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Development of an Oral Recombinant Chancroid Vaccine Delivered
by Attenuated *Salmonella typhimurium* SL3261

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**Development of an Oral Recombinant Chancroid Vaccine Delivered by Attenuated
Salmonella typhimurium SL3261**

A thesis submitted to the School of Graduate Studies
University of Ottawa

In partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

By
Cathy Breau

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ABSTRACT

Chancroid, a sexually transmitted genital ulcer disease caused by the Gram-negative bacterium *Haemophilus ducreyi*, facilitates the acquisition and transmission of HIV. An effective vaccine against chancroid has yet to be developed. We hypothesize that a *Salmonella* vector-based vaccine, expressing *H. ducreyi* antigens, could confer protective immunity in the rabbit model of *H. ducreyi* infection. The *H. ducreyi* outer membrane hemoglobin receptor HgbA has been shown to be a suitable vaccine candidate. HgbA was expressed from *S. typhimurium* SL3261 (pnirBhgbA) but not from the control strain, *S. typhimurium* SL3261 (pnirB). After a single dose or three doses, at two-week intervals of the vaccine, no antibody response to HgbA was detected in the rabbit model. The vaccine administered was immunogenic and survived *in vivo* passage. In this small animal trial, we were unable to induce protective immunity against chancroid. We conclude that the vaccine does not confer protective immunity against chancroid.

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LIST OF ABBREVIATIONS

A₆₀₀	Absorbance at 600 nm
amp	Ampicillin
ASC	Antibody-secreting cell
Asd	Aspartate β -semialdehyde dehydrogenase
CA	Chocolate agar
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CSW	Commercial sex worker
ddH₂O	Double distilled water
DHB	2,3-dihydroxybenzoate
DltA	<i>H. ducreyi</i> lectin A
DsrA	<i>H. ducreyi</i> serum resistance A
DTH	Delayed type hypersensitivity
ECA	Enterobacterial common antigen
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'-tetra-acetic acid
EIA	Enzyme immunoassay
FBS	Fetal bovine serum
flp	Fimbria-like protein
GUD	Genital ulcer disease
HIV	Human immunodeficiency virus
HgbA	Hemoglobin receptor
HlyA	Hemolysin A
IFN	interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
LB	Luria Bertani
LPS	Lipopolysaccharide
Lsp	Large supernatant protein
MAC	MacConkey
MHC	Major histocompatibility complex
Min	Minute

MLN	Mesenteric lymph node
Nca	Necessary for collagen association
NLF	Non lactose-fermenting
NZW	New Zealand White
OMP	Outer membrane protein
pAB	<i>p</i> -aminobenzoic acid
<i>pagC</i>	<i>phoP</i> activated gene C
PAGE	Polyacrylamide gel electrophoresis
PAL	Peptidoglycan-associated lipoprotein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PMN	Polymorphonuclear leukocytes
PPs	Peyer's patches
PVDF	Polyvinylidene fluoride
rHgbA	Recombinant HgbA
rPspA	Recombinant <i>Streptococcus pneumoniae</i> antigen
RT	Room temperature
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
STD	Sexually transmitted disease
TBE	Tris-borate-EDTA
TDRM	Temperature-dependent rabbit model
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGMS	Tris gelatin magnesium sulfate
<i>tetC</i>	tetanus toxin fragment C
Th	T helper
TNF	Tumor necrosis factor
TSI	Triple sugar iron
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Chancroid

Chancroid (reviewed in (173, 183, 209)) is a sexually transmitted genital ulcer disease caused by the Gram-negative bacterium *Haemophilus ducreyi*. As a strict human pathogen, *H. ducreyi* has no animal or environmental reservoir (32). *H. ducreyi* is thought to initiate infection within the genital skin through epidermal microabrasions that occur during sexual intercourse (32). A small inflammatory papule appears 4-7 days after inoculation. The papule progresses to a pustule within 2 or 3 days, followed by rupture of the pustule to form a painful nonindurated ulcer with undermined edges (146). The ulcer, which may be filled with yellow necrotic purulent exudates, has a granulomatous base that bleeds easily upon scraping (146). In up to 50% of cases, inguinal lymphadenopathy occurs and the lymph nodes may develop into buboes, which can then rupture to form inguinal ulcers (146). Extragenital lesions, likely from autoinoculation, can occur and are found on the inner thighs, breasts, fingers and oral mucosa (145, 209). *H. ducreyi* has not been shown to cause systemic infection (209).

1.1.1 Epidemiology

Chancroid is common in many developing countries of Africa, Asia and the Caribbean, where it has been shown to cause 13-62% of genital ulcer disease (GUD) in sexually transmitted disease (STD) clinic populations (9, 27, 47, 93, 166, 204, 205). Yet the true prevalence of chancroid in those resource-poor countries is unknown because of weak public health infrastructure, and the availability of diagnostic tests for *H. ducreyi* is often lacking. The World Health Organization (WHO) excludes chancroid from its STDs

prevalence estimates because of the poor understanding of the epidemiology and the lack of good laboratory testing (216). To get an idea of the magnitude of the chancroid problem, the WHO used the prevalence of syphilis and the ratio of syphilis to chancroid in previous estimates of GUD cases, and estimated in 1997 the annual prevalence of chancroid to 7 million cases worldwide (216).

Chancroid is considered an uncommon disease in North America and Europe, but sporadic outbreaks have occurred in the past. In the 1980s and early 1990s several epidemics were reported in the United States in New York, Louisiana, Florida, Mississippi, California, Georgia, and Texas (68, 86, 184, 209), and were typically associated with prostitution and the exchange of sex for crack-cocaine (209). Reported rates of chancroid in the United States have decreased by more than 80-fold between 1947 and 1997 (194). The shifting patterns of prostitution and the introduction of antibiotics, are two factors that contributed to this decline (194).

Commercial sex workers (CSWs) are the major reservoirs of *H. ducreyi* in chancroid-endemic regions (48, 54, 221). The estimated probability of transmitting chancroid during a single sexual exposure is 0.35 from an infected male to an uninfected female and 0.30 from an infected female to an uninfected male (209). Schmid *et al.* (184) reviewed the 1980s chancroid outbreaks in the United States and showed that the disease occurred primarily among heterosexuals, with a male/female ratio ranging from 3:1 to 25:1.

1.1.2 Chancroid and Human immunodeficiency virus (HIV)

There has been renewed interest in *H. ducreyi* and chancroid since the establishment of genital ulcerative diseases as important risk factors in the transmission of HIV. GUDs in general, are estimated to enhance the transmission of HIV by 10-300

times per sexual exposure (87, 172). There have been several studies showing that chancroid facilitates the transmission of HIV (40, 114, 161, 219). There is also a geographical association between chancroid and HIV infection, particularly in Africa. In 13 African countries where chancroid is common (prevalence of 9.8 to 62%), adult HIV prevalence ranges from 8 to 36 % (194).

Two major mechanisms have been proposed to explain this phenomenon: an increase in both the infectiousness of HIV and the susceptibility to HIV infection (145, 209). Chancroid ulcers increase the infectiousness because there is increased HIV shedding through the ulcers. HIV has been detected in chancroid ulcers from both male and female (117, 162). A study by Dyer *et al.* (59) also shows that there is an increased seminal fluid viral concentration in men with genital ulcer disease, which increases HIV shedding in those patients. Chancroid ulcers also increase the susceptibility to HIV infection because they disrupt the epithelium, providing a portal of entry for HIV, and increasing the number of HIV-susceptible cells (CD4⁺ T cells) at the point of entry. In fact, more CD4⁺ T cells and macrophages are found in the dermis of individuals experimentally infected with *H. ducreyi* (192).

In addition, HIV infections affect the clinical and treatment pattern of chancroid. First, increased numbers of genital ulcers have been described in HIV patients, and these lesions heal at slower rates (112, 162). Second, treatment of chancroid in HIV-infected patients is problematic because of slower healing and increased incidence of treatment failures (28, 132, 213, 214).

1.1.3 Prevention, control and treatment of chancroid

Abstaining from sexual intercourses or consistent correct use of condoms, are the preventive measures recommended by the WHO for prevention of sexually transmitted

infections (223). Simple topical hygiene measures are effective in reducing *H. ducreyi* transmission. Washing with soap and water within a few hours of sexual exposure was effective in reducing the risk of chancroid acquisition during the First World War (194). Male circumcision is also an important factor to lower the risk of acquiring chancroid (220) and HIV infection (102, 154, 215). There is currently no vaccine available for the prevention of chancroid.

Chancroid has features that make it a disease that can be contained. First, the very painful nature of the disease drives patients to seek medical care within a few weeks of ulcerative discomfort. Second, single-dose antibiotic therapies are available and effective at clearing the disease (194). Third, *H. ducreyi* has no non-human reservoir and requires a very active human sexual network to be sustained (194). Bong *et al.* (32) estimated that the sex-partner change rate required to maintain *H. ducreyi* within a population is at least 18 per year. The transmission dynamics suggest that, other than in a CSWs population, the disease is unlikely to be sustained in the general population (32). Therefore, an effort to eliminate *H. ducreyi* infection from CSWs by vaccination or therapeutic treatment, could control outbreaks or possibly eliminate chancroid (194).

The WHO recommends the following regimen for treating chancroid: erythromycin, 500 mg orally, 3 times daily for 7 days (128). The alternate regimens proposed by the WHO are the following: a single dose of ceftriaxone, 250 mg intramuscularly, or a single dose of ciprofloxacin, 500 mg orally, or a single dose of spectinomycin, 2 g intramuscularly (128). The Centers for Disease Control and Prevention (CDC) recommends mostly the same regimens but the frequency and/or duration differs from the WHO recommendation for the erythromycin (500 mg orally, 4 times daily for 7 days), and for the ciprofloxacin (500 mg orally, twice daily for 3 days) (128). The CDC also recommends a single dose of azithromycin, 1 g orally (128).

Treatment efficacy can be affected by several factors such as compliance, HIV infection, and antimicrobial resistance. For compliance reasons single-dose therapies are often preferred, and are highly effective (19, 134). As mentioned above, HIV infection increases the probability of treatment failures, and so HIV-infected patients should be carefully monitored. Antimicrobial resistance to many agents has been documented and are mostly plasmid-mediated (129). Plasmid-mediated resistance to sulfonamides (6), tetracycline (7, 136, 169), chloramphenicol (170, 181), ampicillin (37, 133, 203), and a dual streptomycin-kanamycin (180) resistance have been reported. Chromosomally mediated resistance is not well understood but decreased susceptibilities to ciprofloxacin, ofloxacin, trimethoprim, and penicillin in nonplasmid-containing *H. ducreyi* strains have been reported (147, 182).

Appropriate management of STDs in resource-poor countries has limitations due to the unavailability of laboratory diagnostic tests. Therefore, the WHO recommends a syndromic approach to diagnosis and management of STDs in these settings (53, 128). Basically this means the health care provider will treat the patient at the first visit with a combination of antimicrobials that will treat the most probable etiological agents (128). The guidelines for genital ulceration includes therapy for both chancroid and syphilis (128). This approach was proven to be successful in the management of genital ulcer disease in Rwanda (31). However, there is always a risk of failure due to expanding antibiotic resistance. A vaccine against chancroid, targeted to groups of high-frequency STD transmitters, could eliminate chancroid and may also decrease the incidence of HIV infections, without the risk of failure due to expanding antibiotic resistance or lack of treatment compliance. In areas of chancroid endemicity, the ideal vaccine would require no syringes, no cold chain distribution and could be delivered in a single dose.

1.1.4 A vaccine against chancroid

For effective vaccine development knowledge of the pathogenesis and the immune response to infection are necessary. Characterized virulence determinants, for use as potential vaccine candidates, are also essential. The recognition of chancroid as an important risk factor in the transmission of HIV has renewed interest in this formerly little-studied infection. This has resulted in identification of virulence determinants, development of animal models and a human model of disease, and also in a better understanding of the pathogenesis and the immune response to *H. ducreyi* infection. All of these will help make the development of a vaccine for chancroid more attainable.

1.2 *Haemophilus ducreyi*

H. ducreyi (reviewed in (5, 209)) is a Gram-negative bacillus with an average length of 1.2 to 1.5 μm and width of 0.5 μm (5). On solid media colonies are small, nonmucoid, yellow-grey, semi-opaque, adherent and can be pushed intact across the agar (5). In liquid culture *H. ducreyi* colonies are observed as streptobacillary forming parallel chains described as “railroads tracks” (5). The fact that the cells are adherent and clump together makes single-cell colony isolations and quantitation difficult (5).

H. ducreyi is a fastidious organism that has an absolute requirement for heme, because it lacks the enzymes involved in the conversion of delta-aminolevulinic acid to protoporphyrin, which is essential in the heme biosynthesis pathway (83). It also lacks the enzyme ferrochelatase, which catalyzes the insertion of heme by inserting iron into the porphyrin ring (122). *H. ducreyi* can fulfill its heme requirement by utilizing hemin, or the heme-containing proteins, bovine hemoglobin, human hemoglobin, and bovine catalase, but not equine cytochrome C₁₁₁ (122). The heme requirements of *H. ducreyi* are considerably higher than other heme-requiring *Haemophilus* species (5). The optimal

growth conditions for *H. ducreyi* are the following: a temperature between 33 to 35°C, in a water-saturated atmosphere with the addition of 5% CO₂, and a pH of 6.5 to 7.0 (5, 129, 209). The doubling time is between 1.8 to 4 hours, which is consistent with the 48-72 hours incubation time required for producing visible colonies (146).

1.2.1 Pathogenesis and the immune response to *H. ducreyi* infection

H. ducreyi is thought to initiate infection within the genital epithelium through epidermal microabrasions that occur during sexual intercourse (32). The dose of *H. ducreyi* required to initiate infection in natural disease is not known. However, an estimated delivered dose of 30 colony forming units (CFU) in the experimental infection of human volunteers results in papule and pustule formation rates of 90% and 50%, respectively (12). The mechanisms by which *H. ducreyi* attaches to host skin in the early stage of infection are not fully understood, but *in vitro* *H. ducreyi* has been shown to bind to extracellular matrix proteins of the skin, such as fibrinogen, fibronectin, type I and III collagen, gelatine, and laminin (2, 23).

Studies to describe the interactions of *H. ducreyi* with the host in both experimental infection (22, 24) and in natural infection (25) have been reported. In the experimental human model, *H. ducreyi* colocalizes with polymorphonuclear leukocytes (PMNs), macrophages, collagen, and fibrin within 48 hours, but does not colocalize with keratinocytes, fibroblasts, T cells, Langerhans' cells, laminin, or fibronectin (22). *H. ducreyi* is not phagocytosed throughout experimental infection and remains extracellular (22). Experimental infection of human volunteers only provides information about the first stages of disease because the infection is not allowed to progress to the ulcerative stage of disease, for subject safety reasons (32). Therefore, to understand bacterial-host interaction at the ulcerative stage of disease, Bauer *et al.* (25), examined specimens from

naturally occurring chancroid ulcers. Overall, they found similar results than in the experimental human infection model, the main difference observed was that *H. ducreyi* did not colocalize with collagen (25). Moreover, they were unsuccessful at staining for macrophages and T cells, so they were unable to determine if *H. ducreyi* colocalized with those cells (25).

There is evidence for humoral and cell-mediated immune responses mounted against an *H. ducreyi* infection. Antibodies to *H. ducreyi* are present in serum from naturally infected individuals (8, 36, 148), but this humoral response is not protective since re-infection may occur in the same patient. Histologically, naturally acquired chancroid ulcers demonstrate a T cell infiltrate, which are in majority CD45RO⁺, and a few B cells (113). The cellular infiltrate in the experimental human model is similar to the infiltrate seen in naturally occurring ulcers (32). Over the first 24 hours of infection, PMNs are recruited to the epidermis and the dermis, whereas T cells, macrophages and some B cells infiltrate the dermis (22, 157). The CD4/CD8 T cell ratio found in experimental infection is different from the ratio found in naturally occurring ulcers (32). In naturally occurring ulcers, equal numbers of CD4⁺ and CD8⁺ T cells are observed, whereas in experimental lesions, 60-80% of the T cells are CD4⁺ cells of the $\alpha\beta$ lineage and are CD45RO⁺, and 20-40% of the T cells are CD8⁺ (157).

In the experimental human model, Palmer *et al.* (157) measured cytokine production by reverse transcription- polymerase chain reaction (RT-PCR) of mRNA in biopsies, and saw that both papule and pustule contained interferon (IFN) - γ , interleukin (IL) -8 and tumor necrosis factor (TNF) - α mRNA. In addition to the T helper (Th) 1 cytokine (IFN- γ), other Th1 (IL-2) and Th2 (IL-4) cytokines mRNA were detectable. Most lesions contained mRNA for both Th1 and Th2 cytokines, some contained only Th1 cytokine mRNA, but no lesions contained only Th2 cytokine mRNA.

The mononuclear cell infiltrate and the presence of IFN- γ , IL-8 and TNF- α mRNA is suggestive of a delayed type hypersensitivity (DTH) cell-mediated immune response, but the volunteers have no prior exposure to *H. ducreyi*, which is a prerequisite in this type of immune response. Gelfanova *et al.* (76) hypothesized that because *H. ducreyi* shares antigenic determinants with other members of the *Pasteurellaceae* family, prior colonization with these organisms could have sensitized subjects, resulting in cross-reactivity of memory cells. This proposal was disproved when the same group demonstrated that *H. ducreyi*-specific T cell lines from lesions of experimentally infected human volunteers showed little response to antigens prepared from other members of the *Pasteurellaceae* family, but responded to a variety of *H. ducreyi* antigen fractions (76). This suggest that subjects sensitization to *H. ducreyi* antigens must occur during the course of the experimental infection (32, 76).

Desjardins *et al.* (55, 56) described the histopathology of lesions in the temperature dependent rabbit model of *H. ducreyi* infection at day 4, 10, 15, and 21 post-inoculation. They reported PMNs and histiocytic infiltrate at day 4 and 10, followed by a major lymphoid cell infiltrate at day 15 and a heavy histiocyte infiltrate with very few plasma cells at day 21 (55). Immunocytological analysis also demonstrated that CD5⁺ T cells (the rabbit equivalent of human CD4⁺ T cells) were predominant by day 15 (56).

1.2.2 Animal models

Numerous *in vitro* and *in vivo* models have been used to study *H. ducreyi* infections (209). The *in vitro* models are reviewed elsewhere (209) and will not be discussed here. For testing vaccine candidates, a model that can be used as a quantitative *in vivo* virulence assay is essential. *In vivo* models for the study of chancroid that have been reported include, mice, humans, primates, swine, and rabbits (129, 209).

A mouse model for studying disease is a good tool because the immunology of mice is well understood. The first attempt to develop a mouse model was reported by Tuffrey *et al.* (211), but two years later they demonstrated that ulcer development in their mouse model was not produced by viable organisms or by *H. ducreyi*, as either heat-killed *H. ducreyi* cells or *Neisseria gonorrhoeae* produced the characteristic lesions (212). Trees *et al.* (208) described a mouse subcutaneous chamber model for studying long-term *in vivo* growth of *H. ducreyi*, but to our knowledge this model has never been used for chancroid vaccine studies.

Spinola *et al.* (192) have developed a safe and reliable *H. ducreyi* infection model in humans, which has been very useful in evaluating virulence (11, 34, 99, 120). Human volunteers are inoculated with *H. ducreyi* 35000 on the skin of the upper arm, using a multi-test allergy testing device (192). The model only provides information about the initial papular and pustular stages of disease, because the lesions are not allowed to progress to the ulcerative stage (32). This model is limited for vaccine development studies for ethical reasons that preclude the initial testing of prospective vaccines in humans.

A primate model was developed by Totten *et al.* (206) where adult pigtailed macaques (*Macaca nemestrina*) were inoculated with 10^7 to 10^8 CFU of *H. ducreyi* on the foreskin (males) or the vaginal labia (females). The model was effective in male macaques, which developed ulcers that resemble those seen in humans, but not in female macaques, which did not develop any ulcers (206). Some limitations of this model include limited number of inoculum sites per animal and the high cost of primate husbandry.

A swine model was developed by Hobbs *et al.* (90) in which the ears of pigs were inoculated with 10^7 CFU of *H. ducreyi* with a multi-test allergy testing device. Cutaneous

ulcers histologically resembling human chancroid lesions were observed (90). Cost is also a limiting factor for using this model.

In a rabbit model, Purcell *et al.* (164) investigated whether reducing rabbit skin temperature would facilitate bacterial replication and lesion development. This is based on the observation that optimal *in vitro* growth of *H. ducreyi* occurs at 33-35°C (209). They achieved ulcerative disease after intradermal inoculation with 10⁵ CFU of *H. ducreyi* on shaved backs of rabbits housed at 15-17°C, but not in rabbits housed at 23-25°C (164). Ulcer development was dependent on both viable inoculum and bacterial replication (164). This temperature-dependent rabbit model (TDRM) was later developed for use as an *in vivo* quantitative virulence assay (142), and further adapted by Desjardins *et al.* (55, 56) for the study of inducible immunity.

Both the swine model and the TDRM have been used for vaccine studies (3, 50, 55, 197, 201) and are suitable animal models for the study of chancroid, yet the lower cost of rabbit husbandry and ease of handling, persuaded our laboratory to use the TDRM. The TDRM has several attributes that make it a suitable model for the study of chancroid vaccine development. First, the lesions produced are histologically similar to the ones in natural and experimental human infections (55, 164, 192). Second, the nature and course of the disease is similar to human chancroid (56). Over a period of three weeks lesions develop sequentially an acute suppurative inflammation, a necrotic skin ulceration, sterilization, and reepithelialization during convalescence (56). Third, up to five titred doses that are quantified by viable counts can be used in triplicate on the rabbits back. Intraepithelial injections with a tuberculin syringe permit the confirmation of the inoculum size by doing viable counts with the residual inocula. Finally, the disease can be measured. Three measures of disease severity are recorded: lesions size, lesion score, and culture positivity to *H. ducreyi* over a period of 21 days.

1.2.3 Type of immune responses required for the development of a protective chancroid vaccine

It is generally accepted that humans mount what appears to be a DTH cell-mediated immune response to *H. ducreyi* during natural infection. However, this response is ineffective at preventing subsequent infections since the same patient can acquire chancroid again. Then what kind of immune response could confer protective immunity against chancroid?

The fact that *H. ducreyi* is an extracellular pathogen suggest that a humoral immune response might be effective at conferring protective immunity against chancroid (98). Two studies evaluated the role of a humoral response in protection against *H. ducreyi* infection by passive immunization experiments in rabbits (56) and in pigs (50). In the TDRM, passive transfer of purified polyclonal immunoglobulin G (IgG) from immune sera raised against *H. ducreyi* 35000 whole-cell lysate, did not protect animals against challenge with *H. ducreyi* (56). However, passive transfer of immune sera raised against *H. ducreyi* 35000 after multiple exposures to the organism, protected naïve pigs against challenge with *H. ducreyi* (50). This suggest that, at least in pigs, an effective humoral immune response to *H. ducreyi* can be mounted after multiple exposures to the organism (50).

1.2.4 Virulence determinants

Studies to test the role of putative virulence determinants of *H. ducreyi* have been conducted in the TDRM (195-197, 218), the swine model (49) and the experimental human challenge model (10, 11, 20, 33, 34, 70, 72, 99-101, 120, 158, 189, 202, 225-227), where chancroid disease development is compared between parent and mutant strains.

Isogenic mutants of putative virulence determinants of *H. ducreyi* that were found to be attenuated or partially attenuated are listed below:

Hemoglobin receptor (HgbA) is an outer membrane receptor of the TonB-dependent family involved in heme uptake from hemoglobin (60, 61, 197). It has a calculated molecular weight of 108.6 kDa and its expression is regulated by heme (197). An isogenic mutant of *hgbA* was less virulent than wild-type strain in the TDRM (197) and was attenuated in the human model of experimental *H. ducreyi* infection (11).

Peptidoglycan-associated lipoprotein (PAL) is an 18 kDa outer membrane protein (OMP) with homology to *Haemophilus influenzae* protein 6 (190, 191). An isogenic mutant of PAL was attenuated in the human model of experimental *H. ducreyi* infection (70).

***H. ducreyi* serum resistance A (DsrA)** is a 30 kDa OMP whose expression is required for serum resistance (62). An isogenic mutant of *dsrA* was less virulent than wild-type strain in the swine model of chancroid infection (49) and was attenuated in the human model of experimental *H. ducreyi* infection (34).

***flp* (fimbria-like protein) operon** is a 12.8 kb gene cluster which contains 15 genes and is necessary for microcolony formation *in vitro* (152). A polar mutation in the ninth gene (*tadA*) of the *flp* cluster was slightly attenuated in the TDRM and was attenuated in the human model of experimental *H. ducreyi* infection (189).

Large supernatant protein LspA1 and LspA2 are proteins with predicted molecular weights of 456 and 543 kDa, respectively, and 86% identity (218). An *lspA1 lspA2* double mutant was less virulent than the wild-type strain in the TDRM (218) and was attenuated in the human model of experimental *H. ducreyi* infection (101).

NcaA (necessary for collagen association) is a collagen binding OMP with a predicated molecular weight of 33 kDa (72). Its amino acid sequence is similar to that of

oligomeric coiled adhesins (Oca) a subfamily of the type Vc surface-attached oligomeric autotransporter family (72). A *ncaA* mutant was less virulent than the wild-type strain in the swine model and was attenuated in the human model of experimental *H. ducreyi* infection (72).

***H. ducreyi* lectin A (DltA)** is a 22 kDa OMP that binds fibronectin and plays a role in the organisms evasion from complement-mediated serum killing (121). A DltA mutant was partially attenuated in the human model of experimental *H. ducreyi* infection (99).

WeeA is an enzyme involved in the synthesis of enterobacterial common antigen (ECA), a complex carbohydrate produced by enteric organisms (65). The *H. ducreyi* *wecA* gene is located in a cluster of homologues genes involved in the synthesis of ECA but an ECA-like glycoconjugate in *H. ducreyi* remains to be found (20). A *wecA* mutant was partially attenuated in the human model of experimental *H. ducreyi* infection (20).

1.3 Live attenuated bacteria as vaccine vectors

Live attenuated viral and bacterial vaccines are often considered the most successful human vaccines since they can elicit long-lasting immunity after one or two immunization (39). Bacterial vectors are attractive vaccine vectors because by mimicking natural infections, they generate mucosal, humoral and cellular immune responses (115). Furthermore, bacterial vectors act as intrinsic adjuvant because of immunostimulatory molecules, such as lipopolysaccharide (LPS) and flagella, located on their cell surface (115). Three live attenuated bacterial vaccines are available commercially: *Salmonella enterica* serovar Typhi Ty21a, *Vibrio cholerae* CVD 103-HgR, and *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), which protect against typhoid fever, cholera, and tuberculosis, respectively. Even though many live attenuated bacterial strains have been

successfully used as vaccine vectors to deliver heterologous antigens in animal and human studies, no such vaccine is currently licensed (115).

The advantages of using live attenuated bacteria as vaccine vectors include their simple and low cost of production, their stability without refrigeration (via lyophilization), their simple and safe oral administration, and their potential for increasing compliance through single dose vaccination (39, 115). Different bacterial vectors have been used such as *V. cholerae*, *Shigella*, *Listeria*, and *Salmonella* (115, 139, 153, 178). *Salmonella* strains were among the first to be used and are still the most widely used bacterial vectors (71, 115).

1.3.1 Recombinant expression of pathogen-derived antigens in attenuated

***Salmonella* strains**

Several attenuated *Salmonella* oral vaccines have been constructed to deliver heterologous antigens from other pathogens, for instance antigens of HIV, *Eimeria tenella*, *Streptococcus mutans*, *Helicobacter pylori*, hepatitis B virus, *V. cholerae*, Enterotoxigenic *E. coli* (ETEC), *Bacillus anthracis*, *Clostridium tetani*, *Streptococcus pneumoniae*, *Leishmania major*, and *Shigella dysenteriae* (14, 15, 17, 45, 80, 94, 111, 116, 141, 150, 151, 163, 198, 224). Many of those studies were successful at delivering protective recombinant heterologous antigens (15, 45, 80, 141, 151, 198, 224). Different recombinant expression systems were developed to balance both the need for immunogenicity with the requirement for attenuation in the *Salmonella* strain and the need for antigen stability in the vector with the requirement for high-level expression to ensure immune recognition of the antigen.

1.3.1.1 Immunogenicity and attenuation in *Salmonella* strains

Bacterial strains are attenuated in order to render them avirulent while maintaining their immunogenicity. The commercially available live oral typhoid fever vaccine, is a preparation of *S. typhi* Ty21a, which was created by chemically attenuating the virulent *S. typhi* strain Ty2 (77). The strain still contains uncharacterized mutations and requires multiple doses to induce acceptable levels of protection (67). In an analysis of studies on vaccine efficacy by Engels *et al.* (64), the three year cumulative efficacy for three doses of the Ty21a vaccine was 51% (ranging from 35% to 63%). Therefore, many researchers focused on developing better *Salmonella* strains to elicit protective immunity in a single dose by introducing defined mutations into the bacterial genome.

Among the first described was *Salmonella typhimurium* SL3261, a *aroA* mutant of the virulent strain *S. typhimurium* LT2 (92). The mutation disrupts the aromatic biosynthetic pathway blocking the synthesis of chorismate, the final product of the pathway. This renders the organism auxotroph for two metabolites; *p*-aminobenzoic acid (*p*AB) and 2,3-dihydroxybenzoate (DHB), which are normally synthesized from chorismate (92). Since the chorismate pathway is absent in human and mammals, these essential metabolites are not available in mammalian tissues resulting in *in vivo* attenuation of the organism (67). *S. typhimurium* SL3261 is avirulent in mice and vaccination by intraperitoneal injection of live bacteria in mice has been shown to confer protection against a virulent strain of *S. typhimurium* (92).

Other attenuations in *Salmonella* strains that were constructed include mutations that alter important regulatory genes (e.g., *cya/crp*, *phoP/phoQ*), mutations in secretory function (e.g., *ssaV*), mutations in stress-induced proteins (e.g., *htrA*), and mutations in porins (e.g., *ompR*) (46, 57, 67, 115).

Curtiss *et al.* (52) constructed an attenuated *S. typhimurium* strain by the introduction of mutations in the adenylate cyclase (*cya*) and the cAMP receptor protein (*crp*) genes. These genes are required for the transcription of genes involved in carbohydrate and amino acid metabolism (67). The attenuated strain was avirulent in mice and was also protective against subsequent challenge with the virulent wild-type strain (52). The *S. typhimurium* *phoP/phoQ* system regulates the expression of both virulence genes and genes involved in the survival of the organism within macrophages (143). The *pagC* (*phoP* activated gene C) is one of the genes regulated by the *phoP/phoQ* system, whose constitutive expression attenuates virulence of *S. typhimurium* (144).

An *aroC* mutation combined with a mutation in a specific gene (*ssaV*) of the *Salmonella* pathogenicity island 2 type III secretion system, was tested by Hindle *et al.* (89). *S. typhi* (Ty2 Δ *aroC* Δ *ssaV*) and *S. typhimurium* (TML Δ *aroC* Δ *ssaV*) were orally administered to human volunteers and evaluated for their safety and immunogenicity. Volunteers received single escalating doses of the constructs and both strains were well tolerated. Volunteers elicited anti-serovar Typhi or Typhimurium LPS immunoglobulin A (IgA) antibody-secreting cell responses, and LPS-specific serum IgG. However, the serovar Typhi provided better immune responses than the serovar Typhimurium (89).

HtrA is a periplasmic protease that degrades malformed proteins and may contribute to the survival of *S. typhimurium* in murine macrophages (26, 103). An *htrA* mutant of *S. typhimurium* was attenuated in mice and was protective against challenge with wild-type strain following a single oral dose of *S. typhimurium* Δ *htrA* (46). OmpR is a positive regulator for the expression of *ompC* and *ompF*, genes that encode two of the three major porins of *S. typhimurium* (57). An *ompR* mutant of *S. typhimurium* was attenuated in mice and orally immunized mice were protected against a challenge with wild-type strain (57).

1.3.1.2 Optimal antigen expression

The efficiency of a recombinant *Salmonella* vaccine relies on obtaining high-level expression of the heterologous antigen *in vivo* to ensure optimal presentation of the antigen to the host immune system. However, high-level expression of foreign proteins from high-copy number plasmids, often results in rapid plasmid loss from the vaccine vector. This is due to the metabolic burden that this high expression imposes on the vector (74). To maintain and express the foreign DNA, the cell must utilize its own resources, such as energy (ATP or GTP) and metabolites (amino acids) (78). This generates a selective pressure on the vaccine vector that can result in plasmid-less bacteria outgrowing the population of plasmid-bearing bacteria (74). Plasmid size and copy number and the rate of gene expression shape the magnitude of this burden (74, 75, 188).

An alternative strategy to express recombinant antigens, which eliminates plasmid loss issues, is chromosomal integration. The gene and a suitable promoter are cloned into a suicide vector and introduced into the chromosome by homologous recombination (35). The gene is stably expressed from the chromosome, but the level of antigen produced is low since a single copy of the gene is inserted (168). This often negatively affects the extent of the immune response resulting in a lack of protective immunity (168, 199).

Different approaches have also been developed to improve plasmid stability in the vaccine vector. Balanced-lethal systems ensure that any bacterium replicating contains a plasmid because the plasmid encodes a specific protein essential for bacterial survival. Plasmid loss would result in the death of the bacterium (75). Two balanced-lethal systems have been previously described, the *asd* system and the *hok-sok* system (115). Asd (aspartate β -semialdehyde dehydrogenase), an enzyme involved in the synthesis of structural components of the cell wall of gram-negative bacteria, is deleted from the

chromosome of the bacterial vector to then complement the lethal mutation with a plasmid encoding the *asd* gene (75, 149). This system has been used in attenuated *S. typhimurium* and *S. typhi* to express a variety of antigens in both murine and human studies (51, 109, 150, 193). Similarly, the *hok-sok* system is a naturally occurring post-segregational killing system on the *E. coli* antibiotic resistance factor pR1 (75, 115). This toxin-antitoxin system works by supplying the cell with sufficient intracellular levels of antisense *sok* mRNA, which will bind to *hok* mRNA, so that synthesis of the lethal pore-forming Hok protein is blocked. *sok* mRNA is highly susceptible to degradation by nucleases while *hok* mRNA is more stable. Therefore, when the bacterium loses the *hok-sok* plasmid the levels of the protective *sok* mRNA drop and Hok synthesis occur, which results in cell death (75, 115). This system has been less successful than the *asd* system (115).

Since plasmid stability is directly influenced by the amount of antigen produced, which in turn is influenced by the promoter, choosing the right promoter is important. In vivo-inducible promoters are particularly appealing. The protein expression from these promoters is limited to certain sites because they are activated by specific environmental stimulus. Ideally, a promoter activated only when bacteria reaches the intracellular environment of host cells would be desirable. The well-characterized *nirB* promoter from the nitrate reductase gene of *E. coli* is induced by nitrites in the environment or by anaerobic conditions (159). The promoter was modified to retain its capacities of induction by anaerobic conditions but not by nitrites (156). Chatfield *et al.* (45) first used this modified *nirB* promoter to regulate the expression of *tetC* (tetanus toxin fragment C) in attenuated *S. typhimurium* and showed that a single-dose of this vaccine was protective against tetanus toxin challenge in mice. The *nirB* promoter is also induced by entry of *Salmonella* into eukaryotic cells, including macrophages (66). This promoter was used in

different studies to express a series of antigens and also antigens fused to C fragment (13, 21, 43, 73, 79, 110, 131).

Studies to evaluate the efficacy of other in vivo-inducible promoters have also been conducted. Dunstan *et al.* (58) evaluated the ability of three in vivo-inducible promoters (*nirB*, *pagC*, and *katG*) to stabilize antigen expression in *S. typhimurium* and as a result, potentially enhance the immunogenicity of the vaccine. Promoter *pagC*, induced by low Mg^{+2} concentration, was most efficient at eliciting a humoral response against TetC in mice (58). The promoter for the stress response gene *htrA* was compared to the *nirB* promoter for directing the expression of TetC, and found to be equally protective against lethal tetanus toxin challenge when in *S. typhimurium* ($\Delta aroA \Delta aroD$) (168), but more protective when in *S. typhimurium* ($\Delta aroA \Delta htrA$) (167). Another less commonly used anaerobically induced promoter, *dmsA*, was evaluated by Orr *et al.* (155). They expressed tetC under the control of a derivative promoter of *dmsA* (P_{dmsA3}) in *S. typhi* CVD 908-*htrA* and demonstrated that the vaccine provided protection against lethal tetanus toxin challenge in mice (155).

1.3.1.3 Location of the recombinant antigen

The cellular location of the recombinant antigen within the *Salmonella* vaccine vector is also something to consider for efficient presentation to the host immune system. The heterologous antigens can be expressed within the cytoplasm, in the periplasm, at the cell surface or can be secreted into the extracellular environment of the bacterial vector (74, 115). Several groups have suggested that a recombinant antigen secreted from the cytoplasm of *Salmonella* vaccines would be more immunogenic. The latter is supported by two studies that reported that secretion of an antigen out of the bacterial vector via a *Salmonella* type III secretion system (176) or via an *E. coli* hemolysin A (HlyA) export-

system conferred protective immunity, whereas the cytoplasmic expression of the antigen did not. Another study where recombinant *S. pneumoniae* antigen (rPspA) was expressed from attenuated *S. typhimurium* compared expression in the cytoplasm with secretion into both the periplasm and culture supernatant (106). Mice that received the strain bearing the secreted antigen had anti-rPspA IgG titer 10^4 times higher than those who received the strain bearing the cytoplasmic antigen (106).

However, a study by Haddad *et al.* (81) suggest that while surface display of the antigen requires less amounts than the intracellular location to elicit immune responses, it may not be absolutely required. In their study a malarial antigen was targeted to the periplasm or at the bacterial cell surface of *S. typhimurium* (Δ aroA), and both were equally immunogenic, even though surface expression was 10-100 times lower than periplasmic expression (81). While in some systems protective immune responses are improved with surface display and secretion, cytoplasmic expression can also elicit protective immunity. For example, TetC is soluble in the cytoplasm (156) and provides protection against tetanus toxin challenge (45).

Engineering surface expression and secretion export systems is complex and has limitations. Surface expression, especially at high levels, may compromise the integrity of the cell membrane, whereas secretion techniques introduce more metabolic burden on the bacterial vector from the expression of the secretion system itself (74).

1.3.2 The immune response to attenuated *S. typhimurium* vaccines

Naïve mice inoculated with attenuated *S. typhimurium* are capable of clearing infection and are protected against subsequent infection with virulent *Salmonella*. These two models have been used to study the immune responses to attenuated *S. typhimurium*. The class II major histocompatibility complex (MHC II), T cells expressing the α and β

chains of the T cell receptor ($\alpha\beta$ T cells), and the gamma interferon receptor (IFN- γ R) have been shown to be necessary for clearing infection in mice (88). In contrast, the class I major histocompatibility complex (MHC I) and T cells expressing the γ and δ chains of the T cell receptor ($\gamma\delta$ T cells) are not necessary for clearing infection in mice (88). This emphasizes the role of CD4⁺ T cells in the clearance of attenuated *S. typhimurium* infections. Furthermore, depletion of CD4⁺ T cells or of Th1-like cytokines (IFN- γ , TNF- α , and IL-12) in mice previously vaccinated with *S. typhimurium* SL3261 impairs their resistance to virulent *Salmonella* infections (137, 138). McSorley *et al.* (140) evaluated the role of B cells in clearance of infection with attenuated *S. typhimurium* and in vaccine-induced resistance to virulent *Salmonella* infections. They demonstrated that clearance of attenuated *S. typhimurium* requires co-stimulation via the surface molecule CD28, and is B cell independent. Long-lasting antibody responses however, are induced to attenuated *S. typhimurium* (140). For the vaccine-induced resistance to virulent *Salmonella* infections, they demonstrated that serum antibody produced by B cells during primary infection was required for subsequent protection (140). CD8⁺ T cells might also be involved, since CD8⁺ T cells are induced in response to malarial antigens expressed from attenuated *Salmonella* and are required for protection against the virulent organism (4).

Following oral vaccination, specific antibody-secreting cells (ASC) appear in human blood (107). Kantele *et al.* (108) demonstrated that following oral vaccination with attenuated *S. typhi* Ty21a, ASC express homing receptors guiding them to mucosal surfaces, their final effector site.

The protective immune response to attenuated *S. typhimurium* SL3261 (Δ aroA) was described as a Th1 cellular response because of the high levels of IL-2 and IFN- γ detected, with serum antibodies of the IgG2a subclasses (85). However, another group

described the immune response against a heterologous antigen delivered by attenuated *S. typhimurium* SL3261 ($\Delta aroA$) as a Th1 cellular response with a Th2 component because they detected significant levels of a Th2 type cytokine (IL-5) (43). The antibodies detected in the orally immunized mice were of the IgG1, IgG2a and IgA subclasses (43).

1.3.3 Application of live recombinant attenuated *Salmonella* to chancroid vaccine development

Most of the studies that were successful at delivering protective recombinant heterologous antigens from other pathogens, were conducted in mice models (15, 45, 80, 141, 151, 198, 224). In our laboratory we use the rabbit model of chancroid, as there is no mouse model for chancroid. In the rabbit model of chancroid, we have determined that it is feasible to use oral attenuated *Salmonella* as a vector for the delivery of recombinant antigens in rabbits. The study revealed a framework that uses attenuated *S. typhimurium* SL3261 as a vector for an unrelated recombinant antigen (TetC expressed from a plasmid; pTETnir15) in the TDRM of *H. ducreyi* infection (16). Here we use pTETnir15 and *S. typhimurium* SL3261 to construct a *Salmonella* vector-based *H. ducreyi* vaccine expressing an *H. ducreyi* antigen.

For construction of the recombinant chancroid vaccine a proper vaccine candidate was necessary. We chose the HgbA protein, which is necessary for virulence in humans and was recently shown to protect pigs against subsequent *H. ducreyi* infections (3).

1.4 Hypothesis

The hypothesis of this research is that a *Salmonella* vector-based *H. ducreyi* vaccine expressing *H. ducreyi* antigens confer protective immunity against *H. ducreyi* infection in the temperature-dependent rabbit model (TDRM).

1.5 Objectives

To address this hypothesis, we have pursued the following objectives.

- 1) Cloning the *hgbA* gene into pTETnir15 in place of the *tetC* gene.
- 2) Introducing the recombinant plasmid into attenuated *S. typhimurium* SL3261.
- 3) Assessing the ability of the *S. typhimurium* recombinant vaccine strain to produce protective immunity against chancroid in the TDRM of *H. ducreyi* infection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Strains and culture conditions

H. ducreyi 35000 was isolated from a 1975 chancroid outbreak in Winnipeg, Canada (84) and was subcultured from stock cultures frozen at -70°C onto chocolate agar (CA) plates [GC Medium Base (Difco/Becton Dickinson, Sparks, MD, USA) with 1% (w/v) bovine hemoglobin (BBL/ Becton Dickinson, Cockeysville, MD, USA), supplemented with 1% (v/v) IsoVitalax (BBL/ Becton Dickinson, Cockeysville, MD, USA) and 5% (v/v) heat inactivated fetal bovine serum (FBS; Gibco/Invitrogen, Burlington, ON, Canada)] for 24 to 48 hours at 35°C in an atmosphere of 5% CO_2 and saturated humidity prior to inoculation of GC broth [1.5% (w/v) Protease peptone 3 (Difco/Becton Dickinson and Company, Sparks, MD, USA), 0.4% (w/v) potassium phosphate dibasic (Sigma-Aldrich, Oakville, ON, Canada), 0.1% (w/v) potassium phosphate monobasic (EMD Chemicals Inc., Gibbstown, NJ, USA), 0.5% (w/v) sodium chloride (NaCl; BDH Inc., Toronto, ON, Canada), 0.1% (w/v) starch (EMD Chemicals Inc., Gibbstown, NJ, USA), supplemented with 5% (v/v) FBS and 1% (v/v) IsoVitalax]. Broth cultures were grown at 35°C in an atmosphere of 5% CO_2 for 16-20 hours with shaking at 225 rpm.

S. typhimurium SL3261 (*his* G46 (del) *aroA* 554) (92), *S. typhimurium* LB5010 (*galE* r- m+) (38), phage P22 (HT int-) (185, 186), and phage P22.c2 (c2 mutation) (125, 228) were purchased from the *Salmonella* Genetic Stock Centre (SGSC/University of Calgary, Calgary, AB, Canada). Plasmid pTETnir15 (amp^r) was kindly supplied by Dr. D. Pickard (The Wellcome Trust Sanger Institute, Hinxton, UK). *E. coli* strain BL21 (DE3), pUNCH 672 pLysS and pUNCH 1202 pLysS (kan^r , Cm^r) (63) were kindly

provided by Dr. C. Elkins (University of North Carolina, Chapel Hill, NC, USA). *S. typhimurium* and *E. coli* were grown on Luria Bertani (LB) agar plates [LB broth (Difco/Becton Dickinson, Sparks, MD, USA), with 1.5% (w/v) Bacto-agar (Difco/Becton Dickinson, Sparks, MD, USA)] for 16-20 hours at 37°C, or in LB broth. LB broth cultures were inoculated from a single colony isolated from stock cultures frozen at -70°C or, inoculated directly from -70°C frozen stock cultures, and grown at 37°C with shaking at 225 rpm for 16-20 hours. When required, antibiotics were added to the following concentrations: ampicillin (IBI-Shelton Scientific, Peosta, IA, USA) (LB+amp) 100 µg/mL, chloramphenicol (Sigma-Aldrich, Oakville, ON, Canada) 20 µg/mL and kanamycin (MP Biomedicals, Solon, OH, USA) (LB+cm;kan) 30 µg/mL. For anaerobic growth, an overnight LB broth culture was diluted 1:100 in fresh LB broth and then incubated with agitation by a magnetic stir bar in an anaerobic jar with AnaeroGenTM (Oxoid/Thermo Fisher Scientific, Nepean, ON, Canada) sachets at 37°C. An anaerobic indicator (Oxoid/Thermo Fisher Scientific, Nepean, ON, Canada) was placed inside the jar to confirm that an anaerobic atmosphere was achieved.

2.2 Molecular biology techniques

2.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described previously (18) where agarose (Invitrogen, Burlington, ON, Canada) was added to a final concentration of 1% (w/v) in 1x TBE buffer [1.08% (w/v) Tris Base (Acros Organics, Morris Pla, NJ, USA), 0.55% (w/v) Boric Acid (Bioshop Canada Inc., Burlington, ON, Canada), 0.4% (v/v) of 0.5M ethylene diamine tetraacetic acid (EDTA) (Sigma-Aldrich, Oakville, ON, Canada), pH 8.0]. Prior to gel loading, 2 µl of 10x gel loading buffer [30% (v/v) glycerol (EMD Chemicals Inc., Gibbstown, NJ, USA), 70% (v/v) 5mM EDTA, pH 8.0, 2.5% (w/v)

bromophenol blue (Fisher Scientific, Ottawa, ON, Canada), 2.5% (w/v) xylene cyanol (Sigma-Aldrich, Oakville, ON, Canada)] was added to each DNA sample. Gels were electrophoresed at 90 to 110V using a Hoefer HE Mini Submarine (Amersham Biosciences, Piscataway, NJ, USA) or a Horizontal Electrophoresis Systems model FB-SB-1316 (Fisher Scientific, Ottawa, ON, Canada) and DNA bands were visualized by adding 0.005% (v/v) ethidium bromide (Invitrogen, Burlington, ON, Canada) to the melted agarose prior to casting. Agarose gels were visualized and photographed under UV light using a Multimage Light Cabinet (Alpha Innotech Corp., San Leandro, CA, USA).

2.2.2 DNA digestion

Plasmid DNA or DNA fragments obtained from polymerase chain reaction (PCR) amplification were digested, when necessary, with restriction endonucleases purchased from New England BioLabs (Ipswich, MA, USA) according to manufacturer's conditions.

2.2.3 Purification of DNA fragments

DNA fragments obtained from restriction enzyme digests were separated by agarose gel electrophoresis and fragments of interest were recovered using the QIAquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada), following manufacturer's instructions. DNA fragments obtained from PCR amplification were purified using the QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON, Canada), following manufacturer's instructions.

2.2.4 DNA isolation/quantification and DNA sequencing

Genomic DNA was isolated using a genomic DNA extraction kit (Qiagen Inc., Mississauga, ON, Canada) and plasmid DNA was recovered using a QIAprep Spin Miniprep kit (Qiagen Inc., Mississauga, ON, Canada), as directed by the manufacturer's protocol. DNA concentrations were estimated by comparing an equivalent volume of DNA preparation to the High DNA Mass Ladder (Invitrogen, Burlington, ON, Canada) on an agarose gel.

Extracted plasmid DNA was suspended in sterile double distilled water (ddH₂O) to a final concentration of 12.5 ng/μL and sent to the Ontario Genomics Innovation Centre (OGIC; Ottawa, ON, Canada) for fluorescent DNA sequencing with the Applied Biosystems 3730 DNA Analyzer. For sequencing the expression vector (pnirL) (see below) the following primers were used: forward 5'-CGTATCACGAGGCCCTTTC-3'; reverse, 5'-GTCCTCAACGACAGGAGCAC -3'. For sequencing the *hgbA* gene inside the *S. typhimurium* SL3261 clone the following primers were used: forward 5'-ACATTTCCCCGAAAAGTGC-3'; and the same reverse primer as above (5'-GTCCTCAACGACAGGAGCAC -3').

2.2.5 PCR amplification

PCR amplification using plasmid DNA or genomic DNA as template was performed in 25 μL reaction volume, containing 1x PCR buffer, 0.6 units of Platinum[®] *Taq* DNA polymerase or 2.5 units of *Pfx50*[™] DNA Polymerase, 0.2mM (for PCR with Platinum[®] *Taq*) or 0.3mM (for PCR with *Pfx50*[™]) dNTPs (Invitrogen, Burlington, ON, Canada), 0.5μM of each primer and 2 ng of plasmid DNA or 25 ng of genomic DNA. The PCR was amplified using a Touchgene Gradient Thermocycler (Techne, Burlington, NJ, USA) or a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA,

USA). The following conditions were used for the amplification of the *hgbA* gene: an initial denaturation for 3 min at 94°C, 35 cycles of denaturation at 94°C for 15s, annealing at 68°C for 30s, extension at 68°C for 3 min and a final extension step at 68°C for 5 min. To confirm the presence of plasmid in the transduced *Salmonella* strain SL3261, the same conditions were used with the following modifications: 30 cycles of denaturation at 94°C for 30s, annealing at 53°C for 45s, extension at 72°C for 3 min and the final extension step at 72°C. When transformants were analyzed by colony PCR, the PCR conditions listed above were used. Selected colonies were suspended in the PCR reaction solution and incubated at 100°C for 10 min to release the plasmid DNA. The mixture was cooled for 10 min prior to the addition of the Platinum[®] *Taq* polymerase. All primers were designed using Primer3 design tool (174).

2.2.6 Transformation of *E. coli*

OneShot[®] TOP10 chemically competent *E. coli* cells or DH5a *E. coli* competent cells (Invitrogen, Burlington, ON, Canada) were transformed using the calcium chloride method (179) according to the manufacturer's instructions. Briefly, one vial of competent cells was thawed on ice, and 1.5 µL of ligation reaction was added to 25 or 50 µL of thawed cells. The mixture was incubated on ice for 30 min prior to subjecting to heat shock at 42°C for 30s followed by the addition of 250 µL of SOC medium. The reaction mixture was shaken at 200 rpm for 1 hour at 37°C. Two different volumes (20 and 100 µL) of the transformed cells mixture was plated onto pre-warmed LB+amp agar plates. After overnight incubation at 37°C, several transformants were selected for further analysis.

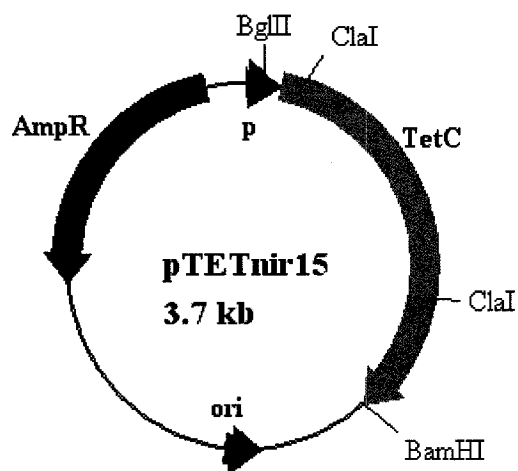
2.3 Production and characterization of the recombinant *Salmonella* strains

2.3.1 Construction of pnirL, cloning of *hgbA*, and construction of pnirB

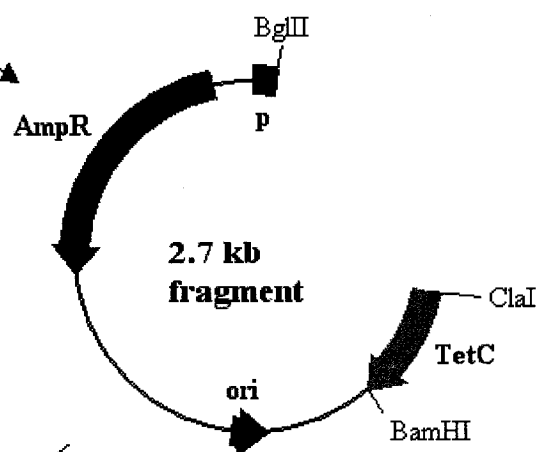
Plasmid pTETnir15 (3.727 kb) contains the *tetC* gene under the control of the anaerobically-induced *nirB* promoter from the nitrate reductase gene of *E. coli* (156), and an ampicillin resistance gene cassette. To facilitate the cloning and expression of the *H. ducreyi hgbA* gene, the plasmid was modified using the approach described by McSorley *et al.* (141) in order to provide a suitable restriction site for the insertion of *hgbA* (Figure 1). Using restriction enzymes *Bgl*III and *Clal*, all but 392 bp at the 3'-end of *tetC* was excised from pTETnir15. A 34-bp double stranded DNA linker [5'-GATCTTAATCATCCACAGGAGACTTTTCATATGAT-3' and 3'-AATTAGTAGGTGTCCTCTGAAAGTATACTACG-5' (synthesized by Invitrogen, Burlington, ON, Canada)] incorporating *Bgl*III and *Clal* restriction enzyme sites at the 5' and 3'-end, respectively and containing a Shine-Dalgarno (SD) sequence (AGGAG); and a unique restriction enzyme site *Nde*I, which encloses a start codon (ATG), downstream of the *nirB* promoter region, was subsequently ligated to the 2.7 kb plasmid fragment. The DNA linker was prepared by first annealing the two oligonucleotides according to a previously described method (187) whereby a mixture comprising equimolar concentrations of each oligonucleotide was incubated at 95°C for 15 min and allowed to cool to room temperature (RT). The DNA linker was then phosphorylated using T4 polynucleotide kinase (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions using a prolonged incubation of 45 min at 37°C. A 10:1 linker:vector ratio with 200 ng of the 2.7 kb purified fragment was incubated overnight at 16°C in the presence of 2 units of T4 DNA ligase (Invitrogen, Burlington, ON, Canada). To remove possible DNA linker concatamers, the plasmid was subjected to *Clal* digestion. The 2.7

Figure 1: Schematic representation of the construction of pnirL.

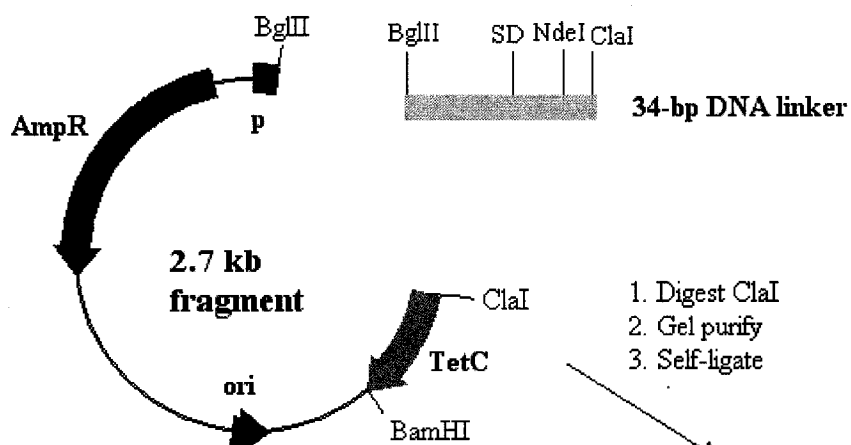
Plasmid pTETnir15 (3.727 kb), which contains the tetanus toxin fragment C gene (*tetC*) under the control of the *nirB* promoter and an ampicillin resistance gene cassette, was digested with restriction enzyme *Bgl*II and *Cl*aI. The 2.7 kb gel purified fragment was then ligated to a 34-bp double stranded DNA linker downstream of the *nirB* promoter region. The DNA linker provides a Shine-Dalgarno (SD) sequence, and a unique restriction enzyme site *Nde*I which also encloses a start codon. The ligated plasmid was digested with *Cl*aI to remove possible DNA linker concatamers, gel purified and self-ligated. The resulting 2.764 kb plasmid was designated pnirL.



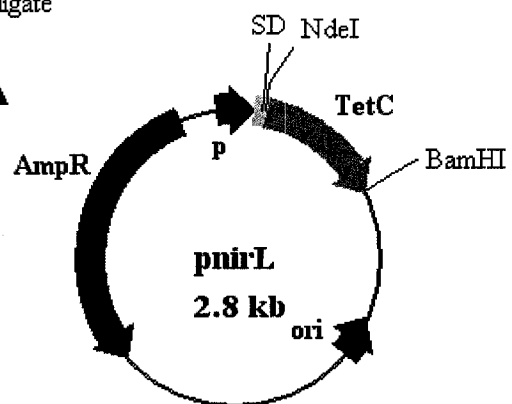
Digest:
BglII + ClaI



1. Gel purify
2. Ligate with DNA linker



1. Digest ClaI
2. Gel purify
3. Self-ligate



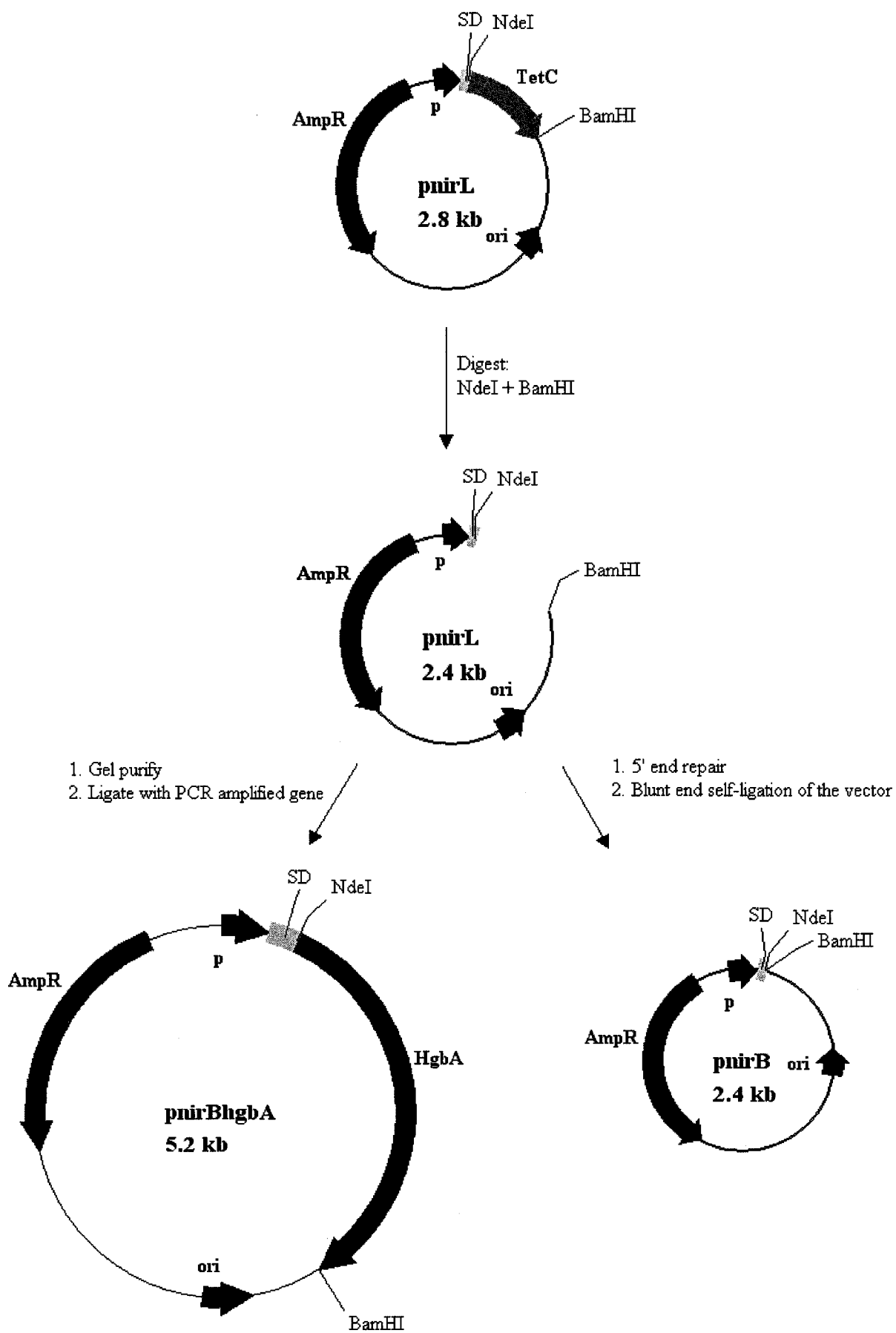
kb gel extracted fragment was self-ligated for 24 hours at 16°C using 2 units of T4 DNA ligase and was introduced into OneShot® TOP10 chemically competent *E. coli* cells. An ampicillin resistant clone was sequenced to confirm the appropriate location of the DNA linker in the vector. This resulting expression vector was designated pnirL.

The *hgbA* gene was PCR amplified from *H. ducreyi* 35000 genomic DNA using the following primers, based on the sequence of *hgbA* in the NCBI GenBank (accession # U17281): forward 5'-GGAATTCCATATGGAAAGCAATATGCAAACAG-3'; reverse, 5'-CGGGATCCTTAGAAAGTGATCTCTGCATTAC-3'. As HgbA is toxic when heterologous expression of the protein includes the leader sequence (Dr. I. Leduc, personal communication) (63), this domain was excluded in the primer design. An *NdeI* restriction site and a *BamHI* site were added to the 5'-end of the forward primer and to the 5'-end of the reverse primer, respectively, to allow directional cloning of the *hgbA* gene. The 2853 bp PCR product was amplified using *Pfx50*™ DNA Polymerase. Following gel purification, the amplicon was ligated into pnirL, which had been restricted with *NdeI* and *BamHI* in order to remove the remaining 392 bp 3'-end of the *tetC* gene (Figure 2). Ligation was performed using a 1:1 linker: vector ratio with 25ng of the expression vector and the ligation mixture was incubated overnight at 16°C in the presence of 2 units of T4 DNA ligase. Following transformation of the plasmid construct into OneShot® TOP10 chemically competent *E. coli* cells, an ampicillin resistant clone was identified by restriction enzyme screening, in which the plasmid was subjected to both *AvrII* digestion and a double digest with *AvrII* and *AlwNI*. The appropriate 5.225 kb vector was termed pnirB*hgbA*.

To construct the vector that would serve as the empty control plasmid, pnirL was digested with *NdeI* and *BamHI*, followed by repair of the 5'-ends with the Klenow fragment DNA polymerase I (New England BioLabs, Ipswich, MA, USA) (Figure 2).

Figure 2: Schematic representation of the construction of pnirBhgbA and pnirB.

pnirL (2.764 kb) was digested with *Nde*I and *Bam*HI to remove the remaining 392 bp 3'-end of the *tetC* gene and gel purified. For construction of pnirBhgbA (left panel), the 2.372 kb gel purified fragment was ligated to the 2.853 kb PCR amplified *hgbA* gene. The resulting 5.225 kb plasmid was designated pnirBhgbA. For construction of pnirB (right panel), the 2.372 kb gel purified fragment was repaired at the 5-ends with the Klenow fragment DNA polymerase I in order to execute blunt end self ligation of the vector. The resulting 2.372 kb plasmid was designated pnirB.



Blunt end self-ligation of the vector was achieved by mixing 80 ng of vector with 40 units of T4 DNA ligase (400U/ μ L; New England BioLabs, Ipswich, MA, USA). Following incubation for 24 hours at 16°C, the ligation mixture was transformed into DH5 α *E. coli* competent cells. Ampicillin resistant transformants were selected and the presence of the appropriate sized plasmid was confirmed by agarose gel electrophoresis. The 2.372 kb control vector was termed pnirB.

2.3.2 Introduction of the plasmids into *Salmonella*

Plasmids pTETnir15, pnirB or pnirBhgba were introduced into *Salmonella* as described by Bowe *et al.* (35). *S. typhimurium* LB5010 was used as an intermediate host as this strain is more readily transformed because of its partial LPS expression (*galE*), and the isolate is restriction incompetent, but modification competent (r-, m+) (38). These traits ensure that the transforming DNA, categorized as foreign by its distinct methylation pattern, is not digested (r-), and that the plasmid DNA methylation pattern will be modified to match that of *S. typhimurium* (m+) (127). To facilitate future electroporation, LB5010 cells were washed in 10 mL of sterile ice cold 10% (v/v) glycerol/water solution, and harvested by centrifugation (CR3i centrifuge, Jouan, Winchester, VA, USA) at 2500 x g for 5 min at 4°. The pelleted cells were resuspended in 400 μ L of sterile ice cold 10% (v/v) glycerol/water solution and 60 μ L aliquots were stored at -70°C.

For electroporation, 50-150 ng of plasmid DNA suspended in a maximal volume of 5 μ L of sterile water was added to 60 μ L of LB5010 electrocompetent cells. The suspension was transferred to an ice cold 0.1 cm gap gene pulser electroporation cuvette (Bio-Rad Laboratories, Mississauga, ON, Canada), and exposed to a current of 1.75 kV, 25 mF capacitance and 600 Ω resistance using a Micropulser™ (Bio-Rad Laboratories, Mississauga, ON, Canada). One mL of LB-broth enriched with 20mM glucose (Sigma-

Aldrich, St. Louis, MO, USA) was immediately added to the cell suspension. The mixture was then transferred to a microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada) and incubated at 37°C for 2 hours with shaking prior to inoculating LB+amp agar plates. Ampicillin resistant transformants were selected and the presence of the appropriate sized plasmid was confirmed by agarose gel electrophoresis.

S. typhimurium LB5010 transformants were infected with phage P22 (HT int-) and the resultant P22 lysate was used to introduce the plasmids into the *S. typhimurium* SL3261 vaccine strain by transduction. Briefly, an overnight culture of transformed *S. typhimurium* LB5010 was supplemented with galactose (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.4% (w/v) and incubated for 20 minutes (min) at 37°C. Serial dilutions of phage P22 stock from 10^{-1} to 10^{-6} were prepared in TGMS buffer [10mM Tris-HCL, pH 7.4, 0.25% (w/v) $MgSO_4$ (both from BDH Inc., Toronto, ON, Canada), 0.1% (w/v) gelatin (Sigma-Aldrich, St. Louis, MO, USA), and 0.58% (w/v) NaCl]. One hundred μ L of bacterial culture was added to 10 μ L of each phage dilution and incubated at 37°C for 45 to 90 minutes. Following the infection, 3 mL of melted top agar [0.7% (w/v) Bacto-agar in LB broth] was added and the mixture was poured onto an LB+amp plate. The agar plates were incubated at 37°C for 5 hours in the case of *Salmonella* LB5010 containing pnirB and pTETnir15 or 22 hours for *Salmonella* LB5010 containing pnirBhgbA. To recover the P22 lysate, 3 mL of TGMS buffer was added to the agar plate containing the lowest phage dilution and the top agar layer was scraped into a 15 mL centrifuge tube (Fisher Scientific, Ottawa, ON, Canada) To lyse the bacteria, 50 μ L of chloroform (EMD Chemicals Inc., Gibbstown, NJ, USA) was added and the mixture was agitated for 30 min at RT. The clear supernatant obtained from centrifugating (Optima L-90K Ultracentrifuge, Beckman Coulter, Mississauga, ON, Canada) the mixture at 15 000 x g for 15 min at 4°C, was passed through a 0.22 μ M

cellulose acetate filter (Whatman, Clifton, NJ, USA) to remove residual bacteria. The resultant P22 lysate was stored in glass tubes in 50 μ L of chloroform at 4°C.

For transduction into *S. typhimurium* SL3261, serial dilutions of P22 lysate from 10^0 to 10^{-4} were prepared in TGMS buffer. Ten μ L of each P22 lysate dilution was added to 100 μ L of an overnight culture of strain *S. typhimurium* SL3261 and the culture was incubated at 37°C for 25 min. One mL of LB-broth containing 5mM Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'-tetra-acetic acid (EGTA; Sigma-Aldrich, St. Louis, MO, USA) was added and the cells were incubated for 1 hour at 37°C. One hundred μ L of the bacterial mixture was inoculated onto LB+amp agar plates containing 5mM EGTA and incubated overnight at 37°C. Colonies were passaged twice on this agar medium to ensure the absence of non-internalized phage particles as EGTA chelates calcium which is required for phage adsorption (135). To preliminarily identify and isolate *S. typhimurium* SL3261 strains bearing phage introduced plasmid, colonies grown on EGTA plates were cultured onto green indicator plates [0.8% (w/t) tryptone (Difco/Becton Dickinson, Sparks, MD, USA), 0.1% (w/t) yeast extract (Oxoid/Thermo Fisher Scientific, Nepean, ON, Canada), 1.5% (w/v) NaCl, 1.5% (w/v) Bacto-agar, to which each 1 L autoclaved agar solution was supplemented with 34 mL of filter sterilized 40% (w/v) glucose, 25 mL of 2.5% (w/v) Alizarin yellow G (Acros Organics, Morris Pla, NJ, USA), and 6.6 mL of 2% (w/v) aniline blue (Fisher Scientific, Ottawa, ON, Canada)] and incubated at 37°C for 24 hours to obtain phage free colonies. The pH indicator present in the plates imparts a green colour to the agar at neutral pH and a dark blue colour at acidic pH. Light-coloured colonies, indicating the presence of either a nonlysogen or a lysogen (135), were streaked onto LB+amp plates and stocks were prepared in glycerol (0.15% v/v) and frozen at -70°C for future testing of phage P22 sensitivity. Colonies that contained a pseudolysogen appeared dark blue because of cell

lysis, which lowers the pH of the medium (135), and were discarded. Presence of the appropriate plasmids *pnirB* and *pnirBhgbA*, in the transduced strain *S. typhimurium* SL3261 was confirmed by PCR amplification using recovered plasmid DNA as template, and presence of *pTETnir15*, in the transduced strain *S. typhimurium* SL3261 was confirmed by restriction enzyme analysis. *S. typhimurium* SL3261 transductants bearing the *hgbA* gene were confirmed by nucleotide sequencing.

2.3.3 Analysis of HgbA and TetC expression

Whole cell lysates derived from aerobic and anaerobic cultures of *E. coli* and *S. typhimurium* SL3261 were assessed for HgbA and TetC expression. Cells were grown and an aliquot was taken at 9 hours for *E. coli*, at 1, 2, 3, 6, 12, and 24 hours for *S. typhimurium* SL3261 (*pnirBhgbA*) and at 2, 4, 6, 12, and 24 hours for *S. typhimurium* SL3261 (*pTETnir15*) and diluted to an absorbance at 600nm (A_{600}) of 0.2, as measured on a Septronic 20 Genesys spectrophotometer (Thermo Scientific, Waltham, MA, USA). A 1 ml aliquot of the A_{600} 0.2 culture was pelleted by centrifugation at 13000 rpm (Sorvall Legend Micro 17, Thermo Fisher Scientific, Nepean, ON, Canada) and the cells were resuspended in 100 μ L of 2x sodium dodecyl sulphate (SDS) sample buffer [1.52% (w/v) Tris Base, 20% (v/v) glycerol, 2% (w/v) SDS (MP Biomedicals, Solon, OH, USA), 2% (v/v) 2-mercaptoethanol (BDH Inc., Toronto, ON, Canada), 0.001% (w/v) Bromophenol blue, pH 6.8]. Samples were heated for 5 min at 100°C prior to separation by one dimensional denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system described by Laemmli (118) using a 8 % acrylamide resolving gel [0.375M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 26.7% (v/v) ProtoGel (30% (w/v) acrylamide/methylene bisacrylamide solution (National Diagnostics, Atlanta, GA, USA), 0.1% (w/v) ammonium persulfate (Bio-Rad Laboratories, Mississauga, ON,

Canada), 0.1% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, St. Louis, MO, USA)] and a 4.5 % acrylamide stacking gel [0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 15% (v/v) ProtoGel, 0.5% (w/v) ammonium persulfate, 0.2% (v/v) TEMED]. The electrophoresis was done using a BioRad MiniProtean II electrophoresis apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada) at 100-150V in electrophoresis buffer [5mM Tris base, 38.4mM glycine (MP Biomedicals, Solon, OH, USA), 0.02% (w/v) SDS] for 1 hour. Proteins were visualized by RapidStain TM (G-Biosciences, St. Louis, MO, USA) according to manufacturer's instructions.

A duplicate gel was electro-transferred at 20V with amperage of 0.5A for 40 min onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using a BioRad Trans-blot semi-dry electrophoretic transfer cell. The gel and membrane were first equilibrated in fresh Towbin transfer buffer [25mM Tris base, 192mM glycine, 20% (v/v) methanol (Fisher Scientific, Ottawa, ON, Canada)] for 30 min (207). The transfer-blot apparatus was assembled according to the manufacturer's instructions (29). After the transfer was complete, the blot was stained with 0.1% (w/v) Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) in 5% (v/v) glacial acetic acid (Fisher Scientific, Ottawa, ON, Canada) to identify the molecular weight markers. The stain was removed by washing the membrane several times in ddH₂O followed by overnight incubation in a 2% skimmed milk blocking solution in 1x sterile phosphate buffered saline [PBS; 0.8% (w/v) NaCl, 0.02% (w/v) potassium chloride (BDH Inc., Toronto, ON, Canada), 0.115% (w/v) sodium phosphate dibasic heptahydrate (Sigma-Aldrich, St. Louis, MO, USA), 0.02% (w/v) potassium phosphate monobasic] at RT. The blot was rinsed with ddH₂O followed by incubation for 30 min at RT in a 1% skimmed milk solution [1% (w/v) skimmed milk, 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in 1x sterile PBS]. After rinsing with ddH₂O, the membrane was probed for 1 hour at RT with a 1:20000 dilution

of rabbit polyclonal antibody against recombinant *H. ducreyi* HgbA (anti-rHgbA) (kindly provided by Dr. I. Leduc, University of North Carolina, Chapel Hill, NC, USA) (222), or a 1:16000 dilution of rabbit polyclonal antibody against tetanus toxin peptides 1300-1314 conjugated to KHL (anti-TetC; Biogenesis Ltd, Raleigh, NC, USA) in 0.1% (v/v) Tween 20 in sterile PBS solution. After three washes for five minutes each with 1x sterile PBS, the membrane was incubated with a 1:10000 dilution (for anti-HgbA or a 1:5000 for anti-TetC) of goat anti-rabbit immunoglobulin (IgG) horseradish peroxidase conjugated second antibody (anti-rabbit IgG; BioSource International, Camarillo, CA, USA) solution [0.1% (v/v) Tween 20 in sterile PBS]. After three washes with PBS for five minutes each, the membrane was developed with 3, 3', 5, 5' - Tetramethylbenzidine (TMB peroxidase substrate; KPL Inc., Gaithersburg, MD, USA) for 1-5 min. The reaction was stopped by rinsing the blot with ddH₂O.

2.3.4 Silver staining of lipopolysaccharides

To verify that the *S. typhimurium* SL3261 transductants exhibited intact LPS profiles, an SDS-PAGE gel was silver stained using the method of Tsai and Frash (210). Briefly, *Salmonella* strains were suspended in 1 mL of 2x SDS sample buffer and boiled for 5 min. Five µL of 20 mg/mL Proteinase K (Invitrogen, Burlington, ON, Canada) was added to the sample and incubated at 60°C for 1 hour prior to loading onto a 15% acrylamide resolving gel and a 4.5% acrylamide stacking gel. Electrophoresis was conducted at amperage of 30mA, power of 250W and voltage starting at 65V and gradually ramping to 182V. The gel was fixed overnight at RT in 40% (v/v) methanol and in 5% (v/v) acetic acid in ddH₂O. The LPS was oxidized in a solution of 0.7% (w/v) periodic acid (Sigma-Aldrich, St. Louis, MO, USA) in 40% (v/v) ethanol (Commercial Alcohols Inc., Brampton, ON, Canada) and in 5% (v/v) acetic acid for 5 min, followed by

three washes for 15 min each in ddH₂O. To prepare the silver staining reagent, 2 mL of ammonium hydroxide (Sigma-Aldrich, St. Louis, MO, USA) was added to 28 mL of 0.1M NaOH (Sigma-Aldrich, St. Louis, MO, USA). Five mL of a 20% (w/v) silver nitrate (Fisher Scientific, Ottawa, ON, Canada) solution was added to the mixture with constant stirring, and ddH₂O was added to produce a final volume of 150 mL. The gel was incubated with silver stain reagent with vigorous agitation for 10 min at RT. To remove excess silver nitrate solution, the gel was rinsed 3 times for 10 min each in ddH₂O. To develop the LPS bands, the gel was soaked in formaldehyde developer [0.005% (w/v) citric acid, 0.05% (v/v) of 37% formaldehyde (both from BDH Inc., Toronto, ON, Canada)] for 2-5 min until the LPS bands appeared. The reaction was terminated by rinsing briefly in ddH₂O followed by the addition of a 5% (v/v) acetic acid solution.

2.3.5 Sensitivity to phage P22

In order to distinguish between nonlysogenic and lysogenic *Salmonella* SL3261 transductants, the transduced *S. typhimurium* strains were tested for phage sensitivity as described by Maloy (135) with modifications (Dr. K. Sanderson, University of Calgary, Calgary, AB, Canada). An LB+amp agar plate was flooded with 500 uL of an overnight culture, grown at 37°C with shaking at 225 rpm for 17 hours, of the transduced strain to be tested. After the bacterial culture diffused into the agar, 10 uL of undiluted phage P22.c2 was placed onto the agar plate. Following incubation at 37°C for 24 hours, the plate was examined for plaque formation. Phage P22.c2 contains a c2 mutation which results in clear plaque formation (125, 228). Bacterial cells that are P22 sensitive are nonlysogens, but lysogens are P22 resistant because they cannot be re-infected with P22 (135).

2.3.6 Growth curves (*S. typhimurium*)

To ensure that there was no growth defects in the *S. typhimurium* (pnirBhgbA) vaccine construct *in vitro*, which would result in altered growth *in vivo*, growth assays were conducted. Strains were grown under both aerobic and anaerobic conditions in 50 mL of LB broth at 37°C. Cultures were continuously agitated with a magnetic stir bar. Growth was monitored at 0, 2, 4, 6, 8, 10, 13 or 14, and 24 or 26 hours using a Septronic 20 Genesys spectrophotometer at A₆₀₀. For cultures grown under anaerobic conditions, the AnaeroGenTM sachet was replaced after each aliquot was removed.

2.3.7 Pulse field gel electrophoresis (PFGE)

PFGE was performed by Ian In Coultish in collaboration with Dr. K. Ramotar (The Ottawa Hospital, Ottawa, ON, Canada) using their standard protocol (30). Briefly, *Salmonella* isolated from rabbit stool samples were grown on LB+amp agar and suspended in CS solution [10mM Tris-HCl pH 7.6, 1M NaCl]. Equal volumes of CS-bacterial cell suspension and 1.6% low-melt agarose (Bio-Rad Laboratories, Mississauga, ON, Canada) were mixed and transferred into the plug mold wells (Bio-Rad Laboratories, Mississauga, ON, Canada). The agar plugs were solidified at RT and then transferred to a 50 mL sterile polypropylene tube containing 10 mL of plug lysis solution [1M NaCl, 100mM EDTA pH 7.5, 0.5% Brij-58 Sigma, 0.5% Sarcosyl, 0.2% Deoxycholate, 6mM Tris-HCl, pH 7.6, 1 mg/mL lysozyme, 20 µg/mL RNase (all from Sigma-Aldrich, St. Louis, MO, USA)] and incubated for 4 hours at 37°C with gentle shaking. The plug lysis solution was replaced with 10 mL of PK solution [0.5M EDTA pH 9-9.5, 1% Sarcosyl, 50 µg/ml proteinase K (Sigma-Aldrich, St. Louis, MO, USA)] and the agar plugs were incubated overnight at 50°C. The plugs were washed three times for 30 min each at 37°C

with gentle shaking with 40 mL of TE buffer [10mM Tris-HCl, pH 7.5, 0.1mM EDTA, pH 7.5] before proceeding with DNA digestion using *Xba*I. A 2 mm slice of the gel plug was transferred into a microfuge tube containing 1x enzyme buffer (supplied from the manufacturer), 100 µg/ml of bovine serum albumin and 20 units of *Xba*I and incubated at 37°C for 4 hours. After the digestion, the reaction solution was removed, the gel plug was melted at 65°C before loading onto a 1% agarose gel (Bio-Rad Pulsed Field certified agarose in 0.5x TBE buffer). Electrophoresis was conducted in a cooling module at 14°C for 22 hours at 6 V/cm, 120°C angle with an initial switch time of 3 seconds and a final switch time of 30 seconds, using the PFGE apparatus (CHEF [Clamped Homogenous Electric Fields] Mapper power supply, Bio-Rad Laboratories, Mississauga, ON, Canada). The DNA bands were visualized and photographed after immersing the gels in 0.00005% (v/v) ethidium bromide solution under UV light using a Multimage Light Cabinet (Alpha Innotech Corp., San Leandro, CA, USA).

2.4 Animal inoculations by gavage

2.4.1 Animal strains and husbandry

Animal experiments were approved by the Animal Care Committee at the University of Ottawa (Protocol BMI-80). Twelve age-matched male New Zealand white (NZW) rabbits, between 2.2 and 2.5 kg in weight (Charles River Co., St. Constant, QC, Canada), were used for this study. Upon arrival at the animal care facility, the rabbits were allowed to acclimatize for six days. Rabbits were housed individually in cages (Hoeltge Inc., Cincinnati, OH, USA), in an 11.7m² room, and given *ad libitum* access to food and water. Rabbits were supplied with alfalfa hay cubes or carrots every other day of the week, and when eating poorly a treat of frozen cubes of pineapple or pumpkin was

given. For environmental enrichment, “bunny blocks” and “jingle balls” (Bio-Serv, Frenchtown, NJ, USA) were also provided.

2.4.2 Preparing the inocula

For the preparation of the inocula and the oral gavage, the method of Ashby *et al.* (16) was used with slight modifications. On the day of the oral inoculation, 5 mL aliquots of a 12 % (w/v) filter-sterilized aqueous bicarbonate solution (NaHCO_3 ; Sigma-Aldrich, St. Louis, MO, USA) was drawn in a 10 mL syringes (Becton Dickinson and Company, Cockeysville, MD, USA). A 50 mL culture of *S. typhimurium* SL3261 (pnirB), *S. typhimurium* SL3261 (pnirBhgbA) or *S. typhimurium* SL3261 (pTETnir15) was grown to mid-log phase (A_{600} between 0.5 and 0.8) and chilled overnight at 4°C. The chilled culture was used to start a 50 mL fresh LB broth culture that grown to an A_{600} of approximately 0.8. The bacteria were harvested by centrifugating at 2500 x g for 15 min at 4°C in a 50 mL polypropylene centrifuge tubes (Fisher Scientific, Ottawa, ON, Canada), and the pellet was resuspended in PBS to achieve the pre-determined bacterial dose for the oral inoculation. Bacterial suspensions and PBS (for sham-immunized controls) were held on ice until needed for the oral gavage. The inoculum was warmed for 5 min before administration, and 5 mL was fed to each rabbit. The remaining bacterial solution was kept on ice until completion of the oral gavage, and tenfold serial dilutions in PBS were plated on LB+amp agar to determine the viable inoculum.

2.4.3 Oral gavage

Twelve rabbits were inoculated in groups of six in two different vaccination trials. In the first vaccination trial, the rabbits were inoculated with a single dose of the

vaccine. A second vaccination trial was later carried out, where the rabbits were inoculated three times two weeks apart (booster regimen) with the vaccine, because no serum antibody response to the guest antigen was achieved in the first trial, as measured by enzyme immunoassay (EIA) against recombinant HgbA (rHgbA see 2.5.1) of serum at 2, 4, 5, and 8 weeks, described in section 2.5.2. For the oral gavage, food was removed from the cages 12 hours before the procedure. Rabbits were sedated with 25-35 mg/kg ketamine-HCl ("ketalean" Bimeda/MTC, Cambridge, ON, Canada), 2 mg/kg midazolam ("versed", Sabex, Boucherville, QC, Canada), and 0.5 mg/kg atropine sulphate (MTC pharmaceuticals, Cambridge, ON, Canada) by intra-muscular injection. Three mL of baseline blood samples were drawn from the ear artery of each rabbit. To ensure correct placement of the feeding tube (French #12 infant feeding tube, Benlan Inc., Oakville, ON, Canada) into the stomach, the distance from the rabbit's mouth to its last rib was marked on the tube. The tube, lubricated with K-Y jelly (Johnson and Johnson, New Brunswick, NJ, USA), was introduced into the stomach to the mark. Correct placement of the tube was confirmed when stomach contents were seen, drawn by capillary action into the tube. To neutralize stomach acidity, 5 mL of 12% bicarbonate solution was introduced into the tube, and flushed into the stomach with 10 mL of air before removing the tube. Thirty minutes was allowed to elapse prior to reinsertion of the tube to ensure that the bicarbonate solution had sufficiently neutralized gastric acidity. Five mL of PBS or bacterial suspension was administered, and the tube was again flushed with 10 mL of air before removing. When required, sedation was maintained by administering Isoflurane ("Aerrane", Janssen, Toronto, ON, Canada), with 100% O₂ (Praxair, Ottawa, ON, Canada).

2.4.4 Oral vaccination with three doses of *Salmonella*

To evaluate whether a booster-vaccination regimen would generate a humoral response to HgbA and subsequently provide protective immunity against chancroid in the TDRM, the rabbits were fed three doses two weeks apart of the *S. typhimurium* SL3261 (pnirBhgbA) vaccine. For controls, two rabbits were fed either three doses two weeks apart of the *S. typhimurium* SL3261 (pnirB) control strain or of PBS for sham-immunization. One rabbit was also fed a single dose of *S. typhimurium* SL3261 (pTETnir15) as an internal control. As prior work in our laboratory showed that rabbits generated antibodies to the heterologous antigen TetC delivered orally by *S. typhimurium* SL3261 (pTETnir15) (16), the humoral response to this protein was used as an internal control of the validity of the oral vaccination protocol. The projected dose for the oral immunization was the same as the first trial, between 10^8 - 10^9 CFU, as this dose was well tolerated in the first trial.

2.4.5 Wellness monitoring

The rabbits were monitored closely the day of the gavage, and daily for 7 days following the gavage. The animal care staff recorded wellness data on a chart containing 10 indicators of wellness. Body mass, rectal temperature, hydration, attitude and demeanour, nasal discharge, piloerection, volume of fluid consumed, volume and type of food consumed, and fecal production (volume and consistency) were recorded daily. Treats were provided.

2.4.6 Detection of *Salmonella* in rabbit stools

A protocol for the isolation of *Salmonella* organism in rabbit feces was developed with the help of Dr. K. Ramotar. Stool suspensions were inoculated onto MacConkey (MAC; BBL/ Becton Dickinson, Cockeysville, MD, USA) agar with the addition of 100 µg/mL of ampicillin (MAC+amp). MAC agar contains bile salts and crystal violet that inhibits the growth of most gram-positive bacteria. The incorporation of neutral red as a pH indicator allows for the selection of lactose-fermenting organisms which appear red due to the acid pH (69). *Salmonella* is a non lactose-fermenting (NLF) bacterium and colonies display a beige colour on MAC agar.

Rabbit feces were collected from the pan underneath the cages on the day that the pans were changed by the Animal Care Staff. Collection was done on day 1, 3, 6, 8, 10, 13, 21, and 27 post-gavage for the first vaccination trial or on day 1, 6, and 10 after each oral gavage for the second vaccination trial. The collection days were minimized from every 2-3 days in the first trial to every 4-5 days in the second trial for convenience purposes. Fecal pellets were collected and placed in a 1.5 mL microcentrifuge tube with forceps sterilized in 70% ethanol. After resuspension in 1.2 mL of PBS, 100 µL of serial tenfold dilutions were inoculated onto MAC and MAC+amp agar plates and incubated at 37°C in ambient air for 16-20 hours. Plates were examined for the presence of NLF organisms. To determine the identity of the ampicillin resistant NLF organisms, colonies underwent further phenotypic analysis described as follows.

For the first vaccination trial, urea hydrolysis (Oxoid/Thermo Fisher Scientific, Nepean, ON, Canada), triple sugar iron (TSI) agar (PML Microbiologicals, Mississauga, ON, Canada), and motility medium (Oxoid/Thermo Fisher Scientific, Nepean, ON, Canada) tests were done according to protocols established at the Eastern Ontario Regional Laboratory at The Ottawa Hospital General Campus (Ottawa, ON, Canada).

Briefly, approximately half of a single colony present on a MAC+amp plate was picked with a straight wire loop. Streaking the surfaces of the urea agar and the TSI agar followed by stab inoculation of the TSI agar and the motility agar were performed sequentially. The loosely capped tubes were incubated at 37°C in ambient air for 16 to 24 hours before recording the results. When necessary the TSI tests were incubated for up to nine additional days, and H₂S production was recorded again at the final incubation day. The characteristic reaction of *Salmonella* on the urea hydrolysis test is negative (yellow), on the motility medium is positive (pink cloudy agar), and on the TSI agar is an alkaline (red) slant over an acidic (yellow) bottom, with gas and H₂S production. The remaining portion of the colony was re-streaked onto LB+amp agar and grown overnight at 37°C. Colonies were suspended in freezing media [2.4% (w/v) trypticase soybroth (Difco/Becton Dickinson, Sparks, MD, USA), 20% (v/v) glycerol] and stored at -70°C.

For the second vaccination trial, MAC plates were also examined for the presence of NLF organisms and one colony was re-streaked onto LB+amp agar and grown overnight at 37°C. Colonies were suspended in freezing media and stored at -70°C. Identification of the colonies was done using a more specific and sensitive bacterial identification method than the urea hydrolysis, triple sugar iron agar, and motility medium tests (95). The VITEK[®] 2 system (BioMérieux Canada Inc. St. Laurent, Québec, Canada) is an automated system that performs bacterial identification by reading an identification card, which has been pre-inoculated with the bacterium to be tested. The gram-negative identification card, which can identify 159 gram-negative taxa with its 47 colorimetric biochemical tests, was used. The tests measures carbon sources utilization, enzymatic activities, and resistance to inhibitory substances (165). The VITEK[®] tests were done according to protocols established at the Eastern Ontario Regional Laboratory at The Ottawa Hospital General Campus (Ottawa, ON, Canada).

The isolates identified as *Salmonella* were further characterized to both determine the presence of the recombinant plasmid, and for expression of HgbA or TetC. Restriction digest analysis was performed on plasmid DNA isolated from *Salmonella* recovered from rabbit stool. For the second vaccination trial, PCR amplification to identify the plasmid construct bearing the *hgbA* gene was also performed using recovered plasmid DNA as template. Whole cell lysates were examined for HgbA and TetC expression by Western immunoblotting as described in section 2.3.3.

2.5 Enzyme immunoassay and delayed type hypersensitivity skin test

2.5.1 Antigen preparation

rHgbA was provided by Dr. I. Leduc, a previous student in our laboratory, produced using the method described by Elkins *et al.* (63, 119). The protein was expressed from *E. coli* strain BL21 (DE3), pUNCH 672 pLysS (A672-10). rHgbA is expressed from pUNCH A672-10 as a fusion protein containing an N-terminal hexahistidine leader. The hexahistidine leader of the expression plasmid pET30a (Novagen, EMD Chemicals Inc., Gibbstown, NJ, USA), which adds 40 amino acids to the mature protein sequence of HgbA, is fused to the first mature amino acid of HgbA. Despite the lack of its native leader sequence (63), the rHgbA is recognized in Western immunoblots probed with antibodies derived from individuals naturally infected with *H. ducreyi* (63). rHgbA is expressed from pUNCH A672-10 under denaturing conditions and re-natured by dialysis. The complete rHgbA extraction method is detailed in the Appendix.

Salmonella and *E. coli* (pUNCH1202) crude soluble antigens were prepared as follows: *S. typhimurium* SL3261 or *E. coli* (pUNCH1202) was grown to an A₆₀₀ of approximately 1.0 and bacterial cells were harvested by centrifugation at 2500 x g for 10

min at 4°C. Cells were washed 4 times with PBS and then resuspended in 5 mL of PBS with 1% (w/v) SDS, before sonicating (60 Sonicator dismembrator, Fisher Scientific, Ottawa, ON, Canada) on ice 3 times for 30 seconds with 15 seconds intervals between pulses at 15W. The solution was incubated for 2 hours at RT with rotation and then centrifuged (Optima L-90K Ultracentrifuge, Beckman Coulter, Mississauga, ON, Canada) at 50 000 x g for 90 min at 4°C. The supernatant, which contains the crude soluble antigens, was collected and stored at -20°C.

Tetanus toxin C fragment from *Clostridium tetani* (TetC) was purchased from Sigma-Aldrich.

Protein concentration for rHgbA, *Salmonella* and *E. coli* crude soluble antigens was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.5.2 EIA procedure

For the first vaccination trial, 3 ml of blood was collected prior to and weekly for eight weeks after the oral gavage, and for the second vaccination trial blood was collected on week 2, 4, 5, and 8 after the first oral gavage. A different collection schedule was used in the second trial to simultaneously perform blood collection and oral gavages, and to minimize the stress induced from the invasive procedures for the animals. Blood also was collected prior to the challenge with *H. ducreyi* at week 5 instead of week 6 (which would be four days after the challenge), to avoid any interference with the natural development of the infection due to the manipulation of the rabbits for blood collection. The blood was left to clot at RT for approximately one hour. After centrifugation at 2500 x g for 5 min at 4°C, serum aliquots were stored at -70°C. Test antigens were diluted in 0.1M of carbonate buffer [30mM Na₂CO₃ (BDH Inc., Toronto, ON, Canada), 70mM NaHCO₃, pH 9.6]. *Salmonella* crude soluble antigens were diluted to a concentration of 50 ng/μL, TetC

was diluted to 10 ng/ μ L and rHgbA to 2 ng/ μ L. The test volume for the EIA was 150 μ L for *Salmonella* crude soluble antigens, 50 μ L for TetC and 100 μ L for rHgbA. One volume of the appropriate antigen was added to each well of a 96 well flat-bottomed polystyrene plate (Corning Life Sciences, Lowell, MA, USA) and allowed to bind overnight at 4°C. The volume of antigen was removed and the plates were washed 3 times with wash buffer [0.1% (v/v) Tween 80 (BDH Inc., Toronto, ON, Canada) in PBS], before adding one volume of blocking solution [2% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS] to each well. After 1 hour of incubation at 37°C, the plates were washed 3 times in wash buffer. One volume of sample buffer [1% newborn calf serum (NBCS; Gibco/Invitrogen, Burlington, ON, Canada), 0.1% (v/v) Tween 80 in PBS] was added to all wells except for the first row. For *Salmonella* EIAs, two volumes of a 1:250 dilution in sample buffer of each serum tested was loaded in triplicate columns in the first row. The serum was then serially twofold diluted from 1:250 to 1:32000 down the plate. For the negative control, three columns received the same two-fold dilutions of serum from a rabbit inoculated with PBS. For rHgbA EIAs, two volumes of a 1:50 dilution in sample buffer of each serum tested was loaded in triplicate columns in the first row, and similarly diluted two-fold from 1:50 to 1:6400. For the positive control, two wells received a 1:4000 or 1:8000 dilution of anti-rHgba. For TetC EIAs, a 1:50 dilution in sample buffer of each serum tested was loaded in duplicate columns. For the positive control, two wells received a 1:250 dilution of anti-TetC. For negative control of rHgbA and TetC EIAs, three columns received the same two-fold dilutions of serum from a rabbit inoculated with *S. typhimurium* SL3261 (pnirB). For all EIAs, at least two wells received sample buffer only to measure background signal. Sera were allowed to bind for 30 min at 37°C, and plates were washed 3 times with wash buffer. One volume of a 1:2000 dilution in sample buffer of anti-rabbit IgG was added to

each well and incubated at 37°C for 30 min. After 3 washes with wash buffer, one volume of colour substrate [ABTS, 2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonate) (KPL Inc., Gaithersburg, MD, USA)] was added to each well. Plates were incubated at RT for 10 min for *Salmonella* EIAs and for 20 min for rHgbA and TetC EIAs, and the optical density of each well was analyzed at 405 nm in a microplate reader (Tecan Systems Inc., San Jose, CA, USA). Incubation times were shorter for *Salmonella* EIAs because the colour developed much faster and it was necessary to read the plate before the wells were no longer recordable.

2.5.3 DTH procedure

To evaluate the cell-mediated immune response in immunized rabbits, we used the DTH to a vaccine antigen in a skin test. A DTH reaction to the antigen, which is distinguished by an indurated lesion due to lymphocytic infiltrate at the site of injection, reveals the induction of a cell-mediated immune response. In a DTH response, animals that have been previously exposed to the antigen will have primed T cells that will migrate to the site of injection and become activated. These cells will release mediators, which results in the recruitment of phagocytes and plasma, producing a visible lesion (98). Three rabbits inoculated with *S. typhimurium* SL3261 (pnirBhgbA) and one rabbit inoculated with *S. typhimurium* SL3261 (pnirB) were challenged with 0, 1, 10, and 100 µg of both enriched rHgbA preparation, and *Salmonella* crude soluble antigens suspended in 100 µL of PBS, by intra-epithelial injections on the shaved rabbit's skin. The injections were done three days after the last oral vaccination (third dose of the vaccine). Similarly, one rabbit inoculated with PBS and one rabbit inoculated with *S. typhimurium* SL3261 (pTETnir15) were challenged with *Salmonella* crude soluble antigens only. Rabbits inoculated with *S. typhimurium* SL3261 (pnirBhgbA) or with *S. typhimurium* SL3261

(pnirB) were also injected with 10 and 100 µg of *E. coli* (pUNCH1202) crude soluble antigens. *E. coli* (pUNCH1202) expresses the ferric enterobactin receptor (FetA) of *N. gonorrhoeae* (41, 42). Except for the FetA gene, this strain is genotypically identical to the recombinant *E. coli* strain expressing *H. ducreyi* rHgbA and was used to control for a skin test reaction arising from antigens other than rHgbA. Forty-eight hours after the challenge, the size of the induration was measured using electronic calipers (Mitutoyo Canada Inc., Mississauga, ON, Canada).

2.6 Animal inoculations with *H. ducreyi*

2.6.1 Growth curves for *H. ducreyi*

H. ducreyi 35000 was grown in 50 mL of supplemented GC broth for 25 hours. Aliquots were taken at 0, 2, 4, 6, 8, 10, 12, and 24 or 25 hours, and the A_{600} was measured. Serial tenfold dilutions of each aliquot were plated onto CA to determine viable counts. The values for the A_{600} were plotted against the plate counts (CFU/mL) to approximate the CFU/mL of the culture used for the preparation of the inocula to infect the rabbits in the temperature-dependent rabbit model of *H. ducreyi* infection.

2.6.2 Preparation of the *H. ducreyi* inocula

H. ducreyi was grown to late log phase (A_{600} between 0.3-0.4) and 10 mL of bacterial culture was harvested by centrifugation at 2500 x g for 10 min at RT. The pellet was resuspended to 10^7 CFU/mL in GC broth, and then serially diluted to obtain the 10^6 , 10^5 , 10^4 CFU/mL suspensions. Each bacterial suspension was drawn into a 1mL tuberculin syringe (Becton Dickinson, Cockeysville, MD, USA). To estimate the

delivered inoculum size, each suspension was plated onto CA to determine viable colony counts.

2.6.3 Rabbit inoculation with *H. ducreyi*

Rabbits from the second vaccination trial were inoculated with *H. ducreyi* ten days after the third oral gavage. Rabbits from the first trial were not challenged with *H. ducreyi*, as that first trial was designed to determine the immunization protocol that would generate a humoral response to HgbA. The rabbit model of *H. ducreyi* infection has been previously described in detail (56, 164). Rabbits were challenged with *H. ducreyi* as described by Desjardins *et al.* (55). The temperature in the room was gradually reduced to $14 \pm 1^\circ\text{C}$ (Thermo Air Plus air conditioning unit) during the week prior to the infection, and maintained at this temperature for the remainder of the experiment. To ensure that a uniform temperature without temperature gradients was achieved in the room, a ducted Turbo-Aire™ fan (Turbo-Aire; Seabreeze Electric Corp., Toronto, ON, Canada) was used to continuously circulate the air in the room. The day before the infection, the rabbit backs were shaved and a 3x4 grid was drawn. Triplicate inocula of estimated 10^6 , 10^5 , and 10^4 CFU in 100 μL volumes were injected intra-epithelially on the rabbit backs, and residual inocula were cultured to confirm cells remained viable. After the inoculation the rabbit backs were shaved every day to maintain the skin temperature by exposure, and the lesions were observed every two days for 21 days. Two lesions at each inoculum were measured for size using the electronic calipers, and assigned a clinical score (1= erythema or redness, 2= induration or swelling, 3= suppuration or fluctuence, 4= ulceration). The third lesion was used for culture of *H. ducreyi* by injecting 100 μL of sterile PBS into the lesion from the side, and back-aspiration of the liquid back into the syringe. This suspension of PBS, blood and

inflammatory exudates was plated onto CA, and colonies were enumerated in forty-eight hours. Culture positivity was determined by the presence of one or more characteristically small gray cohesive colonies, coupled with characteristic streptobacillary appearance of *H. ducreyi* on Gram's stain (5).

CHAPTER THREE

RESULTS

3.1 Constructing a *Salmonella* vector-based *H. ducreyi* vaccine

3.1.1 Construction of pnirL

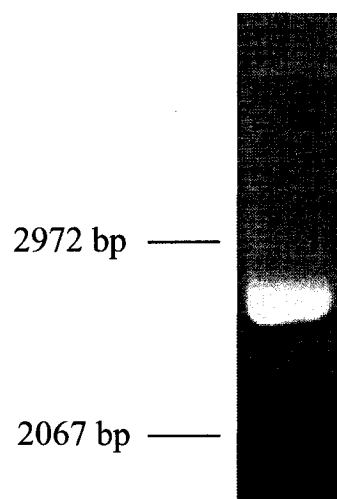
We used plasmid pTETnir15 (3.727 kb), which contains the *tetC* gene under the control of the anaerobically-induced *nirB* promoter, to construct pnirL. The pTETnir15 plasmid does not have a suitable restriction site directly upstream of the *tetC* gene for the insertion of foreign genes. In order to provide a suitable restriction site for the insertion of *hgbA*, the plasmid was modified using the approach described by McSorley *et al.* (141) and described in section 2.3.1.

The plasmid was constructed by excising from pTETnir15 all but 392 bp at the 3'-end of the *tetC* gene, followed by ligation of the purified plasmid fragment to a 34 bp double stranded DNA linker. The DNA linker provided a SD sequence, and a unique restriction enzyme site *NdeI*, that enclosed a start codon. The recombinant plasmid was isolated from an *E. coli* transformant and the appropriate sized plasmid was confirmed by agarose gel electrophoresis (Figure 3A). Nucleotide sequencing verified the expected location of the DNA linker in the vector (Figure 3B). The 2.764 kb expression vector was designated pnirL, and was used to clone the *hgbA* gene and to construct the empty plasmid; pnirB, that served as the negative control in subsequent experiments.

Figure 3: Agarose gel electrophoresis and sequencing results for pnirL.

Plasmid DNA was recovered from an ampicillin resistant clone and applied to a 1% (w/v) agarose gel (A). Sizes of the molecular mass markers (in bp) are shown on the left. Sequences obtained from nucleotide sequencing of plasmid pnirL (B). The first nucleotides of the vector encode the *nirB* promoter, and are shown in blue. The DNA linker is shown in red and the remaining segment of *tetC* is shown in black. The restriction enzyme sites, *Bgl*II and *Cla*I, used to introduce the DNA linker in the vector, and the Shine-Dalgarno (SD) sequence are underlined.

A



B

nirB promoter

5'....CATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGTAG

DNA linker

GGCCCCAGATCTTAATCATCCACAGGAGACTTTCATATGATCGATTC

*Bgl*II SD sequence *Cla*I

TTTCGTTAAATCTGGTGACTTCATCAAACGTACGTTTCTTACAACA

Segment of *tetC* gene

ACAACGAA 3'...

3.1.2 Cloning the *hgbA* gene into *pnirL* and verifying HgbA expression

The *hgbA* gene was PCR amplified from *H. ducreyi* 35000 chromosomal DNA and the gel-purified product was ligated into *pnirL*. A recombinant plasmid isolated from an ampicillin-resistant *E. coli* transformant underwent restriction digest analysis. A single digest using *AvrII* yielded the anticipated single fragment of 5.225 kb (Figure 4). An *AvrII* and *AlwNI* double digest of the plasmid construct generated the two expected fragments of 2.924 and 2.301 kb (Figure 4). The replicon was named *pnirBhgbA*.

To determine whether the *E. coli* recombinant strain bearing *pnirBhgbA* expressed HgbA, Western blots of whole cell lysates grown under aerobic (not induced) and anaerobic (induced) conditions were probed with anti-rHgbA (Figure 5). When *H. ducreyi* is grown in low heme conditions (GC broth supplemented with 5% (v/v) FBS and 1% (v/v) IsoVitallex), a band representing HgbA was detected (Figure 5). An immunoreactive band of 108.6 kDa, corresponding to the size of HgbA, was present in whole cell lysates prepared from cells grown under anaerobic conditions (Figure 5). No such band was seen when whole cell lysates from aerobic grown cultures were reacted with anti-rHgbA (Figure 5). These results indicated that HgbA was expressed from *E. coli* bearing *pnirBhgbA* and that the expression of HgbA was activated by an anaerobic atmosphere.

3.1.3 Construction of the control vector *pnirB*

To construct the vector that would serve as the empty control plasmid, *pnirL* was digested with *NdeI* and *BamHI* in order to remove the remaining 392 bp 3'-end of the *tetC* gene. The digest was followed by repair of the 5' ends and blunt end self-ligation.

Figure 4: Agarose gel electrophoresis of a restriction digest analysis for *pnirBhgbA*. Plasmid DNA was recovered from an ampicillin resistant clone, and digested with both *AvrII* and a double digest with *AvrII* and *AlwNI* before applying to a 1% (w/v) agarose gel. Legend: undigested *pnirBhgbA* (first lane), restriction digest of *pnirBhgbA* with *AvrII* (second lane), and with both *AvrII* and *AlwNI* (third lane). Sizes of the molecular mass markers (in kb) are shown on the left for the supercoiled ladder and on the right for the linear DNA fragments ladder.

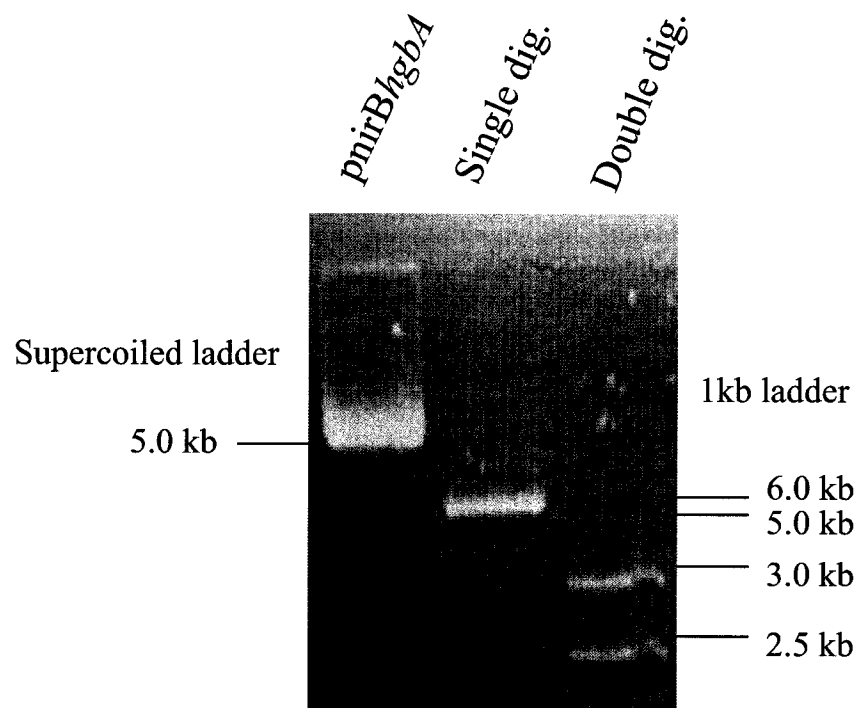
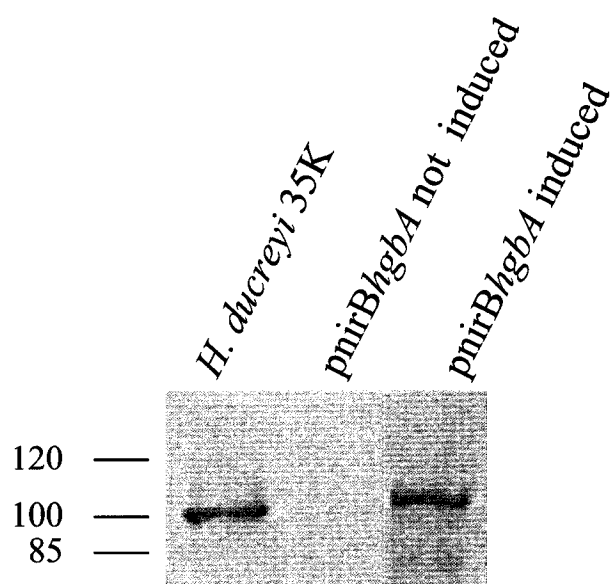


Figure 5: HgbA expression analysis in *E. coli* (pnirBhgbA).

E. coli cells bearing the pnirBhgbA plasmid grown aerobically (= not induced) or anaerobically (= induced) were lysed and separated by SDS-PAGE followed by Western blotting with polyclonal antibody against recombinant *H. ducreyi* HgbA. For positive control, *H. ducreyi* cells were grown in low heme broth and lysed before loading onto the SDS-PAGE. Sizes of the molecular mass markers (in kDa) are shown on the left.



Plasmid DNA was recovered from an ampicillin resistant clone and analyzed by agarose gel electrophoresis. The resultant 2.372 kb plasmid was termed pnirB (Figure 6).

3.1.4 Construction of the recombinant *S. typhimurium* SL3261 strains

Plasmids pnirBhgbA, pnirB or pTETnir15 were electroporated into the intermediate strain *S. typhimurium* LB5010. Ampicillin resistant clones were selected and the presence of the appropriate sized plasmid was confirmed by agarose gel electrophoresis (data not shown). The *S. typhimurium* LB5010 clones were infected with phage P22 (HT int-), and the resultant P22 lysate was used to transduce the plasmids into the *S. typhimurium* SL3261 vaccine strain. The presence of pnirBhgbA and pnirB in the *S. typhimurium* SL3261 transductants was confirmed by PCR amplification using primers annealing to vector sequences immediately upstream and downstream of the hgbA gene. Two amplicons of 3169 bp and 295 bp PCR were generated when pnirBhgbA (Figure 7A) and pnirB (Figure 7B), respectively were used as templates. Nucleotide sequencing of pnirBhgbA recovered from *S. typhimurium* SL3261 corroborated the presence of the hgbA gene (Figure 8).

To confirm that plasmid pTETnir15 was present in the *S. typhimurium* SL3261 transduced strain, an NcoI restriction enzyme digest was performed on recovered plasmid DNA, producing a 3.727 kb fragment (Figure 7C, lane 2). The negative control consisted of the 7.454 kb undigested dimeric form of pTETnir15 (Figure 7C, lane 1).

3.1.5 HgbA and TetC expression in *S. typhimurium* SL3261 transductants

To detect the expression of HgbA in *S. typhimurium* SL3261 containing the plasmid construct pnirBhgbA, a Western blot of whole cell lysates prepared from a *S. typhimurium* SL3261 transductant grown under aerobic and anaerobic conditions was

Figure 6: Agarose gel electrophoresis analysis for pnirB.

Plasmid DNA was recovered from an ampicillin resistant clone and applied to a 1% (w/v) agarose gel. Sizes of the molecular mass markers (in bp) are shown on the left.

2972 bp —

2067 bp —

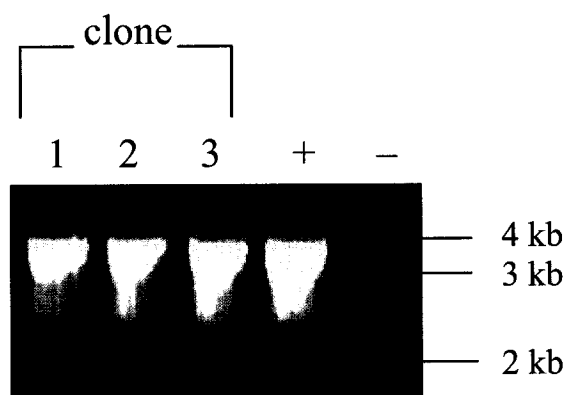


Figure 7: PCR amplification and restriction digest analysis of the recombinant *S. typhimurium* SL3261 strains.

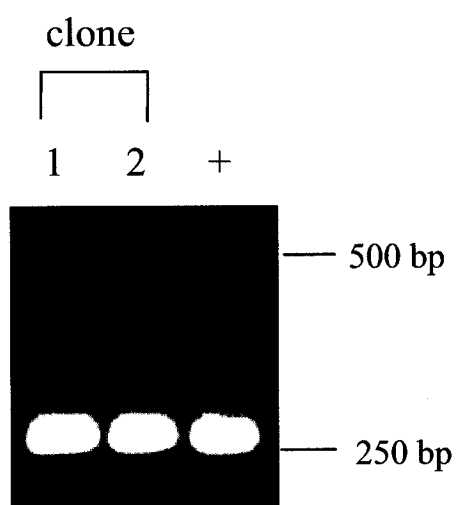
Plasmids pnirBhgbA or pnirB, recovered from transduced strain *S. typhimurium* SL3261, were used as template for PCR amplification using primers that anneal to the backbone vector upstream and downstream of the *hgbA* gene. The amplified products from pnirBhgbA (Figure 7A) and from pnirB (Figure 7B) were applied to a 1% (w/v) agarose gel. Legend (A and B): PCR products amplified from different clones (Lane 1, 2, and/or 3), PCR products amplified from plasmid pnirBhgbA or pnirB recovered from the *E. coli* host (Lane +), and PCR reaction with H₂O as template (Lane -)

Plasmid pTETnir15, recovered from transduced strain *S. typhimurium* SL3261, was digested with *Nco*I before applying onto a 1% (w/v) agarose gel (Figure C). Legend (C): undigested pTETnir15 (Lane 1), and restriction digest of pTETnir15 with *Nco*I (Lane 2). Sizes of the molecular mass markers (in kb) are shown on the left for the supercoiled ladder and on the right for the linear DNA fragments ladder .

A



B



C

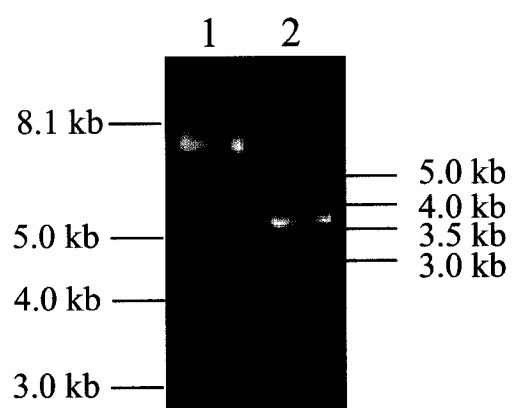


Figure 8: Sequencing results for pnirBhgbA.

Sequences obtained from nucleotide sequencing of plasmid pnirBhgbA, which was recovered from transduced strain *S. typhimurium* SL3261. The first nucleotides representing the *nirB* promoter, are shown in blue. The DNA linker is shown in red with the underlined restriction enzyme site *NdeI*, which was used to clone the *hgbA* gene. The *hgbA* gene is represented in black. * indicates the first sequenced nucleotide of *hgbA*, which corresponds to the first nucleotide of the mature HgbA peptide (NCBI GenBank accession # U17281).

nirB promoter

5'...TTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAAT

DNA linker

CGTTAAGGTAGGCGGTAGGGCCCAGATCTTAATCATCCACAGGAG

*

ACTTTCATATGGAAAGCAATATGCAAACAGAAAAATTAGAACTAT

NdeI

TGTTGTTTCGTCGGAGGACGACTCTGTACATAACAAAAATGTAGGC

GAAATCAAAAAAATGCTAAAGCGCTCAGCAAACAGCAAGTTCAA

GATAGCCGTGATTTAGTCCGTTATGAAACCGGGGTAACCGTAGTA

GAAAAAGGTCGCTTTGGTAGCTCAGGTTATGCTATTCGTGGTGTG

ATGAAAACCGTGTGGCGGTAGTAGTTGATGGCTTACACCAAGCTG

AAACGATTCATCACAAGGGTTTAAAGAATTATTTGAAGGTT 3'...

hgbA gene

probed with anti-rHgbA (Figure 9). An immunoreactive band of 108.6 kDa was seen in cultures at 1 h and 2 h after exposure to an anaerobic environment (Figure 9A, lane 3; Figure 9B lane 2 and 3). No expression was seen in whole cell lysates derived from cells grown anaerobically for 6 h and 9 h (data not shown). Similarly, no immunoreactive band corresponding to HgbA was present in aerobically grown cell lysates (Figure 9A, lane 5-7) nor in lysates prepared from *S. typhimurium* SL3261 containing the bare vector pnirB (Figure 9A, lane 8; Figure 9B, lane 5-7). These results indicated that the *S. typhimurium* SL3261 (pnirBhgbA) expressed HgbA and that the production of this protein was activated by anaerobic conditions. TetC expression was detected at 2, 4, 6, 12, and 24 hours in both anaerobic (Figure 10A) and aerobic (Figure 10B) grown cultures of Western blots reacted with anti-TetC. An increase in the expression of TetC was witnessed only during anaerobic growth. In contrast, the 50 kDa band corresponding to TetC was not seen in Western blots of cell lysates arising from anaerobic cultures of control strain *S. typhimurium* SL3261 (pnirB) (Figure 10A, lane 7). These results indicated that TetC is stably expressed in *S. typhimurium* SL3261 (pTETnir15) for 24 hours, and that the anaerobic atmosphere does not tightly regulate expression.

3.2 Characterization of the recombinant *S. typhimurium* SL3261 strains

3.2.1 Characterization of LPS

The integrity of the bacterial LPS profiles was visualized by silver staining (Figure 11). Strains with intact LPS will depict a ladder of LPS fragments, whereas strains with defects in LPS will reveal low molecular weight fragments (171). The parent strain SL3261 (Figure 11, lane 3) and the recombinant strains *S. typhimurium* SL3261 (pnirBhgbA), SL3261 (pnirB) or SL3261 (pTETnir15) (Figure 11, lane 4-6) displayed an

Figure 9: HgbA expression analysis in *S. typhimurium* SL3261 (pnirBhgbA).

S. typhimurium SL3261 cells carrying the pnirBhgbA plasmid or the control vector (pnirB) grown for 1, 2, or 3 hours aerobically or anaerobically, were lysed and separated by SDS-PAGE followed by Western blotting with polyclonal antibody against recombinant *H. ducreyi* HgbA. *S. typhimurium* SL3261 (pnirBhgbA) cells were grown anaerobically (Figure 9A, lane 1-3) or aerobically (Figure 9A, lane 4-6). In another experiment, *S. typhimurium* SL3261 (pnirBhgbA) (Figure 9B, lane 1-3) or *S. typhimurium* SL3261 (pnirB) (Figure 9B, lane 4-6) were grown anaerobically. For positive control (Lane +), *H. ducreyi* cells were grown in low heme broth and for negative control (Lane –) *S. typhimurium* SL3261 (pnirB) cells were grown under anaerobic conditions. Sizes of the molecular mass markers (in kDa) are shown on the left.

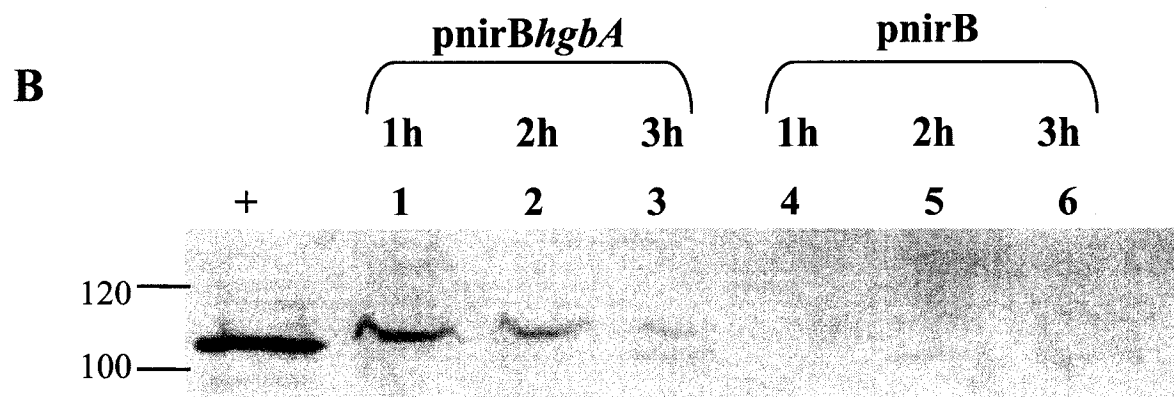
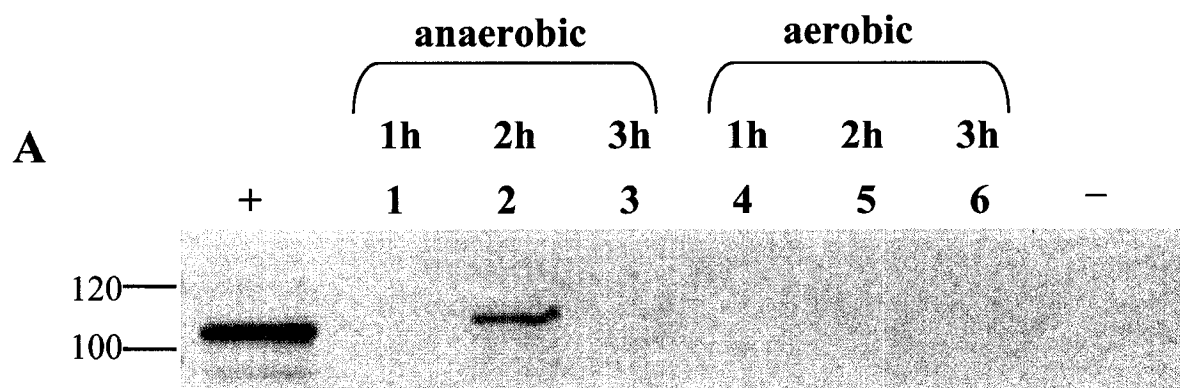
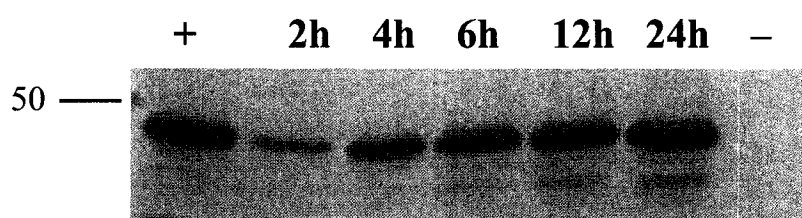


Figure 10: TetC expression analysis in *S. typhimurium* SL3261 (pTETnir15).

S. typhimurium SL3261 cells carrying the pTETnir15 plasmid or the control vector (pnirB) grown for 2, 4, 6, 12, and 24 hours anaerobically (Figure 10A) or aerobically (Figure 10B) were lysed and separated by SDS-PAGE followed by Western blotting with rabbit polyclonal antibody against tetanus toxin peptides (anti-TetC). For positive control (Lane +), *E. coli* (pTETnir15) cells and for negative control (Lane -) *S. typhimurium* SL3261 (pnirB) cells, both grown under anaerobic conditions. Sizes of the molecular mass markers (in kDa) are shown on the left.

A



B

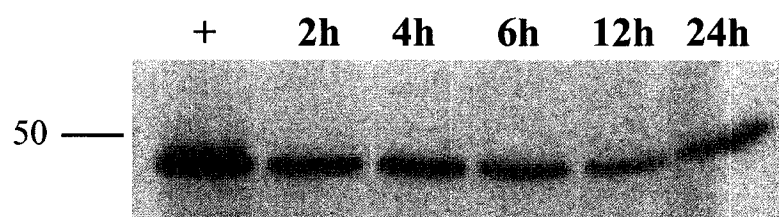
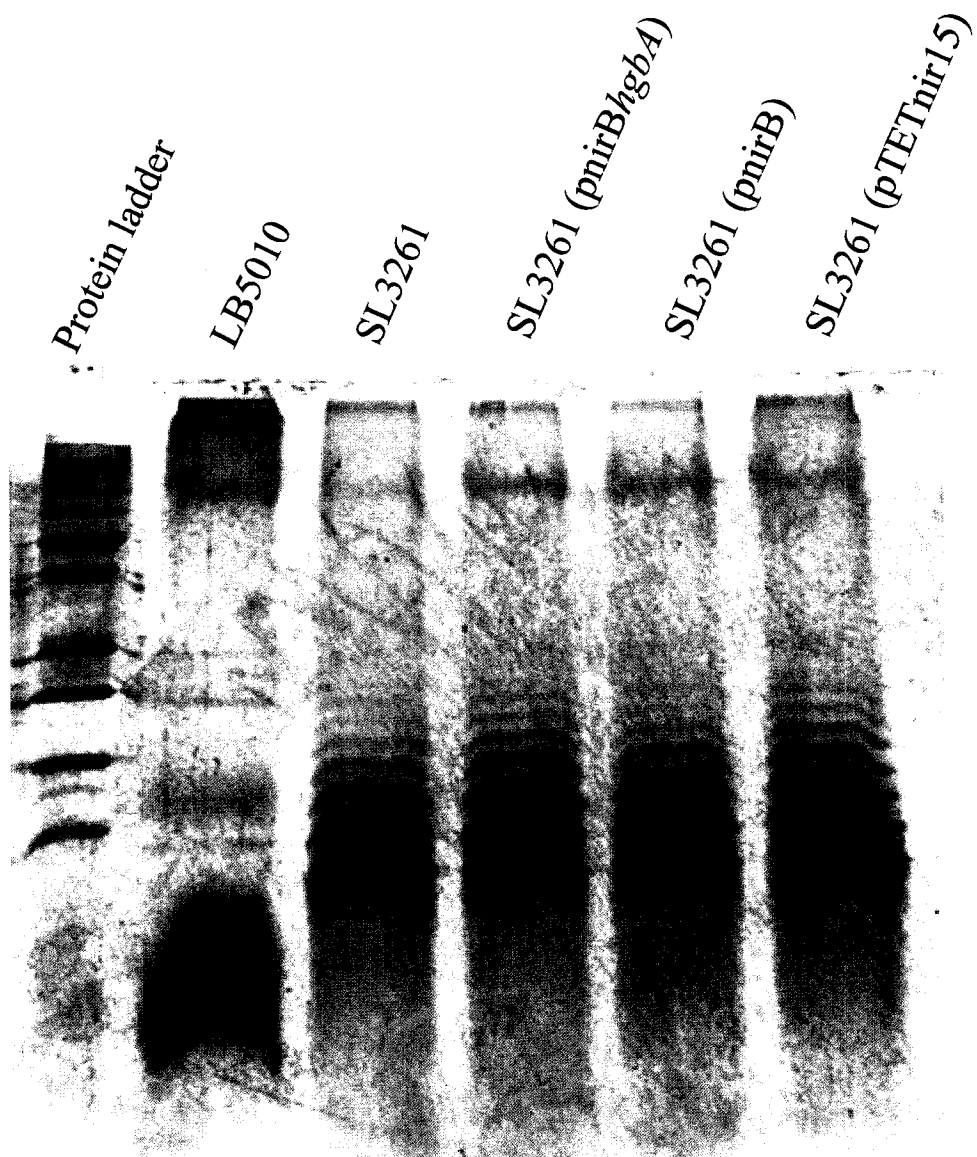


Figure 11: Silver staining analysis of the LPS profile of the recombinant *S. typhimurium* SL3261 strains.

Proteinase K treated whole cell lysates of recombinant strain *S. typhimurium* SL3261 (pnirBhgbA), (pnirB) or (pTETnir15), parent strain *S. typhimurium* SL3261 and *galE* mutated strain *S. typhimurium* LB5010, were separated by SDS-PAGE and LPS were visualized by silver staining.



intact LPS profile compared to the altered LPS profile of *S. typhimurium* LB5010 (Figure 11, lane 2).

3.2.2 Growth curve of the *S. typhimurium* SL3261 parent strain and the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain

To ensure the absence of a growth defect in the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain, growth assays were conducted. The *Salmonella* strains were grown under both aerobic and anaerobic conditions at 37°C, with continuous agitation, and growth was monitored by measuring the A₆₀₀. No major differences in growth between *S. typhimurium* SL3261 and *S. typhimurium* SL3261 (pnirBhgbA) were seen (Figure 12). The slower growth of both strains observed under anaerobic conditions (Figure 12B) compared to that seen under aerobic conditions (Figure 12A) was expected, as anaerobic metabolism reduces the rate of bacterial replication (82). These results indicated that a metabolic burden is not incurred by the presence of the *hgbA* gene in the *S. typhimurium* SL3261 vaccine strain.

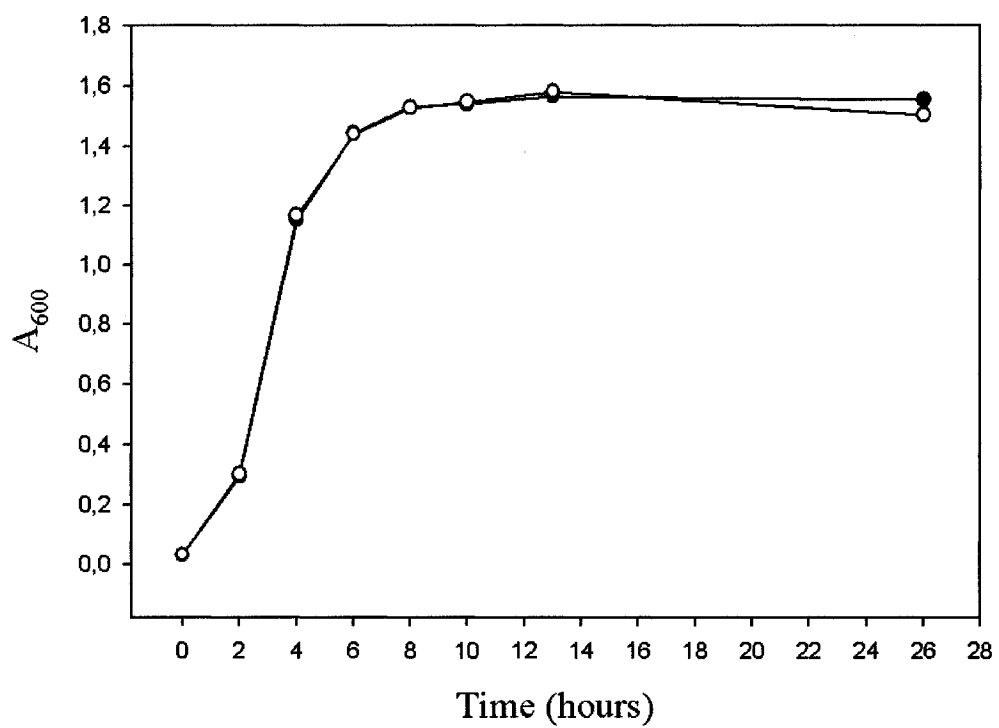
3.2.3 Phage sensitivity of the recombinant *S. typhimurium* SL3261 strains

The plasmids were introduced into the *S. typhimurium* SL3261 vaccine strain by transduction using a phage that contained an *int* mutation, which prevents the formation of stable lysogens (186). To confirm that the *Salmonella* SL3261 transductants were nonlysogens, the bacteria were tested for phage sensitivity to phage P22.c2. This virus contains a c2 mutation, which results in clear plaque formation (125, 228). Bacterial cells that are nonlysogens are expected to be P22 sensitive, whereas lysogenized strains are

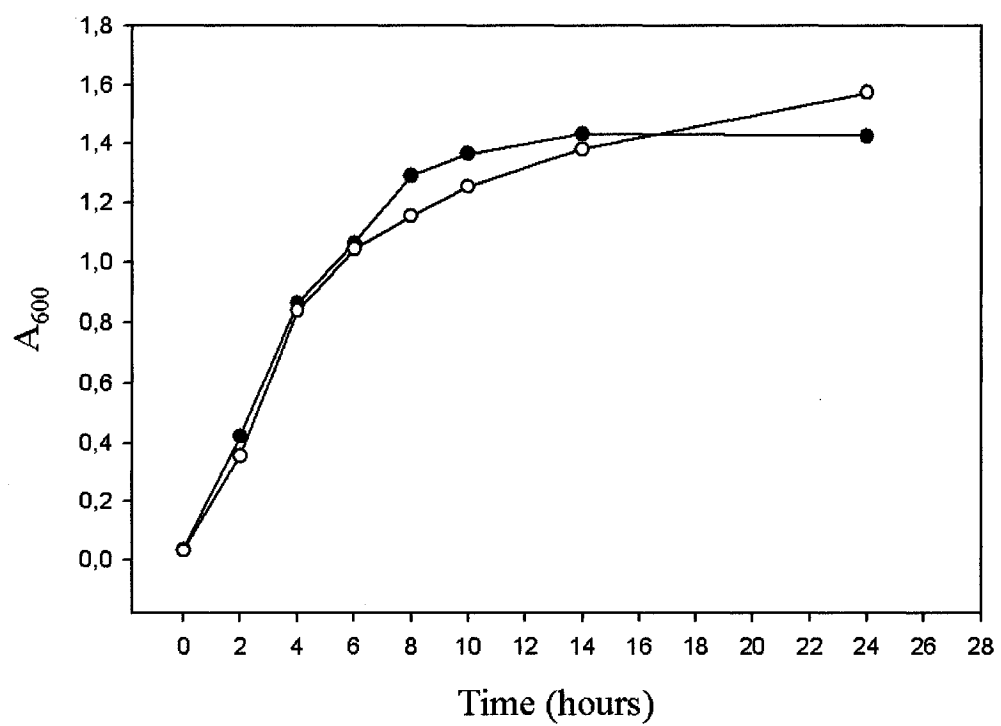
Figure 12: Growth curve of *S. typhimurium* SL3261 and *S. typhimurium* SL3261 (pnirBhgbA) in aerobic and anaerobic environments.

S. typhimurium SL3261 and *S. typhimurium* SL3261 (pnirBhgbA) were grown aerobically (A) or anaerobically (B) in LB broth for 24-26 hours. Growth was monitored by measuring A600 at time points. Legend: *S. typhimurium* SL3261 (closed circles), and *S. typhimurium* SL3261 (pnirBhgbA) (open circles).

A. Aerobic growth



B. Anaerobic growth



P22 resistant (135). Plaque formation was seen when *S. typhimurium* SL3261 (pnirBhgbA) (Figure 13A), *S. typhimurium* SL3261 (pnirB) (Figure 13B), and *S. typhimurium* SL3261 (pTETnir15) (Figure 13C) was infected with phage P22.c2, indicating that all these strains were nonlysogens.

3.3 Rabbit inoculation by gavage

3.3.1 Oral gavage procedure

A total of twelve rabbits were used in this study, in groups of six in two different vaccination trials. In the first vaccination trial the rabbits were inoculated with a single dose of the vaccine, but no humoral response to the recombinant antigen was detected. Therefore, in an attempt to achieve a humoral response to HgbA, a second trial was performed, where the rabbits were inoculated with the recombinant *S. typhimurium* SL3261 strains on three occasions at two-week intervals (booster regimen).

3.3.2 Rabbit dose and wellness monitoring

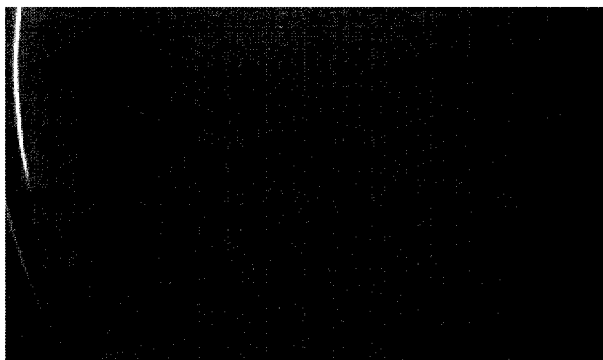
Prior experiments in our laboratory had determined that the maximum tolerated orally administered dose of *S. typhimurium* SL3261 in rabbits was 10^9 CFU, and the minimum immunogenic dose was 10^8 CFU (16). Accordingly, the dose for the oral gavage in these trials was targeted between 10^8 - 10^9 CFU. The delivered dose, assessed by viable colony counts, was within this range for both vaccination trials (Tables 1 and 2).

The rabbits were monitored closely the day of the gavage, and daily for 7 days following the gavage. None of the rabbits exhibited signs of respiratory difficulties, nasal discharge or piloerection. A temporary decrease in water consumption and fecal production were occasionally seen for 1 or 2 days following oral gavage. A single rabbit suffered from diarrhea that resolved within 2 days.

Figure 13: Sensitivity of the recombinant *S. typhimurium* SL3261 strains to phage P22.

S. typhimurium SL3261 (pnirBhgbA) (A), *S. typhimurium* SL3261 (pnirB) (B), and *S. typhimurium* SL3261 (pTETnir15) (C) were grown for 17 hours at 37°C with agitation and 500mL of the culture was placed onto LB+amp agar plates. The bacterial solution was left to diffuse into the agar before placing 10mL of phage P22.c2 onto the agar plate. After an incubation of 24 hours at 37 °C, the plates were examined for plaque formation.

A



B



C

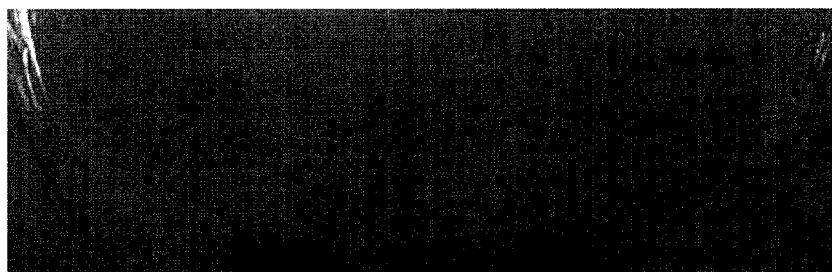


Table 1. Inocula fed by oral gavage in the single dose vaccination trial.

Rabbit Number	Delivered Dose
26	5.6×10^8 CFU
27	6.9×10^8 CFU
28	6.4×10^8 CFU
29	6.4×10^8 CFU
30	6.4×10^8 CFU
31	6.4×10^8 CFU

Legend: #26 *S. typhimurium* SL3261 (pTETnir15)-fed, #27 *S. typhimurium* SL3261 (pnirB)-fed, and #28-31 *S. typhimurium* SL3261 (pnirBhgbA)-fed

Table 2. Inocula fed by oral gavage in the booster regimen vaccination trial.

Rabbit Number	1 st Delivered Dose	2 nd Delivered Dose	3 rd Delivered Dose
1	PBS control	PBS control	PBS control
2	6.9×10^8 CFU	7.1×10^8 CFU	7.3×10^8 CFU
3	4.7×10^8 CFU	8.4×10^8 CFU	8.0×10^8 CFU
4	4.7×10^8 CFU	8.4×10^8 CFU	8.0×10^8 CFU
5	4.7×10^8 CFU	8.4×10^8 CFU	8.0×10^8 CFU
6	5.4×10^8 CFU	N/A	N/A

Legend: #1 PBS-fed, #2 *S. typhimurium* SL3261 (pnirB)-fed, #3-5 *S. typhimurium* SL3261 (pnirBhgb4)-fed, and #6 *S. typhimurium* SL3261 (pTETnir15)-fed. Not applicable (N/A) this rabbit received a single dose of the *Salmonella* strain.

3.4 Oral vaccination with a single dose of *S. typhimurium* SL3261 (pnirBhgbA)

3.4.1 Serum antibody response to antigens

Blood was collected from rabbits prior to and weekly for eight weeks following oral immunization with the *S. typhimurium* SL3261 (pnirB) control strain, the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain and with *S. typhimurium* SL3261 (pTETnir15) to monitor the humoral response. As prior work in our laboratory showed that rabbits generated antibodies to the heterologous antigen TetC delivered orally by *S. typhimurium* SL3261 (pTETnir15) (16), the humoral response to this protein served as internal control of the validity of the oral vaccination protocol.

No significant antibody response to HgbA was detected by EIA in both immunized and non-immunized rabbits (Figure 14). This result indicated that a single oral dose of the vaccine strain was insufficient to produce a humoral response against HgbA. However, a serum EIA against a crude soluble antigen preparation of *S. typhimurium* SL3261 (Figure 15), shows that the recombinant bacteria administered is immunogenic. Furthermore, a serum EIA against TetC (Figure 16), in a rabbit fed *S. typhimurium* SL3261 (pTETnir15), shows that an antibody response to an antigen delivered by the attenuated *Salmonella* vector can be detected in this system. Small numbers of rabbits in the control group preclude comparative statistical analysis.

Figure 14: Serum EIA against rHgbA in rabbits fed a single dose of *S. typhimurium* SL3261 (pnirBhgbA).

Blood from four rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain and one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain, collected at week 0, 2, 4, 5, and 8, was tested against rHgbA in an EIA to measure the antibody response to the vaccine candidate. Sera from a 1:50 dilution was analyzed for substrate colour development by measuring the optical density at 405 nm (A₄₀₅). Legend: Mean $\pm$ SD for *S. typhimurium* SL3261 (pnirBhgbA) (open circles), and *S. typhimurium* SL3261 (pnirB) (closed circles).

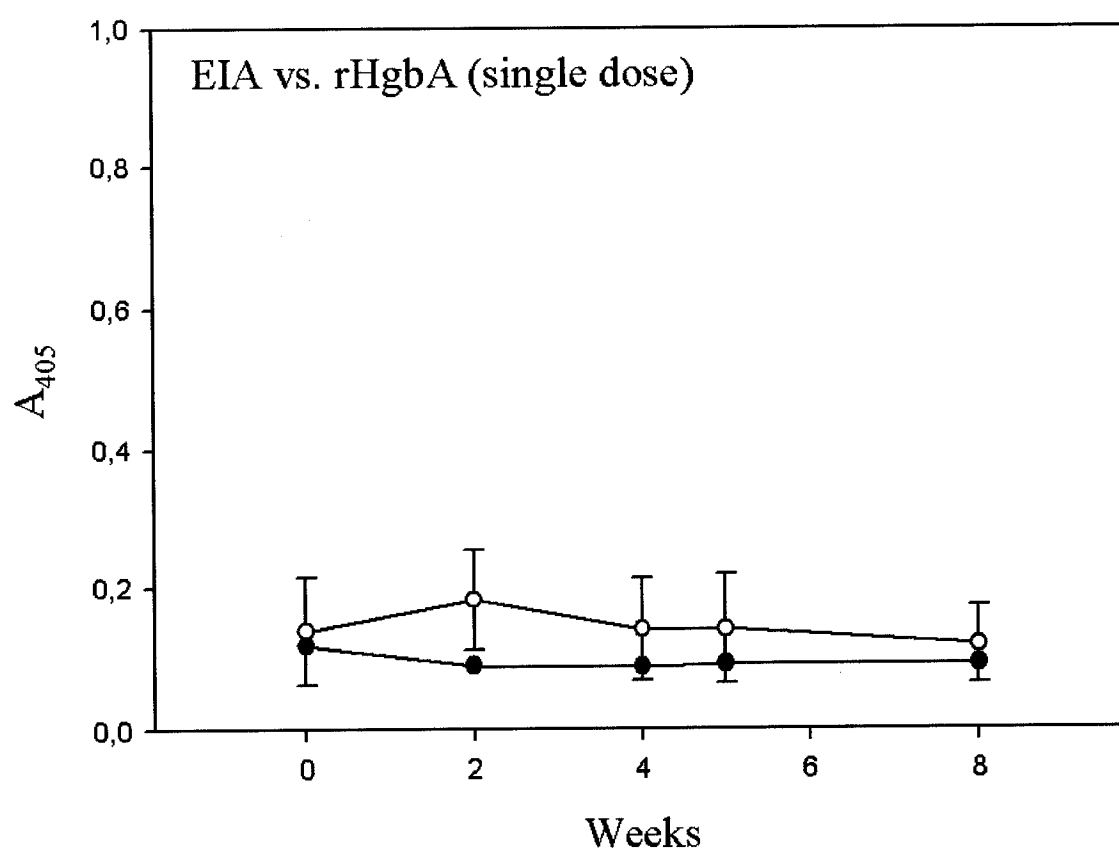


Figure 15: Serum EIA against *Salmonella* SL3261 crude soluble antigens in rabbits fed a single dose of *S. typhimurium* SL3261 (pnirBhgbA).

Blood from four rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain, collected at week 0, 2, 4, 5, and 8, was tested against crude soluble antigens to *Salmonella* SL3261 in an EIA to measure the antibody response to the attenuated *Salmonella* vaccine vector. Sera from a 1:4000 dilution was analyzed for substrate colour development by measuring the optical density at 405 nm (A405). Legend: Mean $\pm$ SD for *S. typhimurium* SL3261 (pnirBhgbA) (closed circles).

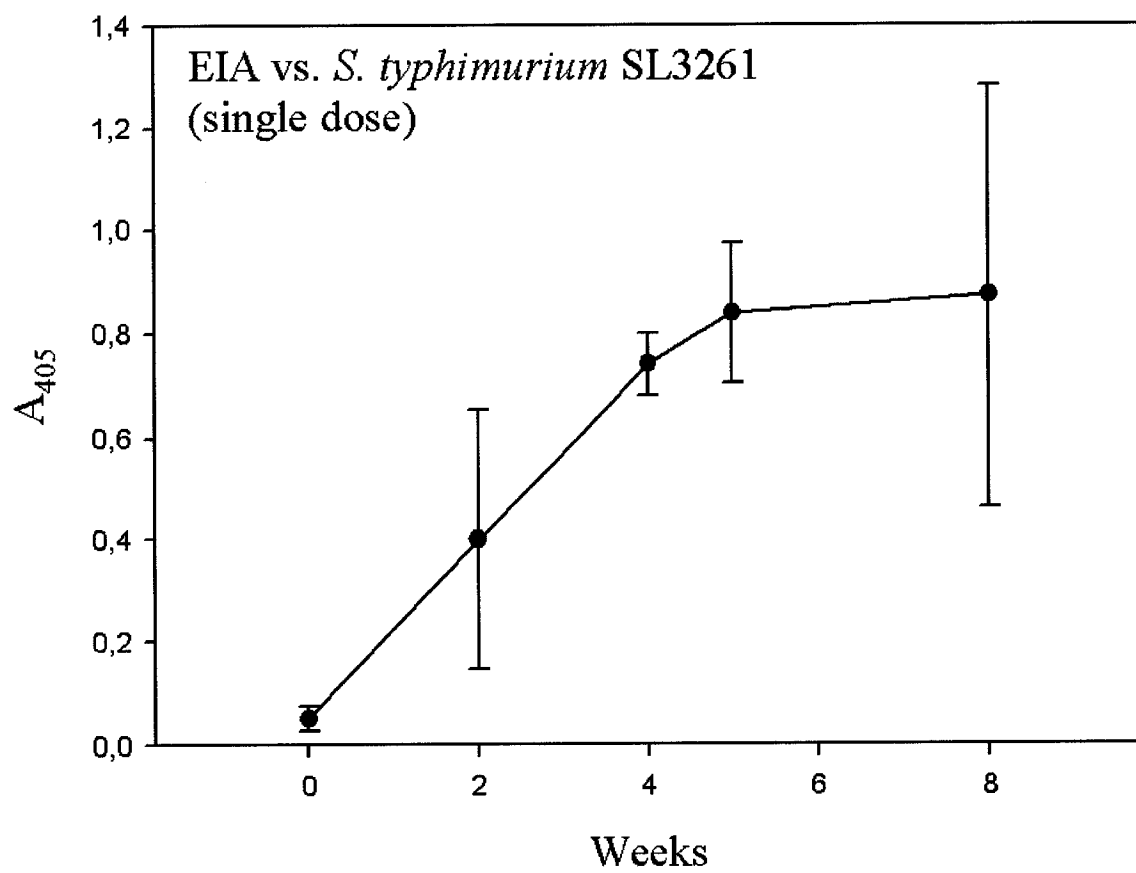
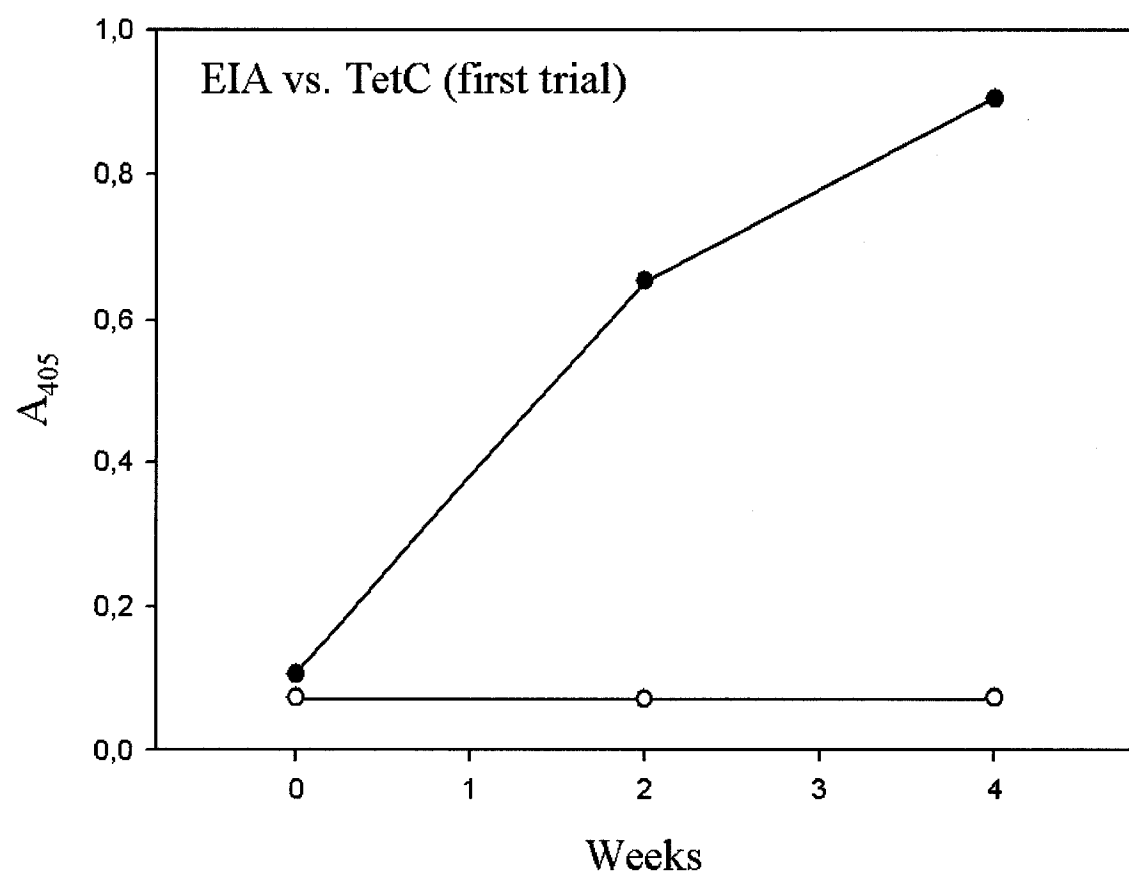


Figure 16: Serum EIA against TetC in a rabbit fed *S. typhimurium* SL3261 (pTETnir15).

Blood from one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain and one rabbit fed *S. typhimurium* SL3261 (pTETnir15), collected at week 0, 2, and 4, was tested against TetC in an EIA to measure the antibody response to TetC. Sera from a 1:50 dilution was analyzed for substrate colour development by measuring the optical density at 405 nm (A405). Legend: *S. typhimurium* SL3261 (pTETnir15) (closed circles), and *S. typhimurium* SL3261 (pnirB) (open circles).



3.4.2 Detection of *Salmonella* in rabbit stools after oral immunization with a single dose of the vaccine strain

Fecal shedding of the *Salmonella* strains was observed in all the orally challenged rabbits and ranged from 13-21 days for *S. typhimurium* SL3261 (pnirBhgbA), 10 days for *S. typhimurium* SL3261 (pnirB), and 6 days for *S. typhimurium* SL3261 (pTETnir15) (Table 3). No ampicillin resistant *Salmonella* species were isolated from stools collected 1 and 5 days prior to oral immunization (data not shown). The macrodigestion profiles of *Salmonella* isolates recovered from the stool of all six immunized rabbits were identical to the strain used for immunization, indicating preservation of the original genotype (Figure 17).

3.4.3 Characterization of *Salmonella* from rabbit stools after oral immunization with a single dose of the vaccine

Salmonella strains recovered from rabbit stool on the last day of fecal shedding underwent further genotypic and phenotypic characterization. To confirm the presence of the appropriate recombinant plasmid, restriction digest analyses of plasmid DNA isolated from *Salmonella* strains recovered on the last day of documented stool shedding were performed (Figure 18). An *AvrII* restriction digest of pnirBhgbA produced the anticipated 5.225 kb linearized fragment (Figure 18A, lane 3-6). When pTETnir15 was digested with *NcoI*, which recognizes a single restriction site within the *tetC* gene, the expected 3.727 kb fragment was generated (Figure 18B, lane 3). Finally, restriction of pnirB with *NheI*, which digests the pnirB vector once, yielded a single band of the predicted size of 2.372 kb (Figure 18B, lane 8). These results indicated that no obvious changes in the plasmid

Table 3. Days of *Salmonella* shedding in the single dose vaccination trial.

Rabbit Number	Days Shed
26	6
27	10
28	15
29	13
30	21
31	13

Legend: #26 *S. typhimurium* SL3261 (pTETnir15)-fed, #27 *S. typhimurium* SL3261 (pnirB)-fed, and #28-31 *S. typhimurium* SL3261 (pnirBhgbA)-fed.

Figure 17: PFGE on organisms recovered from rabbit stools in the first vaccination trial.

Salmonella identified organisms, isolated from rabbit stools, were analyzed for their DNA profile using PFGE analyses. Rabbits #28-31 were fed *S. typhimurium* SL3261 (pnirBhgbA), #27 was fed *S. typhimurium* SL3261 (pnirB), and #26 was fed *S. typhimurium* SL3261 (pTETnir15). Legend: + represents the -70° frozen culture stocks of *S. typhimurium* SL3261 (pnirBhgbA), (pnirB) or (pTETnir15), respectively. Isolates recovered on day 6 post-gavage (Lane #28a, 29a, 30a, and 31a), and on the last day of shedding (Lane #28b, 29b, 30b, 31b, #27, and #26). PFGE performed by Ian In Coulthick.

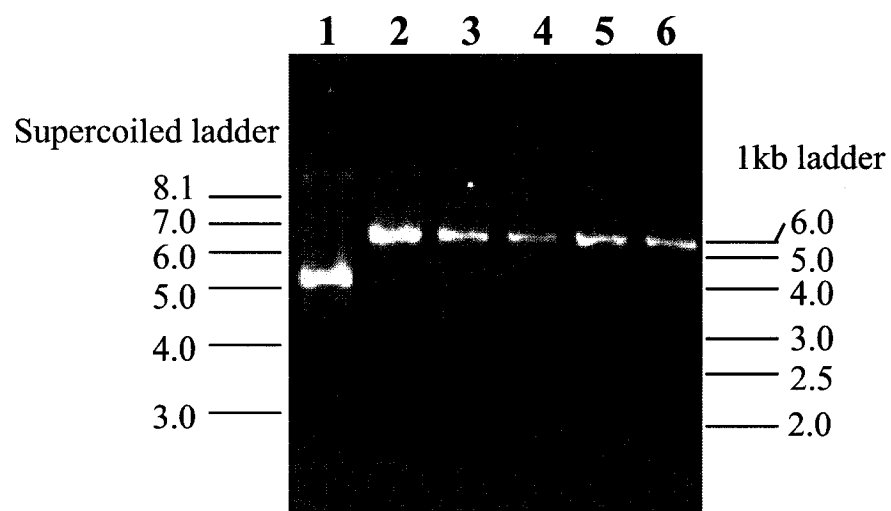
$$\begin{array}{cccc}
 \text{\#28} & \text{\#29} & \text{\#30} & \text{\#31} \\
 a \quad b & a \quad b & a \quad b & a \quad b \\
 + \boxed{} & \boxed{} & \boxed{} & \boxed{} + \text{\#27} + \text{\#26}
 \end{array}$$



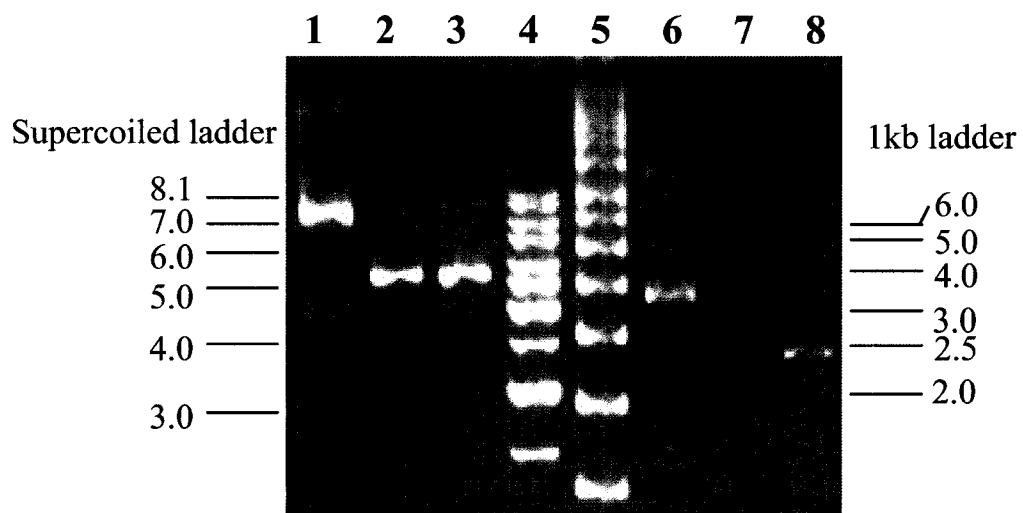
Figure 18: Restriction digest analysis of plasmid DNA isolated from *Salmonella* recovered from rabbit stools in the first vaccination trial.

Plasmid DNA isolated from stool-recovered *Salmonella* strains of rabbits fed *S. typhimurium* SL3261 (pnirBhgbA) (A), a rabbit fed *S. typhimurium* SL3261 (pnirB) (B), and a rabbit fed *S. typhimurium* SL3261 (pTETnir15) (B) were digested with *AvrII*, *NheI*, or *NcoI* respectively, before applying onto a 1% (w/v) agarose gel. Legend (A): undigested (Lane 1) and *AvrII* digested pnirBhgbA (Lane 2) isolated from -70°C frozen cultures stock. *AvrII* digested plasmids recovered from rabbits #28, 29, 30, and 31 respectively, at their last day of shedding (Lane 3-6). Legend (B): Undigested pTETnir15 (Lane 1), *NcoI* digested pTETnir15 (Lane 2), undigested pnirB (Lane 6) and *NheI* digested pnirB (Lane 7) isolated from -70°C frozen cultures stock. *NcoI* digested plasmid recovered from rabbit #26 (Lane 3) and *NheI* digested plasmid recovered from rabbit #27 (Lane 8), at the last day of shedding. Lane 4 and 5; ladders. Sizes of the molecular mass markers (in kb) are shown on the left for the supercoiled ladder and on the right for the linear DNA fragments ladder.

A



B



nucleotide sequences occurred during *in vivo* passage of the recombinant *Salmonella* strains.

To assess whether the retrieved plasmid constructs retained the ability to express either HgbA or TetC, Western blots of *Salmonella* whole cell lysates were probed with the respective specific polyclonal antisera. PVDF membranes for Western blotting were probed with anti-rHgbA (Figure 19A and B) or with anti-TetC (Figure 19C). In all four of the rabbits (#28, #29, #30, and #31) challenged with *S. typhimurium* SL3261 (pnirBhgbA), an immunoreactive band corresponding to the 108.6 kDa HgbA was seen in cell lysates from organisms recovered on the first day of fecal shedding (Figure 19A and B). In one rabbit (#31) the expression of HgbA was lower. No protein was detected in lysates from *S. typhimurium* SL3261 (pnirBhgbA) retrieved on the last documented day of fecal shedding (data not shown). In contrast, HgbA expression was absent as expected in the control strain *S. typhimurium* SL3261 (pnirB) (Figure 19A, lane #27). These results indicated that HgbA expression was preserved following early *in vivo* passage. The expression of TetC was more persistent as the 50 kDa peptide fragment was present in lysates obtained from *S. typhimurium* SL3261 (pTETnir15) isolated from stool on both the first day (day 1) and the last day (day 6) of fecal shedding (Figure 19C).

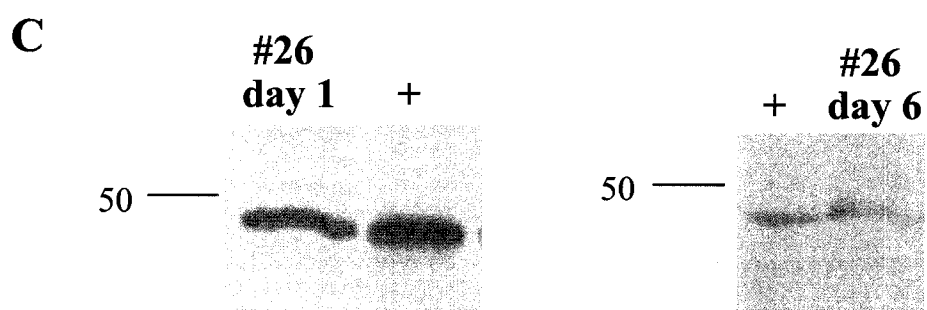
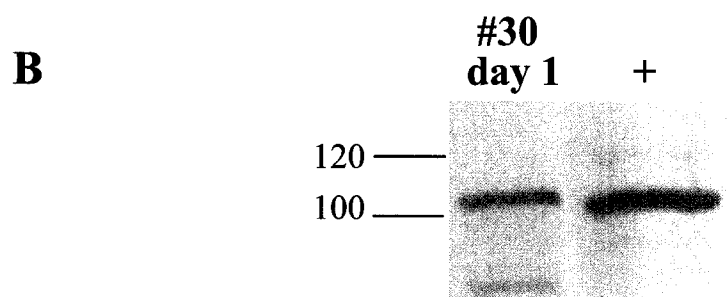
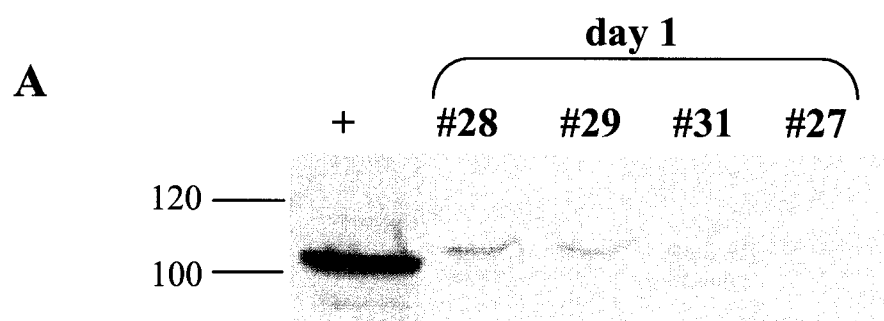
3.5 Oral vaccination with three doses of *S. typhimurium* SL3261 (pnirBhgbA)

3.5.1 Serum antibody response to antigens

Blood was collected prior to and at week 2, 4, 5, and 8 after the first oral gavage from rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain or the control strains (PBS-fed or *S. typhimurium* SL3261 (pnirB)-fed or *S. typhimurium* SL3261 (pTETnir15)-fed) to monitor the humoral response. A serum EIA against rHgbA was used to measure the antibody response to the vaccine candidate. No significant antibody

Figure 19: HgbA and TetC expression analysis in *Salmonella* identified isolates recovered from rabbit stools in the first vaccination trial.

Salmonella identified isolates recovered from rabbit stools of rabbits fed *S. typhimurium* SL3261 (pnirBhgbA) (#28, 29, 30, 31 in A and B), a rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain (#27 in A), or a rabbit fed *S. typhimurium* SL3261 (pTETnir15) (#26 in C), were grown anaerobically, lysed, and separated by SDS-PAGE followed by Western blotting with anti-rHgbA (A and B), or anti-TetC (C). Legend (A and B): for positive control (Lane +), *H. ducreyi* cells were grown in low heme broth. Legend (C): for positive control (Lane +), *S. typhimurium* SL3261 (pTETnir15) cells were grown under anaerobic conditions. The days represent the day the isolate was recovered post-gavage. Sizes of the molecular mass markers (in kDa) are shown on the left.



response to HgbA was detected in both immunized and sham-immunized rabbits, up until week five (Figure 20A). However, a very strong antibody response is detected at week eight (Figure 20B) in rabbits that were experimentally challenged with *H. ducreyi* but not in a rabbit that was not challenged (Figure 20B, open triangles). This demonstrates that three doses of the vaccine is insufficient to produce a humoral response against HgbA in the rabbit model, but that HgbA antibodies raised in rabbits experimentally challenged with *H. ducreyi* can recognize rHgbA in an EIA. Small numbers of rabbits in the control group preclude comparative statistical analysis.

Once again, a serum EIA against a crude soluble antigen preparation of *S. typhimurium* SL3261 (Figure 21) shows that the recombinant bacteria administered is immunogenic. We also see an obvious antibody response to *Salmonella* crude soluble antigens at week 8 in the PBS sham-immunized control (Figure 21, open circles), which is absent at week 0, 2, 4, and 5. This response is likely do to cross reactivity between antibodies raised against *H. ducreyi* from the experimental challenge perform after week 5, and crude soluble antigens of *S. typhimurium* SL3261. In the second vaccination trial rabbits received three doses of the recombinant *Salmonella* strains, whereas in the first vaccination trial rabbits received a single dose. We compared the antibody response to *Salmonella* crude soluble antigens in the different vaccination trials (Figure 22). We observed a statistically significant (Student's t-test, $p < 0.01$) difference between the single dose trial (open squares) and the booster regimen trial (closed squares).

Furthermore, a serum EIA against TetC (Figure 23), in a rabbit fed *S. typhimurium* SL3261 (pTETnir15), shows that once again an antibody response to an antigen delivered by the attenuated *Salmonella* vector can be detected in this system. We also observed an apparent higher response to TetC in the second vaccination trial (Figure 24, closed circles) than in the first trial (Figure 24, open circles).

Figure 20: Serum EIA against rHgbA in rabbits fed three dose two weeks a part of *S. typhimurium* SL3261 (pnirBhgbA).

Blood from three rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain, one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain, one rabbit fed PBS (sham-immunized control) (A and B), and one rabbit fed *S. typhimurium* SL3261 (pTETnir15) (B), collected prior to and at week 2, 4, 5, and 8 after the first oral gavage, was tested against rHgbA in an EIA to measure the antibody response to the vaccine candidate. Sera from a 1:50 dilution was analyzed for substrate colour development by measuring the optical density at 405 nm (A405). Legend: Mean $\pm$ SD for *S. typhimurium* SL3261 (pnirBhgbA) (open circles), *S. typhimurium* SL3261 (pnirB) (inverted closed triangles), *S. typhimurium* SL3261 (pTETnir15) (open triangles), and PBS sham-immunized control (closed circles). The arrow indicates when the rabbits were experimentally challenged with *H. ducreyi* (three days after the blood collection of week 5).

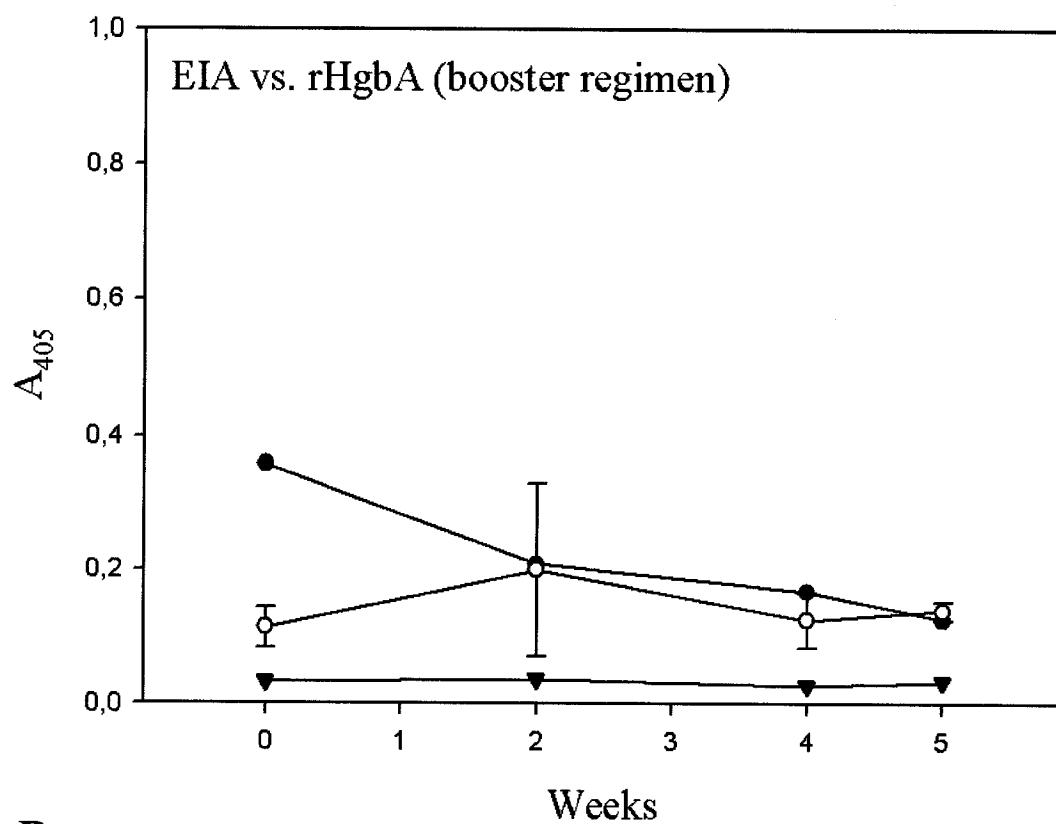
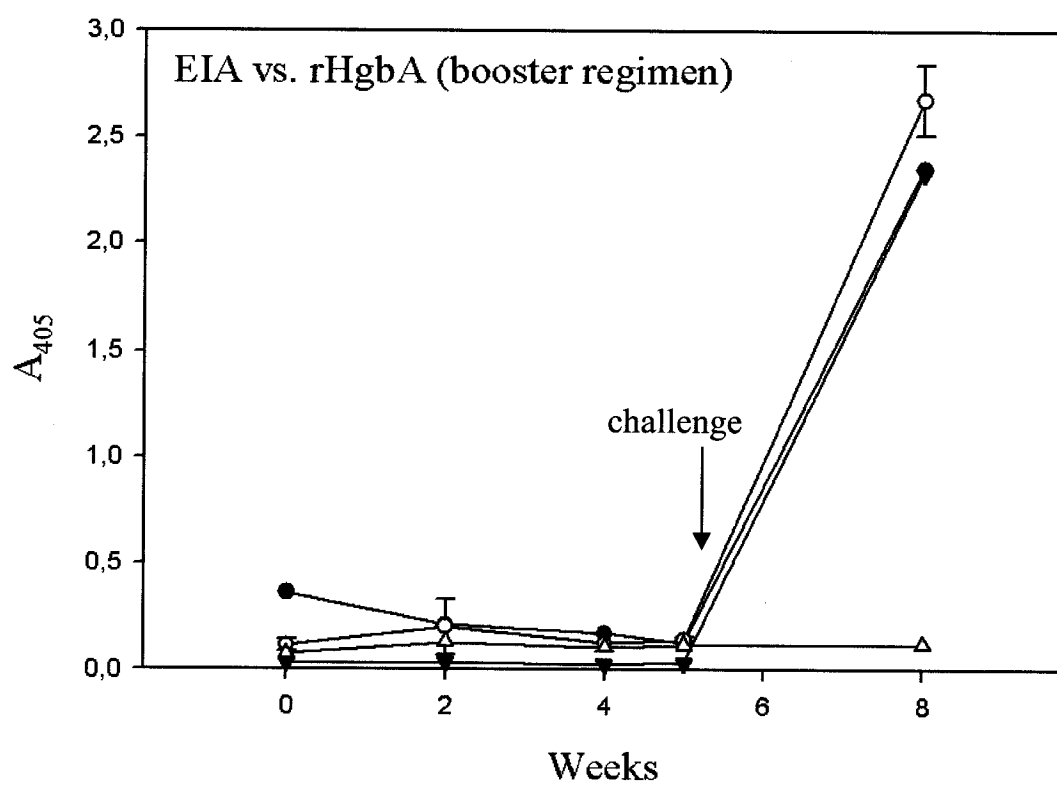
A**B**

Figure 21: Serum EIA against *Salmonella* SL3261 crude soluble antigens in rabbits fed three doses two weeks apart of *S. typhimurium* SL3261 (pnirBhgbA).

Blood from three rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain, and one rabbit fed PBS (sham-immunized control) collected at week 0, 2, 4, 5, and 8 was tested against crude soluble antigens to *Salmonella* SL3261 in an EIA to measure the antibody response to the attenuated *Salmonella* vaccine vector. Sera from a 1:4000 dilution was analyzed for substrate colour development by measuring the optical density at 405 nm (A405). Legend: Mean $\pm$ SD for *S. typhimurium* SL3261 (pnirBhgbA) (closed circles), and PBS sham-immunized control (open circles).

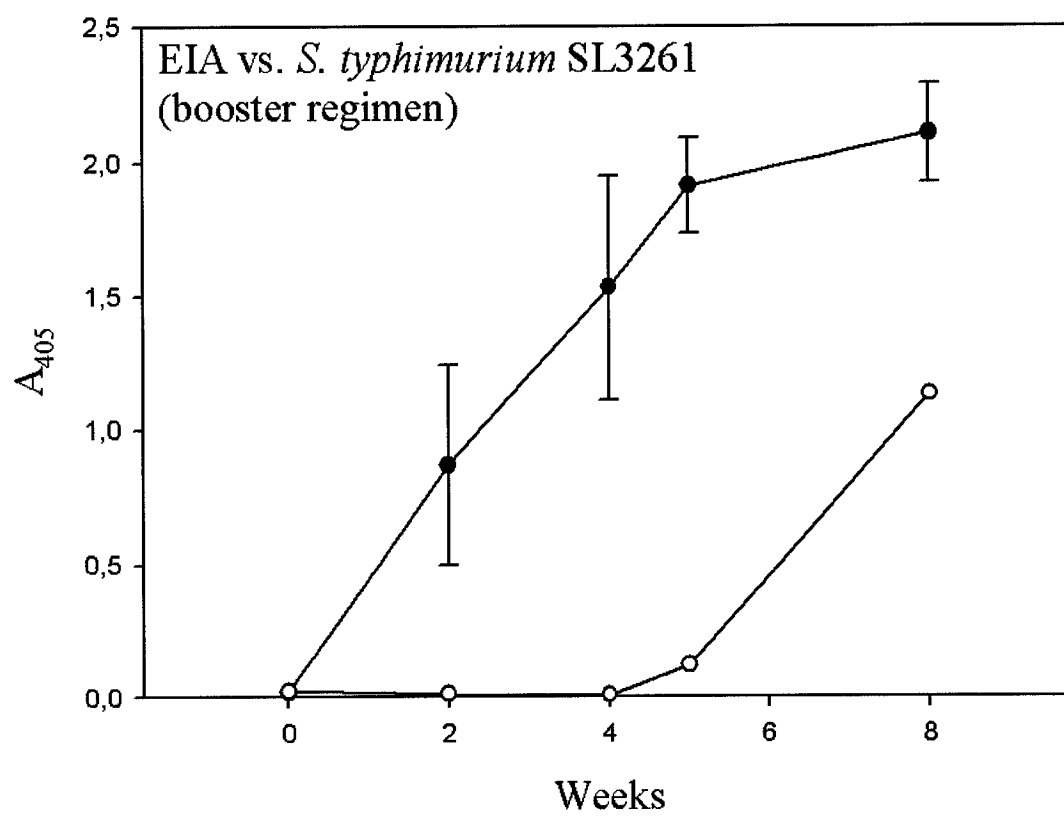


Figure 22: Comparison of the antibody response against *Salmonella* SL3261 crude soluble antigens from the single dose and the booster regimen vaccination trials.

Blood from three rabbits fed a single dose of the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain (open squares), and from four rabbits fed three doses two weeks apart of the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain (closed squares), was collected at week 0, 2, 4, 5, and 8 and the sera at a 1:4000 dilution was reacted against *Salmonella* SL3261 crude soluble antigens in an EIA to measure the antibody response to the attenuated *Salmonella* vaccine vector. Differences in the optical density (mean $\pm$ SD) at 405 nm (A405) were analyzed by the Student's paired t test.

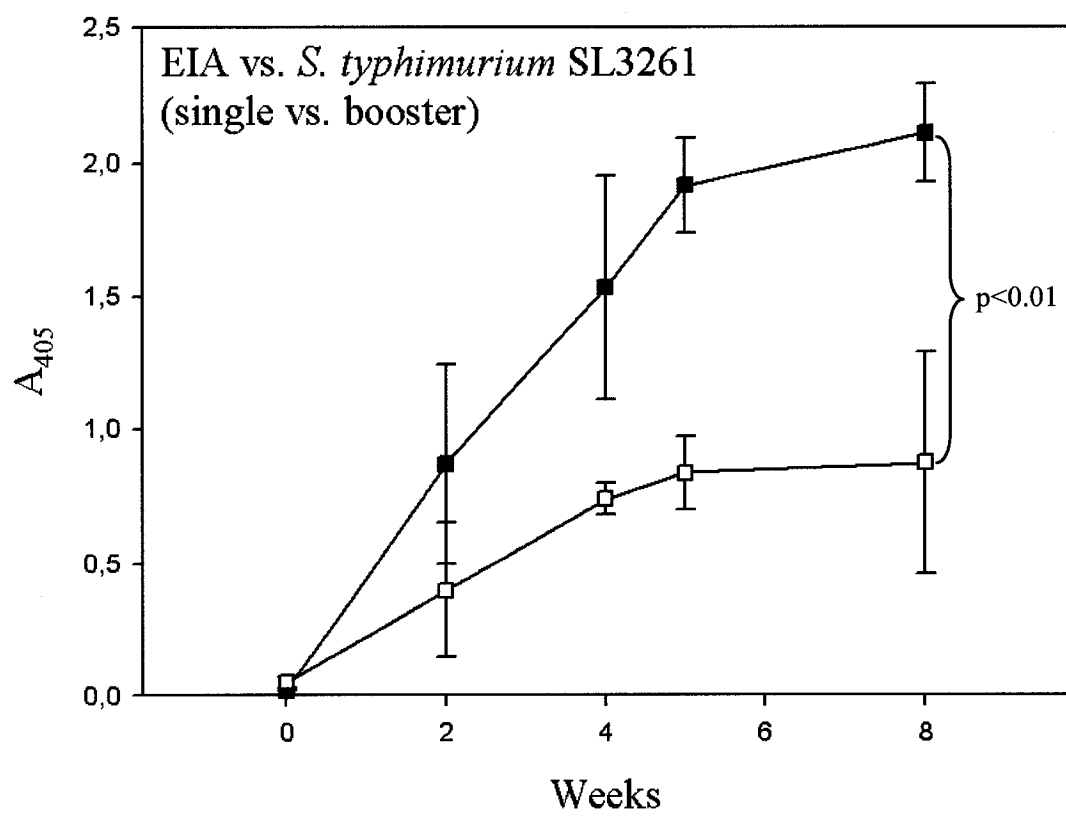


Figure 23: Antibody response against TetC in a rabbit fed *S. typhimurium* SL3261 (pTETnir15).

Sera from one rabbit fed *S. typhimurium* SL3261 (pTETnir15), collected at week 0, 2, and 4 were tested at a 1:50 dilution against TetC in an EIA to measure the antibody response to TetC. The optical density was measured at 405 nm (A₄₀₅).

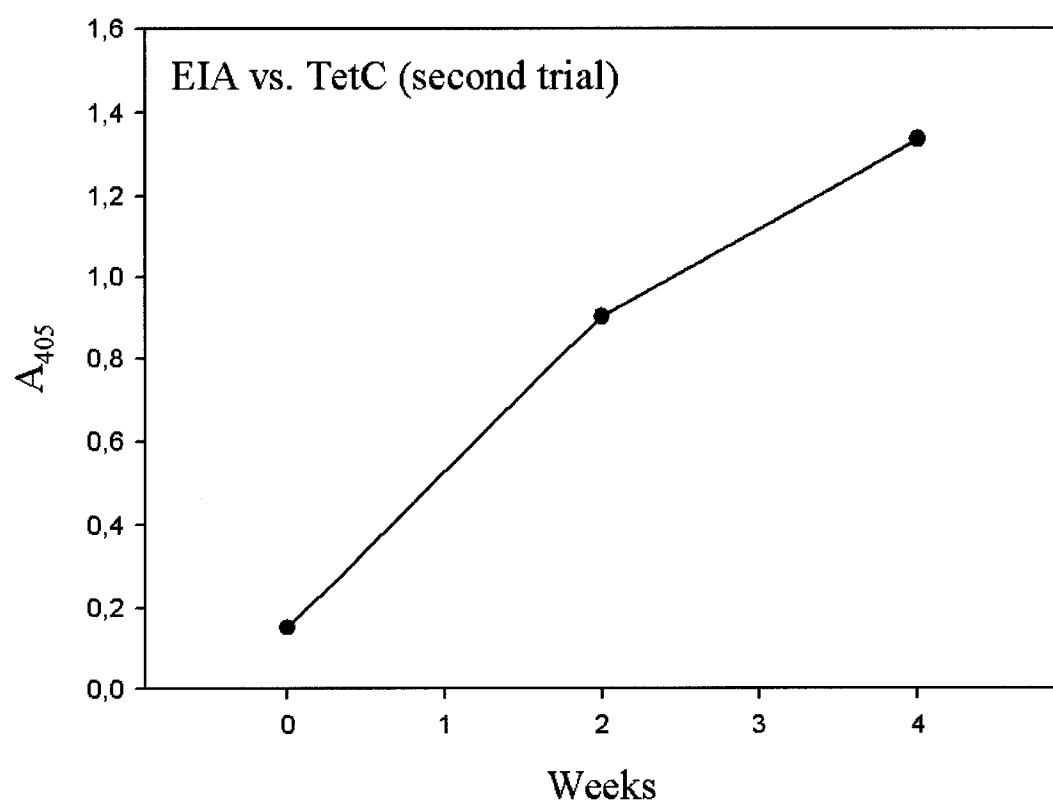
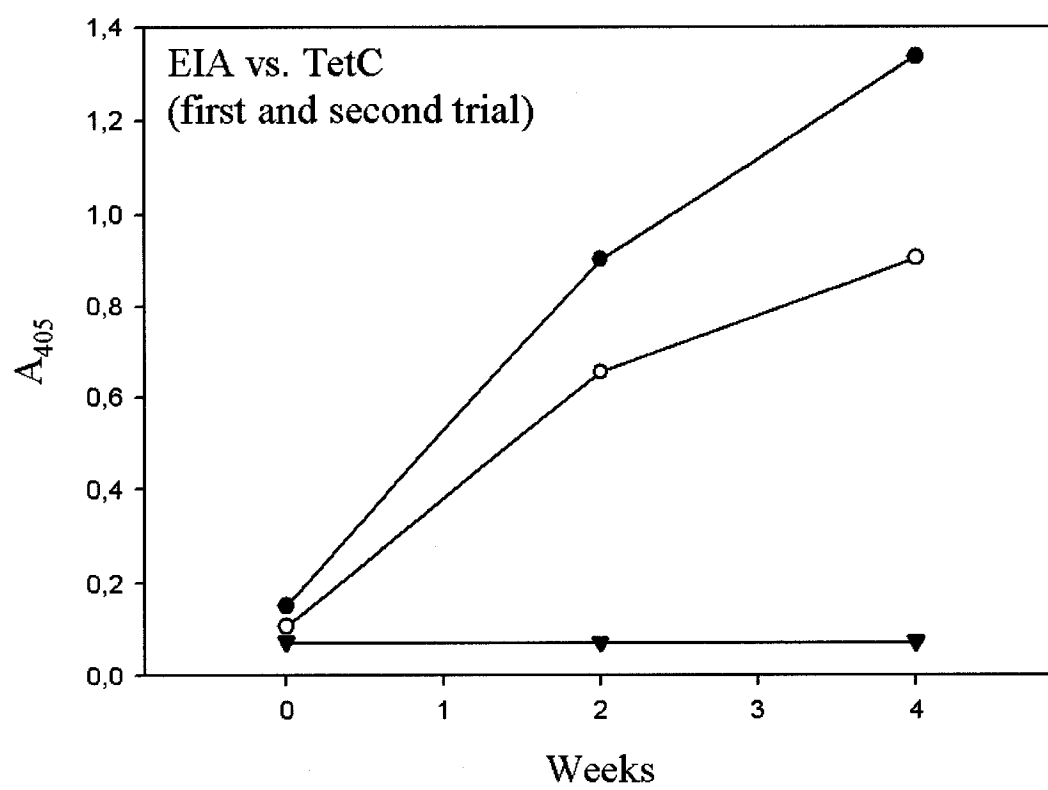


Figure 24: Comparison of the antibody response against TetC with sera from the first and the second vaccination trial.

Blood from one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain in the first vaccination trial (inverted closed triangles) and two rabbits fed *S. typhimurium* SL3261 (pTETnir15) (open circles, first trial) (closed circles, second trial), was collected at week 0, 2, and 4 and the sera were tested against TetC at a 1:50 dilution in an EIA. The optical density was measured at 405 nm (A405).



3.5.2 Detection of *Salmonella* in rabbit stools after oral immunization with three doses of *S. typhimurium* SL3261 (pnirBhgbA)

Ampicillin resistant NLF organisms that were identified on MAC+amp plates underwent further biochemical characterization by VITEK[®] testing. As shown in Table 4, several other enteric bacteria other than the expected *Salmonella* strains were isolated, including *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa*. These organisms likely represented environmental contamination of the stool pellet arising from the water or the stool collecting pan. As displayed in Table 5, all of the recombinant *Salmonella* strains survived *in vivo* passage. The number of days these organisms were isolated from stool ranged between 6-20 days *S. typhimurium* SL3261 (pnirBhgbA), 24 days for *S. typhimurium* SL3261 (pnirB), and 10 days for *S. typhimurium* SL3261 (pTETnir15).

3.5.3 Characterization of *Salmonella* in rabbit stools after oral immunization with three doses of *S. typhimurium* SL3261 (pnirBhgbA)

Salmonella strains isolated from the three rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) were examined for the presence of the plasmid pnirBhgbA. To identify pnirBhgbA, PCR amplification using oligonucleotides annealing to the *hgbA* gene was performed with purified plasmid DNA as template. The expected 2853 bp PCR product was identified in plasmids recovered from 1 to 10 days following oral immunization in all three rabbits [rabbit #3 at day 10 (Figure 25A, lane 2), rabbit #4 at day 1 and 6, and rabbit #5 at day 1 and 10 (Figure 25A, lane 4-7). In plasmids retrieved at subsequent days in two of the three immunized rabbits, no amplicon corresponding to

Table 4. VITEK[®] tests results of organisms recovered from MAC+amp plates in the booster regimen vaccination trial.

Rabbit #2	Organism	Probability	Confidence level
Day 1	<i>Klebsiella pneumoniae</i>	99%	Excellent
Day 6	<i>Klebsiella pneumoniae</i>	99%	Excellent
Day 10	<i>Klebsiella pneumoniae</i>	99%	Excellent
Day 15	<i>Salmonella</i> group	98.53%	Excellent
Day 20	<i>Salmonella</i> group	97.26%	Excellent
Day 24	<i>Salmonella</i> group	98.53%	Excellent
Rabbit #3			
Day 1	<i>Klebsiella pneumoniae</i>	99%	Excellent
Day 6	<i>Stenotrophomonas maltophilia</i>	98.78%	Excellent
Day 10	<i>Salmonella</i> group	97.26%	Excellent
Day 15	<i>Salmonella</i> group	97.17%	Excellent
Rabbit #4			
Day 1	<i>Salmonella</i> group	97.26%	Excellent
Day 6	<i>Salmonella</i> group	94.35%	Very good
Rabbit #5			
Day 1	<i>Salmonella</i> group	98.63%	Excellent
Day 10	<i>Salmonella</i> group	97.26%	Excellent
Day 15	<i>Salmonella</i> group	97.26%	Excellent
Day 20	<i>Salmonella</i> group	97.26%	Excellent
Rabbit #6			
Day 1	<i>Salmonella</i> group	97.17%	Excellent
Day 6	<i>Pseudomonas aeruginosa</i>	97.54%	Excellent
Day 10	<i>Salmonella</i> group	97.17%	Excellent

Legend: #2 *S. typhimurium* SL3261 (pnirB)-fed, #3-5 *S. typhimurium* SL3261 (pnirBhgbA)-fed, and #6 *S. typhimurium* SL3261 (pTETnir15)-fed.

Table 5. Days of *Salmonella* shedding in the booster regimen vaccination trial.

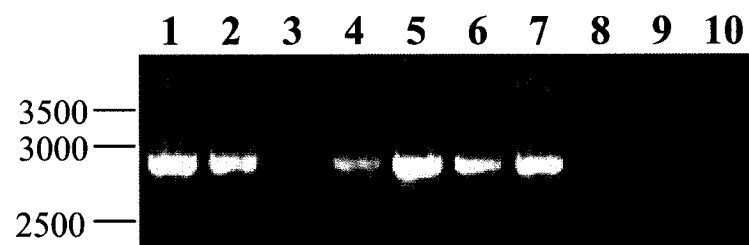
Rabbit Number	Days Shed
1	0
2	24
3	15
4	6
5	20
6	10

Rabbits were administered by oral gavage PBS (rabbit 1), *S. typhimurium* SL3261 (pnirB) (rabbit 2), *S. typhimurium* SL3261 (pnirBhgbA) (rabbits 3-5), and #6 *S. typhimurium* SL3261 (pTETnir15) (rabbit 6).

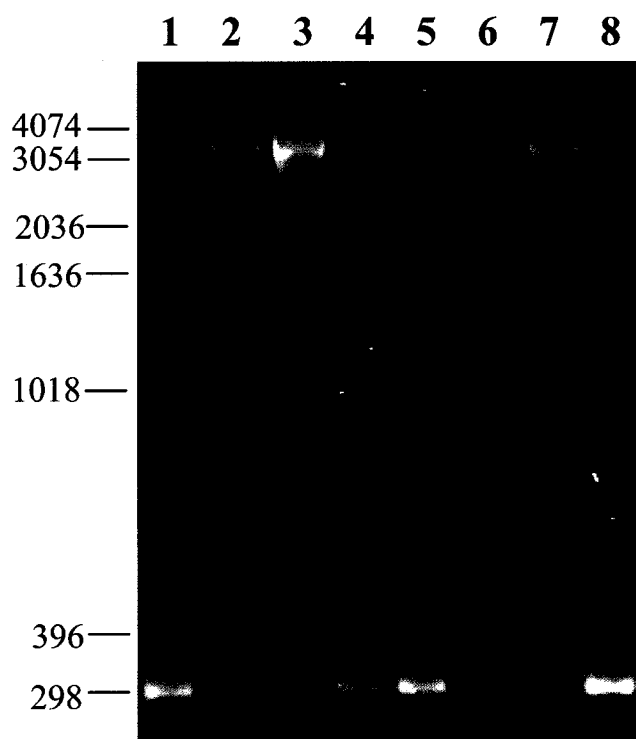
Figure 25: PCR amplification analysis of plasmid DNA isolated from *Salmonella* recovered from rabbit stools in the second vaccination trial.

Plasmids isolated from *Salmonella* recovered from stools of rabbits fed *S. typhimurium* SL3261 (pnirBhgbA), were used as template for PCR amplification using primers that annealed to the *hgbA* gene sequence (A), or primers that bracketed the *hgbA* gene (B). The amplified products were applied to a 1% (w/v) agarose gel. For Panel A, the following templates were used: pnirBhgbA recovered from -70°C frozen stocks *S. typhimurium* SL3261 (pnirBhgbA) (Lane 1), plasmid recovered from rabbit #3 at day 10 and 15 (Lane 2 and 3), plasmid recovered from rabbit #4 at day 1 and 6 (Lane 4 and 5), plasmid recovered from rabbit #5 at day 1, 10, 15, and 20 (Lane 6-9), H₂O (Lane 10). For Panel B, the following templates were used: pnirB recovered from -70°C frozen stocks of *S. typhimurium* SL3261 (pnirB) (Lane 1), plasmid recovered from rabbit #5 at day 1, 10, 15, and 20 (Lanes 2-5), H₂O (Lane 6), *S. typhimurium* SL3261 (pnirBhgbA) from frozen stock (Lane 7), plasmid recovered from rabbit #3 at day 15 (Lane 8). Sizes of the molecular mass markers (in bp) are shown on the left.

A



B



hgbA was present (Figure 25A, lane 3 and lanes 8 and 9). Although plasmids were recovered from these isolates, their size on agarose gel electrophoresis did not match that of *pnirBhgbA* but rather the dimeric form of *pnirB* (data not shown). As loss of the cloned *hgbA* gene from these plasmids represented a likely explanation for these observations, PCR amplification using primers complementary to vector sequences bracketing the *hgbA* gene was conducted. The presence of the insert would generate a 3169 bp PCR product (Figure 25B, lane 7) whereas the absence of *hgbA* would result in an amplicon of 295 bp (Figure 25B, lane 1). As depicted in Figure 25B, a PCR product of 3169 bp was amplified from plasmids previously shown to contain *hgbA* (lanes 2 and 3). However, a 295 bp amplicon was produced from the plasmids extracted from stool isolates of *Salmonella* strains recovered beyond 10 days of oral gavage (Figure 25B, lanes 4, 5, and 8).

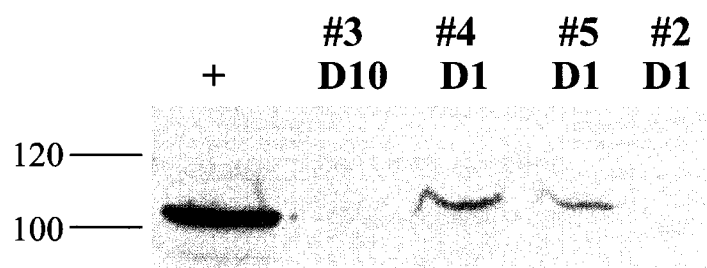
The expression of HgbA was restricted to *S. typhimurium* SL3261 (*pnirBhgbA*) recovered 1 day after immunization except in one rabbit where the expression was present 6 days after immunization (Figure 26A and B). However, despite the presence of the corresponding gene, HgbA was not detected in *S. typhimurium* SL3261 (*pnirBhgbA*) isolated beyond 24 h for two of three rabbits. As expected, in the *S. typhimurium* SL3261 strains carrying either *pnirB*, or *pnirBhgbA* in which *hgbA* was spontaneously deleted, no HgbA expression was detected (Figure 26A, lane #2, and data not shown). These results indicated that although the *hgbA* gene was stably maintained in recombinant plasmids for at least 10 days of *in vivo* passage, protein production occurred only in *S. typhimurium* SL3261 (*pnirBhgbA*) strains with a more limited *in vivo* exposure.

TetC expression was identified in *S. typhimurium* SL3261 (pTETnir15) recovered on the last day that fecal shedding of this strain was observed (Figure 26C).

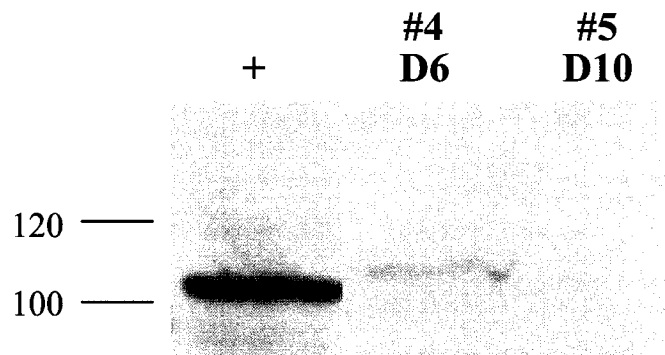
Figure 26: HgbA and TetC expression in *Salmonella* recovered from rabbit stools in the second vaccination trial.

Whole cell lysates, prepared from anaerobically grown *Salmonella* strains recovered from rabbit stools, were analyzed by Western immunoblots. In Panels A & B, the blots were probed with anti-rHgbA. In Panel C, the transferred proteins were reacted with anti-TetC. Sizes of the molecular mass markers (in kDa) are shown on the left.

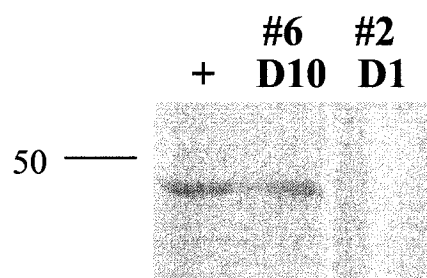
A



B



C



3.5.4 Cell-mediated immune response in immunized rabbits

Following intra-epithelial challenge with 100 µg rHgbA, no difference in the size of induration was noted between rabbits fed *S. typhimurium* (pnirBhgbA) [8.09 ± 0.99 mm] and the *S. typhimurium* (pnirB) control strain [9.88 mm] (data not shown). Following intra-epithelial challenge with 100 µg of *E. coli* (pUNCH1202) crude soluble antigens, no difference in the size of induration was noted between rabbits fed *S. typhimurium* (pnirBhgbA) [6.71 ± 1.53 mm] and the *S. typhimurium* (pnirB) control strain [8.95 mm] (data not shown). *E. coli* (pUNCH1202) expresses the ferric enterobactin receptor (FetA) of *N. gonorrhoeae* (41, 42). Except for the FetA gene, this strain is genotypically identical to the recombinant *E. coli* strain expressing *H. ducreyi* rHgbA and was used to control for a skin test reaction arising from antigens other than rHgbA. The absence of a difference between the control and the vaccinated rabbit in the rHgbA challenge, and because the response elicited in the *E. coli* (pUNCH1202) crude soluble antigens is almost equal to the response in the rHgbA challenge, indicates that there is no specific HgbA cell-mediated immune response in immunized rabbits.

3.6 Evaluating the response of immunized rabbits to *H. ducreyi* challenges. Does the vaccine provide protective immunity against chancroid in the TDRM?

3.6.1 Experimental *H. ducreyi* challenge

One rabbit fed PBS for sham-immunization, one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain, and three rabbits fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain were experimentally challenged with intraepithelial triplicate injections of 2×10^6 , 10^6 , 10^5 or 10^4 CFU of *H. ducreyi* 35000. Furthermore, the minimum inoculum that consistently produces ulcers in the TDRM of *H. ducreyi*

infection is 10^5 CFU (55). We intended to infect the rabbits with 10^6 , 10^5 , 10^4 , and 10^3 CFU, but after estimating the delivered inoculum size on CA, we actually had infected the rabbits with 2×10^6 , 10^6 , 10^5 and 10^4 CFU. The disease of interest, ulcers development and presence of viable *H. ducreyi* cells in the lesions (culture positivity), was observed in both immunized and non-immunized rabbits (Data presented for day 6 only, Figure 27). Progression of the disease, induration, suppuration followed by ulcer formation, was observed in all of the rabbits (representative data for a PBS sham-immunized rabbit, Figure 28).

To evaluate the severity of infections in rabbits, two lesions at each inoculum were measured for size using the electronic calipers, and assigned a clinical score (1= redness, 2= induration, 3= suppuration, 4= ulceration). The course of experimental challenge infection in the immunized and control rabbits is described in Figure 29 for the 2×10^6 CFU inocula, in Figure 30 for the 10^6 CFU inocula, and in Figure 31 for the 10^5 CFU inocula. The 10^4 CFU inocula were not graphed, as this inoculum did not produce ulcerative disease in the PBS sham-immunized control rabbit (data not shown). The severity of infections in the controls (PBS-fed and *S. typhimurium* SL3261 (pnirB)-fed rabbits) and in the vaccinated (*S. typhimurium* SL3261 (pnirBhgbA)-fed rabbits) rabbits was similar (Figure 29-31).

To evaluate the duration of the infection, the third lesion was used for culture of *H. ducreyi* by injecting 100 μ L of sterile PBS into the lesion and followed by aspiration of the liquid back into the syringe. This suspension of PBS, blood and inflammatory exudate was plated onto CA, and colonies were enumerated in forty-eight hours. Culture positivity was determined by the presence of one or more small gray colonies characteristic of *H. ducreyi* coupled with Gram's stain of the organism (5). The duration

Figure 27: Lesion appearance on day 6 following inoculation with *H. ducreyi* in the TDRM of chancroid infection in rabbits immunized with *S. typhimurium* SL3261 (pnirB), PBS and *S. typhimurium* SL3261 (pnirBhgbA) from the second vaccination trial.

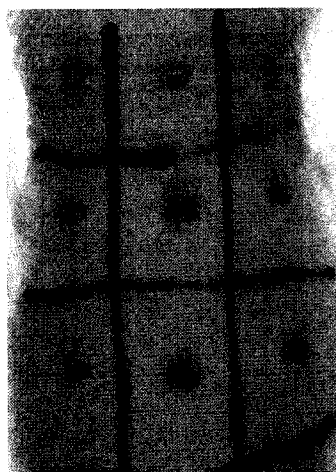
Experimental *H. ducreyi* lesions produced by intraepithelial injections of 2×10^6 , 10^6 or 10^5 CFU on the rabbit backs are shown for day 6. Rabbit #1 was sham-immunized with PBS, rabbit #2 was immunized with the *S. typhimurium* SL3261 (pnirB) control strain, and rabbits #3-5 were immunized with the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain.

Rabbit #1

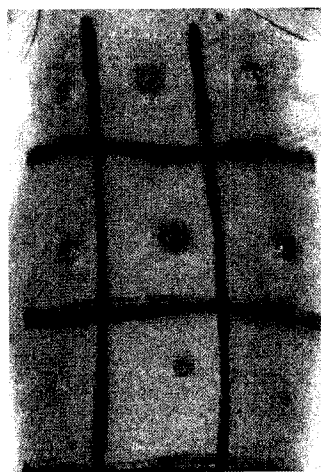
10^6

10^6

10^5



Rabbit #2

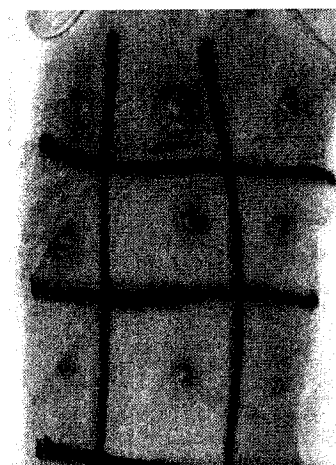


Rabbit #3

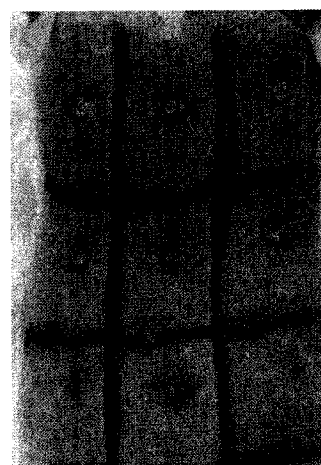
10^6

10^6

10^5



Rabbit #4



Rabbit #5

10^6

10^6

10^5

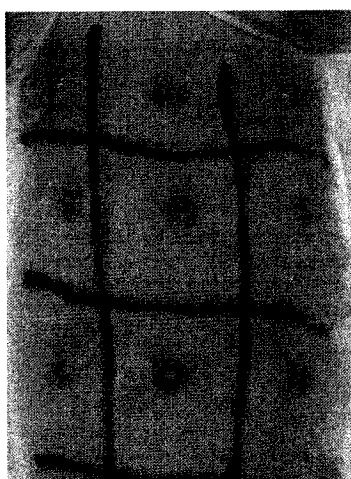
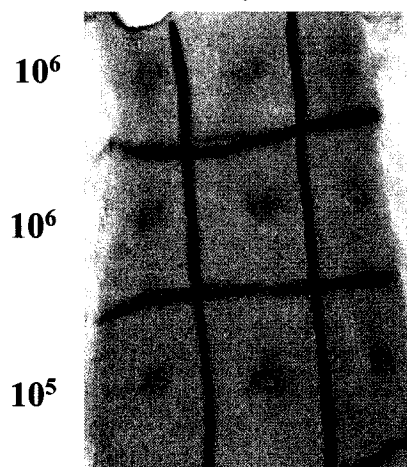


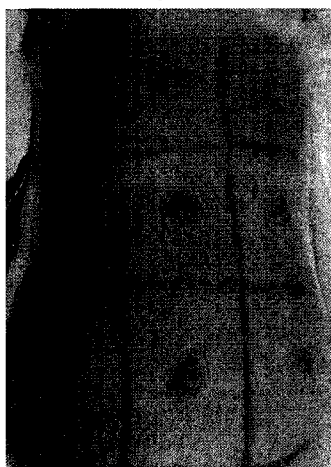
Figure 28: Lesion appearance on days 2 to 18 following inoculation in the TDRM of *H. ducreyi* infection in the rabbit sham-immunized with PBS from the second vaccination trial.

Experimental *H. ducreyi* lesions produced by intraepithelial injections of 2×10^6 , 10^6 or 10^5 CFU on the rabbit backs are shown for days 2 to 18 following challenge with *H. ducreyi* 35000.

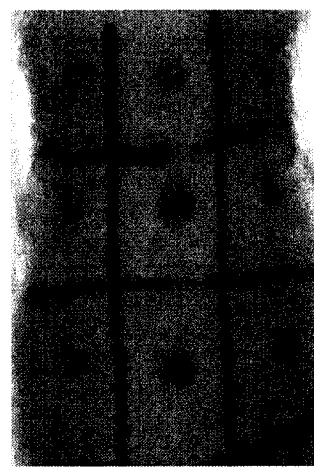
Day 2



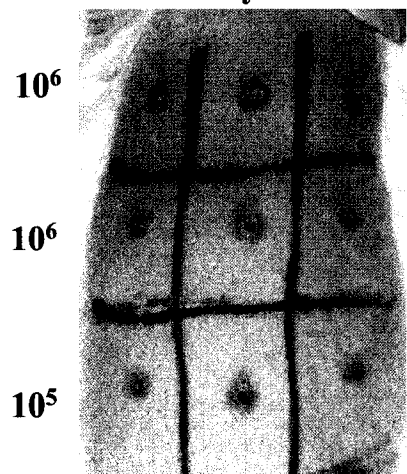
Day 4



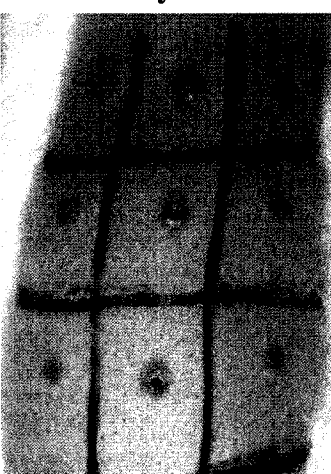
Day 6



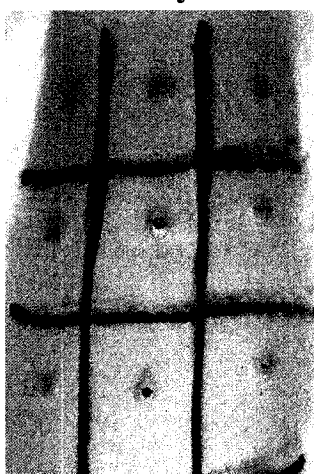
Day 8



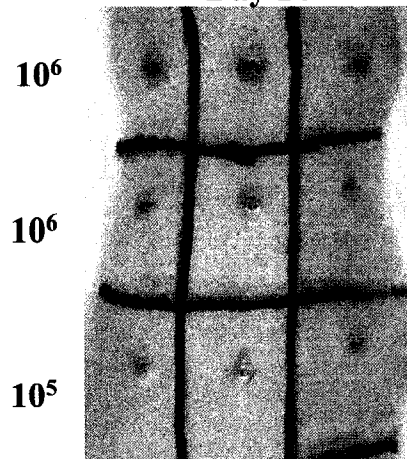
Day 10



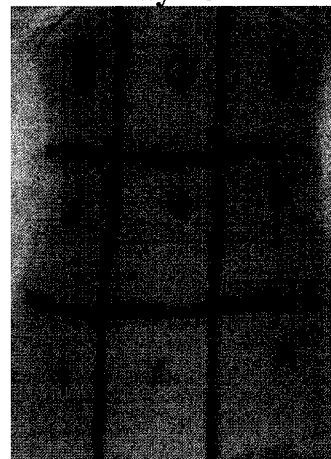
Day 12



Day 14



Day 16



Day 18

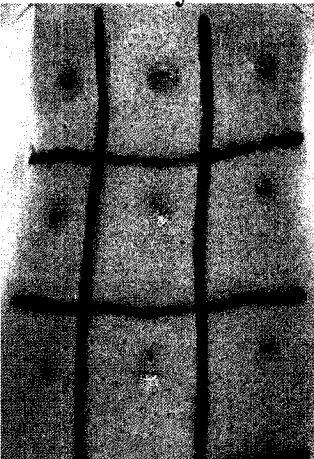


Figure 29: Lesion size and lesion score in the TDRM of *H. ducreyi* infection in rabbits immunized with *S. typhimurium* SL3261 (pnirB), PBS and *S. typhimurium* SL3261 (pnirBhgbA) and challenged with 2 x 10⁶ CFU of *H. ducreyi* 35000. Experimental *H. ducreyi* lesions produced by intraepithelial injections of 2 x 10⁶ CFU on the rabbit backs were compared. In Panel A, the lesion size was measured with electronic calipers. The PBS sham-immunized control is represented by the closed circles, the *S. typhimurium* SL3261 (pnirB) immunized rabbit by the open circles, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the inverted closed triangles. In Panel B, the lesions were scored as 1-redness, 2-induration, 3-suppurative, or 4-ulceration. The PBS sham-immunized control is denoted by the black bars, the rabbit immunized with *S. typhimurium* SL3261 (pnirB) by the light grey bars, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the dark grey bars. The error bars indicate the standard deviation from the mean value obtained from the lesion size and lesion score of 3 rabbits.

A

Experimental *H. ducreyi* lesions produced by 2×10^6 CFU

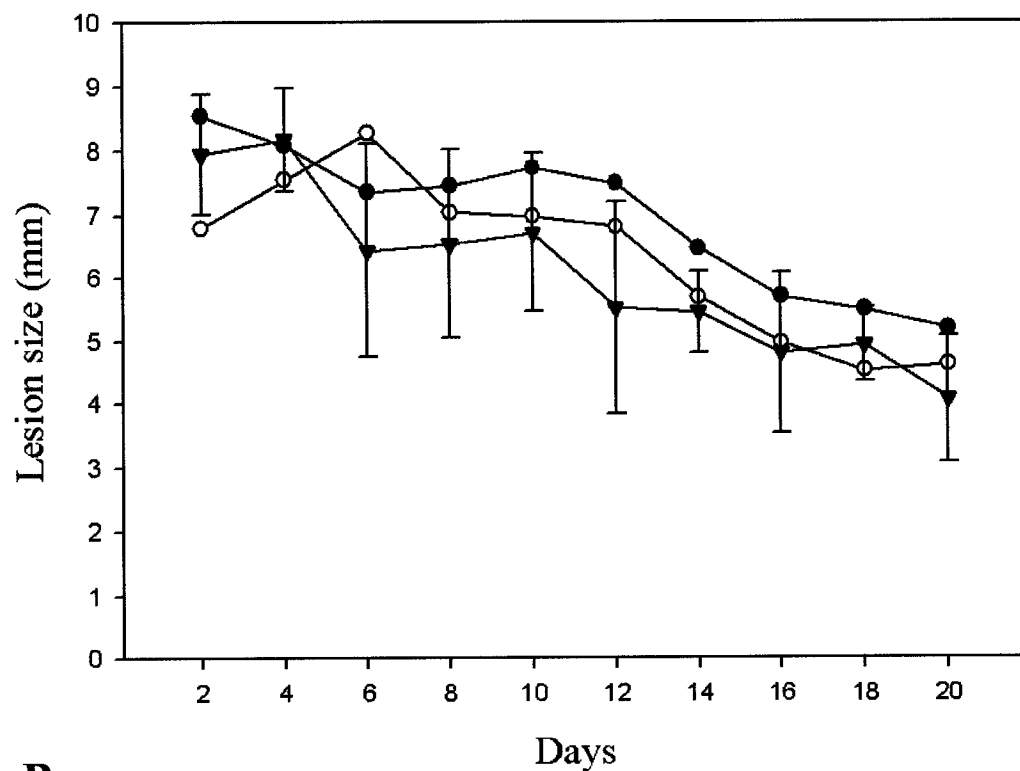
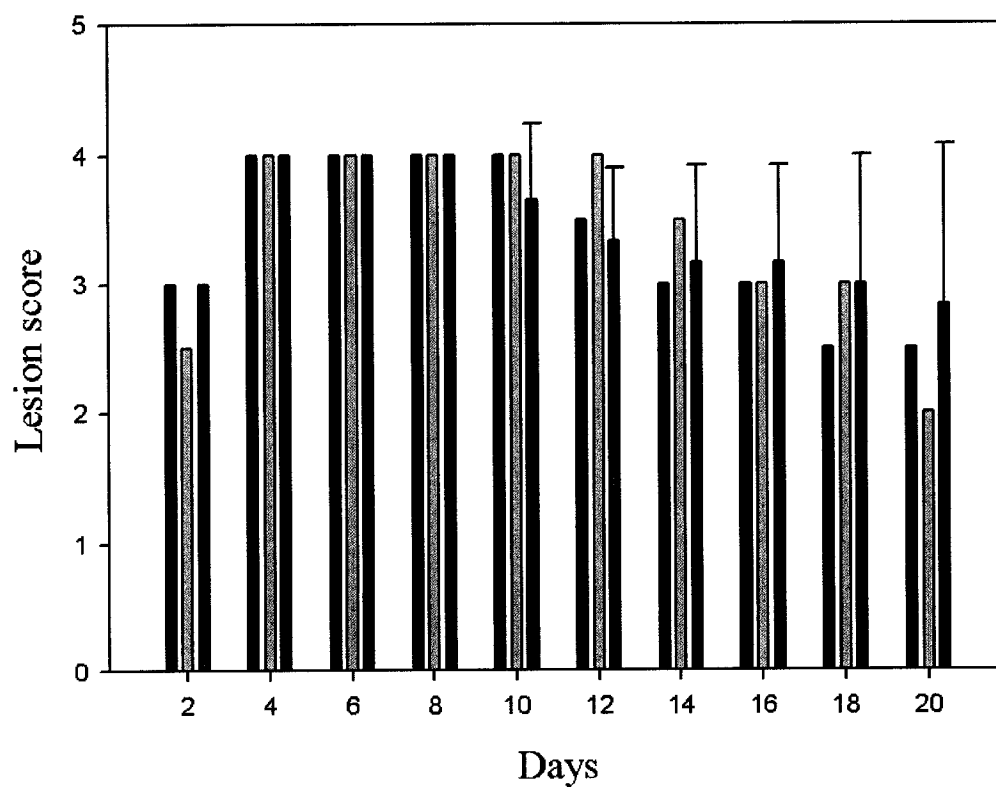
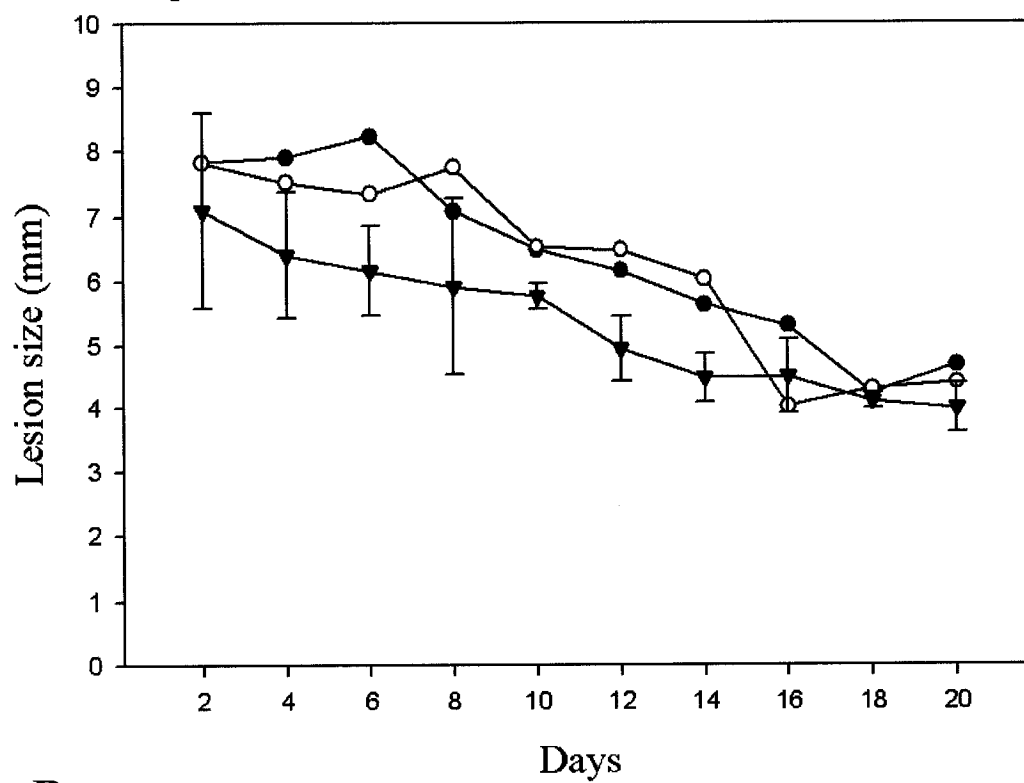
**B**

Figure 30: Lesion size and lesion score in the TDRM of *H. ducreyi* infection in rabbits immunized with *S. typhimurium* SL3261 (pnirB), PBS and *S. typhimurium* SL3261 (pnirBhgbA) and challenged with 10⁶ CFU of *H. ducreyi* 35000.

Experimental *H. ducreyi* lesions produced by intraepithelial injections of 10⁶ CFU on the rabbit backs were compared. In Panel A, the lesion size was measured with electronic calipers. The PBS sham-immunized control is represented by the closed circles, the *S. typhimurium* SL3261 (pnirB) immunized rabbit by the open circles, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the inverted closed triangles. In Panel B, the lesions were scored as 1-redness, 2-induration, 3-suppurative, or 4-ulceration. The PBS sham-immunized control is denoted by the black bars, the rabbit immunized with *S. typhimurium* SL3261 (pnirB) by the light grey bars, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the dark grey bars. The error bars indicate the standard deviation from the mean value obtained from the lesion size and lesion score of 3 rabbits.

A Experimental *H. ducreyi* lesions produced by 10^6 CFU



B

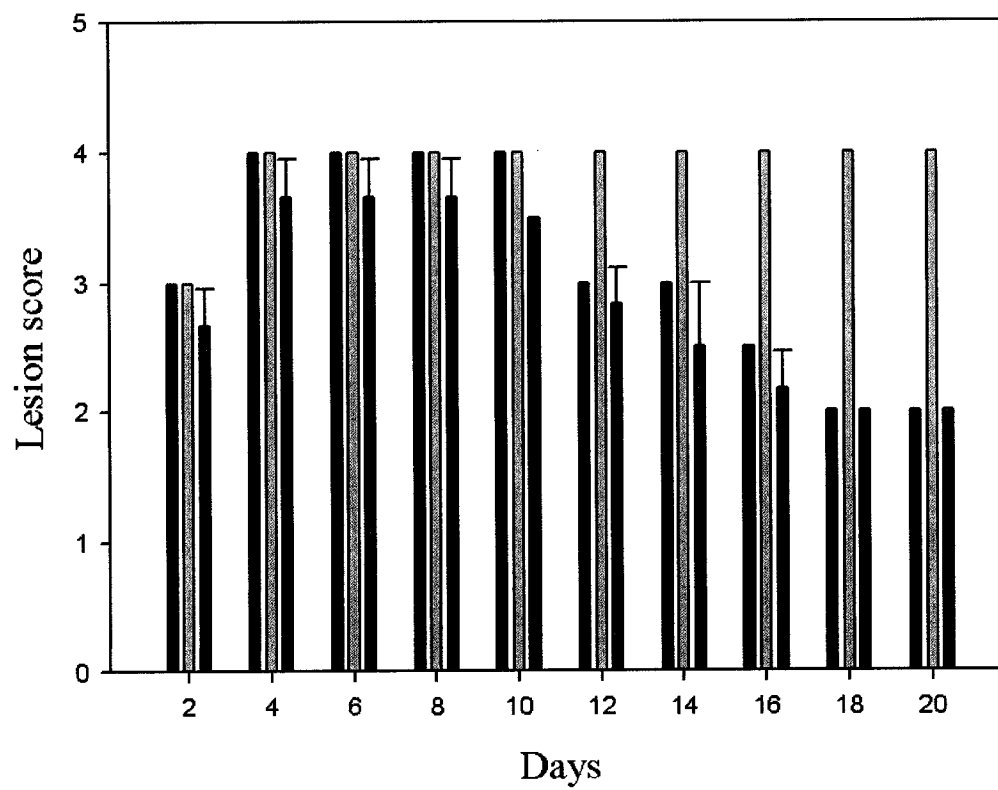
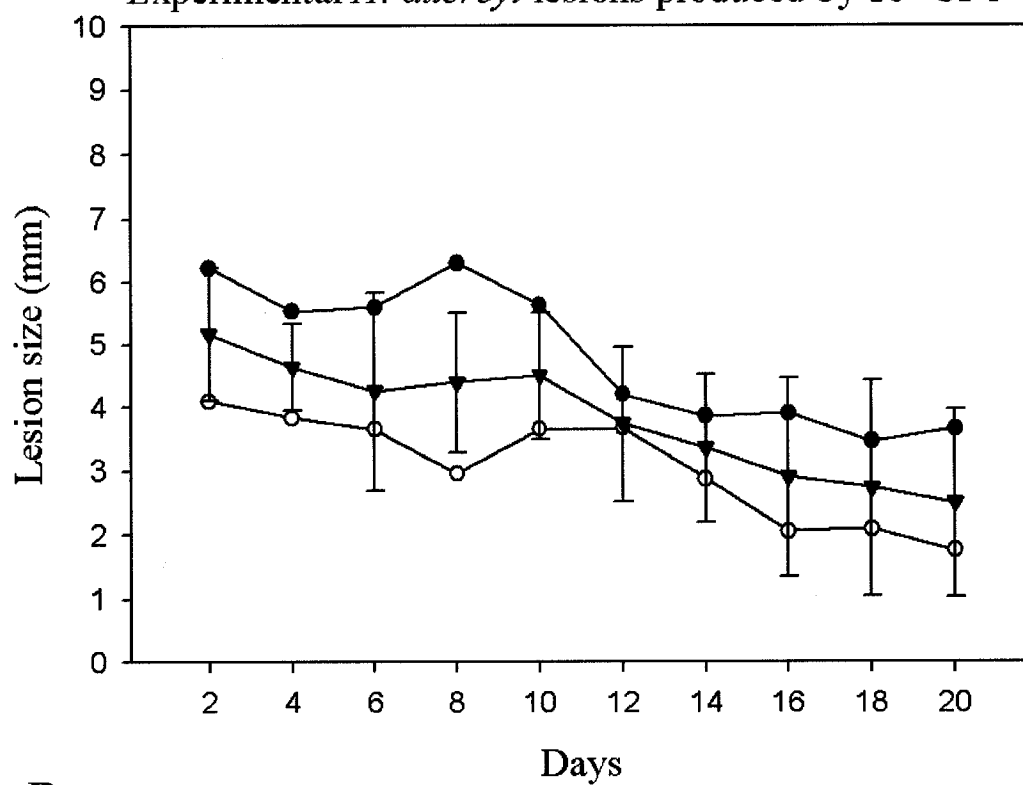
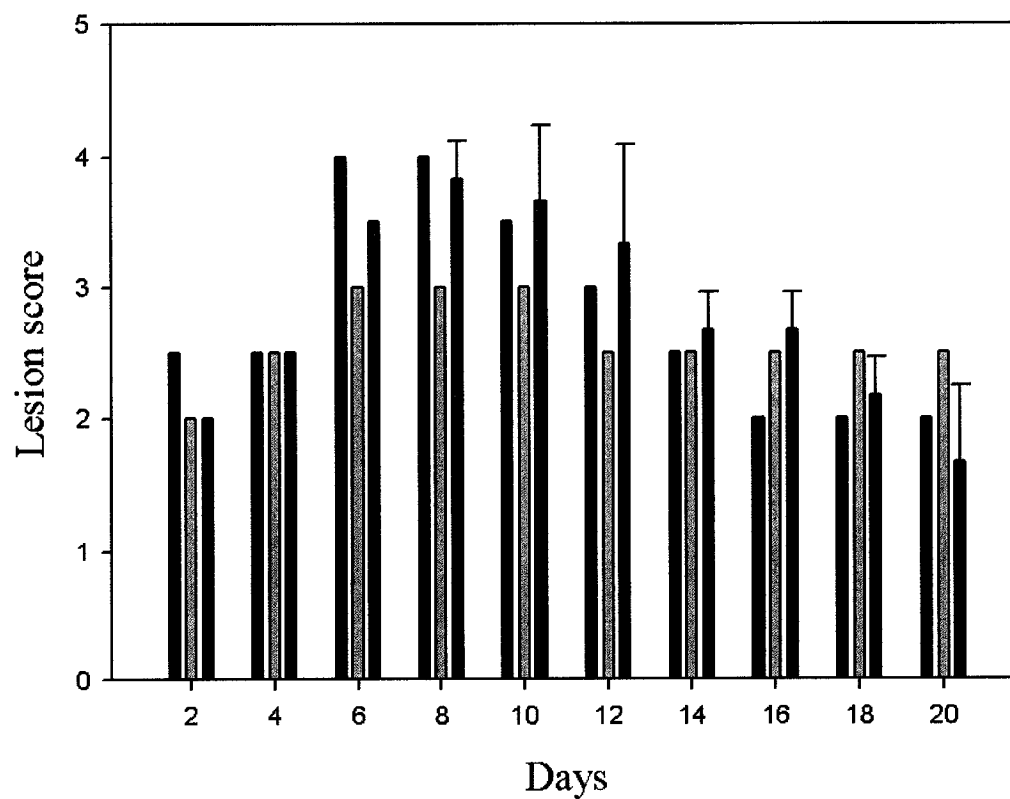


Figure 31: Lesion size and lesion score in the TDRM of *H. ducreyi* infection in rabbits immunized with *S. typhimurium* SL3261 (pnirB), PBS and *S. typhimurium* SL3261 (pnirBhgbA) and challenged with 10⁵ CFU of *H. ducreyi* 35000.

Experimental *H. ducreyi* lesions produced by intraepithelial injections of 10⁵ CFU on the rabbit backs were compared. In Panel A, the lesion size was measured with electronic calipers. The PBS sham-immunized control is represented by the closed circles, the *S. typhimurium* SL3261 (pnirB) immunized rabbit by the open circles, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the inverted closed triangles. In Panel B, the lesions were scored as 1-redness, 2-induration, 3-suppurative, or 4-ulceration. The PBS sham-immunized control is denoted by the black bars, the rabbit immunized with *S. typhimurium* SL3261 (pnirB) by the light grey bars, and the rabbits immunized with *S. typhimurium* SL3261 (pnirBhgbA) by the dark grey bars. The error bars indicate the standard deviation from the mean value obtained from the lesion size and lesion score of 3 rabbits.

AExperimental *H. ducreyi* lesions produced by 10^5 CFU**B**

of infection in the controls (PBS-fed and *S. typhimurium* SL3261 (pnirB)-fed rabbits) and in the vaccinated (*S. typhimurium* SL3261 (pnirBhgbA)-fed rabbits) rabbits was very similar (Table 6), except for the lesions that were sterile (absence of viable *H. ducreyi* cells) for the entire experimental course (day 2-20) (* in table 6). Small numbers of rabbits in the control group preclude comparative statistical analysis for the *H. ducreyi* challenge experiment.

Table 6. Last day that *H. ducreyi* was cultured from lesions of rabbits experimentally challenged with *H. ducreyi*.

Rabbit number	2 x 10 ⁶ CFU inoculum	10 ⁶ CFU inoculum	10 ⁵ CFU inoculum
1	12	18	20
2	12	12	0*
3	18	16	0*
4	0*	18	0*
5	14	20	14

Rabbits were administered by oral gavage PBS (rabbit 1), *S. typhimurium* SL3261 (pnirB) (rabbit 2), and *S. typhimurium* SL3261 (pnirBhgbA) (rabbits 3-5). * No viable *H. ducreyi* was isolated throughout the entire experiment from days 2 to 20.

CHAPTER FOUR

DISCUSSION

A vaccine against chancroid, targeted to groups of high-frequency STD transmitters, could eliminate chancroid in the developing world, and may help decrease the incidence of HIV infections. This approach would be beneficial given that antibiotic resistance to *H. ducreyi* is increasing (96, 177, 217). Also, the WHO acknowledges that controlling sexually transmitted infections is an important HIV prevention strategy (223). The ideal vaccine would require no syringes, no cold chain distribution and could be delivered in a single dose. Oral attenuated *Salmonella* vaccines fulfill these characteristics and have been successful at delivering recombinant antigens from other pathogens in both animal model and human studies (45, 80, 115, 116).

Recombinant attenuated *Salmonella* vaccines are most commonly tested in mice. The immune responses elicited following vaccination would be easier to study in a mouse model of infection, but there is currently no mouse model for chancroid. In our laboratory, vaccine development for chancroid uses the TDRM of *H. ducreyi* infection. We have recently shown that it would be feasible to use an oral *Salmonella* vaccine vector for the evaluation of chancroid vaccine candidates in the TDRM of *H. ducreyi* infection. The study revealed a framework that uses attenuated *S. typhimurium* SL3261 as a vector for an unrelated recombinant antigen (TetC expressed from plasmid pTETnir15) in the TDRM of *H. ducreyi* infection (16). In this work we used pTETnir15 and *S. typhimurium* SL3261 to construct a *Salmonella* vector-based *H. ducreyi* vaccine, and assessed its ability to produce protective immunity against chancroid in the TDRM of *H. ducreyi* infection.

4.1 Constructing an attenuated *Salmonella* vector-based *H. ducreyi* vaccine

4.1.1 Choice of *S. typhimurium* SL3261 and plasmid pTETnir15

Since previous work in our laboratory had determined the well-tolerated immunogenic dose of *S. typhimurium* SL3261 in rabbits and showed that *S. typhimurium* SL3261 did not affect the nature and course of experimental *H. ducreyi* infection in rabbits (16), it was logical to use this strain to continue our work on the development of a chancroid vaccine. This well-characterized strain is attenuated by a mutation that disrupts the aromatic biosynthetic pathway, rendering it auxotroph for *pAB* and DHB, two nutritional requisites that are not available in mammalian tissues (92). Thus, it is expected to be safe to use in immunocompromised individuals because the attenuation is based on nutrient starvation rather than a deletion of virulence genes. Strains with mutation in virulence genes that combat host defences, may possibly regain virulence in immunocompromised individuals. A study by Izhar *et al.* (97) indicated that moderate immunosuppression did not enhance the susceptibility of mice to live *S. typhimurium aroA* vaccines. This is important since a chancroid vaccine would target individuals that are likely to be infected with HIV.

Plasmid pTETnir15 also has interesting features for use in *Salmonella* vaccine vectors. It contains an *in vivo*-inducible *nirB* promoter from *E. coli* that is induced by an anaerobic atmosphere (35). Genes under the control of the *nirB* promoter will be induced once the bacterium reaches the intracellular environment of the host cells. Therefore, expression of an antigen from an attenuated *Salmonella* vaccine would be delayed until it reaches the gut and this would promote plasmid stability. The plasmid also contains a β -lactamase gene (*amp^r*) necessary for *in vitro* selection, and the *tetC* gene, which can be excised to clone a gene of interest.

Plasmid pTETnir15 was chosen to express the vaccine candidate. Experimental evidence has demonstrated that delivery of the plasmid by an attenuated *Salmonella* vaccine vector can produce protective immune responses. Chatfield *et al.* (45) showed that a single-dose of the *S. typhimurium* (pTETnir15) vaccine was protective against tetanus toxin challenge in mice. In addition, excision of the *tetC* gene to express another antigen can provide protective immunity. McSorley *et al.* (141) demonstrated that expression of the *Leishmania major* promastigote surface glycoprotein (gp63) from the pTETnir15 plasmid following *tetC* excision, was protective against *Leishmania major* infection.

The production of a protective immune response is difficult to predict because it is a function of the heterologous antigen, the antigen expression levels, and the plasmid expression system. However, the use of *S. typhimurium* SL3261 and pTETnir15 is a reasonable starting point for the construction of an attenuated *Salmonella* vector-based *H. ducreyi* vaccine.

4.1.2 HgbA as a vaccine

Characterized virulence determinants, for use as potential vaccine candidates, are essential in vaccine development. *H. ducreyi* has numerous characterized virulence determinants but a particular interesting one is HgbA. *H. ducreyi* is a heme obligate organism and can acquire heme through its hemoglobin receptor. HgbA is necessary for virulence in humans and was shown to be an effective vaccine in pigs (3). A recent study also shows that expression of HgbA is sufficient for heme/iron acquisition in humans (120). The study revealed that a double mutant of two other TonB-dependent receptors of *H. ducreyi*; the heme receptor TdhA; and an uncharacterized conserved hypothetical protein TdX, was as virulent as the parent strain. This also suggests that hemoglobin is a

very important source of heme/iron for *H. ducreyi* during infection (120). Overall this shows that HgbA has the potential to be an effective vaccine and we chose to evaluate it as a vaccine candidate in our study.

4.1.3 Expression of HgbA in *E. coli* and *Salmonella*

Since introduction of a recombinant plasmid into *Salmonella* is more difficult than in *E. coli*, we first introduced the plasmid into *E. coli* and verified expression of HgbA. *E. coli* and *Salmonella* are closely related therefore expression in *E. coli* is a reasonably good indication that expression will occur in the attenuated *Salmonella* vector as well. When the entire *hgbA* gene was cloned into the expression vector (pnirL) under the control of the *nirB* promoter, we were unable to demonstrate protein expression of HgbA. Previously, it had been shown that high-level expression of HgbA was possible without toxicity when the *hgbA* gene lacking its leader sequence was cloned into an expression vector (63). We used a similar approach and excluded the leader sequence of *hgbA* from the amplified product. HgbA expression was successfully detected from *E. coli* bearing pnirB*hgbA* and the expression was activated by an anaerobic atmosphere *in vitro*.

The recombinant plasmid pnirB*hgbA* was then introduced into the *S. typhimurium* SL3261 vaccine strain. The resulting construct expressed HgbA and the production of the protein was activated under anaerobic conditions. Yet, the expression was limited to early timepoints (1, 2 and 3 hours) and the levels of expression were somewhat low when compared to another gene under the control of the *nirB* promoter, *tetC* (*S. typhimurium* pTETnir15), which was expressed at high levels for up to 24 hours of culture *in vitro*. This could potentially have an effect on the vaccines ability to elicit immune responses to HgbA *in vivo*. However, there is evidence that the amount of heterologous antigen that a vaccine produces does not necessarily correlate with its ability to induce an antibody

response to the antigen. Dunstan *et al.* (58) compared an *in vivo*-inducible promoter to a constitutive promoter, and observed that even though the two constructs had equivalent amounts of TetC expression, the *in vivo*-inducible promoter produced higher levels of antibodies against TetC. In another study, even if high-levels of TetC were expressed *in vitro* from the *nirB* promoter and from a constitutive promoter, the construct with constitutive expression was a lot less protective than the construct from the *nirB* promoter (45). This suggests that the ability of a vaccine to induce protective immune responses depends on reaching the appropriate levels of antigen expression and so each construct should be evaluated in an animal model to determine its protective capacities.

4.1.4 Characterization of the recombinant *S. typhimurium* SL3261 strains

First we characterized the LPS, the bacterial LPS profile of *S. typhimurium* SL3261 (pnirBhgbA), SL3261 (pnirB) or SL3261 (pTETnir15) was intact compared to the altered LPS profile of *S. typhimurium* LB5010, as demonstrated by silver staining of SDS-PAGE. This is important because defects in the LPS can lead to further attenuation of the *Salmonella* strains *in vivo* (35). Strains with intact LPS will depict a ladder of LPS fragments, whereas strains with defects in LPS will reveal low molecular weight fragments (35). It should be noted that we were unable to visualize the higher molecular weight fragments in the top portion of the gel, including the ones in the parent strain SL3261, but the difference in the profiles of the vaccine and the control strain is evident, indicative of LPS integrity.

Second, we compared the growth of *S. typhimurium* SL3261 (pnirBhgbA) to the parent strain SL3261 to ensure the absence of a growth defect in the vaccine construct. No major differences in growth were seen, indicating that the presence of the *hgbA* gene

in the *S. typhimurium* SL3261 vaccine strain did not cause a metabolic burden on the organism.

Finally, the recombinant strains were tested for phage sensitivity to phage P22 to ensure that the *Salmonella* SL3261 transductants were nonlysogens. Stable lysogens should be avoided because they can later be induced and this would result in bacterial cell lysis. Bacterial cells that are nonlysogens are expected to be P22 sensitive, whereas lysogenized strains are P22 resistant (135). All the recombinant *S. typhimurium* SL3261 strains were indeed found to be nonlysogens.

4.2 Immune response to vaccination

Our laboratory had previously determined the well-tolerated immunogenic dose in this model to be between 10^8 - 10^9 CFU and so the dose for the oral gavage in these trials was targeted to 10^8 - 10^9 CFU. The targeted dose was always achieved as determined by viable colony counts. Predictably, the dose was well tolerated by all rabbits used in this study and the recombinant bacteria administered were immunogenic as determined by serum EIAs against a crude soluble antigen preparation of *S. typhimurium* SL3261.

The first vaccination trial consisted of a single dose of the vaccine orally administered to rabbits. No significant antibody response to HgbA was detected by EIA in all immunized rabbits. However, a serum EIA against TetC, in a rabbit fed *S. typhimurium* SL3261 (pTETnir15), indicated that an antibody response to an antigen delivered by the attenuated *Salmonella* vector was detected in the system. Therefore, we concluded that the absence of an antibody response against HgbA was not due to the delivery system, and that a single dose of the vaccine was insufficient to produce a humoral response against HgbA. Other groups have used multiple immunization schedules for oral delivery of attenuated *Salmonella* vectors, which ranges from 2 to 4

doses given at different intervals (2-28 days) depending on the study (108, 130, 160, 175). We decided to perform a second trial, where the rabbits were inoculated with the recombinant *S. typhimurium* SL3261 strains on three occasions at two-week intervals (booster regimen).

In the booster vaccination trial there was no significant antibody response to HgbA in either immunized or sham-immunized rabbits, up to week five. Rabbits were experimentally challenged with *H. ducreyi* three days after blood was collected, at week five. During experimental *H. ducreyi* infection, rabbits mounted a strong antibody response to HgbA as demonstrated by the EIA from week eight, a response that was absent in a non-challenged rabbit. The serum EIA against TetC confirmed that for the booster regimen, an antibody response to antigen delivered by the attenuated *Salmonella* vector, could be detected. We concluded that three doses of the vaccine were also insufficient to produce a humoral response against HgbA in the rabbit model, and that HgbA antibodies are produced in rabbits experimentally challenged with *H. ducreyi*.

In the second vaccination trial, rabbits received three doses of the recombinant *Salmonella* strains, whereas in the first vaccination trial rabbits received a single dose, it would therefore be predicted that there would be a difference in the amount of antibody raised against the *Salmonella* vector in the two vaccination trials. Indeed we observed a statistically significant higher response in the booster regimen trial than in the single dose trial. This is important because it suggests that multiple doses of the vaccine do not result in immunity to the vector itself caused by multiple exposures to the vector.

We attempted to evaluate the cell-mediated immune response in immunized rabbits using the DTH to a vaccine antigen skin test. A DTH reaction to the antigen, which is distinguished by an indurated lesion due to lymphocytic infiltrate at the site of injection, reveals the induction of a cell-mediated immune response. In a DTH response,

animals that have been previously exposed to the antigen will have primed T cells that will migrate to the site of injection and become activated. These cells will release mediators, which results in the recruitment of phagocytes and plasma, producing a visible lesion (98). Following intra-epithelial challenge with rHgbA, no difference in the size of induration was noted between rabbits fed *S. typhimurium* (pnirBhgbA) and the *S. typhimurium* (pnirB) control strain. Furthermore, following intra-epithelial challenge with *E. coli* (pUNCH1202) crude soluble antigens, no difference in the size of induration was noted between rabbits fed *S. typhimurium* (pnirBhgbA) and the *S. typhimurium* (pnirB) control strain. *E. coli* (pUNCH1202) expresses the ferric enterobactin receptor (FetA) of *N. gonorrhoeae* (41, 42). Except for the FetA gene, this strain is genotypically identical to the recombinant *E. coli* strain expressing *H. ducreyi* rHgbA and was used to control for a skin test reaction arising from antigens other than rHgbA. We were unable to determine if a cell-mediated immune response specific to HgbA was mounted since the control rabbit had similar lesion development than the immunized rabbit and the response observed against the *E. coli* (pUNCH1202) crude soluble antigens was almost equal to the response in the rHgbA challenge. The skin test cross-reaction probably arises from antigens other than rHgbA, because the enriched rHgbA preparation depicts a few contaminants bands on an SDS-PAGE.

4.3 Detection and characterization of *Salmonella* in rabbit stools

To help us establish if the *Salmonella* vector-based vaccine bypassed the stomach and reach the gastrointestinal cells, we performed experiments to detect *Salmonella* in rabbit stools after oral immunization. We observed that *Salmonella* did reach the gut of immunized rabbits and that fecal shedding of *S. typhimurium* SL3261 (pnirBhgbA) ranged from 13-21 days for the single dose vaccination trial and between 6-20 days for

the booster regimen vaccination trial. This is similar to studies in human trials. *S. typhimurium* ($\Delta phoP/phoQ$) was shed for at least 10 days in one volunteer (range 2-10 days), after which antibiotic therapy was given to clear the vaccine bacteria (14). In another study, *S. typhimurium* ($\Delta aroC \Delta ssaV$) was shed for up to 23 days (range 16-23 days) (89). Similarly, in a third study *S. typhimurium* ($\Delta phoP/phoQ \Delta aroA \Delta asd \Delta strA/strB$) was shed for up to 13 days (range 0-13days). *S. typhimurium* have higher levels of shedding than *S. typhi*, the latter ranges from 1 to 4 days (91, 126, 200).

Prolonged colonization of the gastrointestinal tract could be positive because it could increase presentation of the antigen delivered by an oral *Salmonella* vector, which in turn could result in greater immunogenicity. *Salmonella* enters the host through enterocytes or M cells of the Peyer's patches (PPs) in the gut-associated lymphoid tissues of the small intestine (104, 105). It then encounters resident macrophages in which it can survive and thereby provide a prolonged source of the vaccine to antigen presenting cells. However, this is not supported by our study in which *S. typhimurium* SL3261 (pTETnir15) had greater immunogenicity than the *S. typhimurium* SL3261 (pnirBhgbA) vaccine even if it was shed for fewer amounts of days; 6 and 10 days in the single dose and the booster regimen vaccination trials, respectively.

On the other hand, persistence of attenuated *Salmonella* vectors in the gastrointestinal tract could also be negative. Some researchers have raised the concern over releasing genetically modified organisms into the environment (1). Efforts to improve live vaccines with a reduced impact on the environment has been achieved by Abd El Ghany *et al.* (1), where they introduced additional mutations in genes that contributed to intestinal persistence of *S. typhimurium*, which resulted in decrease shedding but did not have an impact on the immunogenicity of the strain.

Salmonella strains recovered from rabbit stool underwent further genotypic and phenotypic characterization. PFGE was performed on isolates recovered in the single dose vaccination trial and confirmed that they were identical to the strain used for immunization, indicating preservation of the original genotype. VITEK[®] testing was performed on isolates recovered in the booster regimen vaccination trial and revealed that *Salmonella* strains and several other enteric bacteria were present, including *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa*. These organisms likely represent environmental contamination of the stool pellet arising from the water or the stool collecting pan rather than true intestinal colonization.

Salmonella strains recovered from rabbit stool were also used to assess plasmid stability *in vivo*. In mice models, both bacterial colonization and plasmid stability *in vivo* are done using homogenates of spleen, liver, mesenteric lymph nodes (MLN), and PPs. This method was not used in our study because collecting rabbit organs would require many animals and this would increase both the difficulties of manipulating higher numbers of animals and the cost of our study. Instead we used restriction digest analyses (single dose vaccination trial) and PCR amplification (booster regimen vaccination trial) of plasmid DNA isolated from *Salmonella* from rabbit stools.

The restriction digest of *pnirBhgbA* produced the anticipated linearized fragment in all immunized rabbits on the last day of documented stool shedding. This shows that the recombinant *S. typhimurium* SL3261 (*pnirBhgbA*) vaccine can retain the plasmid *in vivo* throughout the period of bacterial colonization. Similar results were observed in the control strains *S. typhimurium* SL3261 harbouring *pnirB* or *pTETnir15*. This is in accordance with studies in mice that have demonstrated that *S. typhimurium* Δ *aroA* Δ *aroD* harbouring a plasmid encoding a gene under the control of the *nirB* promoter,

could retain its plasmid *in vivo* at levels approaching 100% throughout the period of bacterial colonization (range 10-21 days) (45, 58, 94, 141).

In the booster regimen vaccination trial, *pnirBhgbA* was identified by PCR amplification using oligonucleotides that annealed to the *hgbA* gene. In this experiment, the expected amplicon corresponding to *hgbA* was not identified in plasmids recovered beyond 10 days of oral gavage. Although plasmids were recovered from these isolates, their size on agarose gel electrophoresis did not match that of *pnirBhgbA* but rather the dimeric form of *pnirB*. As loss of the cloned *hgbA* gene from these plasmids represented a likely explanation for these observations, we conducted PCR amplification using primers complementary to vector sequences bracketing the *hgbA* gene. Our PCR results confirmed our theory and indicated that *hgbA* was lost from plasmid extracted from stool isolates of *Salmonella* strains recovered beyond 10 days of oral gavage. Hence, plasmid stability *in vivo* in this vaccination trial is harder to interpret. The recombinant *S. typhimurium* SL3261 (*pnirBhgbA*) vaccine can retain the plasmid *in vivo* (containing the *amp^r* gene) throughout the period of bacterial colonization, but it cannot retain the *hgbA* gene beyond day 10. The reason for this is unclear.

To our knowledge this has not been reported in the literature for attenuated *Salmonella* vaccine studies. However, we should note that in a number of those studies they use media growth with or without antibiotics as an indicator of plasmid presence, which, as we can see in our study, is not always an indicator of full size plasmid presence. Nonetheless, some groups also verify that the antigen is still present by examining protein expression from colonies recovered from PPs, spleens, livers, and MLNs. Huang *et al.* (94) were able to demonstrate expression of saliva-binding region (SBR) of *Streptococcus mutans* antigen I/II adhesion from *S. typhimurium* Δ *aroA* Δ *aroD* colonies isolated from PPs or spleens for at least 21 days. Similarly, Lee *et al.* (123)

demonstrated expression of *Schistosoma haematobium* glutathione S-transferase (Sh28GST) from *S. typhimurium* SL3261 colonies isolated from livers and spleens for at least 14 days.

We also evaluated whether the colonies retrieved from immunized rabbit stools retained their ability to express either HgbA or TetC, by Western blotting. HgbA expression was preserved following early *in vivo* passage (day 1 in 2 rabbits and day 6 in another rabbit) in all rabbits that were administered *S. typhimurium* SL3261 (pnirBhgbA) in both vaccination trials. However, on the last documented day of fecal shedding, or on the last day of fecal shedding with the confirmed presence of the *hgbA* gene, there was no HgbA expression. This is interesting because it indicates that although the *hgbA* gene was stably maintained in recombinant plasmids for at up to 21 days in the single dose trial and for at least 10 days in the booster trial, of *in vivo* passage, protein production occurred only in *S. typhimurium* SL3261 (pnirBhgbA) strains with a much more limited *in vivo* exposure.

Yet, this is not consistent with the expression of another protein, TetC, from *S. typhimurium* SL3261 (pTETnir15) colonies retrieved from immunized rabbit stools. The expression of TetC was more persistent and was detected on both the first day and the last day (day 6) of fecal shedding in the single dose vaccination trial and also on the last day of fecal shedding (day 10) of the booster regimen vaccination trial.

4.4 Response of immunized rabbits to experimental *H. ducreyi* challenges

The purpose of this study was to see if a *Salmonella* vector-based *H. ducreyi* vaccine expressing the HgbA protein could confer protective immunity against chancroid. Hence, we experimentally challenged the immunized rabbits from the second vaccination trial (booster regimen) with *H. ducreyi* to evaluate their response.

4.4.1 Technical considerations for the temperature-dependent rabbit model of *H.*

***ducreyi* infection**

In the rabbit model of chancroid we use partially outbred NZW rabbits and so there is considerable biological variability between animals. This mimics the anticipated response in an outbred human population, but can sometimes confound experimental results especially in small sample animal trials. This variability can be overcome in large sample animal trials, but in this trial because of the complexity of the intragastric feeding, a small sample was necessary. Therefore, the results of this study should be tested in a large sample animal trial to definitively reveal if this vaccine can induce protective immunity.

4.4.2 Experimental *H. ducreyi* challenge

One rabbit fed PBS for sham-immunization, one rabbit fed the *S. typhimurium* SL3261 (pnirB) control strain, and three rabbit fed the *S. typhimurium* SL3261 (pnirBhgbA) vaccine strain were challenged with *H. ducreyi*. The disease progress was normal; induration, suppuration followed by ulcer formation, in all of the rabbits. The minimum inoculum that produced ulcerative disease was 10^5 CFU, which is consistent with previous work with this model in our laboratory (55).

The severity of infections in rabbits, as measured by lesion size and score, was very similar in the controls and the vaccinated rabbits at each inoculum. In addition, the duration of the infection, as measured by culture positivity to *H. ducreyi* from the lesion, was very similar in the controls and the vaccinated rabbits, except for the lesions that were sterile (absence of viable *H. ducreyi* cells) for the entire experimental course (day 2-

20) (* in table 6 of the result section). Thus, immunization with *S. typhimurium* SL3261 (pnirBhgbA) does not appear to protect rabbits against challenged with *H. ducreyi* in this animal trial.

We speculate that the absence of viable *H. ducreyi* cells in those lesions is due to technical difficulties, as the other lesions at the corresponding inoculum progressed to an ulcerative stage (rabbit #3 and #4) or to a suppurative stage (rabbit #2), indicating that replication of the organism was occurring. There are many technical difficulties in measuring the culture positivity, which could explain why some of the lesions appeared to be sterile. First, when the PBS is injected the liquid diffuses into the lesion and the full volume (100 µL) is almost never aspirated back into the syringe. Second, the suspension of PBS, blood and inflammatory exudates, is very viscous and hard to aspirate into the narrow needle of the syringe. Third, the lesion is hard to pierce because it is crusted due to the infection and/or due to the repeated culture positivity injections, as a result more pressure was applied on the syringe, sometimes piercing the lesion through to the other side, allowing some of the fluid containing viable cells to be lost.

4.5 Future work

The ultimate success of a live bacterial vaccine vector relies significantly on the appropriate synthesis of the heterologous antigen. So in future experiments this attenuated *Salmonella* vector-based *H. ducreyi* vaccine should be optimized in order to reach the proper levels of heterologous antigen expression. This would allow greater exposure of the antigen to the immune system and conceivably provide protection. Several things can optimize antigen expression and include; surface display or secretion of the heterologous antigen, construction of protein fusions, and both the nature of the

attenuation and the strain background of the *Salmonella* vector. I will briefly describe some of those available approaches.

The *Pseudomonas syringae* ice nucleation protein system has been used to display foreign antigens on the surface of *Salmonella*. Lee *et al.* (124) used this system to display the hepatitis B surface antigen/hepatitis C core protein on the surface of *S. typhi* Ty21a and demonstrated that surface-displayed antigens resulted in higher serum antibody levels than those expressed intracellularly.

In addition to surface display, secretion of the heterologous antigen out of the *Salmonella* vector is also effective at enhancing the immune response. Russmann *et al.* (176) achieved protection against lymphocytic choriomeningitis virus (LCMV) infection when the heterologous antigen was secreted via a type III secretion system, but not when it was expressed within the cytoplasm. The HlyA export-expression system of uropathogenic *E. coli* is another system that was used to allow extracellular secretion of listeriolysin from a *Salmonella* vector and as a result conferred protection against a lethal challenge of *Listeria monocytogenes* (88).

Another attractive approach is the expression of antigens as a C-terminal fusion to the highly immunogenic TetC. The idea is that the strong immunogenicity of TetC can have an adjuvant effect on the guest protein to promote the immune response. This approach was capable of rescuing the expression of a bacterial antigen from a *Salmonella* vector for which previous attempts were unsuccessful (79). Moreover, it was shown that when comparing an antigen expressed alone or as a fusion to TetC, only the strain expressing the fusion protein elicited an antibody response against the vaccine antigen (123).

The last point is the nature of the attenuation and the strain background of the *Salmonella* vector. Chabalgoity *et al.* (44) expressed amino acids 8-23 of glycoprotein D

of herpes simplex virus as a fusion protein to TetC under the control of the *nirB* promoter in different live attenuated *S. typhimurium* strains and found that both the nature of the attenuating mutation (*aroA* versus *htrA*) and the strain background influenced the expression of the antigen.

Even though HgbA has good potential to be an effective vaccine other characterized *H. ducreyi* virulence determinants should also be tested in this system. Potential vaccine candidates include the following proteins; PAL, DsrA, NcaA, LspA1 and LspA2, DltA, WecA and proteins of the *flp* operon. Another potential vaccine candidate recently identified in our laboratory is a periplasmic-binding protein component of a putative heme ABC transporter (unpublished data). This periplasmic-binding protein has a presumptive role in heme acquisition for *H. ducreyi*, which is a heme obligate bacterium.

4.6 Conclusion

Our study has resulted in the following conclusions:

- We constructed a *Salmonella* vector-based vaccine expressing an *H. ducreyi* antigen (HgbA) and showed HgbA expression under anaerobic conditions.
- A single dose or three doses, at two-week intervals, of the vaccine does not produce a humoral response against HgbA in the rabbit model.
- The vaccine administered in both trials was immunogenic and the vaccine survived *in vivo* passage. The recombinant plasmid was relatively stable in the *Salmonella* vector.
- In this small animal trial, we were unable to induce protective immunity against chancroid. The results of this study do not exclude the possibility of using an

optimized *Salmonella* vector-based *H. ducreyi* vaccine to confer protection against chancroid.

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APPENDIX

Recombinant HgbA extraction

The rHgbA protein was expressed from *E. coli* strain BL21 (DE3), pUNCH 672 pLysS (A672-10). rHgbA is expressed from pUNCH A672-10 as a fusion protein containing a hexahistidine leader under denaturing conditions and re-natured by dialysis. The strain was grown in LB+cm;kan broth at 37°C until the A₆₀₀ reaches 0.4 to 0.6. Isopropyl-β-D-thiogalactoside [IPTG; 2mM, Sigma-Aldrich, St. Louis, MO, USA) was added to the culture and incubated for 30 min, followed by the addition of Rifampicin (0.2mM) (Sigma-Aldrich, St. Louis, MO, USA). After a further 2 hour incubation, the bacterial cells were harvested by centrifugation at 10 000 x g for 20 min, and the pellet resuspended in 3 mL of cell disruption buffer [5mM Tris base, 1mM EDTA, 100mM NaCl] per gram of pellet weight, 0.8mg of lysozyme stock and 4mg of deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) both per gram of weight pellet, and also 8 µL of 0.1M Phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA). Following overnight storage at -20°C, the solution was French pressed (French Pressure cell press, American Instrument Co., Hartland, WI, USA) 3 times, centrifuged at 10 000 x g for 20 min, and the resulting pellet was rinsed twice and washed 4 times with binding buffer (BB) without urea [BB: Novagen, Madison, WI, USA. 40mM imidazole, 4M NaCl and 160mM Tris-HCl, pH 7.9]. To solubilize the contents of the inclusion bodies, the solution was resuspended in 6 M-urea-BB and incubated at 4°C for 60 min. Following centrifugation at 50 000 x g for 30 min, the supernatant was loaded onto a nickel column (Novagen, Madison, WI, USA). The column was washed with 100 mL of 6 M-urea-BB and the eluted fractions [Elution buffer: Novagen, Madison, WI, USA. 4M imidazole, 2M NaCl and 80mM Tris-HCl, pH 7.9] were collected in dialysis tubes (Tube-o-Dialyzers, MWCO 50 000 kDa, RPI, Mount Prospect, IL, USA). The samples were dialyzed

overnight against 4M urea in PBS, followed by addition of Zwittergen 3,14 (Calbiochem, San Diego, CA, USA) to the dialysis tubes (0.35g of Zwittergent 3,14 per 7mL of eluant) and further dialysed for several days against PBS-EDTA containing decreasing amount of urea. A last overnight dialysis against PBS-EDTA was performed, and the final solution of enriched rHgbA was stored at -20°C. For DTH skin tests, rHgbA was concentrated using Microcon columns (Millipore, Billerica, MA, USA) according to manufacturer's instructions.