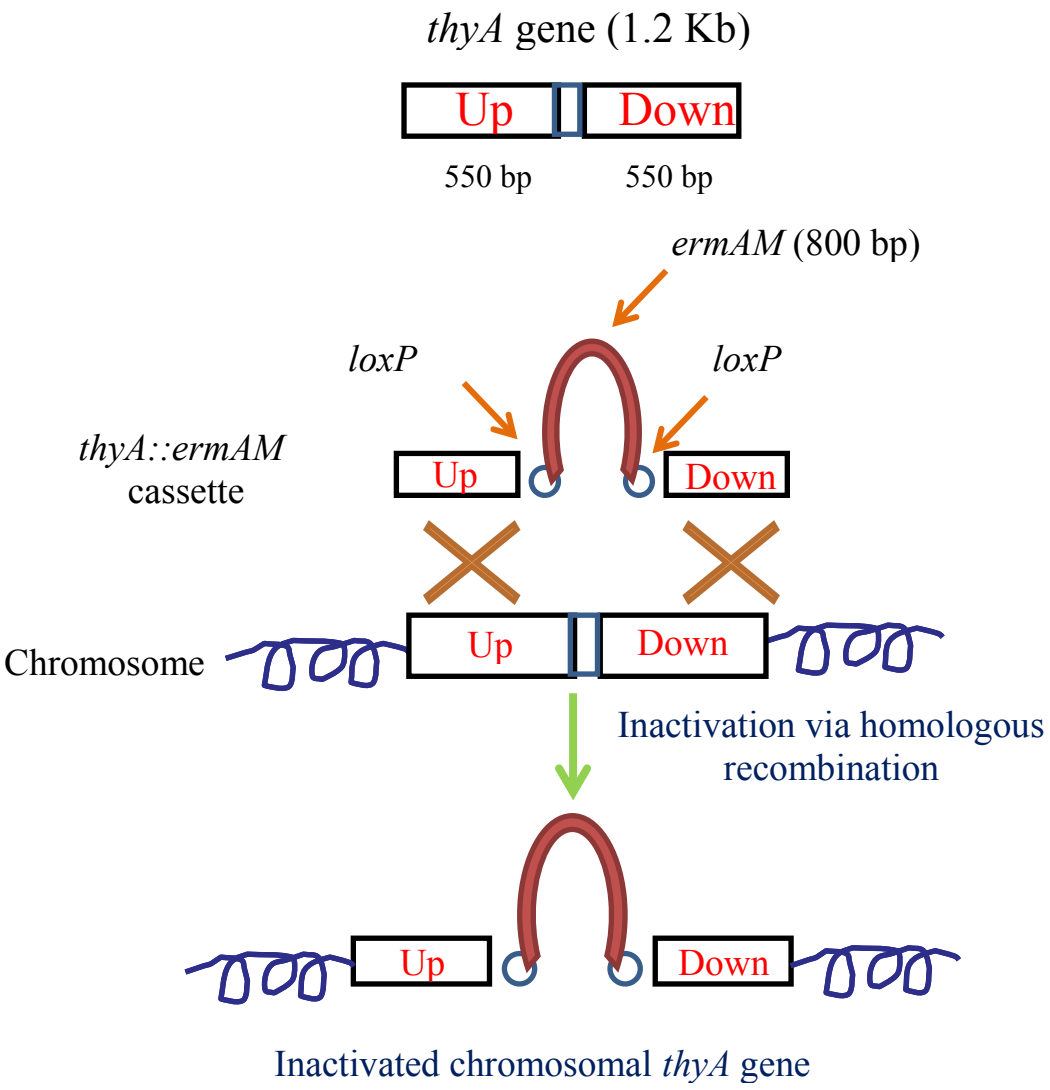


Figure 4. Strategy for the construction of *thyA* mutant by double cross-over homologous recombination.

amplification of the two fragments of *thyA* gene carry *loxP* sequences. The two fragments of the *thyA* gene (550 bp each) were each amplified from the genome of *S. gordonii* via PCR. The 800 bp *ermAM* gene was also amplified and ligated with the two fragments of *thyA* to obtain the 2 kb *thyA::ermAM* construct which was successfully amplified by PCR (Figure 5).

3.2.2. Construction of *S. gordonii thyA* mutant

Following transformation, the chromosomal *S. gordonii thyA* gene was replaced by the *thyA::ermAM* construct via homologous recombination. Inactivation of the *thyA* gene in *S. gordonii* was confirmed phenotypically by replica plating the mutant on TYG agar plates with and without 20 µg/ml thymidine. The *S. gordonii thyA::ermAM* mutant is a thymidine auxotroph and only grows on media supplemented with thymidine, in contrast to the parent strain which has the functional *thyA* gene (Figure 6A). As well, the mutant is resistant to erythromycin due to the insertion of *ermAM* in the *thyA* gene of *S. gordonii*. Similarly, the optical density of the overnight liquid culture of the mutant in TYG broth with and without thymidine was measured at an OD_{600} and indicated that the mutant is a thymidine auxotroph (Figure 6B). Mutation of the *thyA* gene was also confirmed genetically via PCR. The result of the PCR amplification showed that the *thyA* gene in the mutant was larger in size (2 kb) compared to the intact *thyA* gene from the parent strain (1.2 kb) (Figure 7A). The increased size (aproximatly 0.8 kb) of *thyA* from the mutant strain was due to the insertion of *ermAM* cassette, which confirmed the mutation of the *thyA* gene. Based on these results, *S. gordonii thyA* mutant is a thymidine auxotroph.

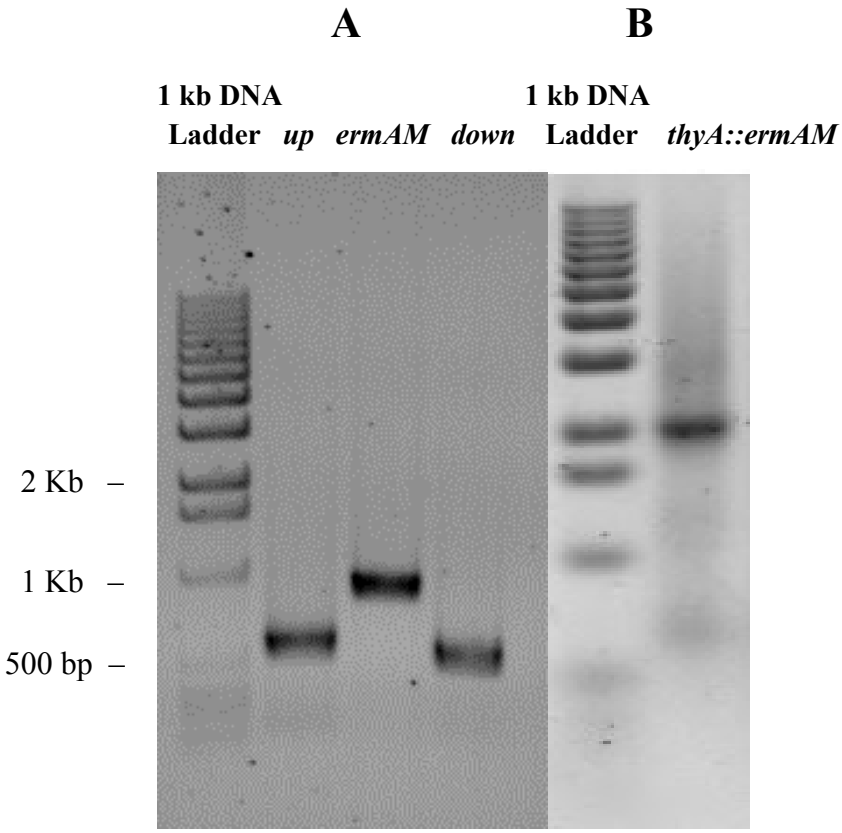


Figure 5. Agarose gel electrophoresis of the PCR amplifications of the thymidylate synthase gene (*thyA*) fragments for construction of an inactive *thyA* gene. (A) The upstream (*up*) and downstream (*down*) portions of the *thyA* gene were PCR amplified from *S. gordonii* genomic DNA, in addition to an erythromycin resistance cassette (*ermAM*). (B) The fragments were ligated together and amplified by PCR with the primers SL841/SL844 to generate a 2 kb construct that was used to transform *S. gordonii*.

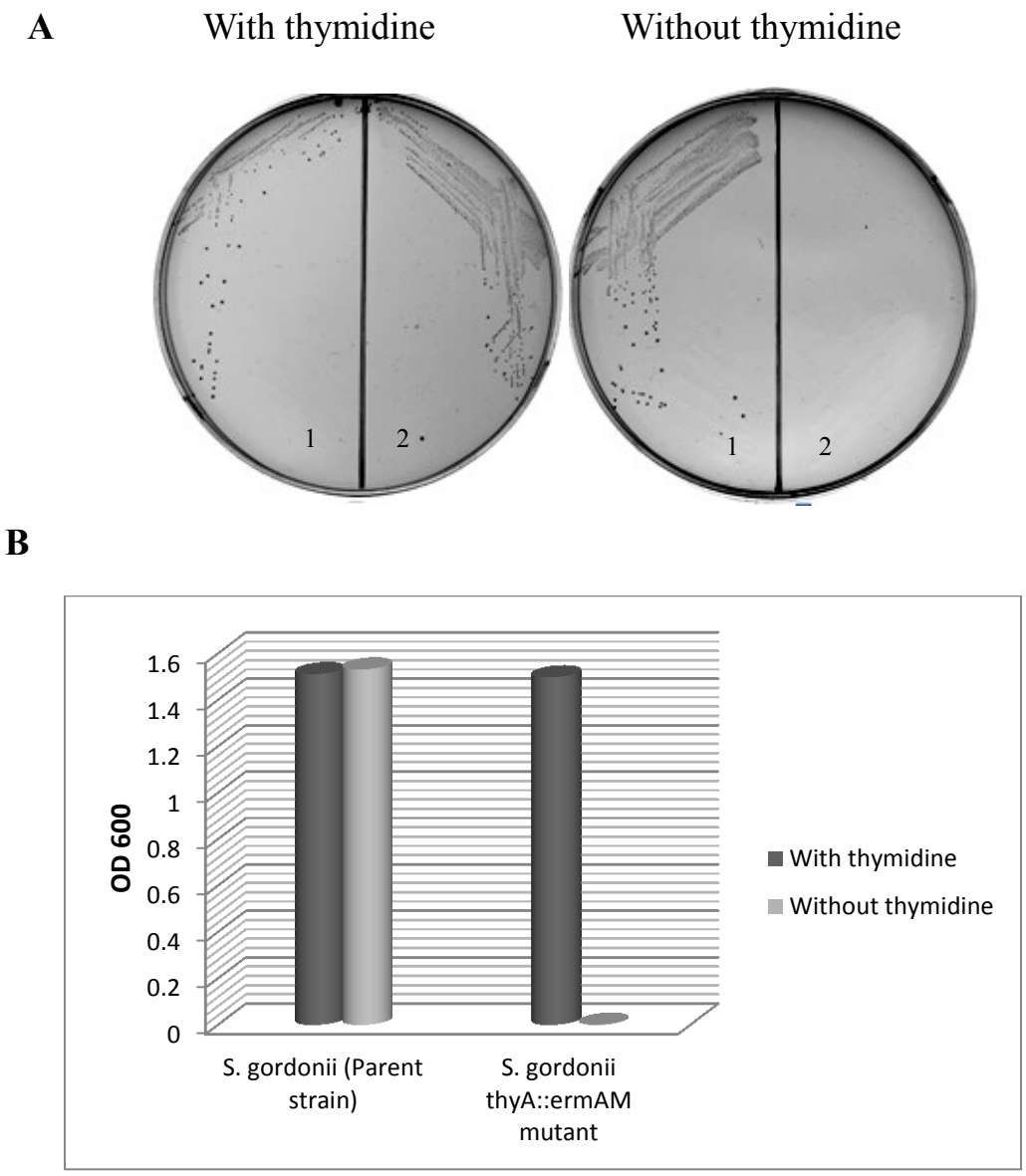


Figure 6. The *S. gordonii thyA::ermAM* mutant is auxotrophic for thymidine. (A) The parent strain (1) and *thyA::ermAM* mutant (2) were grown on TYG agar plates with or without the addition of 20 µg/ml thymidine. (B) Optical density of overnight cultures grown in TYG broth with or without thymidine.

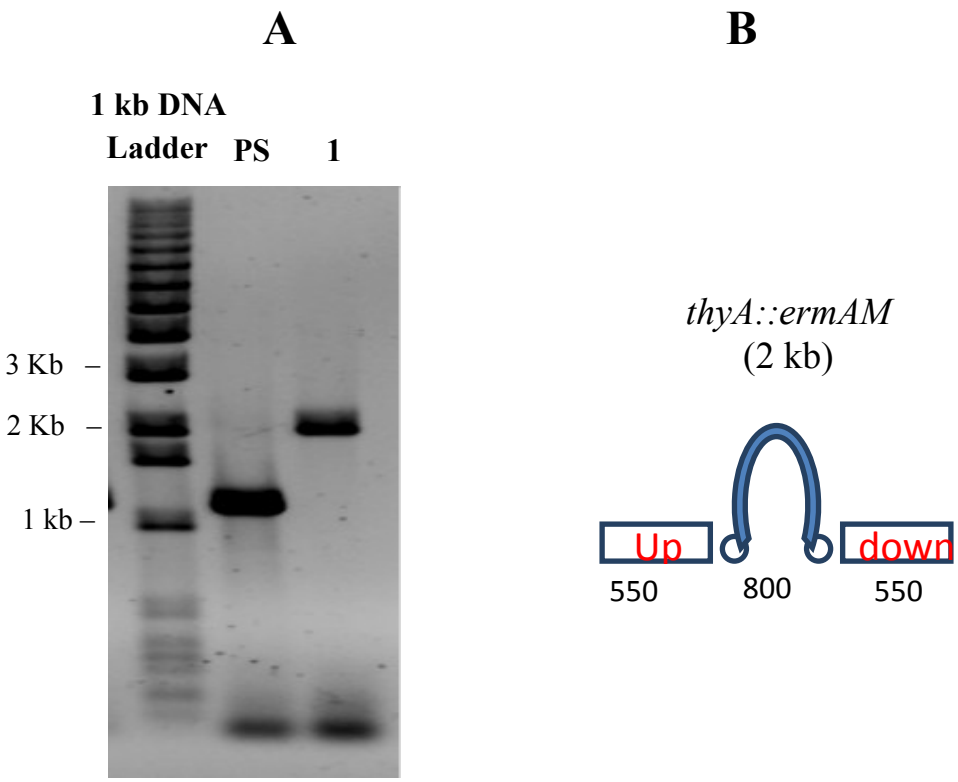


Figure 7. Genetic confirmation of the inactivation of the *thyA* gene in *S. gordonii thyA::ermAM* mutant. (A) PCR amplification of the *thyA* gene from *S. gordonii* parent strain (PS) and the *thyA::ermAM* mutant (lane 1). (B) Diagram depicting the *thyA::ermAM* construct.

3.3. Cloning and complementation of the *thyA*

The *S. gordonii thyA* mutant was successfully constructed and confirmed by the auxotrophic phenotype to thymidine. Maintaining the growth of the mutant depended on exogenous supplementation of thymidine to the media. The mutant would be expected to grow without any supplementation if complemented with a plasmid carrying the intact *thyA* gene.

For the construction of an auxotrophic complementation vector, the intact *thyA* gene with promoter was PCR amplified from the genomic DNA of the *S. gordonii* using the primer pair SL841/SL844 and then cloned onto pDL276, an *E. coli-Streptococcus* shuttle vector. The *thyA* gene was successfully PCR amplified and the 1.2 kb gene was cloned onto pDL276 to produce the pDL276/*thyA* plasmid (Figure 8), which gave a 1.2 kb fragment when restricted with SphI and KpnI (Figure 9). The plasmid, pDL276/*thyA*, was transformed into the *E.coli* Δ*thyA*Δ*dap* mutant strain χ2338 and the resulting transformant was selected on kanamycin-containing LB agar plates and analyzed for its ability to complement *thyA* mutation in *E. coli* Δ*thyA*Δ*dap.* The transformants were able to grow in LB broth containing 100 µg/ml of 2,6-diaminopimelic acid and without adding exogenous source of thymidine. 2,6-diaminopimelic acid was needed in *E. coli* Δ*thyA* Δ*dap* cultures because of the defect in the *dap* gene. The above result demonstrated that the cloned *thyA* gene in pDL276/*thyA* is functional (Figure 10).

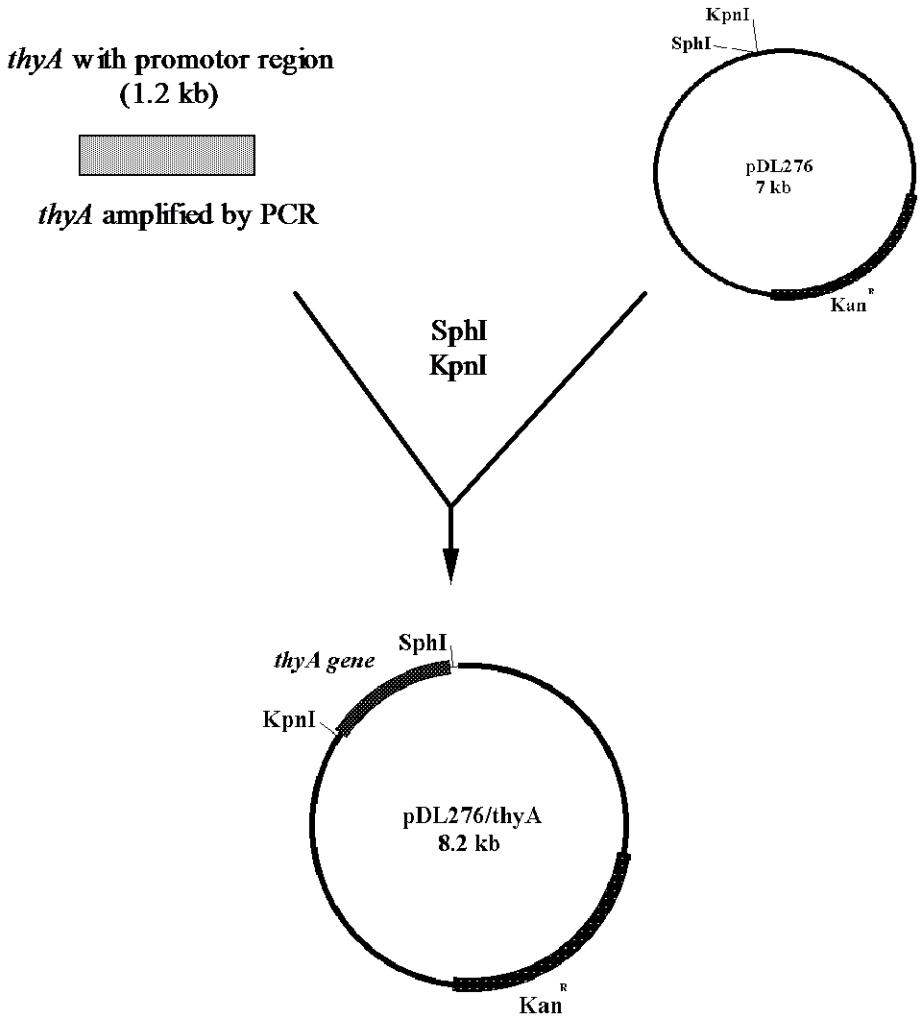


Figure 8. Diagram depicting construction of the pDL276/*thyA* plasmid.

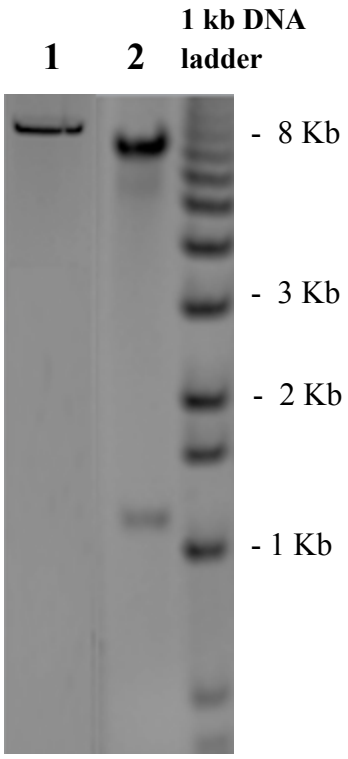


Figure 9. Restriction analysis of pDL276/*thyA*. The single cut plasmid with KpnI (lane 1) shows a band size at 8 kb, while the double cut plasmid with SphI and KpnI (lane 2) shows a drop out of 1.2 kb *thyA*.

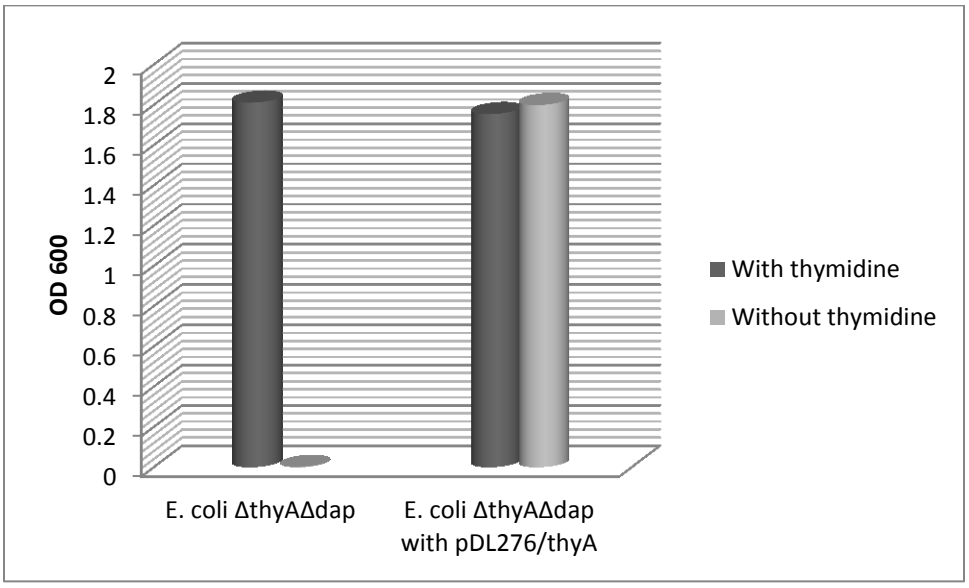


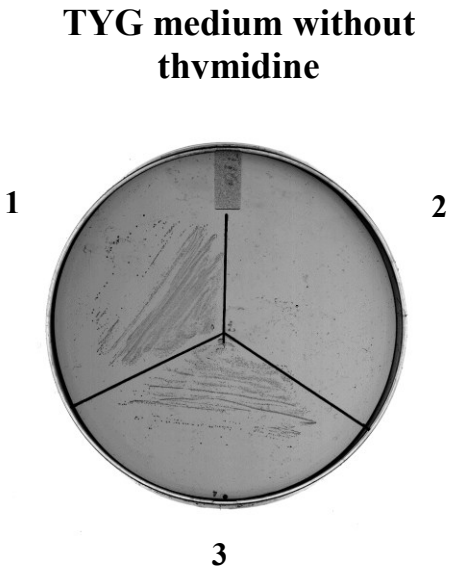
Figure 10. Growth of the *E. coli* Δ*thyA*Δ*dap* mutant complemented with pDL276/*thyA*. Bacteria were grown in LB media with or without addition of 20 µg/ml of thymidine.

The ability of pDL276/*thyA* to complement the mutation in *S. gordonii* was then tested. pDL276/*thyA* was transformed into the *S. gordonii thyA::ermAM* mutant. The resulting transformants were selected on TYG agar plates containing 250 µg/ml of kanamycin and then replica plated on TYG agar plates with or without 20 µg/ml of thymidine. The complemented mutant was able to grow on both agars, confirming the functionality of the cloned *thyA* gene. The ability of the complemented mutant to grow on TYG agar plates, similar to the parent strain and unlike the *S. gordonii thyA::ermAM* mutant, is shown in Figure 11A. The liquid culture of the three different strains of *S. gordonii* DL1 (the parent, the *S. gordonii thyA::ermAM* mutant and the complemented mutant in both TYG broths with and without thymidine) were measured at OD_{600}. The results confirmed the phenotypic conversion of *S. gordonii thyA::ermAM* mutant from a thymidine auxotroph to a thymidine prototroph following complementation with pDL276/*thyA* (Figure 11B).

3.4. Removing the *ermAM* gene from *S. gordonii thyA::ermAM* mutant by *cre/loxP* system

The Cre protein is a site-specific DNA recombinase that originates from the bacteriophage P1 and catalyses the recombination of the DNA between specific sites, called *loxP* sites, in a DNA molecule. It mediates the excision or inversion of specific sequences between two *loxP* sites depending on their relative orientation to each other (Langer et al. 2002). In our case, *loxP* sites were constructed to be in parallel or the same

A



B

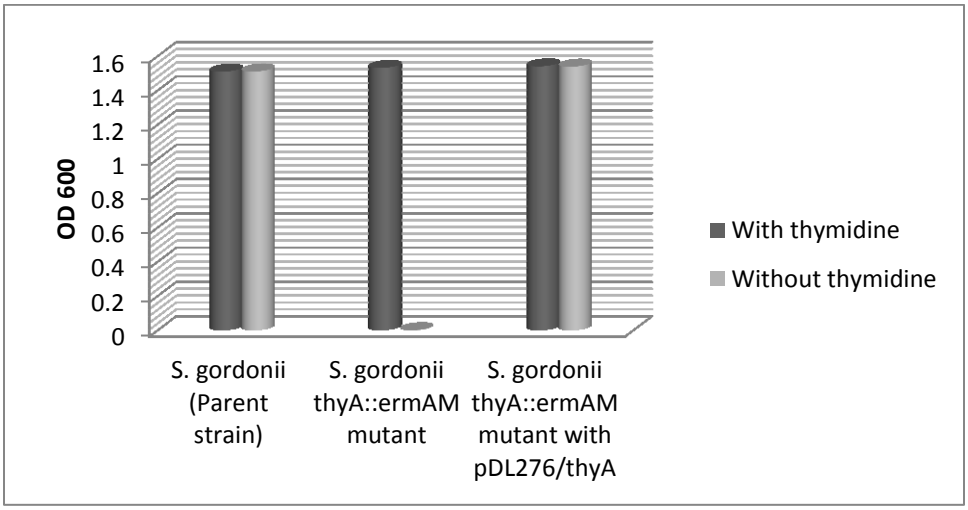


Figure 11. Complementation of *S. gordonii thyA::ermAM* mutant with pDL276/*thyA*. (A) The *S. gordonii* parent strain (1), *thyA::ermAM* mutant (2), and complemented *thyA::ermAM* mutant (3) were grown on TYG agar without exogenous thymidine. (B) Overnight liquid culture of the three *S. gordonii* strains measured at OD_{600}.

direction in order to achieve the deletion of *ermAM* gene. To facilitate the deletion of *ermAM* cassette from the *S. gordonii thyA::ermAM* mutant by the *cre/loxP* system, the *cre* gene was cloned onto a plasmid and then transformed into the *S. gordonii thyA::ermAM* mutant. The strategy used for the deletion of an *ermAM* cassette is outlined in Figure 12.

3.4.1. Construction of pTV1-OKCre and the expression of Cre

Primers were designed to amplify the *cre* gene from the plasmid pLEW100cre-del-tetO and cloned into pTV1-OK, a temperature-sensitive plasmid. The 1.3 kb gene of *cre* was successfully amplified and ligated to the tetracycline-inducible promoter *xyl/tetO* on pTET6 using the KpnI site. The PCR product of $P_{xyl/tetO}$ *cre* DNA (2.2 kb) was then cloned into the EcoRI and PstI sites on pTV1-OK to yield pTV1-OKCre. The resulting plasmid retained the temperature-sensitive origin of replication and kanamycin resistance marker but the transposon *Tn917* was removed (Figure 13). A 2.2 kb fragment was obtained when the plasmid was restricted with EcoRI and PstI, indicating the successful cloning of $P_{xyl/tetO}$ *cre* (Figure 14). The pTV1-OKCre was transformed into the *S. gordonii thyA::ermAM* mutant by natural transformation. The culture was incubated for 1 hour at 30˚C and then incubated for another 3 hours at 30˚C following the addition of pre-warmed BHICS. Transformants were grown at 30˚C and selected on TYG agar plates containing thymidine and kanamycin. The transformants were verified to carry pTV1-OKCre by PCR amplification of the *cre* gene (Figure 15). The excision of the *loxP-ermAM-loxP* gene from the *S. gordonii thyA::ermAM* mutant was facilitated by the action

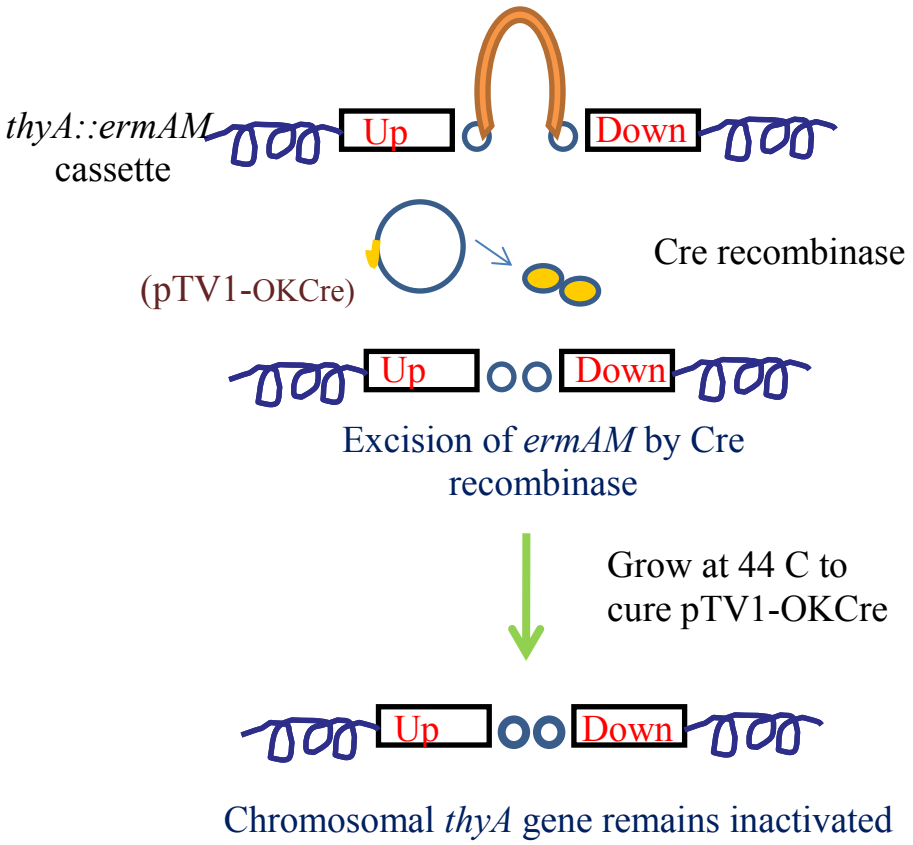


Figure 12. Strategy for the deletion of *ermAM* cassette using the *cre/loxP* system.

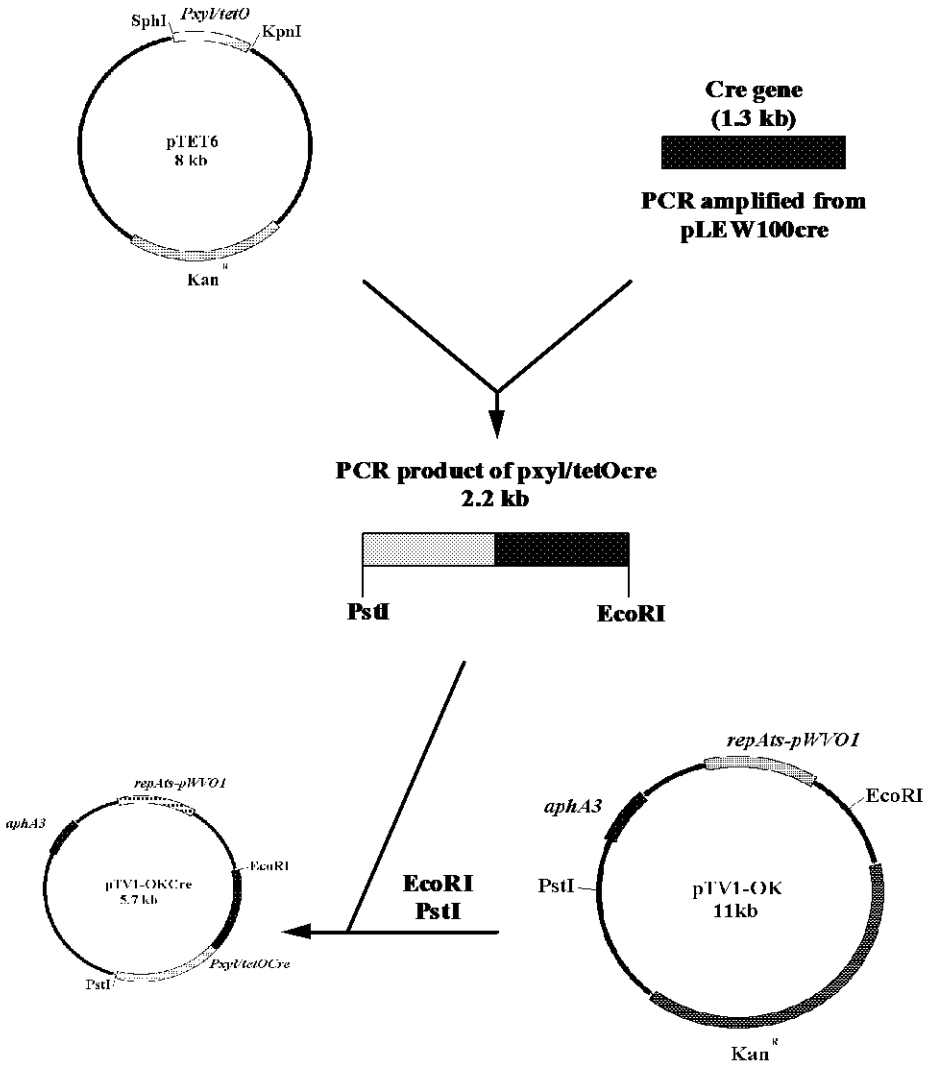


Figure 13. Diagram describing the construction of pTV1-OKCre. *aphA3,* the kanamycin resistance marker; *repAts*-pWVOI, the temperature-sensitive replicon from pWVO1.

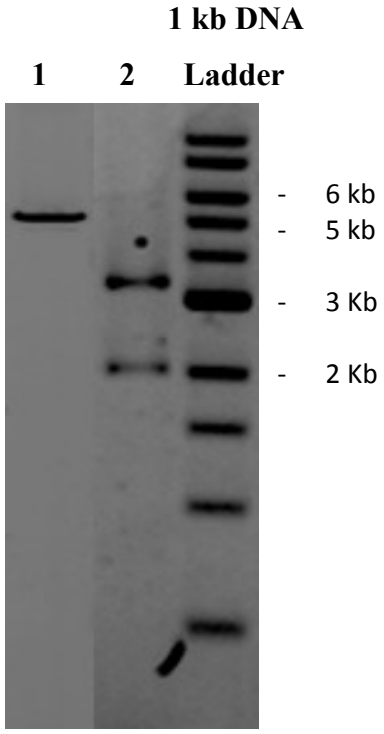


Figure 14. Restriction analysis of the pTV1-OKCre plasmid. The single cut plasmid with EcoRI represents 5.7 kb on the gel (Lane 1), while the drop out of 2.2 kb appeared after the plasmid was double cut with EcoRI and PstI leaving 3.5 kb of the plasmid backbone (Lane 2).

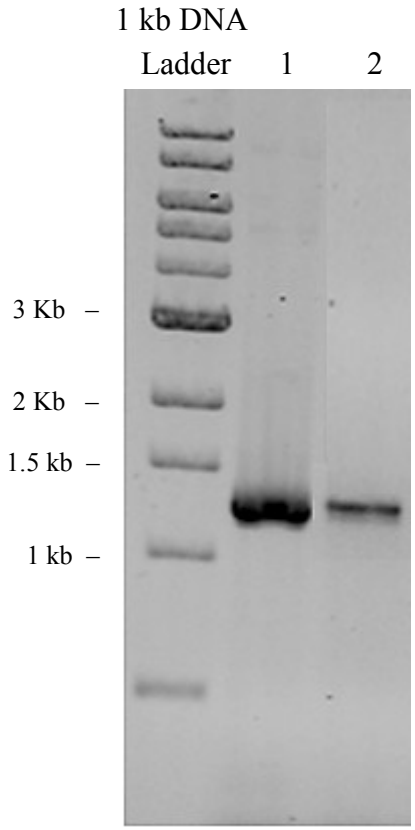


Figure 15. Gel electrophoresis of the *cre* gene following PCR amplification. The *cre* gene was PCR amplified from pTV1-OKCre (Lane 1) and from *S. gordonii thyA::ermAM* carrying pTV1-OKCre (Lane 2) with primers SL849/SL850.

of Cre recombinase. Induction of Cre recombinase was achieved by growing *S. gordonii thyA::ermAM* (pTV1-OKCre) at 30˚C in the presence of a sub-inhibitory concentration of tetracycline (10 ng/ml). The next day, the culture was streaked on TYG agar plates containing thymidine, kanamycin and tetracycline. Approximately 300 colonies were replica-plated onto TYG agar plates with or without erythromycin. After overnight growth at 30˚C, 2 out of the 300 colonies were erythromycin-sensitive, indicating the loss of the *ermAM* gene. The erythromycin-sensitive phenotype of the *S. gordonii* Δ*thyA*Δ*ermAM* mutant was evident on the plate cultures (Figure 16A). Liquid cultures confirmed that the mutant was sensitive to erythromycin, in addition to the retention of the thymidine auxotrophic phenotype (Figure 16B). Deletion of the *ermAM* gene from the antibiotic marker-free mutant was further confirmed genetically by PCR amplification of the *thyA* and *ermAM* genes. The product of the *thyA* gene from the antibiotic marker-free mutant was 1.2 kb, which is smaller than the product (2 kb) from *S. gordonii thyA::ermAM* (Figure 17). In addition, no PCR product was obtained for *ermAM* amplification from the antibiotic marker-free mutant compared to the *S. gordonii thyA::ermAM* (Figure 17). These results confirmed the deletion of the *ermAM* cassette by the *cre/loxP* system.

3.4.2. Curing pTV1-OKCre from *S. gordonii* Δ*thyA*Δ*ermAM* at 44˚C

An erythromycin-sensitive colony of *S. gordonii* Δ*thyA*Δ*ermAM* was selected and grown in TYG broth containing thymidine at 44˚C. As pTV1-OKCre has a temperature-sensitive replicon, the elevated growth temperature promoted the curing of the plasmid.

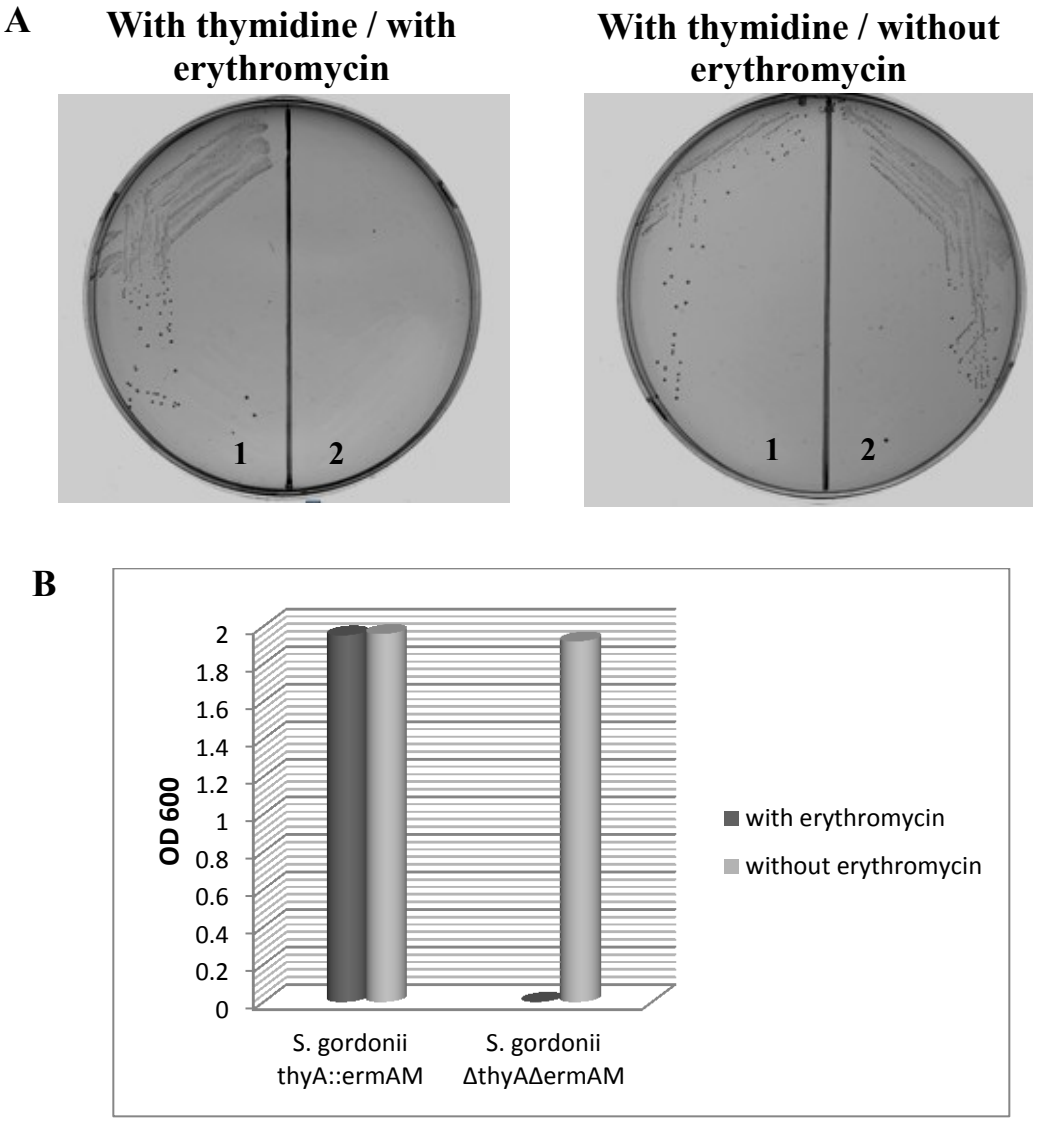


Figure 16. The *S. gordonii* Δ*thyA*Δ*ermAM* mutant is sensitive to erythromycin following the successful deletion of the *ermAM* cassette using the *cre/loxP* system. (A) The *S. gordonii thyA::ermAM* (1) and *S. gordonii* Δ*thyA*Δ*ermAM* (2) were grown on TYG agar supplemented with 20 µg/ml thymidine, with (left) and without (right) 10 µg/ml erythromycin. (B) Optical density of overnight cultures grown in TYG broth with thymidine and with or without erythromycin.

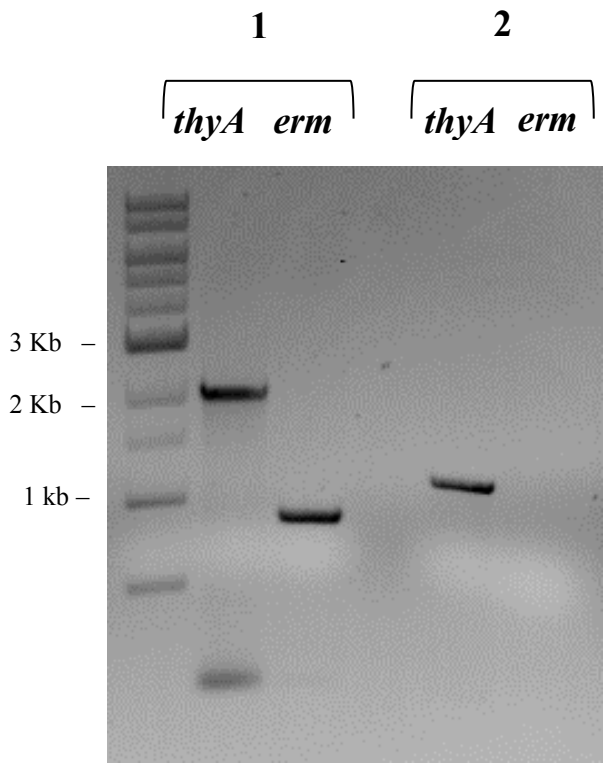


Figure 17. PCR confirmation of the deletion of the *ermAM* cassette. The *thyA* and *ermAM* genes were amplified from genomic DNA isolated from *S. gordonii thyA::ermAM* (1) and *S. gordonii* Δ*thyA*Δ*ermAM* (2) with primers SL841/SL844 and SL609/SL729 for *thyA* and *ermAM* genes respectively.

Following overnight incubation, the culture was streaked on TYG agar plate for subsequent kanamycin sensitivity testing. Fifteen percent of the colonies (65 colonies tested) were kanamycin-sensitive, indicating that pTV1-OKCre had seen cured. The growth of one of the colonies in liquid culture is shown in Figure 18.

3.5. Removal of the kanamycin resistance gene from pDL276/*thyA*

The final step in constructing the antibiotic marker-free system was to delete the kanamycin resistance gene from the auxotrophic complementation vector, pDL276/*thyA*. The kanamycin resistance gene was removed from the plasmid by restriction digestion followed by re-ligation of the vector. The plasmid was restricted with BssHII, blunt ended by Klenow enzyme, and restricted with ScaI (Figure 19). A fragment of 1.5 kb, corresponding to the kanamycin gene, was seen after the restriction digestion with BssHII and ScaI (data not shown). The 7 kb fragment was self-ligated, transformed into *E. coli* Δ*thyA*Δ*dap* competent cells and selected on LB agar containing diaminopimelic acid but without thymidine. Replica-plating the transformants on agar with and without kanamycin confirmed the successful removal of the kanamycin resistance gene. The complemented strain of *E. coli* Δ*thyA*Δ*dap* mutant with the antibiotic marker-free *thyA* plasmid, pDL276/*thyA*del*Kan*, confirmed the success of auxotrophic complementation as a selectable marker. The ability of the complemented mutant to grow in media not supplemented with thymidine indicates that the antibiotic marker-free plasmid had been retained (Figure 20). The pDL276/*thyA*del*kan* plasmid was isolated from the transformant and restricted with SphI. The result showed that pDL276/*thyA*del*kan* has smaller molecular weight (7 kb) than pDL276/*thyA* (8.2 kb), confirming the removal of the kanamycin resistance gene from the plasmid (Figure 21).

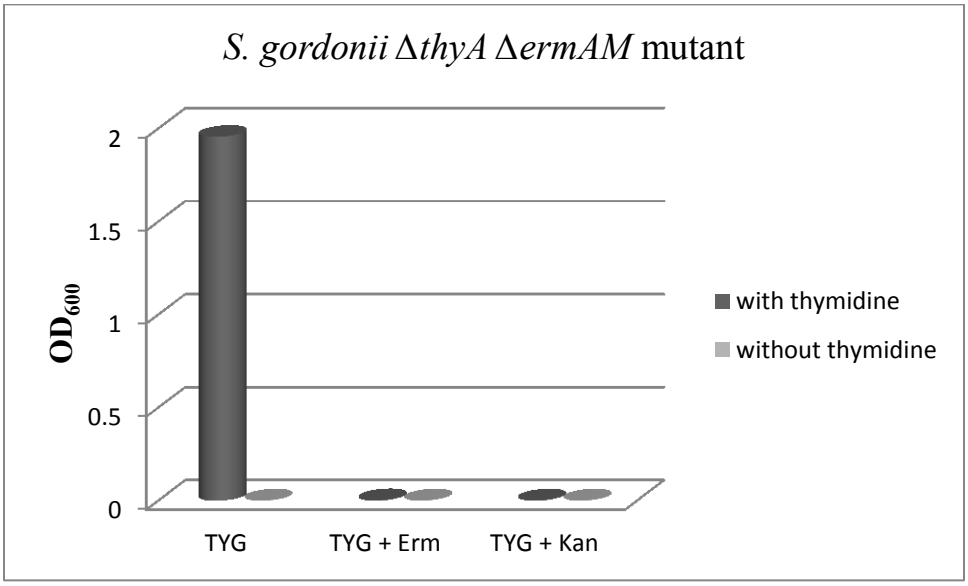


Figure 18. Growth of *S. gordonii* Δ*thyA*Δ*ermAM* mutant after curing the pTV1-OKCre at 44˚C. The mutant was grown in TYG broth with 10 µg/ml erythromycin, and TYG with 250 µg/ml kanamycin**.** The mutant is phenotypically sensitive to both erythromycin and kanamycin, and still auxotrophic for thymidine.

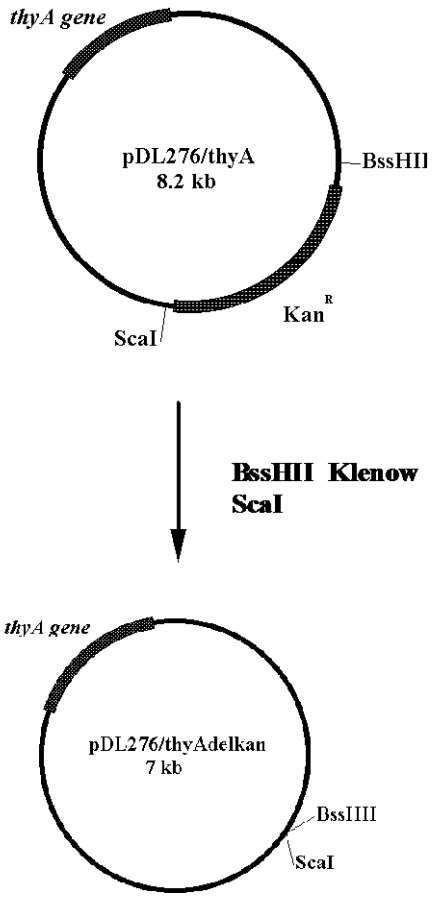


Figure 19. Diagram representing construction of pDL276/*thyA*del*Kan* plasmid.

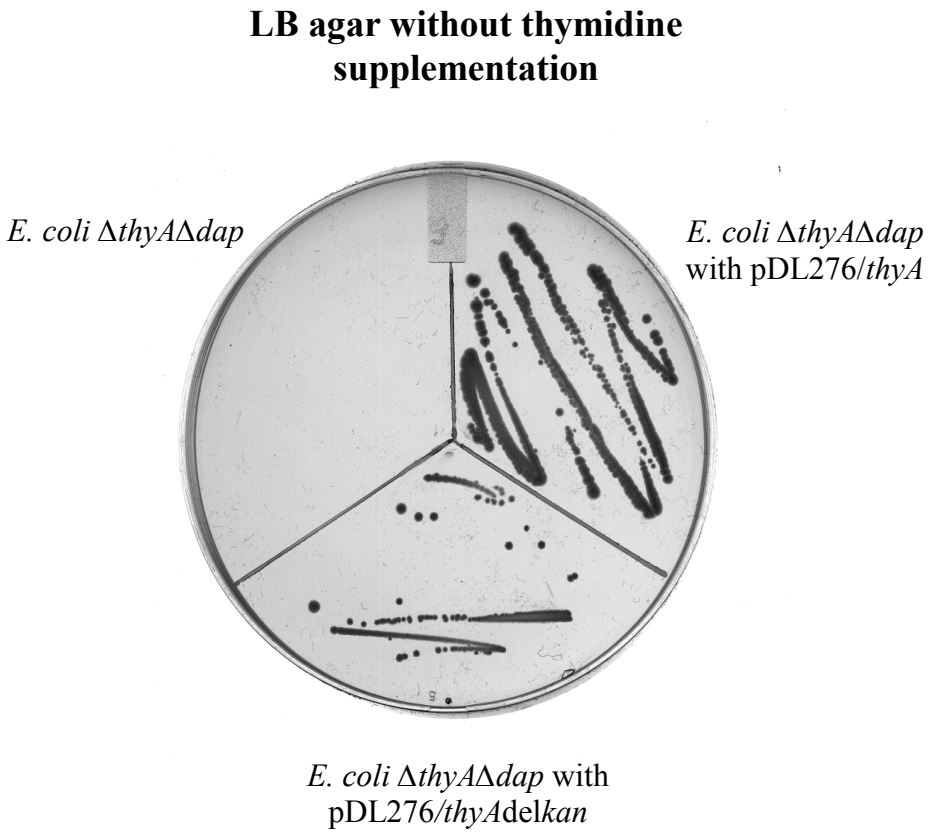


Figure 20. Complementation of the *E. coli* Δ*thyA*Δ*dap* mutant with the antibiotic marker-free plasmid pDL276/*thyA*del*kan.* The *E. coli* mutants were grown on LB agar without thymidine supplementation.

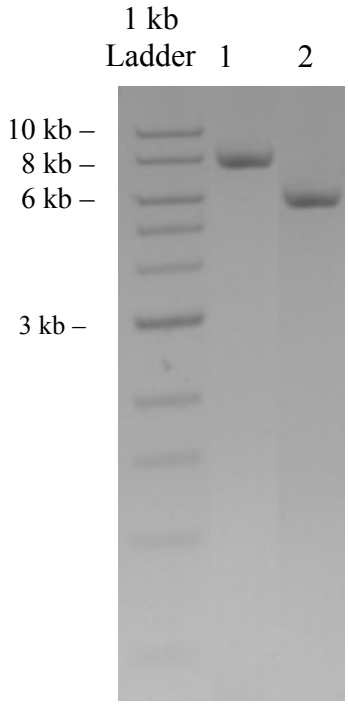


Figure 21. Restriction analysis of pDL276/*thyA*del*kan*. The single cut pDL276/*thyA* (Lane 1) and pDL276/*thyA*del*kan* (Lane 2) with SphI.

3.6. Increased transformation efficiency in *S. gordonii* Δ*thyA*Δ*ermAM*

In order to evaluate the usefulness of auxotrophic complementation as a selectable marker, the antibiotic marker-free *thyA* plasmid pDL276/*thyA*del*kan* was transformed into *S. gordonii* Δ*thyA*Δ*ermAM*. Unexpectedly, an unusually high number of transformants were observed, leading me to assess the transformation efficiency of the pDL276/*thyA*del*kan* plasmid compared to the parent pDL276.

The transformation efficiency was calculated by dividing the number of successful transformants by the amount of DNA used in the transformation. A dilution factor of 10^{-8} was used to dilute the competent cells following transformation with 1µg of pDL276/*thyA*del*kan* in order to facilitate counting of the colonies on the TYG agar plate. The transformation efficiency for pDL276/*thyA*del*kan* was $1.1 \pm 0.1 \times 10^9$ cfu/µg DNA and for pDL276 was $2.35 \pm 0.65 \times 10^7$ cfu/µg DNA. Thus, the antibiotic marker-free *thyA* plasmid gave a 100-fold increase in transformation efficiency compared to pDL276, despite both plasmids having the same size (7 kb) and the same backbone.

In order to determine whether the 100-fold decrease in the transformation efficiency of pDL276 compared to pDL276/*thyA*del*kan* was due to using the antibiotic kanamycin in the media for the selection, another set of transformation was done. The pDL276/*thyA* plasmid was transformed into *S. gordonii* Δ*thyA*Δ*ermAM*, and the transformation efficiency was identified upon counting the number of transformants in three different sets of TYG agar plates. One of these sets did not have any kind of selection and facilitated the growth of the transformants based on *thyA* complementation. The other two sets of plates contained either only kanamycin or kanamycin with thymidine, and used the same selection conditions as with the previous transformation of

pDL276. The results showed that the transformation efficiency for pDL276/*thyA* in media without selection give 6.75×10^7 cfu/μg DNA, while in media with kanamycin and kanamycin with thymidine give 3.9×10^5 cfu/μg DNA and 4.5×10^5 cfu/μg DNA, respectively. The results of the transformation of pDL276/*thyA* revealed the decrease of the transformation efficiency for pDL276 compared to pDL276/*thyA*del*kan* was due to the effect of kanamycin selection in the media.

3.7. Evaluation of pDL276/*thyA*del*kan* maintenance

To investigate whether pDL276/*thyA*del*kan* was maintained as a plasmid in the transformants, PCR amplification of the cloned *thyA* gene on the plasmid was performed (21 colonies tested) using a reverse primer (SL106) designed to bind a sequence on the plasmid and the forward primer for the *thyA* gene (SL841). No products were obtained from any of the 21 transformants, suggesting that pDL276/*thyA*del*kan* was not maintained as a plasmid in the transformants (Figure 22).

3.8. Homologous recombination and thymidine prototrophic phenotype

Although pDL276/*thyA*del*kan* was not maintained in the transformants, *S. gordonii* Δ*thyA*Δ*ermAM* was converted into a thymidine prototroph after the transformation (Figure 23). This phenomenon could be due to homologous recombination between the *thyA* gene on the plasmid and the knockout *thyA* on the chromosome. Either double crossing over, which leads to replacement of mutated *thyA* by intact *thyA* from the plasmid, or single crossing over, with the entire plasmid integrating, could result in a functional *thyA* gene on the chromosome (Figure 24).

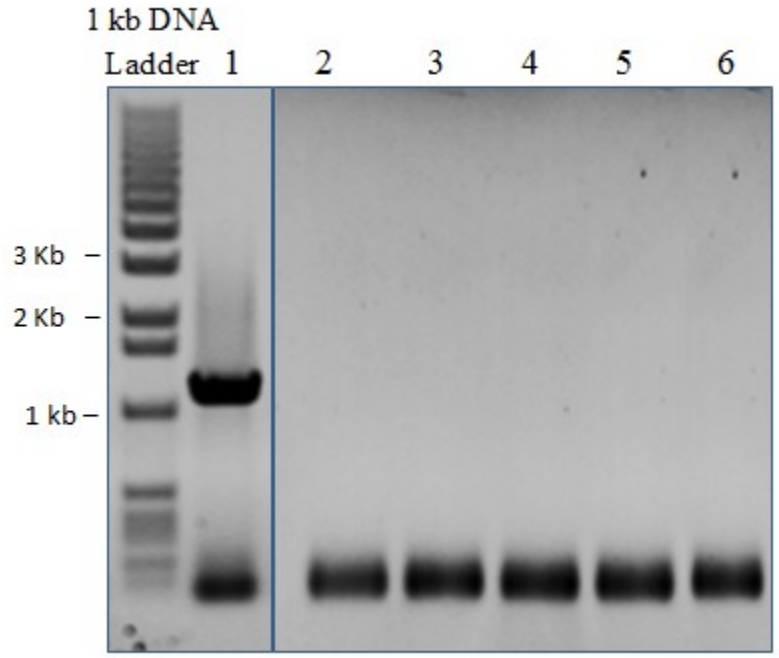


Figure 22. The pDL276/*thyA*del*kan* plasmid is not maintained in *S. gordonii* Δ*thyA*Δ*ermAM* transformants. The *thyA* gene and an adjacent portion of the pDL276 plasmid backbone was amplified with the primer pair SL841/SL106 using pDL276/*thyA*del*Kan* plasmid DNA (Lane 1) or genomic DNA extracted from transformants (Lanes 2-5) as a template.

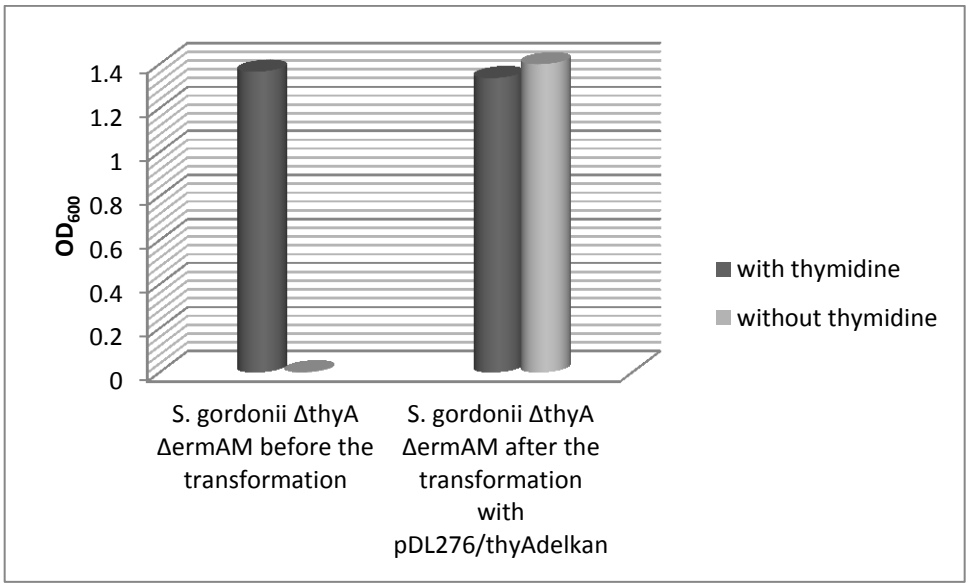


Figure 23. Overnight liquid culture of one representative transformant of the *S. gordonii* Δ*thyA*Δ*ermAM* mutant following transformation with pDL276/*thyA*del*kan* compared to the one before the transformation.

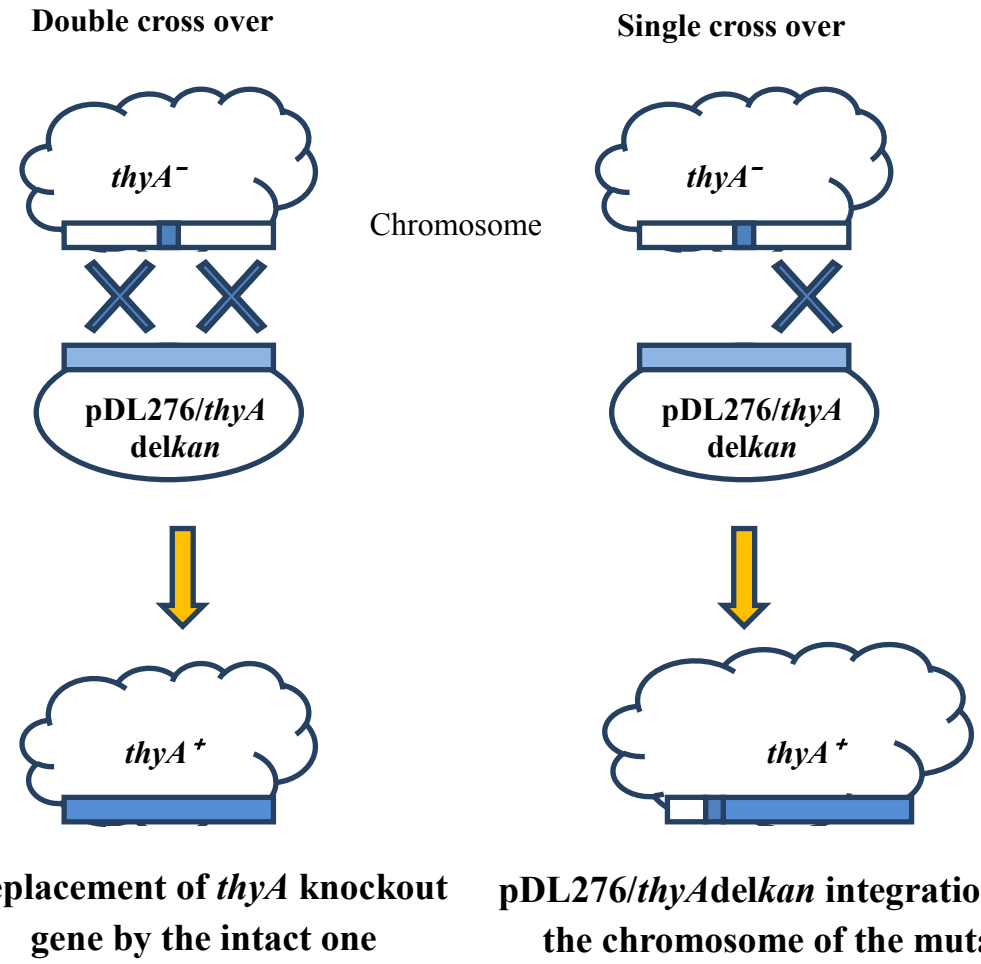


Figure 24. Schematic diagram illustrating the two types of the crossing over between *thyA* genes during homologous recombination.

To test the above notion, pDL276/*thyA*del*kan* was transformed into the *S. gordonii thyA::ermAM* mutant that carries the *ermAM* cassette. The transformants were selected on TYG agar without thymidine. Replica plating of 100 colonies on TYG agar plates with or without erythromycin revealed that 34 of the transformants were erythromycin-resistant, while 66 were erythromycin-sensitive. These phenotypes suggest that two types of crossing over had occurred during the transformation. The erythromycin-resistant colonies were the result of single crossing over between the *thyA* gene on the plasmid and *thyA* DNA on the chromosome. Erythromycin-sensitive colonies resulted from the double crossing over between the *thyA* gene on the plasmid and the *thyA::ermAM* gene.

PCR amplification of the *thyA* gene from 10 transformants each of the erythromycin-resistant (*erm*^{r}) and erythromycin-sensitive (*erm*^{s}) colonies showed the presence of the 1.2 kb *thyA* gene on the chromosome (Figure 25). This result is consistent with the replacement of the *thyA* knockout gene (2 kb) on the chromosome of the mutant with the intact (1.2 kb) *thyA* gene. Another PCR reaction was performed for the amplification of the upstream fragment of the *thyA* gene with the *ermAM* cassette from 5 *erm*^{r} colonies. A product of 1.4 kb was obtained after the amplification, suggesting the occurrence of single crossing over and the integration of the antibiotic marker-free *thyA* plasmid into the chromosome (Figure 26). Additional PCR was performed for eight of the *erm*^{r} transformants using the forward primer (SL1017), which binds to a sequence on the chromosome as a reference point, and the reverse primer for the *thyA* gene (SL844) in order to localize the site of single crossing over (Figure 27). The results showed that the single crossing over of the *thyA* gene in the *erm*^{r} transformants occurred equally within

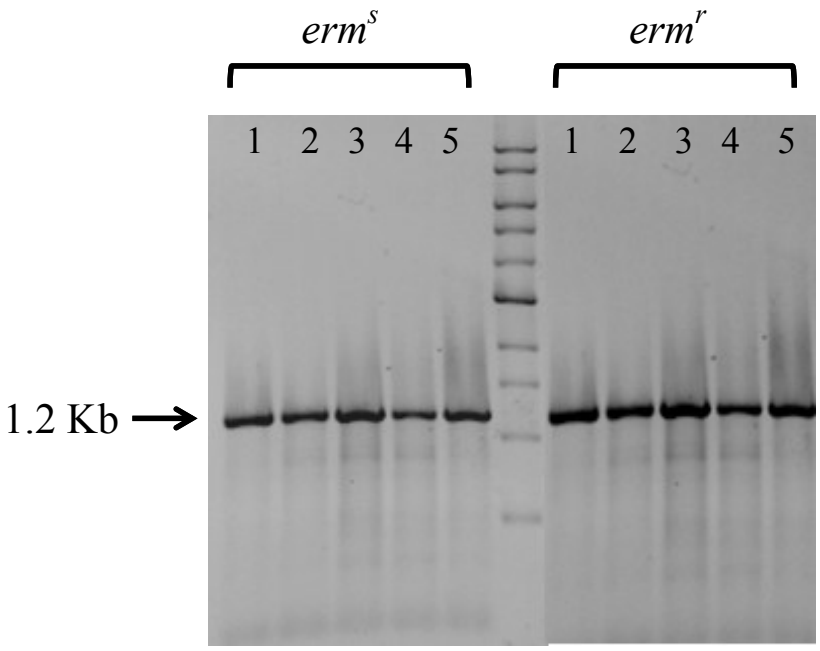


Figure 25. PCR amplification of *thyA* gene from the two types of transformants: erythromycin-sensitive (*erm*^{s}) and erythromycin-resistant (*erm*^{r}) after the transformation with the pDL276/*thyA*del*kan*. The primers used for the amplification are (SL841/SL844).

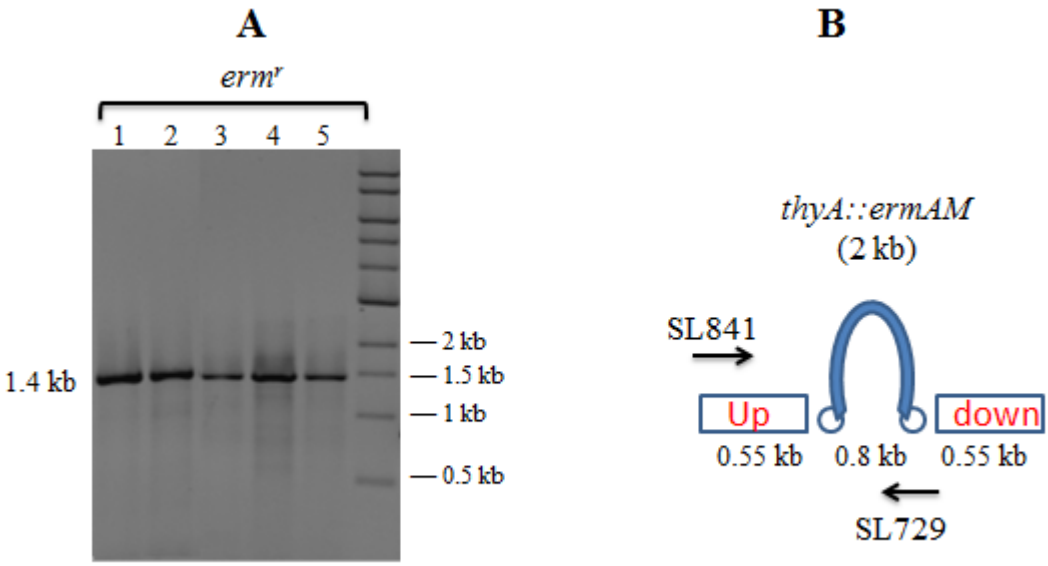


Figure 26. Genetic confirmation of the integration of pDL276/*thyA*del*kan* into the genomic DNA of *S. gordonii* Δ*thyA*Δ*ermAM*. (A) PCR amplification of the upstream fragment of *thyA* gene with the *ermAM* cassette from the erythromycin resistant transformants (Lane 1, 2, 3, 4 &5). The primers used for the PCR are SL841/SL729. (B) Diagram shows the size of *up-ermAM* fragment on *thyA::ermAM* gene.

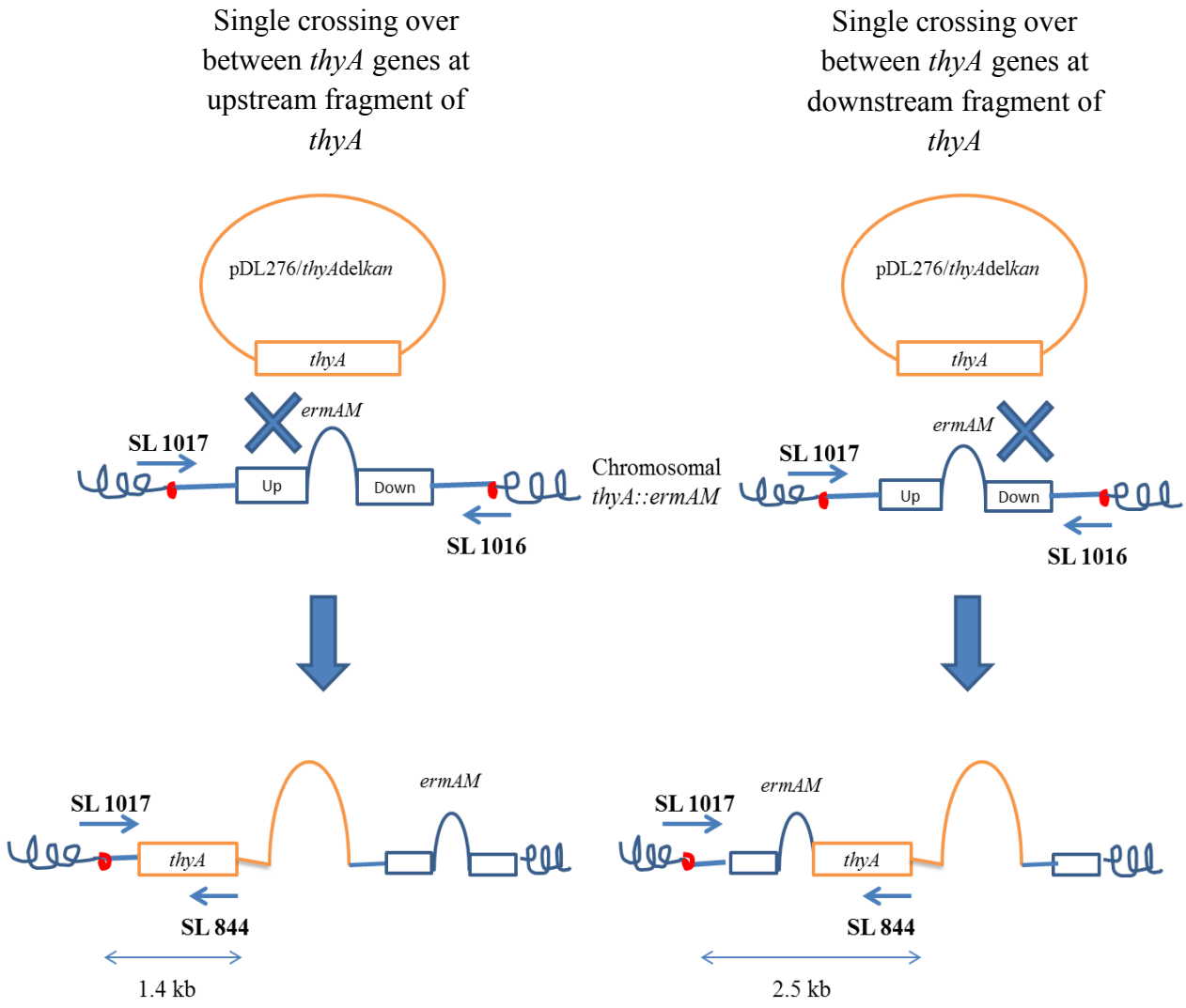


Figure 27. Diagram depicting two types of single crossing over that may occur between *thyA* gene on pDL276/*thyA*del*kan* and the chromosomal *thyA::ermAM*.

both the upstream and downstream portions of *thyA* in chromosomal *thyA::ermAM* (Figure 28).

3.9. A useful tool for the expression of vaccine antigen in *S. gordonii*

The above results indicated that an antibiotic marker-free system to deliver genes to the chromosome using *thyA* complementation has been successfully created. The vaccine antigen gene I decided to test is *fha/cr-1*, previously constructed in our lab (pSecFHA/CR-1 #12 plasmid). The fusion gene is coding for the type 1 domain of FHA of *Bordetella pertussis* and anti-CR1 scFv in addition to the C-terminal histidine and HA tags. The expression of the fusion protein is under the control of the tetracycline-inducible *xyl/tetO* promoter.

To clone the *fha/cr-1* onto the pDL276/*thyA*del*kan* plasmid, the DNA was PCR amplified from pSecFHA/CR-1 #12 using SL985/SL986 primers with Phusion DNA polymerase to generate blunt-ended product. The PCR product of FHA/CR-1 was only restricted with PvuI and cloned into KpnI, which has been blunt ended, and PvuI and sites on pDL276/*thyA*del*kan* (Figure 29). The resulting plasmid, pDL276/*thyA*del*kan*FHA/CR-1, was electroporated into *E.coli* Δ*thyA*Δ*dap* electro-competent cells.

The transformants were grown on LB broth for the plasmid isolation. The 9.5 kb plasmid, pDL276/*thyA*del*kan*FHA/CR-1, was digested with EcoRI and gave two bands, 9.1 kb and 400 bp, which confirmed the correct plasmid (data not shown).

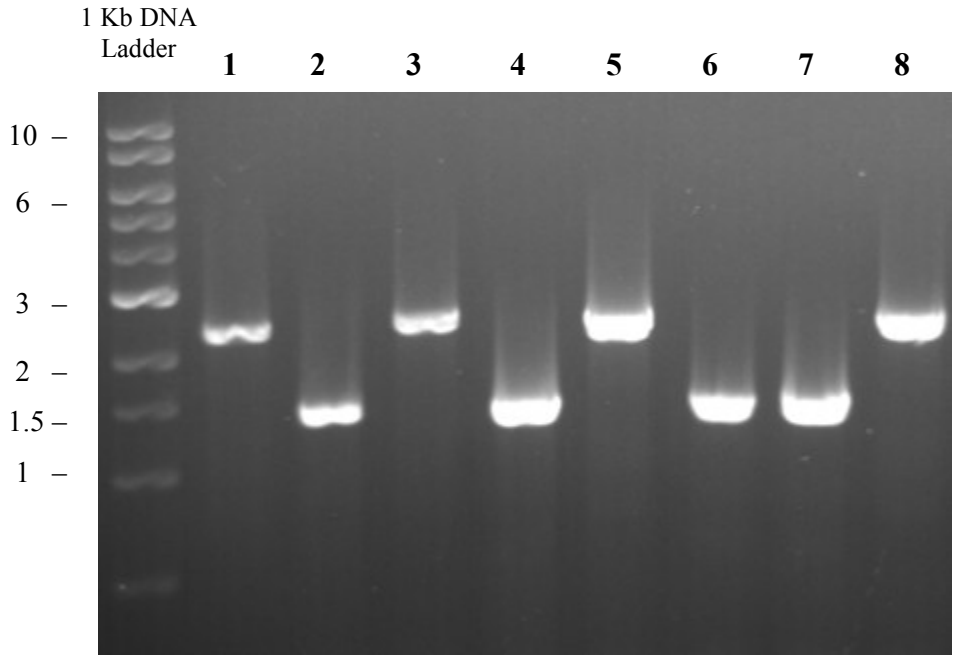


Figure 28. Identification the location where single crossing over between *thyA* gene occurred following the transformation of pDL276/*thyA*del*kan* with *S. gordonii thyA::ermAM* by PCR amplification. Chromosomal DNA of eight erythromycin resistant colonies was subjected to PCR amplification using the primers SL1017/SL844. The 2.5 kb PCR products of 4 colonies (Lanes 1, 3, 5, 8) indicate that the single crossing over was occurred at the downstream fragment of *thyA* gene while in the other 4 colonies (Lanes 2, 4, 6, 7) the crossing over was at the upstream and gave a 1.4 kb band.

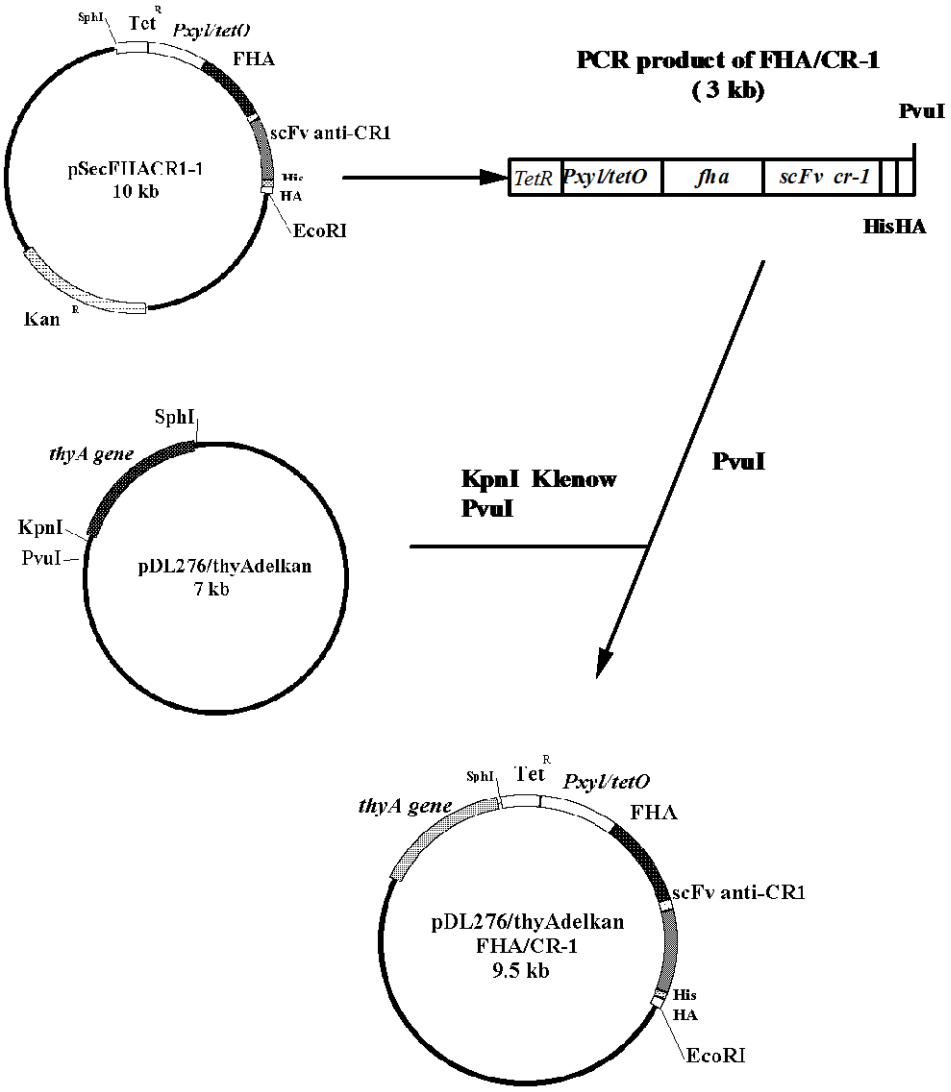


Figure 29. Diagram illustraiting construction of pDL276/*thyA*del*kan*FHA/CR-1.

The transformants carrying pDL276/*thyA*del*kan*FHA/CR-1 were tested for the production of FHA/CR-1 using Western Immunobloting with a monoclonal anti-FHA antibody. The Western blot gave the expected band size of 58 kDa (representing the FHA/CR-1 fusion protein) as well as lower molecular weight bands, presumably representing degradation products (Figure 30). On the other hand, Western blot did not detect proteins from the negative control, *E. coli* Δ*thyA*Δ*dap* carrying pDL276/*thyA*, suggesting that the detection of FHA/CR-1 from pDL276/*thyA*del*kan*FHA/CR-1 was not from cross reactivity. These results indicated that pDL276/*thyA*del*kan*FHA/CR-1 has been correctly constructed.

3.10. The frequency of pDL276/*thyA*del*kan*FHA/CR-1 transformants with single crossing over is very low

The pDL276/*thyA*del*kan*FHA/CR-1 plasmid was transformed into the antibiotic marker-free *S. gordonii* Δ*thyA*Δ*ermAM.* Fifty colonies were isolated and grown in TYG broth containing 10 ng/ml tetracycline and screened for plasmid integration by PCR amplification of FHA/CR-1 using the primer pair SL985/SL986. No PCR products for FHA/CR-1 were obtained from any of the 50 transformants. As well, the same transformants were screened for protein production of FHA/CR-1 by Western blot and no protein bands associated with FHA/CR-1 were recognized. These results indicated that these transformants arose from double crossing over between the *thyA* genes, which is probably the most common event associated with the transformation of the pDL276/*thyA*del*kan*FHA/CR-1.

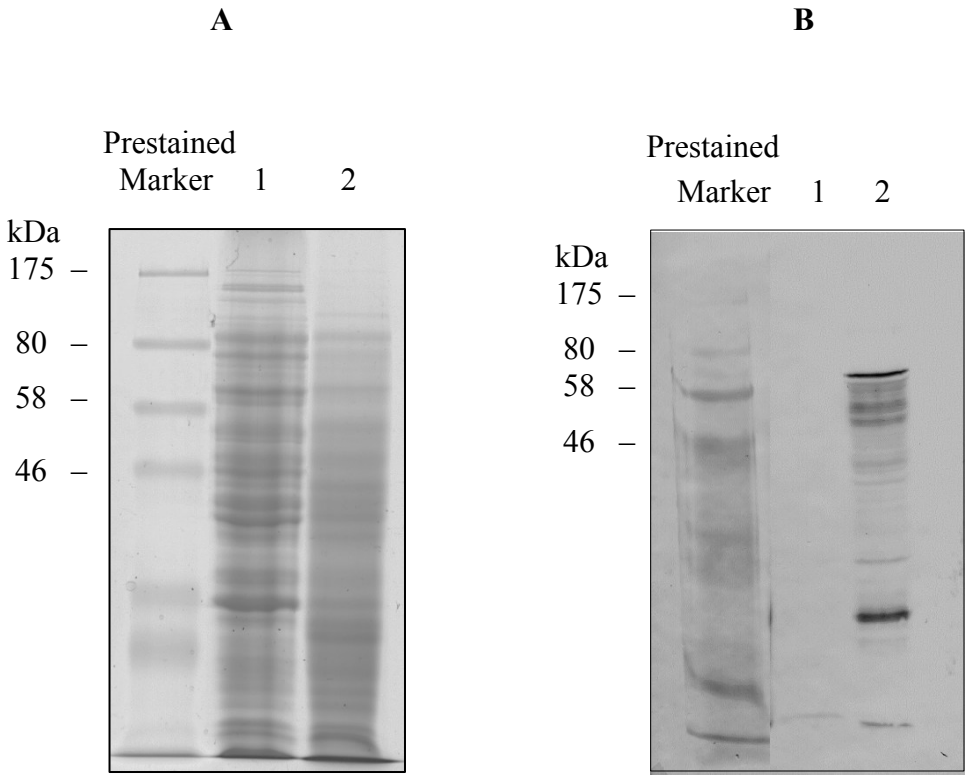


Figure 30. Expression of FHA/CR-1 fusion protein in *E. coli* Δ*thyA*Δ*dap*. (A) A 7.5% SDS-PAGE gel showing equal amounts of protein from *E. coli* Δ*thyA*Δ*dap* carrying pDL276/*thyA* (Lane 1) or pDL276/*thyA*del*Kan*FHA/CR-1 (Lane 2). (B) Immunoblot of FHA/CR-1 from the same samples as in (A). Proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against the FHA.

To further investigate the above result, pDL276/*thyA*del*kan*FHA/CR-1 was transformed into *S. gordonii thyA::ermAM*, which carries an erythromycin antibiotic resistance cassette that can be used for selection. The transformants were grown on TYG agar without thymidine. One hundred transformants were selected and screened by replica plating on TYG agar with or without erythromycin. The results of replica plating showed that all 100 transformants were sensitive to erythromycin. The erythromycin-sensitive thymidine prototroph phenotype indicated that the replacement of *thyA::ermAM* from the chromosome with the intact *thyA* gene from the plasmid must have occurred by double crossing over.

The previous results led me to investigate how frequently pDL276/*thyA*del*kan*FHA/CR-1 transformants with single crossing over in *S. gordonii thyA::ermAM* would occur. The pDL276/*thyA*del*kan*FHA/CR-1 plasmid was transformed into *S. gordonii thyA::ermAM*, and the number of transformants with single crossing over (distinguished by their erythromycin resistance) were counted after plating the 1.5 ml transformation culture onto five TYG agar plates containing erythromycin. The results showed that out of 1×10^9 cfu of thymidine prototroph (from 1μg of pDL276/*thyA*del*kan*FHA/CR-1) only 324 colonies were erythromycin-resistant. This result provided an explanation for the difficulty in obtaining colonies carrying the plasmid DNA in the chromosome when the colonies were screened by PCR and Western blot for the FHA/CR-1 protein. The results collectively reveal that the frequency of transformants resulting from single crossing over is very low with the transformation of pDL276/*thyA*del*kan*FHA/CR-1 compared to that of pDL276/*thyA*del*kan.*

3.11. The expression of FHA/CR-1 fusion protein in *S. gordonii thyA::ermAM*

Six erythromycin-resistant transformants of pDL276/*thyA*del*kan*FHA/CR-1 were grown in 1 ml TYG broth containing 10 ng/ml tetracycline for Western immunobloting. Four out of six transformants showed FHA/CR-1 fusion protein expression, as indicated by Western blot using a monoclonal antibody against FHA (Figure 31).

It is unclear whether those transformants expressing FHA/CR-1 proteins carry the circular plasmids inside the bacteria or if the plasmids were integrated into the chromosome by single crossing over. A series of PCR amplifications was done in order to address this question. The first PCR amplified the *thyA::ermAM* gene from the six transformants using primers SL1017/SL1016, which bind at two references points around the locus of the *thyA* gene on the chromosome (Figure 27). Positive results from this PCR amplification of *thyA::ermAM* gene (a 2 kb band) would indicate that no crossing over occurred at the *thyA* gene locus and that the transformants are carrying the circular pDL276/*thyA*del*kan*FHA/CR-1 as a plasmid. On the other hand, negative results or no PCR products would suggest that the plasmid was integrated into the chromosome of the *thyA* gene, because the fragment to be amplified would be too large. The results of PCR amplification of the *thyA* knockout gene from the six transformants were negative, suggesting chromosomal integration of plasmid DNA by single crossing over (Figure 32).

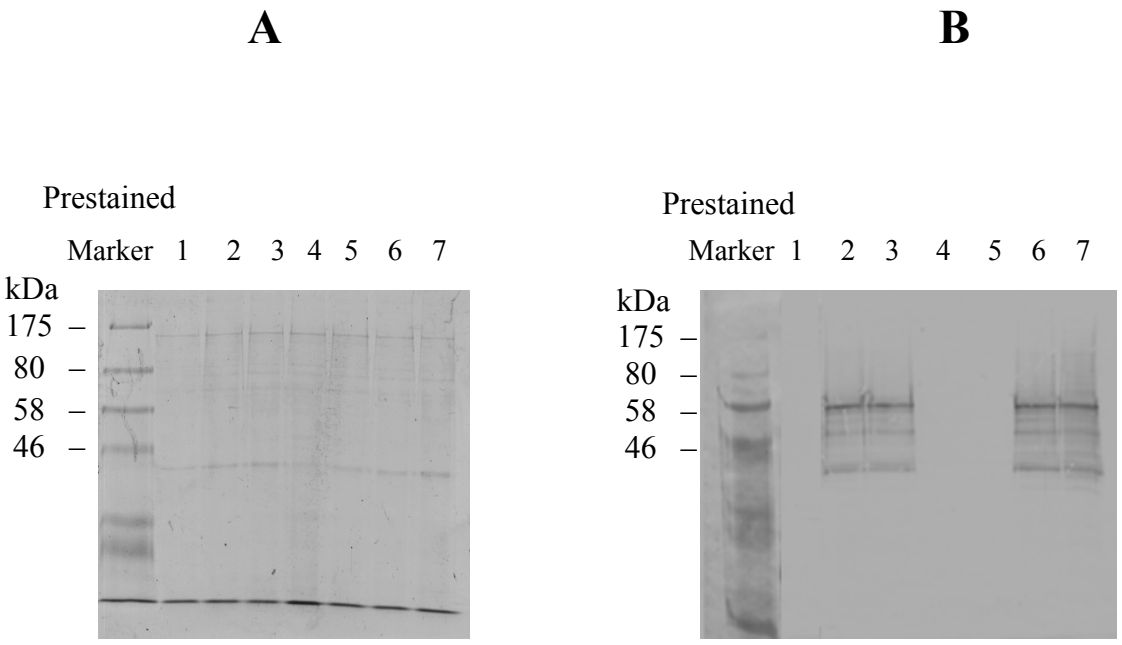


Figure 31. Expression of FHA/CR-1 fusion protein in *S. gordonii thyA::ermAM*. (A) A 7.5% SDS-PAGE gel showing equal amounts of protein from *S. gordonii thyA::ermAM* carrying pDL276/*thyA* (Lane 1) or from six of erythromycin resistant colonies selected following the transformation of *S. gordonii thyA::ermAM* with pDL276/*thyA*del*Kan*FHA/CR-1 (Lanes 2-7). (B) Immunoblot of FHA/CR-1 from the same samples as in (A). Proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against the FHA.

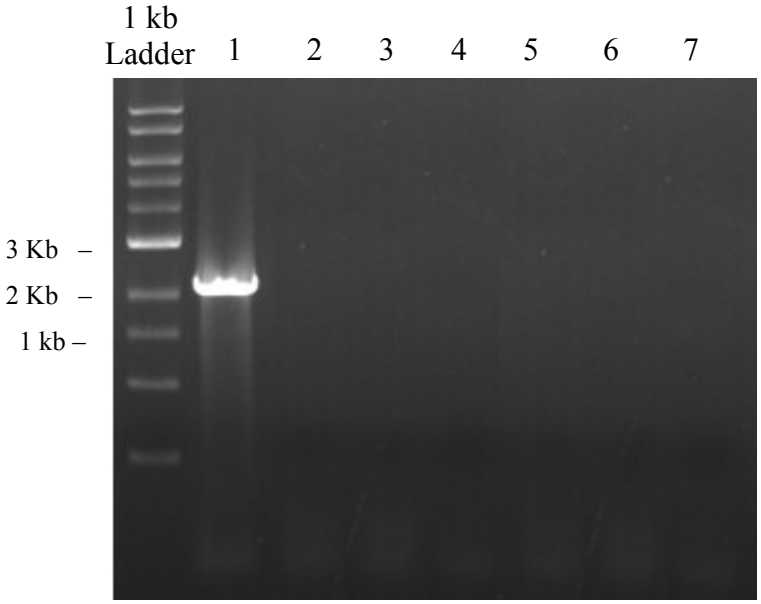


Figure 32. PCR amplification of *thyA::ermAM* gene from erythromycin resistant transformants of pDL276/*thyA*del*kan*FHA/CR-1. The *thyA::ermAM* gene was amplified with the primer pair SL1017/SL1016 using genomic DNA extracted from *S. gordonii thyA::ermAM* (Lane 1) or from the six transformants (Lanes 2-7) as a template.

The location of plasmid integration in the six transformants was also investigated by additional PCR amplification using the primers SL1017/SL844 (Figure 27). The PCR products with a band size of 1.4 kb from the three transformants suggested that the single crossing over occurred at the upstream fragment of that *thyA::ermAM* gene, while the product with a band size of 2.5 kb from one transformant suggested that the crossing over was downstream of the *thyA::ermAM* gene (Figure 33). In the case of the remaining two transformants, PCR amplification produced multiple bands, making the results for these transformants difficult to interpret (Figure 33). The results of the PCR amplification demonstrated that the single crossing over took place at both fragments of *thyA::ermAM*, upstream and downstream, and that the plasmid was successfully integrated in both cases. The results also indicated that the transformants were able to produce an adequate amount of the heterologous proteins (Figure 30).

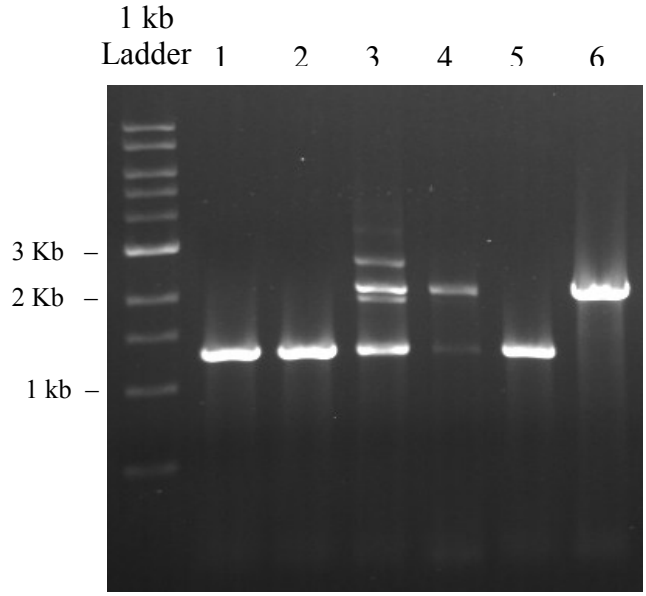


Figure 33. PCR amplification of erythromycin-resistant transformants of pDL276/*thyA*del*kan*FHA/CR-1 showing the location of the single crossing over occurrence on the chromosomal *thyA::ermAM* gene. Genomic DNA of six erythromycin-resistant colonies (Lanes 1-6) was extracted for PCR amplification using the primers SL1017/SL844.

Chapter 4. Discussion

The development of vaccines based on live bacterial vectors can be achieved through genetic engineering by constructing strains that express recombinant vaccine antigens. The genes encoding these antigens may be expressed as a single copy located on the chromosome or from multicopy plasmids. Because plasmids impose a metabolic strain on the cell, plasmid retention is not always maintained.

The most common approach to apply a selective pressure for plasmid maintenance is to include an antibiotic resistance marker. However, the use of antibiotic selection is undesirable due to the risk of spreading antibiotic resistance genes in the environment. Therefore, alternative strategies have been developed to achieve plasmid retention without the use of an antibiotic marker. Such alternatives include toxin-antitoxin systems, operator-suppressor titration, and auxotrophic complementation of essential genes (Galen et al., 1999; Altboum et al., 2003; Garmory et al., 2004; Leckenby et al., 2009; Curtiss et al., 1990; Tacket et al., 1990; Attridge et al., 1991; Fu and Xu, 2000). The objective of this study was to construct an antibiotic antibiotic marker-free expression system in the live vaccine vector *S. gordonii.*

The findings from this study reveal that mutation of the alanine racemase gene (*alr*) in *S. gordonii* may not be possible, as *alr* mutants could not be obtained. However, our results demonstrate that the essential thymidylate synthase gene (*thyA*) can be successfully inactivated and used for auxotrophic complementation. Additionally, the findings reveal that the complementation of the antibiotic marker-free *thyA* mutant with a *thyA*$^{+}$ plasmid (pDL276/*thyA*del*kan*) gave an unexpected increase in the transformation

efficiency as compared to pDL276. Transformants arose from both single and double crossing over between *thyA* genes. The observed increase in transformation efficiency indicates that a highly efficient antibiotic marker-free system to deliver genes to the chromosome has been created using *thyA* complementation.

4.1. *S. gordonii alr*-null mutants could not be obtained

Alanine racemases are important for peptidoglycan biosynthesis in both Gram-positive and Gram-negative bacteria. These enzymes catalyze the racemization of naturally occurring L-alanine to D-alanine, an integral precursor to the formation of peptidoglycan crosslinks. Most bacteria are predicted to encode either one or two alanine racemase genes. For example, *E. coli* and *L. monocytogenes* carry two genes encoding alanine racemases (Wild et al., 1985; Verch et al., 2004), while only one gene has been identified in *S. pneumoniae* and *B. subtilis* (Strych et al., 2007; Xia et al., 2007). The *alr* gene in *S. gordonii* was identified through sequence comparison between known *alr* sequences and the sequenced *S. gordonii* genome (www.oralgen.lanl.gov). This analysis detected a single unambiguous match in the database, suggesting that *S. gordonii* has only one *alr* gene.

Our attempts to inactivate the *alr* gene in *S. gordonii* via homologous recombination with an *alr::ermAM* construct (Figure 2) failed to produce the expected mutation. Phenotypic characterization of the transformants showed that they were erythromycin resistant, but not auxotrophic for D-alanine. The ability of the transformants to grow in media lacking D-alanine was investigated via PCR analysis for

the *alr* gene, which revealed the presence of the intact gene (Figure 3). Although *alr* mutants have been reported previously in other bacteria, such as *E. coli* (Wild et al., 1985), *L. monocytogenes* (Verch et al., 2004) and *S. pneumoniae* (Strych et al., 2007), based on our investigations we concluded that an *alr*-null mutant of *S. gordonii* could not be obtained.

There are two possible reasons for the inability to generate an *alr*-deletion mutant of *S. gordonii* in our study. One possibility would be related to D-alanine uptake. Following the natural transformation of the *alr::ermAM* knockout gene into *S. gordonii*, replacement of the chromosomal *alr* gene via homologus recombination may have occurred. However, due to the lack of D-alanine transport across the cytoplasmic membrane the mutant could not be grown despite the presence of D-alanine in media.

The transport mechanism of the two stereoisomers of alanine, L-alanine and D-alanine, has been investigated in bacterial cells via membrane vesicles in the presence of an electron donor (Konings and Freese, 1971; Short and White, 1971). In *E. coli*, the presence of L- and D-alanine in growth media induces the expression of two membrane-associated enzymes, alanine racemase and D-alanine dehydrogenase (Raunio et al., 1973; Raunio and Jenkins, 1973). These two enzymes have been isolated from cytoplasmic membrane vesicles of *E. coli* and shown to carry out active transport of several amino acids, including D-alanine (Kaczorowski et al., 1975). Alanine racemase catalyzes the racemization of naturally occurring L-alanine to D-alanine. D-alanine is then dehydrogenated by D-alanine dehydrogenase to stimulate its uptake into the cells (Kaczorowski et al., 1975). In the case of *S. gordonii*, it is possible that the absence of

alanine racemase may affect negatively on D-alanine dehydrogenase activity, leading to difficulty in D-alanine uptake.

The fact that the erythromycin-resistant transformants obtained following transformation with the *alr::ermAM* construct were not auxotrophic for D-alanine indicates the presence of sequence homology in the chromosome of *S. gordonii* to the sequence of the erythromycin cassette. The sequence homology facilitates the integration of the *ermAM* cassette into the chromosome of *S. gordonii*, leaving the *alr* gene intact. This phenomenon represents another possible explanation for not obtaining an *alr*-null mutant in *S. gordonii*.

4.2. Successful construction of an *S. gordonii thyA::ermAM* mutant followed by deletion of *ermAM* by *cre/loxP* system

The primary function of thymidylate synthase (*thyA*) is to generate thymidine monophosphate (dTMP), which is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. Because of this gene's critical role in DNA synthesis, it was targeted next for forming an auxotrophic complementation system in *S. gordonii.*

The mutant was successfully constructed, as confirmed both phenotypically and genetically. The *S. gordonii thyA* mutant was totally dependent on exogenous supplementation of thymidine to the media (Figure 6). In addition, PCR amplification of the *thyA* gene from the mutant revealed inactivation of the *thyA* gene due to insertion of the *ermAM* gene, as indicated by a band size larger than the intact *thyA* gene from the

parent strain (Figure 7). This technique of mutation in *thyA* gene has been used successfully in *S. typhi* Ty21a (Tacket et al., 1990; Attridge et al., 1991; Bumann et al., 2002), *L. acidophilus* (Fu and Xu, 2000) and *V. cholerae* (Liang et al., 2003).

To obtain an antibiotic marker-free mutant that is free of antibiotic resistance genes, the *cre/loxP* site specific recombination system was applied to the constructed *S. gordonii thyA::ermAM* mutant. Cre recombinase, a 38.5 kDa protein from bacteriophage P1, catalyzes the recombination of the DNA between two *loxP* sites (Langer et al. 2002). The sequence of *loxP*, ATAACTTCGTATA GCATACAT TATACGAAGTTAT, consists of a 34 bp element composed of two 13 bp inverted repeats separated by an asymmetric 8 bp spacer region (Langer et al. 2000). In addition, Cre recombinase mediates the deletion of specific sequences flanked by *loxP* sites oriented in the same direction (Palmeros et al., 2000). Based on this property, the *ermAM* gene in the *thyA::ermAM* construct was cloned between two parallel *loxP* sites in order to be subsequently removed from the bacterial genome by Cre recombinase. Since Cre recombinase is sufficiently active to promote excision when expressed in bacteria from a plasmid, we constructed plasmid pTV1-OKCre (Figure 13) as the Cre protein-producing component. In this replicon, the Cre recombinase gene is under control of the inducible $P_{xyl/tetO}$ promoter to facilitate the expression of recombinase in *S. gordonii* in the presence of a sub-inhibitory concentration of tetracycline. Moreover this plasmid carries a temperature-sensitive replicon derived from pWVO1 that can replicate at 30˚C or below but not at 37˚C (Gutierrez et al, 1996). Thus, pTV1-OKCre could be cured from *S. gordonii* Δ*thyA*Δ*ermAM* by increasing the growth temperature to 44˚C.

The efficiency of the *cre/loxP* system for gene deletion was investigated via deletion of the erythromycin cassette (*ermAM*) following the natural transformation of pTV1-OKCre into the *S. gordonii thyA::ermAM* mutant. Deletion of this gene resulted in an erythromycin-sensitive phenotype (Figure 16). Of all the transformants screened for erythromycin sensitivity (300 colonies tested), only 0.6 % of colonies (2 colonies) were sensitive, indicating the low efficiency of the *cre/loxP* system in *S. gordonii*. Banerjee and Biswas (2008) reported similar results when using the *cre/loxP* system to generate an antibiotic marker-free gene deletion in *S. mutans*, finding that approximately 13% of the colonies were without the kanamycin resistance gene following exposure to the Cre recombinase (Banerjee and Biswas, 2008). Thus, for unknown reasons, the *cre/loxP* system may not function efficiently for gene deletion in streptococci.

4.3. Auxotrophic complementation of the *S. gordonii thyA* mutant with pDL276/*thyA*

The survival of a viable mutant without any supplementation of the deficient material in media can be achieved using a plasmid carrying that essential gene. In this study, I constructed a plasmid carrying the *thyA* gene of *S. gordonii* (Figure 8). The plasmid was confirmed to carry the *thyA* gene using restriction analysis (Figure 9). The functionality of the cloned *thyA* gene in the plasmid was demonstrated by the transformation of pDL276/*thyA* into *E. coli* Δ*thyA*Δ*dap.* The ability of the *E. coli* Δ*thyA*Δ*dap* mutant to grow in LB media without exogenous supplementation of thymidine revealed that pDL276/*thyA* successfully complemented the *thyA* mutation

(Figure 10). As well, the complementation of *thyA* mutation in *E. coli* with the cloned *thyA* gene from *S. gordonii* in pDL276/*thyA* suggests that *thyA* genes are highly conserved among bacteria.

The successful auxotrophic complementation of *E. coli* Δ*thyA*Δ*dap* with pDL276/*thyA* is not novel and has been observed previously in *S. typhi* Ty21a (Tacket et al., 1990; Attridge et al., 1991; Bumann et al., 2002), *L. acidophilus* (Fu and Xu, 2000) and *V. cholerae* (Liang et al., 2003) when complemented with plasmids carrying the intact *thyA* gene. These bacteria were used as live bacterial vectors to carry cloned vaccine antigen genes on balanced lethal plasmids. The complemented strains were restored to the wild type phenotype of thymidine prototroph and were capable of maintaining the plasmid for long periods. In addition, they continued to express the vaccine antigen in an amount sufficient to induce an immune response against the specific pathogen.

Auxotrophic complementation of *S. gordonii thyA::ermAM* mutant with the balanced lethal plasmid, pDL276/*thyA*, was tested. The complemented mutant grew similar to the parent on media without thymidine supplementation (Figure 11). Our successful complementation of the *thyA* gene in *S. gordonii* with pDL276/*thyA* indicates that auxotrophic complementation can be used as a selective marker for plasmid retention.

4.4. Transformation of *S. gordonii* Δ*thyA*Δ*ermAM* with pDL276/*thyA*del*kan* increases transformation efficiency

Construction of the antibiotic marker-free auxotrophic complementation vector pDL276/*thyA*del*kan* was achieved by removing the kanamycin resistance gene from the constructed *thyA* plasmid pDL276/*thyA*. This plasmid successfully complemented the *thyA* mutation in *E. coli* Δ*thyA*Δ*dap,* confirming the sustainability of the balanced lethal plasmid without the need for antibiotic selection.

Evaluation of the usefulness of auxotrophic complementation as a selectable marker was assessed by the transformation of pDL276/*thyA*del*kan* into *S. gordonii* Δ*thyA*Δ*ermAM.* Surprisingly, the transformation resulted in an unusually high number of transformants, prompting us to further investigate the transformation efficiency of this system.

Transformation efficiency was compared between pDL276/*thyA*del*kan* in *S. gordonii* Δ*thyA*Δ*ermAM* and pDL276, both of which have the same molecular weight and backbone. The transformation efficiency rate obtained for pDL276/*thyA*del*kan* (1.7×10^9 cfu/μg DNA) was 100-fold greater than the rate obtained for pDL276 (1.2×10^7 cfu/μg DNA). This difference was due to the different kind of selection used to select for the transformants of the two different plasmids, as the kanamycin antibiotic selection, which was used to select for the pDL276 transformants, apparently decreased the transformation efficiency of *S. gordonii* Δ*thyA*Δ*ermA*. The effect of kanamycin selection on transformation efficiency was confirmed following the transformation of the pDL276/*thyA* into *S. gordonii* Δ*thyA*Δ*ermA*. The transformation efficiency for

pDL276/*thyA* in the presence of kanamycin was 100-fold less than the efficiency for the same plasmid in an antibiotic-free media, a decrease that is similar to the results obtained previously for the transformation of pDL276 and pDL276/*thyA*del*kan*. It is possible that when the bacteria are forced to maintain a plasmid via antibiotic selection, extra metabolic burden is required resulting in a reduced number of successful transformants.

Interestingly, all of the pDL276/*thyA*del*kan* transformants failed to maintain their plasmid and had become prototrophic for thymidine. The thymidine prototrophic phenotype was due to the phenomenon of integration of the intact *thyA* gene by homologous recombination between the *thyA* gene on the plasmid and the knockout *thyA* on the chromosome of the mutant (Figure 25).

The phenomenon of homologous recombination that occurred between the *thyA* genes was likely a consequence of how the auxotrophic complementation vector was constructed. The pDL276/*thyA* was constructed by cloning the *thyA* gene from *S. gordonii* onto the pDL276, thus creating regions of homologous sequence. One strategy that could be used to eliminate crossing over at *thyA* is to use a *thyA* gene from a different species, with limited homology, for complementation. Alternatively, the *S. gordonii thyA* mutant could be constructed using an approach that eliminates *thyA* sequence from the chromosome entirely.

The increased number of antibiotic marker-free *thyA* plasmid transformants was a result of homologous recombination involving both single and double crossing over between *thyA* genes. That was confirmed by transforming the pDL276/*thyA*del*kan* to the erythromycin-resistant mutant *S. gordonii thyA::ermAM,* from which two different

phenotype colonies (*erm*^{r} and *erm*^{s}) were obtained. The erythromycin-sensitive transformants (66%) resulted from the replacement of the *thyA* knockout gene in the mutant with the intact *thyA* gene from the plasmid by double crossing over. The remaining, erythromycin-resistant transformants (34%) were confirmend to carry the intact *thyA* gene with the *thyA* knockout gene still in the chromosome by single crossing over (Figures 25 & 26). This result is consistent with those of Niaudet et al. (1985), who transformed a plasmid containing a sequence for *thyB* homologous to the chromosomal *thyB* sequence of *B. subtilis* and found that 27% of transformants arose by single crossing over and 73% by double crossing over.

I tested whether the plasmid was maintained in the transformants via PCR amplification for the cloned *thyA* gene on the plasmid using the primers SL106/SL844, and the result was negative with no PCR products obtained. It is likely that the *thyA* gene inserted into the chromosome by double cross over, resulting in the loss of the plasmid. In this scenario, the expected result of the above PCR amplification would be negative. Because this kind of amplification would be expected to work if the transformants had a plasmid integrated into the chromosome by single crossing over, the above explanation is most likely. An additional experiment that could be used to examine plasmid maintenance in the transformants is to carry out plasmid isolation. However, the protocol used for the plasmid extraction from the Gram-positive bacteria is time consuming and does not represent a practical approach for screening purposes.

The event of single crossing over with plasmid integration into the chromosome observed here has also been reported in other systems such as *E. coli* (Gutterson and Koshland, 1983), *B. subtilis* (Duncan et al. 1978, Ferrari et al. 1983), *S. pneumoniae* (Pozzi and Guild, 1985; Pozzi et al., 1988) and *L. lactis* (Casey et al. 1991). The site of integration corresponds to the chromosomal location of the fragment carried by the plasmid. This integration strategy has been exploited to generate insertion mutants (Stahl and Ferrari, 1984; Niaudet et al. 1985) and to clone specific genes (Duncan et al. 1978). In these cases, the insertion of the plasmid into the chromosome was by single crossing over recombination and the integrated plasmids were found to be quite stable within the chromosome (Ferrari et al., 1983; Pozzi et al., 1988; Casey et al., 1991). These studies illustrated that strains maintain the plasmid DNA on the chromosome after 50 generations in the absence of selection for an antibiotic (Ferrari et al., 1983; Pozzi et al., 1988; Casey et al., 1991).

There are several advantages to this integration strategy. One is that it can allow the plasmids, typically unable to replicate in a specific host, to integrate into the host genome. Another advantage is that it allows for the stability of the genes and facilitates the examination of genes present in only a single copy on the chromosome. In our case, the advantage of having a whole plasmid efficiently integrated into the chromosome of the *S. gordonii thyA* mutant can be used as a tool to introduce vaccine antigens. Our experiments have led us to create a reliable expression delivery system in *S. gordonii* that allows genes to be delivered into the chromosome using *thyA* complementation rather than antibiotic resistant markers.

4.5. Low frequency of single crossing over transformants obtained following transformation of pDL276/*thyA*del*kan*FHA/CR-1

The vaccine antigen gene (*fha/cr-1*) was successfully cloned onto the antibiotic marker-free *thyA* plasmid. *E. coli* Δ*thyA*Δ*dap* transformants carrying pDL276/*thyA*del*kan*FHA/CR-1 expressed the FHA/CR-1 fusion protein as demonstrated by Western blot using the antibody against FHA (Figure 30).

Next we attempted to introduce a vaccine antigen onto the chromosome of *S. gordonii* by transforming pDL276/*thyA*del*kan*FHA/CR-1 into *S. gordonii* Δ*thyA*Δ*ermAM*. Negative results were obtained from screening fifty transformants via Western blot, revealing that FHA/CR-1 was not expressed. These results indicate that transformation with pDL276/*thyA*del*kan*FHA/CR-1 is more likely to result in transformants with double crossing over rather than single crossing over.

Transformation of pDL276/*thyA*del*kan*FHA/CR-1 into *S. gordonii thyA::ermAM* was done in order to evaluate the frequency of obtaining transformants with a plasmid integrated into the chromosome by single crossing over. In addition, the frequency was compared to the transformation frequency obtained using the smaller plasmid, pDL276/*thyA*del*kan.* Not surprisingly, the transformation efficiency of both plasmids was similar (1×10^9 cfu/μg DNA). However, the number of transformants (erythromycin-resistant) having the plasmid integrated into the chromosome by single crossing over was different. The frequency of transformants with single crossing over from pDL276/*thyA*del*kan*FHA/CR-1 (324 cfu out of 1×10^9 cfu) was much less compared to that from pDL276/*thyA*del*kan* (34% of 1×10^9 cfu). The low frequency of single cross

over is consistent with that seen by Pozzi and Guild (1985). Comparing the efficiencies of the two different processes (double and single crossing over) in the integration of heterologous DNA from the plasmid into the chromosome of *S. pneumoniae* during transformation, they found that the single crossing over that leads to plasmid integration into the chromosome gave 600-fold fewer insertions than did the double crossing over (Pozzi and Guild, 1985).

The reason that we obtained fewer pDL276/*thyA*del*kan*FHA/CR-1 transformants from a single cross over recombination compared to pDL276/*thyA*del*kan* may be related to the size difference between the two plasmids. Integration of the larger plasmid may be difficult, resulting in preferential replacement of the *thyA* gene by double crossing over and consequently the loss of the FHA/CR-1 antigen.

It is noteworthy that *erm*^{r} transformants of pDL276/*thyA*del*kan*FHA/CR-1 were able to express the FHA/CR-1 fusion protein (Figure 31), regardless of whether the single crossing over had occurred at upstream or downstream fragments of the *thyA* gene. The efficiency of protein expression in those transformants indicated that successful integration of the plasmid into the chromosome had been achieved.

The antibiotic marker-free vaccine delivery system in *S. gordonii* I developed is different from other delivery systems because other systems use the auxotrophic complementation as a selective pressure for plasmid maintenance. In my system, the auxotrophic complementation vector was used as a tool to deliver the vaccine antigen gene into the chromosome. Plasmid maintenance is important for persistent expression of the vaccine antigens in other systems. For plasmid maintenance, the gene must be

carefully selected so that the mutant's growth depends entirely on it and can not be compeomised by materials from the host. If the material exists in the host, the bacteria will not maintain the plasmid. A good example of this was reported by Ryan et al. (2000), who created an auxotrophic complementation system in *V. cholerae* based on auxotrophy in glutamine (*glnA)* and discovered the recovery of $glnA^+$ plasmid free *V. cholerae* in the stool of mice following oral immunization due to the presence of sufficient quantities of glutamine in the intestine. In contrast, our delivery system achieves complete stability of vaccine antigen expression without the potential for plasmid loss.

4.6. Conclusion

In summary, the results of this study indicate, for the first time, that auxotrophic complementation of the *thyA* gene can be used to deliver the vaccine antigen genes in *S. gordonii,* thereby circumventing the need for antibiotic resistance genes. This newly developed delivery system for the oral vaccine candidate *S. gordonii* can be used to deliver and express different vaccine antigen genes based on *thyA* complementation. The FHA/CR-1, vaccine antigen of *B. pertussis*, has been expressed on a *S. gordonii thyA* mutant, although the mutant strain was not the antibiotic marker-free *S. gordonii thyA* mutant.

For future work, it would be interesting to try to increase the probability of obtaining transformants with plasmids integrated into the chromosome by reconstructing the *S. gordonii thyA* mutant to have only one fragment of *thyA* gene, either upstream or downstream. The newly constructed *thyA* mutant should force single crossing over to

occur and eliminate double crossing over. Auxotrophic complementation of *thyA* will be an effective system for use in delivering the vaccine antigen gene from the plasmid to the chromosome of the live bacterial vector, *S. gordonii*.

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