The Role of *hhbp* in Heme Uptake in *Haemophilus ducreyi*

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Abstract

Haemophilus ducreyi is a gram-negative and heme-dependent bactreia. *H. ducreyi* is the responsible of causing chancroid, a sexually transmitted infection forming genital ulcers. Infection with *H. ducreyi* is associated with an increased risk of acquiring HIV-1 as well as increasing the risk of the HIV-1 transmission. Heme acquisition in *H. ducreyi* occur through a receptor mediated process in which it start with binding of hemoglobin and heme to their cognate outer membrane receptors, HgbA and TdhA, respectively. The receptors are energized by the TonB complex. Following that the deposition of heme into the periplasmic area is unclear. Profiling of the periplasmic proteome of the *H. ducreyi* resulted in the identification of a periplasmic- binding protein that highly expressed in heme limitation conditions, and it has been called hHbp. This protein is encoded by a gene resides in a locus of four genes displaying genetic features of an ABC transporter. The gene cluster is organized as an operon comprising an internal membrane protein (IntPro), a sulphate reductase gamma subunit (dsvC), a heme dependant periplasmic binding protein (hHBP), and an ATPase. The purified periplasmic binding protein, hHbp, bind heme in a dose-dependent and saturable manner. Moreover, the binding between heme and hHbp was specifically competitively inhibited by heme. The proposal planned to create an isogenic *hhbp* mutant by insertional inactivation using a kanamycin cassette, to genotypically and phenotypically characterize the mutant and thereby to confirm the crucial role of the *hhbp* gene in heme transport in *H. ducreyi*. Several attempts to ligate a kanamycin resistance cassette into *hhbp* to construct such a mutant were unsuccessful despite the systematic alteration of the ligation conditions and the use of kanamycin resistant genes derived from a variety of

different plasmids. The explanations for this failure are uncertain. In future work, two other approaches to construct an *hhbp* mutant include the FRT-FLP recombinase technology and the use of overlapping extension PCR with a chloramphenicol cassette.

Dedication

To my family who endured all the struggling and all the sufferings of this journey. To my mother and father for breaking their hearts being away from them all this time and not helping them in their daily needs. To my adorable, gorgeous and stunning two boys, Ahmad and Abdullah, you are the light of my day and the joy of my life and all the stress and suffer vanish with your smiles. To my heaven on earth, my lovely wife, you have carried all the weight from me to complete this part of our life with your kindness, caring and devotion. You share this success as much as I do. To my brothers, thank you for all the support that you gave me all this time. To those who endured my constant discussions on *H*. *ducreyi* and to those who encouraged and supported me throughout this endeavor and always.

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List of Abbreviations

ABC	ATP-binding Cassette
AMP	Ampicillin
ATP	Adenosine triphosphate
bp	base pairs
cat	chloramphenicol
CCR5	Chemokine Receptor type 5
CD4	Cluster of Differentiation 4
C	Celsius
CFU	Colony Forming Unit
CO_2	Carbon dioxide
CXCR4	chemokine receptor type 4
ddH20	Double Distilled Water
DNA	Deoxyribonucleic acid
dsvC	sulphate reductase gamma subunit of *H. ducreyi*
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FLP	Fimbria Like Protein
Fur	Ferric Uptake Regulator
FRT	Flippase Recognition Target
GC	Gonococcal
*gro*EL	A protein chaperone
GUD	Genital Ulcer Disease
Hb	hemoglobin
HgbA	Hemoglobin Outer Membrane Receptor of *H. ducreyi*
hHBP	*H. ducreyi* Heme-dedicated Periplasmic Binding Protein
His	Histidine
HIV	Human Immunodeficiency Virus
HP	Human Passaged

HSV	*Herpes simplex* Virus
IntPro	Internal membrane Protein of the *H.ducreyi* ABC system
Kan	kanamycin Resistance
Kb	Kilo base
kDa	Kilodalton
LB	Luria Bertani
LOS	Lipooligosaccharide
M-PCR	Multiplex Polymerase Chain Reaction
NHS	Normal Human Serum
OMP	Outer Membrane Protein
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
rrl (23S)	ribosomal intergenic spacer region (23S)
rrs (16S)	ribosomal intergenic spacer region (16S)
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SodC	Superoxide Dismutase Protein
STI	Sexually Transmitted Infection
STD	Sexually Transmitted Disease
Taq	*Taq* DNA Polymerase
TdhA	Heme Outer Membrane Receptor of *H. ducreyi*
TonB	Protein mediating transport across outer membrane
WHO	The World Health Organization
ZnuA	Periplasmic Zinc Transport Protein of *H. ducreyi*

1 INTRODUCTION

1.1 *Haemophilus ducreyi*

Haemophilus ducreyi is a fastidious Gram-negative pathogenic bacillus with optimal growth in a water-saturated environment at 33 ^{o}C under 5% CO_2 or microaerophilic conditions [1], [2]. On Gram stain, *H. ducreyi* has been described to resemble schools of fish, fingerprints, or railroad tracks of coccobacilli [1]. Recent rRNA analysis indicate that *H. ducreyi* is only slightly related to other species of *Haemophilus* and the reasons for classifying it as part of the *Haemophilus* genus are the absolute requirement of heme, similar antigenic properties, and similar biochemical properties. *H. ducreyi* has been categorised under the *Actinobacillus* genus within the *Pasteurellaceae* family [3], [4]. *H. ducreyi*, strain 35000HP (human-passaged strain), has a single chromosome, 1.7 Mb in length, encoding 1693 open reading frames (ORF) [5].

1.2 Chancroid

H. ducreyi is the bacterium causing the sexually transmitted infection (STI) known as chancroid. The infection by *H. ducreyi* forms soft chancres, which gives the infection its name, on the genital area of men and women. In men, the ulcers are usually present on the frenulum and prepuce while, on women, the ulcer is present on the perianal area, cervix and vulva [1], [6]. Although chancroid is principally a genital ulcer disease (GUD), there have been some reports of non-genital ulcers, thought to be caused by autoinoculation, on

the inner-thighs, breasts, and fingers [1], [7]. Some cases also have been reported of laboratory acquired infection [8]. The infection process starts with tiny abrasions in the skin which occur during sexual intercourse [1], [9]. Four to seven days after the initial infection, erythematous papules appear and progress to pustules [10]. Pustules usually burst producing very painful ulcers after two to three days following the appearance of the papules. The ulcers exhibit a granulomatous base and usually have ragged edges and are covered by putrid secretions [11]. In half of the cases, inguinal lymphadenopathy appears with involved lymph nodes forming buboes in some cases [11]. If not aspirated or drained, buboes may unexpectedly rupture [11].

1.3 Discovery and Antimicrobial Resistance

In 1852, Leon Bassereau was the first scientist who separated chancroid from the chancres of syphilis, but the most important discovery in *H. ducreyi* research was made by Augusto Ducrey for whom the bacterium was named after [12]. He used the forearm autoinoculation technique using pus which had been taken from the soft genital ulcers as he had not succeeded in culturing *H. ducreyi* on solid media. Ducrey demonstrated microscopically that the bacterium has a length of 1.48 microns and a width of 0.5 microns and the ends of the bacterium are rounded [12]. Bezancon was the first person who re-cultured the bacterium from humans who were re-inoculated with *H. ducreyi* cultured from genital ulcers. Recently, *H. ducreyi* has revealed plasmid-mediated resistance to antimicrobial products such as aminoglycosides, chloramphenicol, β-lactams, tetracyclines, penicillins, and sulphonamides [1]. Strains exhibiting chromosomally-mediated resistance to ciprofloxacin, ofloxacin, penicillin, and trimethoprim have also been described [1].

1.4 Epidemiology

The lack of tests to diagnose chancroid is the main reason for the poor comprehension of the epidemiology of the disease [13]. In addition, the special requirements of *H. ducreyi* for growth on artificial media have contributed to the difficulty of recovering the bacterium from clinical ulcers. The expense of culture media for isolating *H. ducreyi* has impaired the identification of chancroid in resource limited areas of the world where the infection is endemic. The World Health Organization has suggested that there is approximately six million cases of chancroid annually worldwide [14]. Chancroid is the primary cause of GUD in economically deprived countries in Asia, Africa, and Latin America [10], [15-20]. Research has estimated that 56% of the cases of GUD in these countries to be caused by *H. ducreyi* [13], [16-19].

In the United States, Canada, and Europe, chancroid is considered an uncommon disease [7], [21-23]. Chancroid is found in persons who have had sexual contact with commercial sex workers and/or have been linked to persons with low socioeconomic status [24-27].

Males are more susceptible to chancroid than females with a ratio between 3:1 and 25:1. Circumcision has been shown to be a risk factor in acquiring chancroid [22]. Engaging in sex with crack cocaine users or using crack cocaine puts individuals at risk for chancroid [21], [28].

1.5 Methods of Diagnosis

1.5.1 Clinical and Cultural Detection

Differentiating the clinical presentation of chancroid from other GUDs caused by *Herpes simplex* virus (HSV) [21] and *Treponema pallidum* is very challenging. Co-infection with human immunodeficiency virus (HIV) also alters the presentation and the course of chancroid infection. Taking these aspects into consideration, the accuracy range of the clinical diagnosis of chancroid resides from 33% to 80% with the highest diagnostic accuracy occurring in regions where chancroid is most prevalent [29-34].

No single media has been considered the optimum media for the isolation of all strains of *H. ducreyi* because of the diversity of nutrient requirements of the strains, especially the nitrogen source [9]. The two media that has the highest recovery rate (71%) from clinical samples are either gonococcal agar supplemented by fetal bovine serum, hemoglobin, vancomycin, IsoVitalex or Mueller-Hington agar supplemented by chocolatized horse blood, vancomycin, and IsoVitalex. Some studies have shown that the monetary expense can be reduced by using activated charcoal or bovine albumin instead of fetal bovine serum without affecting the efficiency of the media [35], [36].

1.5.2 Non-culture Techniques

Polymerase chain reaction (PCR) is one of the best tools to identify *H. ducreyi* by amplifying genes such as *gro*EL [37], *rrl* (23S) and *rrs*(16S) ribosomal intergenic spacer region,16S rRNA, and an anonymous 1.1kb fragment [38-42]. Another form of PCR called multiplex PCR (M-PCR) is able to identify *H. ducreyi* in the presence of other GUD pathogens such as *T. pallidum*, HSV 1 and 2. However, M-PCR has some problems such as the challenge of identifying *H. ducreyi* directly from genital ulcers because of the existence of

Taq polymerase inhibitors in the transport media which lead to 75% sensitivity rate compared to the culture technique but the technique has succeeded in identifying *in vitro* cultured *H. ducreyi* [43].

Other methods to detect *H. ducreyi*, include antigen detection using antisera directed against outer membrane proteins or against lipooligosaccharide (LOS), DNA-DNA hybridization techniques and enzyme immunoassays using whole-cells, outer membrane proteins, or purified LOS. All these non-culture techniques have been only used in research protocols due to the lack of general availability, and the high cost [15], [42-50].

1.6 Links to HIV transmission

Some cross-sectional African studies have unambiguously demonstrated that the spread of heterosexually acquired HIV infection is epidemiologically linked with infection due to *H. ducreyi* [21], [51-53]. Chancroid is a common cause of GUD in some parts of the world that have high rate of HIV infection and the two infections have a strong interaction [54-56]. On the other hand, chancroid infection in countries have a low rate of infection where HIV is uncommon [26]. There are several mechanisms whereby chancroid facilitates the transmission of HIV. Ulcerative STIs, including chancroid, disrupt the mucosal barriers and the epithelial tissues in the genital area [57-61]. Moreover, semen from HIV serologically positive patients who are co-infected with chancroid contains more HIV than semen from patients who are not infected with *H. ducreyi* [62]. Patients infected with both HIV and chancroid have more erosive chancroid ulcers which results in prolongation of antibiotic treatment compared with patients infected with chancroid alone [62]. The vaginal shedding of HIV increases in cases of GUD [63]. Furthermore, the primary cellular target of HIV is the $CD4^+$ T lymphocyte which has been shown to congregate in chancroid ulcers

[29], [30]. HIV is readily cultured from chancroid ulcers in HIV/*H. ducreyi* co-infected individuals [59], [60]. In the ulcers, macrophages have increased expression of CCR5 and CXCR4 receptors together and in CD4 T cells the expression of CCR5 is increased. This increase in HIV-1 co-receptor expression could assist the acquisition of certain strains of HIV such as X4 (CXCR4), dual-tropic strains, and R5 (CCR5) [64].

1.7 Treatment and prevention

The World Health Organization (WHO) has issued a treatment protocol for chancroid that includes several antibiotic options. Either a single oral dose of 500 mg ciprofloxacin, a single intramuscular dose of 2 g spectinomycin, or 500 mg erythromycin orally three times daily for one week [10] is recommended. The single dose treatments have advantage over the daily regimens because increased of patient compliance [26], [65]. Circumcision in men reduces the treatment failure rate in chancroid patients co-infected with either HIV or HSV infection [10], [29], [52], [66], [67]. The WHO protocol has the additional benefit of treating patients with antimicrobial agents that are active against all probable STIs prevalent in the local area. This therapeutic approach removes the necessity of minimal clinical diagnostics, and avoids problems culturing this fastidious bacterium, and the use of expensive culturing media [68].

Targeting sex workers with an interventional therapy in many countries has diminished the rate of chancroid infection. For example, in Thailand, initiation of a 100% condom policy for commercial sex agencies resulted in a 95% decline in chancroid cases in five years [26], [69]. In another example, a decline in the number of chancroid cases to less than 10 % in Kenya was seen after establishing a system to increase condom usage in sex workers by 80% [26]. Chancroid was the most common cause of GUD in Kenya [26]. In

Senegal, the legislation of prostitution offered regular examinations and treatment options to sex workers which significantly reduced the incidence of chancroid [26].

1.8 Virulence factors of H. ducreyi

H. ducreyi possesses several virulence factors such as lipooligosaccharide (LOS) [70-72], a soluble cytolethal distending toxin that has the ability of cytotoxicity linking direct bacterial-cell contact, [73-75] pili that cover the surface of *H. ducreyi* and mediate host cell adhesion [76], [77], hemoglobin-binding outer membrane proteins [75], [78], to shield *H. ducreyi* from exogenous superoxide there is a copper-zinc superoxide dismutase (SodC) [79], a hemolysin [80], a zinc-binding periplasmic protein(ZnuA), [81] and a filamentous hemagglutinin-like protein. [82].

1.9 Animal models of chancroid Infection

1.9.1 Human Challenge Model

Viable *H. ducreyi* with 10^1 to 10^2 colony forming units (CFUs) is used to inoculate the upper arm of human volunteers by a Multi-Test Applicator for lesion development [5]. For ethical and safety purposes, lesions are not allowed to proceed to ulceration. Therefore, this model only provides information of the first few weeks of infection and only antibiotic sensitive strains are used in this study [83].

1.9.2 Temperature Dependent Rabbit Model

Viable *H. ducreyi* at an inoculum of 10^5 CFUs is injected intradermally in the rabbit model of chancroid which is temperature dependent [84]. All the stages of chancroid are

represented in this model starting with the initial lesion formation up to the ulcer development. Viable *H. ducreyi* are recovered from the ulcers. The disadvantage of this model is that *H. ducreyi* have an optimal growth temperature of 33 ^{o}C so the rabbits must be incubated at 15-17 ^{o}C for lesion formation [84].

1.10 Heme and Bacteria

Heme is a prosthetic group found in an abundant range of proteins, which function in the synthesis of bioactive lipid, regulation of gene expression, transporting oxygen, synthesis of steroid, biosynthesis of antibiotic, cellular signaling and transduction of energy. Furthermore, the activation of iron-bound dioxygen, which is heme-dependent, enables a wide range of reactions, including epoxidation, hydroxylation, carbon-carbon bound cleavage and demethylation [85].

Thus, heme is perhaps the most functionally diverse cofactor bound to proteins, taking part in a wide sort of chemical reactions. Transferring electrons, regulation of genes and activation of oxygen are some of the important reactions [86].

Bacterial iron is imported from the host or the environment by specialized uptake mechanisms. The most important role of iron inside the bacterial cell is its participation in enzymatic redox reactions. Moreover, iron can as well play a structural task in proteins [85].

Iron is an essential molecule in several metabolic pathways as it is required by pathogenic bacteria. The amount of iron in the human body is low due to the low abundance

of extracellular free iron and to the binding of iron to lactoferrin and transferrin [87]. Therefore, a high affinity pathway for iron acquisition is required for the bacteria to survive [88]. The low heme and iron environment in the human body works as a defense mechanism against the survival of microorganisms and protects the human cells from ferric iron toxicity [89].

The most abundant iron source in the human body is heme. Therefore, by directly utilizing heme associated with proteins or free heme bacteria can fulfil all the iron necessities [90], [91]. In fact, bacteria appear to be favoring heme containing compounds or heme as sources of iron. *S. aureus* extract heme-iron preferentially over transferrin-iron according to Skaar et, al [92]. Furthermore, Lee has shown, in *H. ducreyi*, that the sole sources of iron are heme and diverse of heme-containing compounds [93]. Extracellular pathogens release the intracellular heme by proteases and hemolysins. Heme binding proteins from the host such as albumin and hemopexin rapidly bind to heme as soon as it is freed.

Bacteria acquire the necessary heme/iron either by direct contact with the exogenous heme/iron source or by relying on synthesized molecules secreted into the extracellular medium. These molecules are called siderophores and hemophores and they extract heme or iron from various sources [94], such as iron from the host iron binding proteins, transferrin (Tf) and lactoferrin (Lf), and heme from hemoglobin. ABC transporters secrete the hemophores by a carboxy-terminal secretion signal. HasA and HxuA are examples of hemophores from *Serratia marcescens* and *H. influenza* respectively. HasA protein removes the heme component from the heme containing proteins in the host such as myo-

globin, hemoglobin, and hemopexin and then delivers the released heme to the outer membrane receptor, HasR, on the outer membrane of *S*. *marcescens*. On the other hand, the HxuA hemophore of *H*. *influenza* has only one substrate which is hemopexin. HxuA binds to hemopexin and delivers the complex to a receptor on the outer membrane. [95-97]. The *P*. *gingivalis* lysin-specefic protease acts as a hemophore as it binds heme and haemoglobin, delivering the molecules to HmuR, a TonB-dependant receptor. The capacity of the bacterial heme uptake is enhanced by hemophores because of their interaction with variety of hemoproteins and heme sources [98].

Specific receptors for the uptake of iron of the outer membrane exist. Several receptors of 75 and 85 kDa are expressed under iron deficient conditions, and have been recognized in many different organisms. The receptors use energy from the TonB-ExbB-ExbB complex to transport compounds against a concentration gradient across the outer membrane in Gram negative bacteria after the specific high affinity binding to their ligands [99], [100]. Outer membrane receptors facilitate the transport of ferric sidophores and heme complex because these molecules are too large to pass through porin channels. As the concentration of heme and ferric siderophores is low in the external environment, the use of receptor proteins with high affinity to ferric siderophores and heme allows the concentration of these substrates at the cell surface facilitating their transportation rate across the outer membrane [85].

Following the specific interaction of heme and heme containing proteins with their cognate outer membrane receptors, the translocation of heme through the outer membrane

into the periplasm occur by an energy dependent process. Iron siderophore complexes similarly use this mechanism [101] [102] There is no source of energy in the outer membrane in the form of an electrochemical gradient nor are there compounds of high energy like ATP in the periplasm. Ferric siderophores or vitamins B_{12} accumulate in the periplasm in mutants lacking the capability of transporting these compounds across the inner membrane [77-79]. The energy is provided to the outer membrane by the TonB-ExbB-ExbD complex [85].

The TonB box is a sequence of five common conserved amino acids in the N-terminus of the receptors that have need of the TonB complex as a source of energy. The sequence tolerates mutations with no functional defect [103-105]. ExbB and ExbD are two proteins localized in the cytoplasmic membrane [86], and they form the TonBExbBExbD complex with the TonB receptor [86], [106], [107]. The formation of the complex is needed for function. The inner membrane is predicted to be spanned three times by the N-terminal region of ExbB with the remainder of the protein fronting the cytoplasm [106], [108]. The N-terminal region of the periplasmic domain of ExbD is anchored in the inner membrane [109]. ExbB is necessary for stabilizing TonB and ExbD is stabilizing ExbB [86], [110].

The mechanism of transporting compounds by the TonB complex is not well characterized. How the TonB complex reacts to the activated state of the cell is unknown [99]. Once deposited into the cytoplasmic area by an ATP transporter, the iron is released from heme. Releasing the iron from heme might occur via an iron removal enzyme mediated mechanism such as the reverse ferrochelatase or by heme oxygenase present in *Corynebacterium diphtheria* [111]. This process is well characterized in eukaryotes and occurs by

cleaving the porphyrin ring in heme and resulting in the production of iron, biliverdin, and carbon monoxide.

To signal the initiation of genes responsible for the iron uptake, pathogens often use low levels of the environmental iron. At the level of transcription the genes expression is controlled. In mycobacteria, the iron-dependent regulator IdeR is a very good example for that. Iron binds IdeR during rich iron concentrations and genes that encode for the acquisition of iron molecules get repressed while initiating the expression proteins for iron storing [112], [90]. Another example for the repression and activation of the genes according to the saturation is the protein responsible of regulating ferric-uptake (Fur), which is a homodimer composed of 17 KDa subunits [90]. This protein acts as a positive repressor, so it get the transcription of the genes responsible of iron acquisition repressed upon interaction with iron, which work as a co-repressor in this situation, and resulting in de-repression in the absence of iron. When the Fur is absent, free iron levels become excessive [90]. The iron dependent expression of many genes is mediated and controlled by the Fur protein in bacteria. For example, the expression of higher than 90 genes in *E. coli* is controlled by the Fur protein [90]. Moreover, the expression of other genes not straightly related to the metabolism of iron is mediated by the Fur protein. As an example, the expression of Pvds which is a sigma factor, interact with the expression of other iron-regulated genes. The Pvds expression is controlled by Fur [85]. The iron-Fur complex attached on the genome somewhere after the site –35 and before –10 site on the promoters of the affected genes to repress them after binding to iron by Fur [90]. Originally, a 19 bp sequenced consensually in a palindromic sequence called the "fur box" were thought to be the Fur binding sites. However, recent studies of the DNAse I footprinting resulted in confirming that the fur box

is smaller than the binding site [90]. Lavrrar et al [113] assumed that the sites where the Fur bind are overlapping 13 bp "6-1-6" motifs that permit two dimers of Fur to be binding in on opposite faces at each box of the DNA double strands, permitting one extra Fur dimer to attach, causing the expansion of the binding site of the Fur, thus complementing the studies of the DNAse I footprinting [90].

1.11 ABC Transporter

ATP-dependent transporter systems (ABC transporter systems) usually consist of three components, a transmembrane permease, a periplasmic protein, and an ATP-binding lipoprotein. The ABC transport systems are used for the transport of many diverse ligands [114]. The three proteins share sequences that identify them as a specific transporter family [18], but in different systems they cannot substitute each other. There are various ABC transport systems which transport iron, iron complexes, amino acids, sugar molecules, peptides and other important nutrients [98]. The mechanism of transporting ligands across the inner membrane by ABC transporter systems can be correlated and extracted from other homologous systems [85]. However, the stoichiometry of these proteins may vary [85]. In gram-positive bacteria, an N-terminal lipid anchor attach the periplasmic component to the cell membrane. As an example, in *Bacillus subtilis* it has been shown in the system of ferrichrome uptake [85]. Otherwise, in the gram-positive bacteria, the ABC transport systems are similar to the systems of gram-negative bacteria [85]. The genes encoding the ABC system are usually organized as an operon but sometimes the permease encoding gene is not located within the operon. The periplasmic component of the ABC transporter differ greatly in nucleotide sequence [115]. The other components of the ABC transporter, ATPase and the permease, however share kind of similarity in sequence [85]. There is a

very conserved motif localized approximately hundred residues from the C terminus "EAA(3X)G(9X)I(X)LP". This motif is a little different where residues "E(X)A(3X)G" are exist in heme permeases [91]. The proteins binding ATP have a homologous domain of amino acids around 200 edged by the known residues Walker A:GPNGAGKSTLL and Walker B:hhhD consensus sequences. Any change in the sequence of the 200 amino acid results in failure in the ATP hydrolytic activity such as observed in changes in this sequence of FhuC in *E. coli* [115]. Furthermore, a linker motif in all ATPases prior to the Walker B sequence is believed in heme transporting systems to interact with the permease component through the EAA(3X)G motif.

1.12 Heme and *H. ducreyi*

Not much is really acknowledged about the way *H. ducreyi* obtains heme. *H. ducreyi* is an organism obligated to consume very great amount of heme to survive. Between 200 and 500 ug of heme/ml is required for growing optimally while to initiate the growth, 10 ug of heme/ml is obligatory [116]. The organism does not have the ability to synthesize heme because of its lacking the enzyme ferrochelatase. *H. ducreyi* does not produce siderophores and does not have receptors for transferrin and lactoferrin. Bovine haemoglobin, bovine catalase and human haemoglobin, human serum albumin, and human haptoglobin, and heme have been shown to be heme sources for *H. ducreyi*. It is possible that *H. ducreyi* uses hemolysins and/or cytotoxins to extract heme to gain access to these sources as many of these molecules are intracellular. Additionally, the most likely heme-iron source seems to be hemoglobin, as an *H. ducreyi* mutant incapability of using hemoglobin as a source of heme-iron was totally diminished in virulence [116].

Three outer membrane receptors, which are Ton B dependent, have been recognised in *H. ducreyi*, the TdhA receptor, the receptor of haemoglobin (HgbA), and an undescribed preserved proposed outer membrane protein TdX [117], [118]. In conditions of low heme levels, the expression of the preserved HgbA receptor, 100 KDa, shown to be upregulated. An *H. ducreyi* mutant did not grow on plates, where the hemoglobin blood is the source of iron, or attach hemoglobin because it lacks the expression of *hgbA* indicating that the HgbA receptor is indispensable for hemoglobin utilization. The mutant has the capability to use heme as a source of iron, showing that for utilization of heme the HgbA receptor is not obligatory, another receptor has been postulated to be responsible for heme utilization [88], [119-121]. The 75KDa TdhA protein only under conditions of heme deficiency is expressed. An *E. coli* mutant was able to use heme as the sole source of iron while it express the TdhA receptor and an integral Ton system isolated from *H. ducreyi*. An isogenic TdhA *H. ducreyi* mutant retained the capability to use heme as a source of iron, thus indicating, in *H. ducreyi,* heme acquisition involves another receptor or mechanism [121]. The outer membrane protein Tdx, is such a candidate receptor but its specific function remains uncharacterized, and a postulation that it binds heme was proposed in *H. ducreyi* [118].

In the human challenge model of *H. ducreyi*, an isogenic *hgbA* mutant was inoculated and the patients were infected [88]. No difference in virulence compared to the parental strain was observed in the double *tdx*/*tdhA* mutant, supporting the important role of HgbA in the pathogenesis of chancroid [88], [118]. The mechanism of how heme is deposted through the periplasmic area and the inner membrane of *H. ducreyi* remains indefinable. Findings from our laboratory have provided evidence for a heme dedicated ATP transporter involved in this process.

A periplasmic binding protein called hHbp was recognized under heme limiting condition using profiling protein expression of the periplasmic proteome of the *H. ducreyi* [122]. The gene encodes for the hHbp was located in a locus of genes that have an ABC system characteristics. This suggested an operon specific for heme transport consists of four genes in *H. ducreyi*. Instantly upstream of the first gene in the suggested operon, a promoter is suggested to be located and a likely site for a termination of the transcription follows the last gene in the operon. A permease anchored to the inner membrane is assumed to be encoded by the first gene of this suggested operon. A dissimilatory desulfoviridin gamma subunit is suggested to be encoded by the second gene because of its homology to *dsvC* in *Desulfovibrio vulgaris* encoding the dissimilatory desulfoviridin gamma subunit (DSG). The *D. vulgaris* DSG is a part of the dissimilatory sulfite reductase, desulfoviridin. desulfoviridin catalyzes the six-electron reduction of sulfite to sulfide in sulfate-reducing bacteria, and work in the sulfate-reduction pathway as the terminal oxidase [122] , [123]. In bacteria that reduce sulfate, the role of the gamma subunit is indistinct. Some studies suggest that the activity of the thiosulfate reductase is conferred by the gamma subunit within *Desulfovibrio desulfuricans* [124]. Moreover, the gamma subunit and the delta subunit is suggested to work together, the delta subunit is encoded by *dsvD* and it is suggested to function as an independent thiosulfate reductase [124] [92]. In non-sulfate reducing bacteria, it is highly unlikely to observe the presence of product of the *dsvC* gene. However, the stabilization of siroheme cofactors is suggested to be encountered by the existence of the *dsvC* gene product [123-125]. Therefore, we believe that the product of the transcription of *dsvC* may play a role in increasing the affinity between the hHbp (a periplasmic protein encoded by gene before the last one in the operon) and the heme molecule. The final gene in the suggested operon is strongly pustulated to be encoding an ATPase [126], [127].

The role of hHbp in heme uptake has been supported by experimental proof provided by our laboratory. First, hHbp is proven to be homologous to proteins transport iron or metal iron. Under heme-limiting conditions, hHbp was consistently upregulated and the addition of exogenous iron has not affected the expression in vitro. hHbp bounding hemin-agarose depending on the concentration of heme and recognized the heme molecule without considering the existence of the internal metal moiety. Finally, among *H. ducreyi* clinical isolates, hHbp was ubiquitous and structurally preserved.

Furthermore, our laboratory has shown that hHbp attaching to heme was saturable as determined by enhanced chemiluminescence (ECL). An *E. coli* mutant with growth defect was repaired by introducing the *hhbp* gene for heme utilization as a source of iron. The difference in growth between the mutant before and after the introduction of *hhbp* was comparable with the complementation with the intact Dpp permease. This *E. coli* recombinant mutant FB827 dppF::Km(pAM238-hasR), which expresses the heme receptor HasR, allows heme to be translocated into the periplasmic area but denies heme to be introduced into the cytoplasmic area because of the existence of the dppF::Kam mutation that inactivates the Dpp heme/peptidepermease. In contrast, rescuing the growing of the mutant with the empty plasmid was not performed. The hHbp protein of *H. ducreyi* functionally attaches heme. The experiments in the *E. coli* mutant support the suggestion of the participating of the hHbp in transporting heme in *H. ducreyi*.

The focus of this research will generate the results of characterizing the function of *hhbp* genetically in heme acquisition in *H. ducreyi*.

1.13 Hypothesis

The gene that encodes for the periplasmic heme binding protein, *hhbp*, is involved in heme uptake in *H. ducreyi*.

1.13.1 Objectives

The goal of this proposal is to determine the role of *hhbp* in heme acquisition in *H. ducreyi*. Therefore, to achieve this goal, the following objectives will be pursued:

1. Construction of an *H. ducreyi hhbp* mutant via insertional inactivation
2. Phenotypic and genotypic characterization of the mutant
3. Reconstitution of the wild-type phenotype

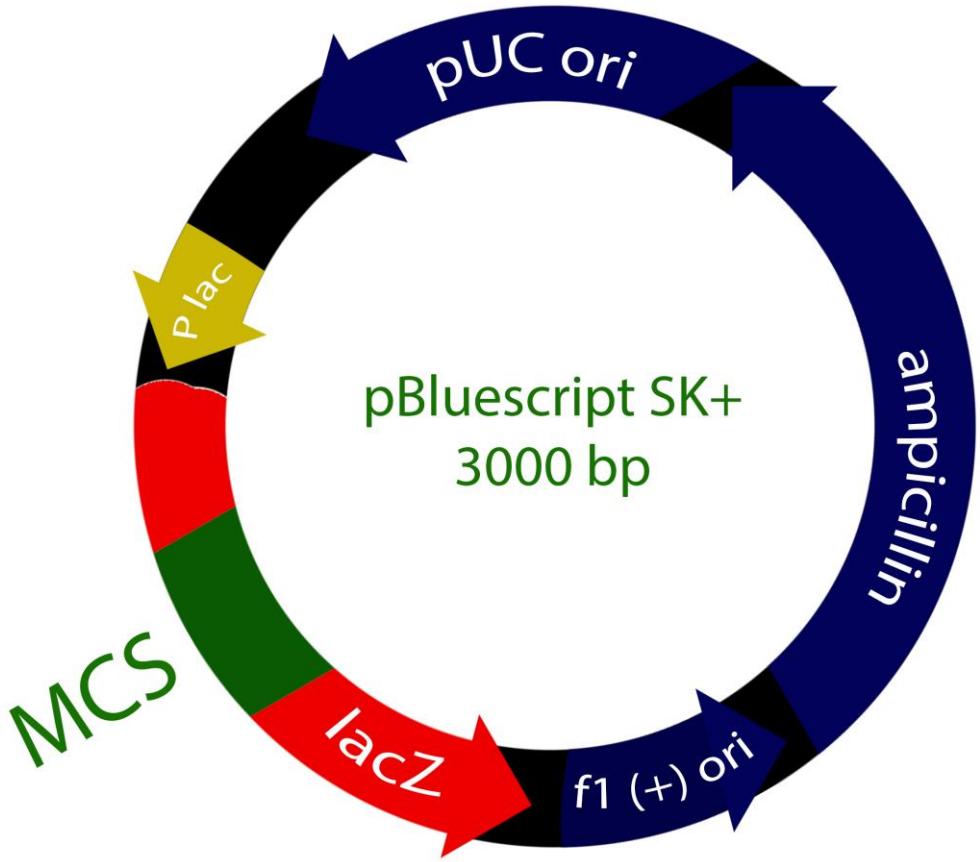
2 MATERIALS AND METHODOLOGY

2.1 Bacterial Strains and Growth Conditions:

The *H. ducreyi* 35000 strain was acquired from frozen bacterial glycerol stocks stored at -80 ºC. *H. ducreyi* was grown on chocolate agar plates (Oxoid) in an environment of 5% CO_2 at 35ºC and the environment was also humid. *Escherichia coli* TOP10 cells were used as the host cell for cloning and manipulating the DNA of the plasmids and genes from *H. ducreyi*. The TOP10 cells were purchased from Invitrogen. The cells were grown in aerobic conditions in ambient air at 37 ºC on Luria-Bertani (LB) plates [LB broth (Difco/Becton Dickinson, Sparks, MD, USA) with 1.5% (w/v) Bacto agar (Difco/Becton Dickinson, Sparks, MD, USA)] or with shaking vigorously at 225 round per minute (rpm) in LB broth at 37 ºC. Antibiotics were added when necessary at the subsequent final concentrations: ampicillin 100 mg/ml (Sigma, St. Louis, MO, USA), and kanamycin 50 mg/ml.

2.2 Plasmids:

pBluescript KS, 3.0 kb, (Figure 1) was used as cloning vector because of its ability to work as a suicide vector when introduced into *H.ducreyi*. The *hhbp* gene was cloned into pET151 by a previous student in our laboratory. Three plasmids were used as a source for the kanamycin resistant cassette. The plasmid pCMV6-Entry, 4.9 kb,(Figure 2) also encodes a gene for neomycin resistance. The map for pET30a, 5.4 kb, is shown in (Figure 3). Thirdly, the kanamycin cassette from pUC18K2 has also been used to mutate the *hhbp* gene.



In the Multiple cloning site (MCS) resides the *EcoRV* site where we digested the pBluescript

.5`.....AAGCTTGATATCGAATT.....3

Figure 1. Vector map for pBluescript

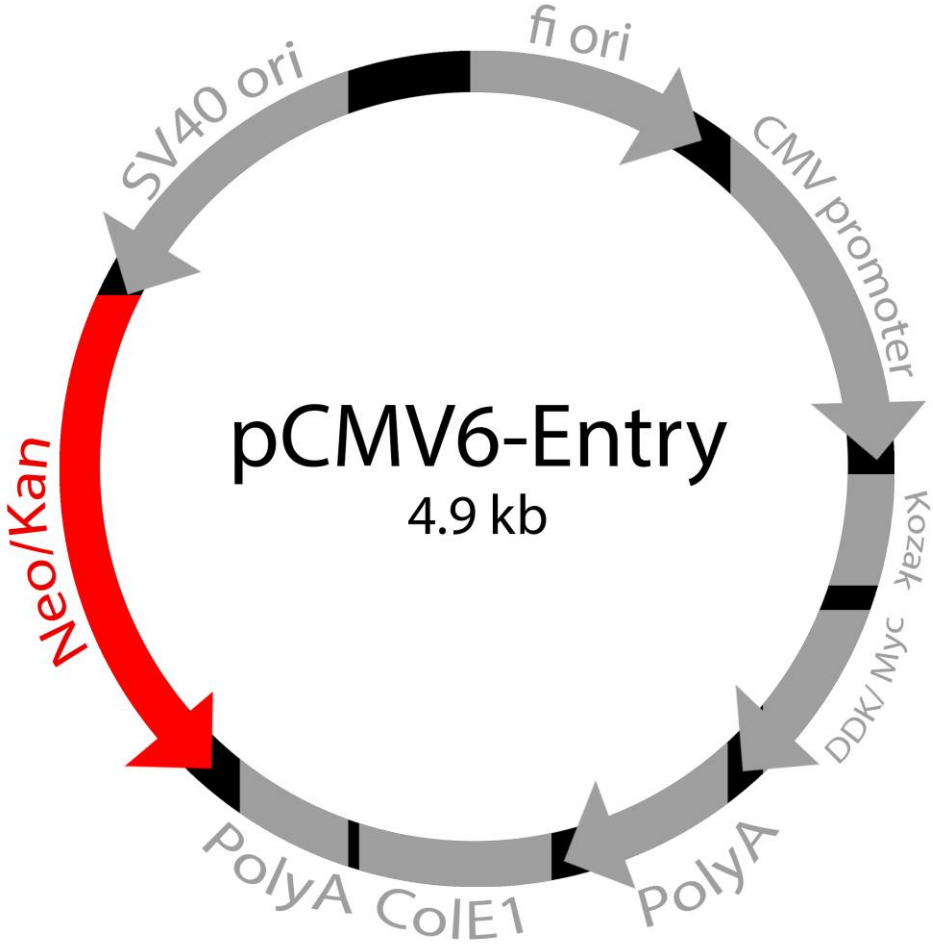

fi ori
CMV promoter
Kozak
DDK/ Myc
PolyA
PolyA
ColE1
Neo/Kan
SV40 ori
pCMV6-Entry
4.9 kb

Figure 2. Vector map of pCMV6-Entry

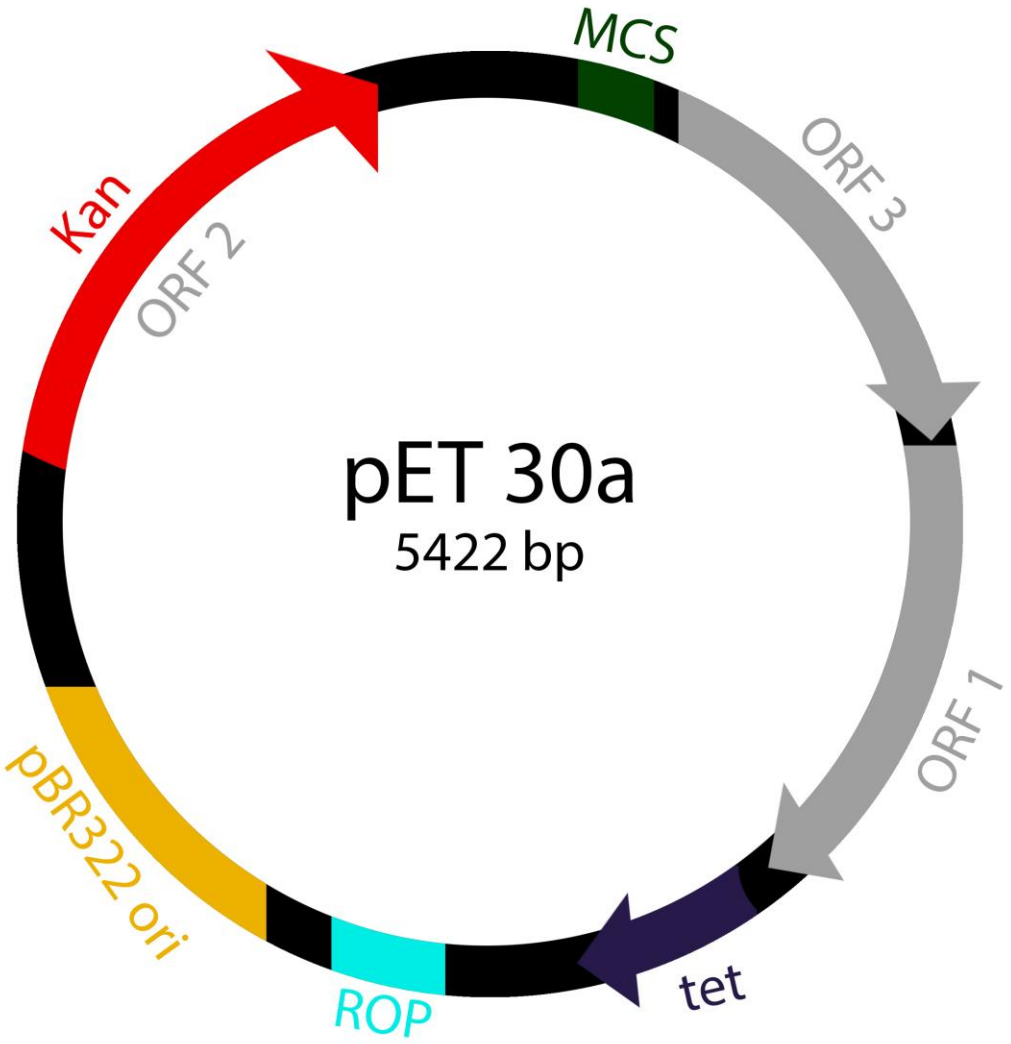

MCS
ORF 3
ORF 1
tet
ROP
pBR322 ori
Kan
ORF 2
pET 30a
5422 bp

Figure 3. A vector map of the pET30a

2.3 Molecular Biology Techniques

2.3.1 DNA Quantification

The spectrophotometer RNA/DNA calculator GeneQuant II (Pharmacia Biotech, Cambridge, England) was used to quantify the plasmid DNA.

2.3.2 Agarose Gel Electrophoresis:

Plasmid and DNA fragment separation were performed by agarose gel electrophoresis. The Hoefer HE 33 Mini Submarine (Amersham Biosciences, Piscataway, NJ, USA) was used to electrophoreses Gels composed of agarose in a percentage of 1.0% (w/v) as per the instructions of the manufacturer. Briefly, 1x Tris-Borate-EDTA buffer [TBE; 0.55% (w/v) boric acid (BioShop, Burlington, ON, Canada) 0.4% (v/v) 0.5M EDTA, Tris base in a percentage of 1.1% (w/v), and pH 8.0)]. Moreover, to every sample, the amount of 1µl was add from 10X DNA gel loading dye (Eppendorf, Westbury, NY, USA) prior to gel loading. Fter that, Ehidium bromide (EtBr; Invitrogen, Carlsbad, CA, USA) was been added to the melted agarose in a percentage of 0.005% (v/v) before casting the gel to visualize the DNA bands. Gels were electrophoresed at 110 V. Markers for the sizes of DNA fragments were loaded with every running of a gel. Different markers were used and included 1 kb, supercoiled DNA Ladder (Invitrogen, Carlsbad, CA, USA), 1000 bp marker, 500-10000 bp marker (Bio Basic Canada Inc., Markham, ON, Canada). Agarose gel images were observed under UV and photographed by a MultiImage Light Cabinet (Alpha Innotech Corp., San Leandro, CA, USA).

2.3.3 PCR

Isolated plasmid containing the isolated hhbp gene were used as a DNA template to amplify the *hhbp* using hHBP-F: 5'- ATGAATCTTTCCTTTCTAAA and hHBP-R: 5'- TCATTTAGGCTCTTTTTTGCTCG primers (Eurofins MWG Operon LLC, Louisville, KY). A 46.25 µl PCR master mix tube was equipped with 5 µl of *Pfx50* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.3 mM dNTP solution, 0.3 µM solution of each primer, 50 µl of 10X *Pfx50* PCR Mix . After that, sterile ddH2O and DNA template were added to complete 25 µl. DNA concentrations, as the manufacturer suggested, always were in the range of 10- 50 ng/25 µl PCR reaction if the amplified DNA was a DNA of a plasmid. The DNA templet was amplified by the machine in our lab called Touchgene Gradient Thermocycler (Techne; Cambridge, UK) by the conditions suggested by the polymerase manufacturer as follow: an initial denaturation of 94 ºC for 2 minutes; 35 cycles of 94 ºC for a quarter of a minute, 48 ºC for a half of a minute, and 68 ºC for 54 seconds; and a final extension of 68 ºC for 5 minutes. For specific PCR amplification reactions, the profile was customized by differentiating the reaction amount, ratio of primers to DNA template, the primer extension duration, and the annealing temperature. These steps were alternated systematically to succeed to obtain the desired results. The PCR amplification conditions for each specific experiment are listed in (Table 1). The size of the amplified gene was confirmed by gel electrophoresis of the PCR products. The identity of PCR products was determined by nucleotide sequencing.

During this research different DNA polymerases were used. *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), Platinum®*Taq* DNA Polymerase (Invitrogen, Carlsbad, CA),

PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA), and Phusion Flash High-Fidelity (Thermo Scientific, Affibody AB, Sweden) were used in different stages of the research.

Taq DNA polymerase and Platinum *Taq* DNA polymerase used the same protocol except that the final concentration of the primers in each reaction was 0.5 uM in *Taq* DNA polymerase and 0.2 uM final concentration with Platinum®*Taq* DNA Polymerase. In these PCR protocols, 50 mM $MgCl_2$ was added separately to a final concentrations of 1.5mM in 1X concentration of PCR buffer. There was no final extension step when the Platinum®*Taq* DNA Polymerase was used. The Phusion Flash High-Fidelity generated a PCR product of 1 kb size in less than 12 minutes.

2.3.3.1 Designing Oligonucleotide Primers for PCR

All the primers were designed by the software provided on Invitrogen website and are listed in (Table 1**).** The sequences were ordered from either (Eurofins MWG Operon LLC, Louisville, KY) or (Invitrogen, Carlsbad, CA).

#	Name	Sequence in 5`-3` orientation	Restriction site	Source
1	hHBP-F	ATGAATCTTTCCTTTCTAAA		Operon
2	hHBP-R	TCATTTAGGCTCTTTTTTGCTCG		Operon
3	Int Pro-F	ATGGAAAATCGTTTAATTGCTAACA		Operon
4	Int Pro-R	TTAATCGTCCTTGCTTAATACACCA		Operon
5	dsvC-F	ATGCATATGATCGAATGTAATGG		Operon
6	dsvC-R	TTATAAACATTTTATAGGTTTAGGCAA		Operon
7	ATPase-F	ATGAACAATATAACACAAGCTACAGCA		Operon
8	ATPase-R	TTAACTAGCTTGCAAATAATGTACAAT		Operon
9	(Kan-r) 1 F	ATGCATCTCAGAAGAACTCGTCAAGAAGG	*Nsi1*	Operon

10	(Kan-r) 1 R	ATGCATTGATTGAACAAGATGGATTGC	*Nsi1*	Operon
11	hHBP-Mid-F	GCTGTCGTCGTAACTGAAGG		Operon
12	hHBP-Mid-R	ATCTTGTGCAAGGTAGCTAAATG		Operon
13	pcmv-6-entry-KanF	ATGCATACAAATAAAGCAATAGCATCACA	*Nsi1*	Operon
14	pcmv-6-entry-KanR	ATGCATAGCCCAGCTTGGAGCG	*Nsi1*	Operon
15	hhbp+kan-r plas F	TAAAACGACGGCCAGTGAG		Operon
16	hhbp+kan-r plas R	CACAGGAAACAGCTATGACCAT		Operon
17	ATPaseMed+kan-F	GCAGGCTTAGGGGATGCA		Operon
18	ATPaseMed+kan-R	AGGACATATCTTTTTTGGTGGTTAAG		Operon
19	IntProMed+kan-F	CTTTTTCAGCGATTGTCGC		Operon
20	IntProMed+kan-R	ATTAGTATTCGTTAAGAATAATAAGCC		Operon
21	IntproQEcor1FFF	GATATCATGGAAAATCGTTTAATTGC	*EcoR1*	Operon
22	IntproQKpn1RRR	GGTACCTTAATCGTCCTTGCTTAATACA	*Kpn1*	Operon
23	Kan-sty1-Puc18KF	CCWWGGCTAAAACAATTCATCCAGTAAAA	*Sty1*	Invitrogen
24	Kan-sty1-Puc18KR	CCWWGGATGGCTAAAATGAGAATATCACC	*Sty1*	Invitrogen
25	Hhbp+Kan+res1F	GAATTCGAATTCGATATGAATCTTTCCTTTCT		Invitrogen
26	Hhbp+Kan+res1R	AAGCTTAAGCTTGATTCATTAGGCTCTTTT		Invitrogen
27	Kan-STY1 1 F	CCWWGGATGAGCCATATTCAACGGGA	*Sty1*	Invitrogen
28	Kan-STY1 1 R	CCWWGGGAAAAACTCATCGAGCATAAAT	*Sty1*	Invitrogen
29	hhbp-res-new 1 F	GAATTCATGAATCTTTCCTTTCTAAAAAAGACC		Invitrogen
30	hhbp-res-new 1 R	AAGCTTTCATTTAGGCTCTTTTTTGCTCG		Invitrogen
31	Kan-pet30a 1 F	ATGCATATGAGCCATATTCAACGGGA	*Nsi1*	Invitrogen
32	Kan-pet30a 1 F	ATGCATGAAAAACTCATCGAGCATCAAAT	*Nsi1*	Invitrogen

Table 1. A list of the primers used in the thesis.

2.3.3.2 PCR Purification

To purify the PCR products, the QIAquick® PCR Purification Kit (Qiagen, Mississauga, ON, Canada) was used according to the manufacture instructions. In brief, 125 ul of buffer PB was mixed with 25 ul of the PCR product. The sample was then applied to the middle of the QIAquick column and centrifugated for 1 min to attach the DNA to the membrane in the column tube. The flow-through was discarded and washing the DNA was the next step with the addition of 0.75 ml Buffer PE. After centrifugation for a minute, discarding the flow through was the next step. Residual wash buffer was removed by a further centrifugation for 1 min. The column was then placed in a 1.5 ml microcentrifuge tube to which was added 50 ul of ddH_2O followed by centrifugation for 1 min to elute the PCR product. The DNA concentration was diluted with the addition of 30 ul of ddH_2O and after a 1 min incubation, the column was centrifuged for 1 min. All centrifugations, if not otherwise specified, were performed at 13,000 round per minute (rpm) at room temperature.

2.3.4 DNA Digestion:

New England Biolabs (NEB, Beverly, MA) was the source of all the restriction enzymes used in this study and reactions were performed according to the instructions of the manufacturer. 1 μg plasmid DNA was in the Reaction mixtures with, 1X NEB Reaction buffer, 10 U restriction enzyme and filling the tube to 50 μl with ddH_20. Incubating the mixtures were performed in a 37 °C water bath 1-16 hours. The following enzymes were used for digestion during this study (*Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nsi*I, and *Sty*I).

2.3.5 Gel Purification of DNA Fragments

DNA fragments resulted from the PCR amplification were segregated on a gel with the percentage of 1% agarose. Extraction of the DNA fragments from the agarose gel by using the QIAquick Gel Extraction Kit (Qiagen, Alameda, CA) according to the manufacturers' instructions. Re-suspension of the DNA samples were performed by the addition of 25 to 50 μl of ddH_20. To confirm purity and sizes DNA fragments produced from the gel extraction were electrophoresed on an agarose gel.

2.3.6 Phosphorylation of DNA

Phosphorylation of the PCR products of the *hhbp* gene and the digested pBluescript prior to blunt-end ligation was performed using the End-It™ DNA End-Repair Kit according to the manufacturer's protocol. Briefly, 5 ug of the targeted DNA was mixed with 5 ul of the 10X End-Repair Buffer, 5 ul dNTP mix, 5 ul of ATP, 1 ul of the End-Repair Enzyme Mix. The reaction volume was increased to 50 ul with sterile ddH_2O. Following incubation for 45 minutes at room temperature, the reaction was stopped by heat shock at 70 ^oC for 10 minutes.

2.3.7 DNA Blunt End Ligation

Blunt-end ligation of the PCR product of the *hhbp* gene into pBluescript was achieved using T4 DNA Ligase. The reaction mixture comprised 4 ul of 5X Ligase Reaction Buffer, a 3:1 molar ratio between the insert and the vector DNA, 0.1 unit of the T4 DNA ligase, and the addition of sterile ddH_2O to a final volume of 20 ul. The ligation reaction was incubated at 14 ^oC overnight. Alteration of the incubation time and temperature and increasing the amount of the T4 DNA ligase were performed as necessary.

2.3.8 Transformation of *E. coli* TOP10 Cells

Competent *E. coli* Top10 cells tubes were obtained from the -70°C freezer and positioned on ice to thaw. Every tube contains 200 µl of competent cells. An appropriate amount of the plasmid DNA of interest (typically 50 ng) was added to the cell suspension and was mixed gently by inversion. The reaction mixture was incubated on ice for 30 minutes. After that, a 90 second heat shock at 100 °C. Then 0.5 ml of SOC media (Invitrogen, Carlsbad, CA) was added and the mixture was obtained at 37 °C for 45 min with vigorous shaking at 225 rpm by an orbital shaker (Forma Scientific, Marietta Ohio). On LB agar containing the appropriate antibiotic(s), a volume of 200 µl of the transformed cells were plated. After overnight incubation at 37 ºC, single colonies were placed in 5 ml of LB broth and incubated at 37 ºC with shaking at 225 rpm overnight.

2.3.9 Plasmid DNA Isolation

Plasmids were extracted from TOP10 cells by the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada) according to the the manufacturer instructions. Cells were harvested by centrifugation at 8000 rpm for 3 mins at room temperature. The pellet was resuspended with 250 ul of Buffer P1 Qiagen, Mississauga, ON, Canada). Then, 250 ul was added of Buffer P2 (Qiagen, Mississauga, ON, Canada), then inverted 4-6 times to mix the reaction. The tubes were left for 5 mins at room temperature. After that, 350 ul of buffer P3 (Qiagen, Mississauga, ON, Canada) was suplemented and mixed by inversion 4 - 6 times. Lysate was decanted into the tub of the QIAPrep spin column and cenrifuged for 1 min. The flow-through was discarded. The colunm was washed with the addition of 500 ul Buffer PB. A second wash step was performed with centrifugation for 1 min with the addition of 750 ul Buffer PE., and the flow-through was discarded. The DNA was eluted

with the addition 50 ul of ddH_2O followed by a 1 min incubation and centrifugution for 1 min.

2.3.10 DNA Sequencing

All experiments of sequencing the nucleotide were performed in the facility of sequencing in Ottawa Hospital Research Institute - Ontario Genomic Innovation Centre (OHRI-OGIC). PCR products and inserted DNA fragments were sequenced by the Big Dye Terminators v 3.1 Chemistry following the instructions of the manufacturer. Furthermore, the data resulting from the sequence was produced on the Applied Biosystems 3730 DNA analyzer (ABI Biosystems, Columbia, MD). The analyzation of the sequences configurations were subjected to MacVector (Oxford Molecular, Palo Alto, CA). Also, BlastN (DNA sequences) was used for similarity searches of GenBank and BlastN were used to perform searches of the Project Database of the *H. ducreyi* genome.

3 RESULTS

3.1 Cloning the *hhbp* gene:

The gene encoding the hHbp protein has been previously cloned in our laboratory into the pET151/D-TOPO® vector and maintained in *E. coli* TOP10 cells. Following plasmid isolation using the Qiagen QIAprep Spin Miniprep Kit, the *hhbp* gene was PCR amplified using the Pfx50 DNA Polymerase from the plasmid template with primers hHBP-F and hHBP-R (Table 1). The size of the resulting DNA fragment corresponded to the known size of the *hhbp* gene, 907 bp, when analyzed by agarose gel electrophoresis (Figure 4). After gel purification performed by the QIAquick® PCR Purification Kit, the identity of the product was confirmed by nucleotide sequencing.

3.2 Cloning the *hhbp* gene into pBluescript II KS+:

The pBluescript plasmid was used as the cloning vector because it cannot multiplied in *H. ducreyi* and replicate, and thus would function as a suicide vector facilitating integration via homologous recombination of the mutated *hhbp* gene into the chromosomal locus. The backbone vector pBluescript was digested by the restruction enzym *Eco*RV- to linearize the plasmid preparing it for blunt end ligation of *hhbp*. The manufacturer's directions were altered by increasing the digestion time to 4 hours. The gel purified *hhbp* 907 bp product was blunt ended and phosphorylated using the End-It™ DNA End-Repair Kit and ligated into pBluescript using T4 DNA ligase. After an incubation at 14°C for 18 hours

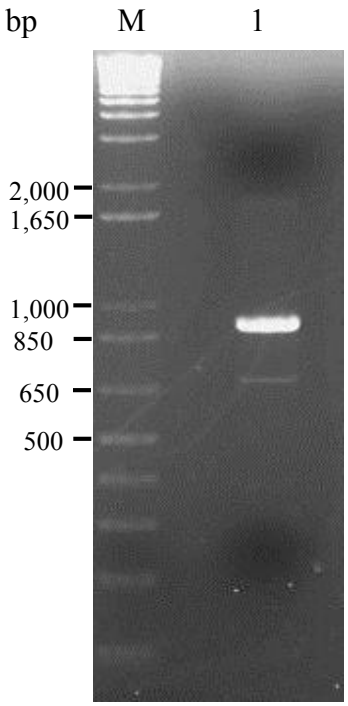

bp
M
1
2,000
1,650
1,000
850
650
500

Figure 4. A 1% agarose gel electrophoresis of the purified *hhbp* gene PCR amplified from pET-151. M is the 1 kb plus ladder from Invitrogen. Lane 1 is the purified PCR product. The *hhbp* gene is 907 bp in size.

using a molar ratio between the insert and the vector of 2:1, the recombinant plasmid was transformed into *E. coli* TOP10 cells on LB agar containing ampicillin.

Plasmids were extracted from single colonies using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada) and a construct of 3907 bp containing the *hhbp* gene was detected on agarose gel electrophoresis (Figure 5, lanes 1,3,5,8). The existence of the *hhbp* gene insert was verified by nucleotide sequencing. The plasmid resulting from the ligation was named pBluHhbp, 3907 bp. In lane 4 the band around the 2500 bp size approximates the size of three inserts (2721 bp) ligated end to end. The 6 kb band in lane 6 likely represents the ligation of two pBluescript plasmids. In lane 7 the 3 kb band corresponds to the size of the vector alone without the insert.

3.3 Construction of an *hhbp* mutant by insertional inactivation

The strategy to construct a mutation in the *hhbp* gene employed insertional inactivation using a kanamycin resistance cassette. Plasmid pBluHhbp was first linearized with *Nsi*1 which targeted a unique restriction site located in the middle of the plasmid (Figure 6). The kanamycin cassette was PCR amplified from pCMV6-Entry using primers whose 5' and 3' ends contained the *Nsi*1 restriction sites (Table 1). The appropriate size kanamycin 794 bp gene was successfully PCR amplified using Taq DNA Polymerase (Figure 7).

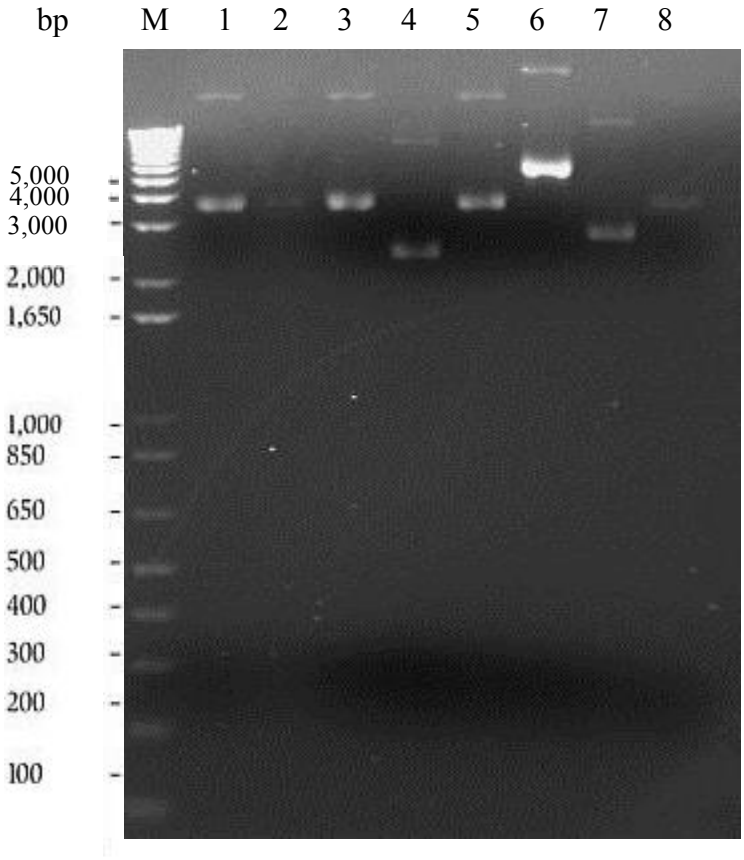

bp
M
1
2
3
4
5
6
7
8
5,000
4,000
3,000
2,000
1,650
1,000
850
650
500
400
300
200
100

Figure 5. A 1% agarose gel electrophoresis of pBluHhbp isolated from transformed TOP10 cells. M is the 1 kb plus ladder from Invitrogen. Lanes 1, 3, 5, and 8 represent pBluHhbp constructs isolated from four separate transfomants. The 3907 bp size of pBluHhbp conforms to insertion of the 907 bp hhbp into the 3.0 kb pBluescript. In lane 4 the band around the 2500 bp size which is close to the size of three inserts (2721 bp) combined back to back. The 6 kb band in lane 6 likely represents the ligation of two pBluescript plasmids. In lane 7 the 3 kb band corresponds to the size of the vector alone without the insert.

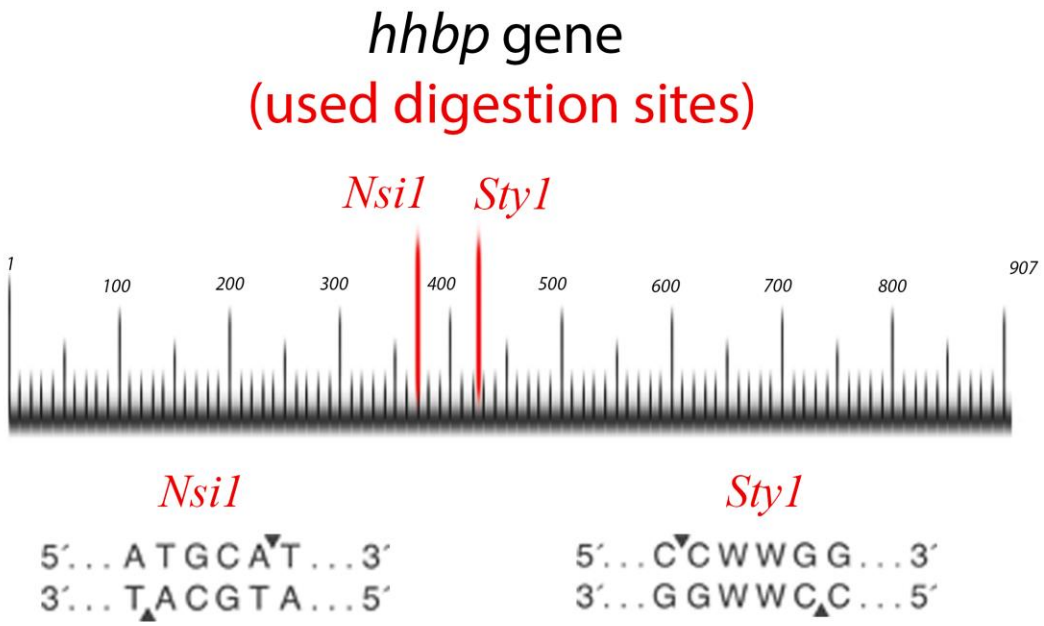

hhbp gene
(used digestion sites)
Nsi1
Sty1
1
100
200
300
400
500
600
700
800
907
Nsi1
5′. . . A T G C A T . . . 3′
3′. . . T A C G T A . . . 5′
Sty1
5′. . . C C W W G G . . . 3′
3′. . . G G W W C C . . . 5′

Figure 6. The map of restriction sites of the *hhbp* gene with *Nsi*1 and *Sty*1 marked

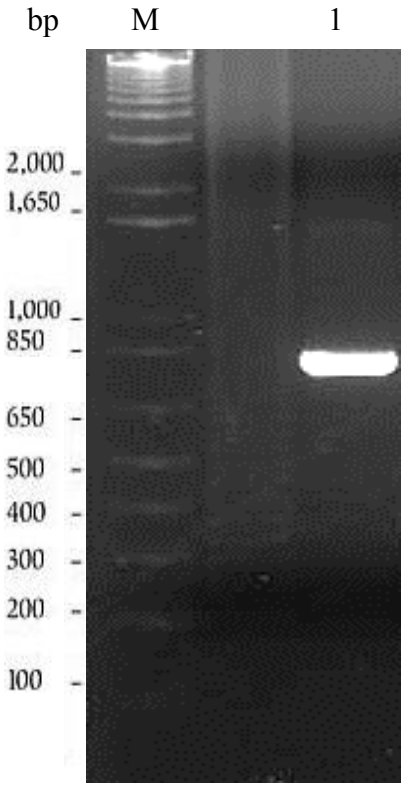

bp
M
1
2,000
1,650
1,000
850
650
500
400
300
200
100

Figure 7. A 1% agarose gel electrophoresis of the kanamycin cassette PCR amplified from pCMV6-Entry. M is the 1 kb plus ladder from Invitrogen. Lane 1 is the purified 794 bp kanamycin cassette.

3.3.1 Ligation of the kanamycin cassette within the *hhbp*

Ligations were performed at 20 °C for 1h. The process was performed using an insert to vector molar ratio of 3:1. Several different ligations produced no ligation products when aliquots were assessed on a 1% agarose gel with a negative control of the digested pBluescript (data not shown).

3.3.1.1 Varying the ligation conditions

As the initial attempts to ligate the kanamycin cassette into the *Nsi*1 site were unsuccessful, the ligation conditions were systematically altered. First, three incubation temperatures of 37 °C, 14 °C, and 4 °C were used. No yielded ligation products of the expected size were produced (Figure 8). Several faint bands were seen of ligation products analyzed in lanes 1, 2, and 3. The two most prominent bands represented the 3907 bp vector alone and an approximate 5.5 kb band representing the vector containing two inserts.

Second, varying the incubation periods (4 h, 16 h and 24 h) at a fixed incubation temperature of 20 °C was also unsuccessful in producing ligation products of the correct size (Figure 9). Although ligation products in lanes 5 and 6 were of the expected 4701 bp size, no kanamycin resistant colonies were produced following transformation of the gel purified extracted bands into TOP10 cells. In lanes 1, 2, 3, and 4, the three prominent bands represent the 3907 bp pBluHhbp, the 5495 bp pBluHhbp containing two kanamycin cassettes, and the upper band corresponds to the size of the empty vector ligated end to end. These three bands are also seen in lanes 5 and 6.

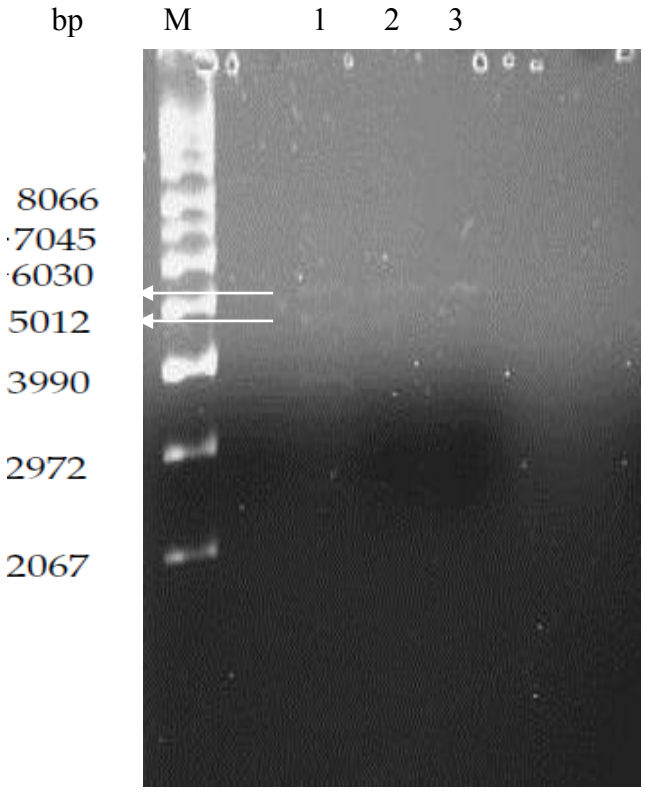

bp
M
1
2
3
8066
7045
6030
5012
3990
2972
2067

Figure 8. A 1% agarose gel electrophorisis of ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. Ligations were incubated for one hour and the ratio of insert to plasmid was 3:1. M is the Supercoiled DNA Ladder from Invitrogen. The incubation temperature for the reaction was 37 °C in lane 1, 14 °C in lane 2, and 4 °C in lane 3. The expected size of the construct is 4701 bp. Several faint bands were seen of ligation products analyzed in lanes 1, 2, and 3. The two most prominent bands represented the 3907 bp vector alone and an approximate 5.5 kb band representing the vector containing two inserts.

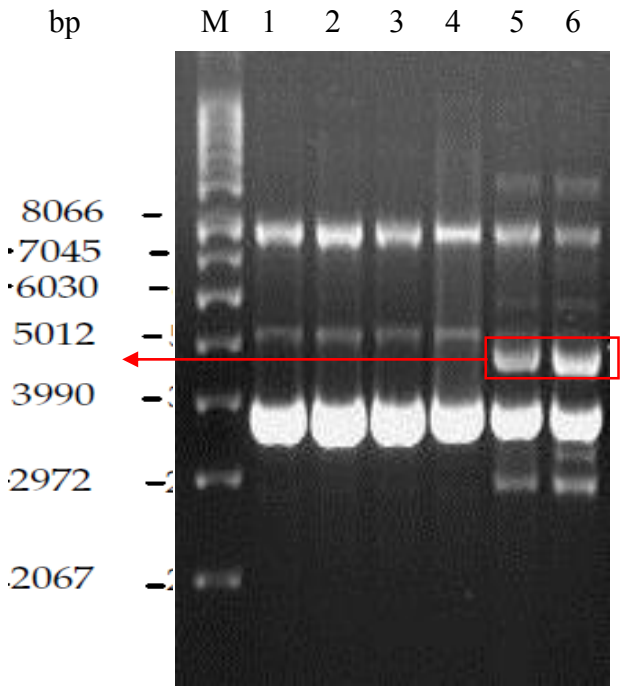

bp
M
1
2
3
4
5
6
8066
7045
6030
5012
3990
2972
2067

Figure 9. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. The ligation reactions were incubated at 20 °C and the ratio of insert to vector was 3:1. M is the Supercoiled DNA Ladder from Invitrogen. The incubation time was 4 h in lanes 1 and 2, 16 h in lanes 3 and 4, and 24 h in lanes 5 and 6. The expected size of the construct is 4701 bp.

Similarly, no ligation products containing a single copy of the kanamycin cassette resulted when the concentration of the T4 ligase was systematically increased (Figure 10) while maintaining a fixed incubation time and temperature of 1h and 20°C, respectively. The multiple bands seen in lanes 1, 2, 3, and 4 represent the empty 3907 bp vector, pBluHhbp containing multiple copies of the kanamycin cassette, and concatamers of the kanamycin cassette.

To examine whether the molar ratio between the insert and the vector may have been responsible for the inability to generate a *H. ducreyi hhbp* mutant, three different ratios of 2:1, 1:1 and 0.5:1 were used. The incubation time was for 1h and the reactions were incubated at room temperature.

Ligation products of the appropriate size were seen on agarose gels and these were subsequently transformed into TOP10 cells. Plasmids were extracted from single kanamycin resistant colonies and a construct of 4701 bp was detected on a agarose gel (Figure 11, lanes 1 to 8), corresponding to the anticipated size of pBluescript containing the kanamycin cassette ligated within *hhbp*. However, PCR amplification analysis using primers bracketing the 5' and 3' junctions of the *hhbp* insertion revealed only amplicons larger than the 1764 bp *hhbp*:Kan product or no PCR products were generated using several different *Taq* polymerases (Figure 12, lanes 1 to 3). Restriction digestion of several plasmid constructs with *Nsi*1 failed to release the kanamycin cassette (data not shown).

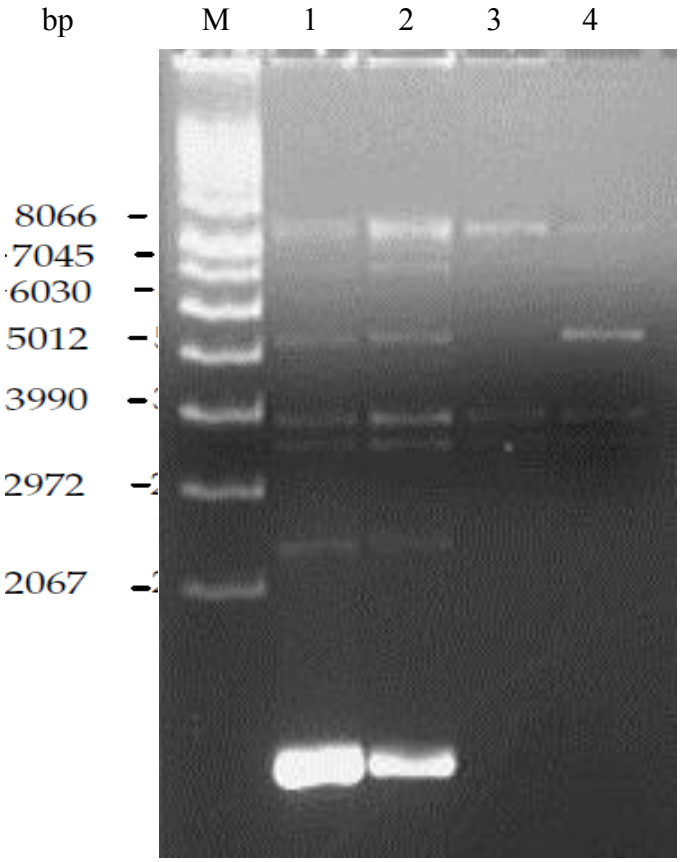

bp
M
1
2
3
4
8066
7045
6030
5012
3990
2972
2067

Figure 10. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. The ligation reactions were incubated at 20 °C for 24 h and the ratio of insert to vector was 3:1. The expected size of the construct is 4701 bp. (M) is the Supercoiled DNA Ladder from Invitrogen. The T4 ligase used was 0.2 U in lane 1, 1 U in lane 2, 2.5 U in lane 3, and 5 U in lane 4. The multiple bands seen in lanes 1, 2, 3, and 4 represent either the empty 3907 bp vector, pBluHhbp containing multiple copies of the kanamycin cassette, or concatamers of the kanamycin cassette.

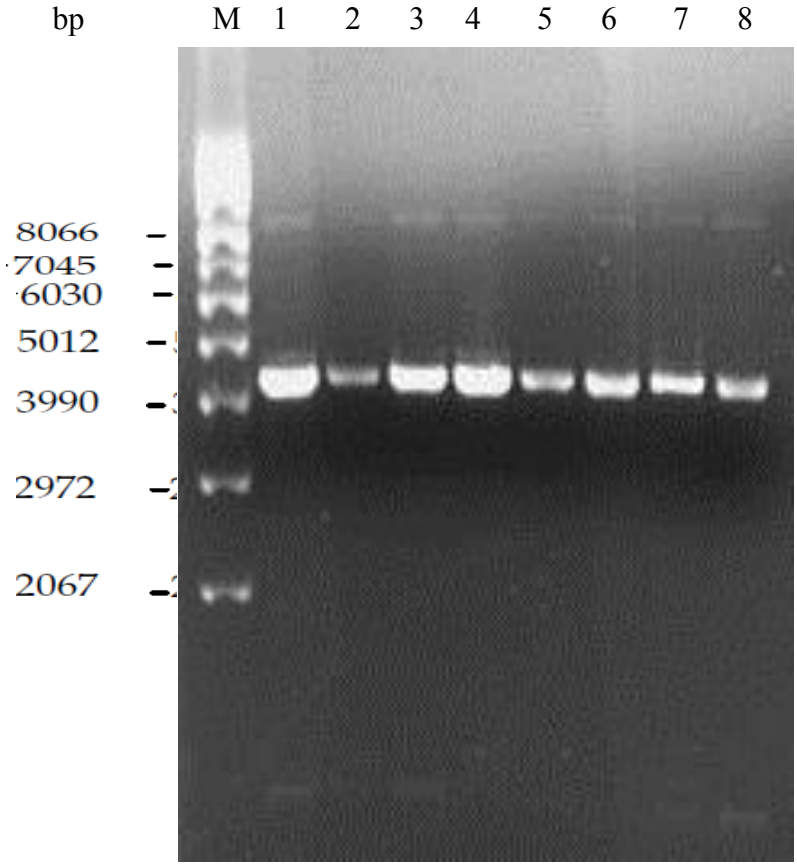

bp
M
1
2
3
4
5
6
7
8
8066
7045
6030
5012
3990
2972
2067

Figure 11. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. Ligations were performed at 20 °C for 24 h. The expected size of the construct is 4701 bp. M is the Supercoiled DNA Ladder from Invitrogen. The ratio between the insert and vector was 2:1 in lanes 1, 2, and 3, 1:1 in lanes 4, 5, and 6, and 0.5:1 in lanes 7 and 8.

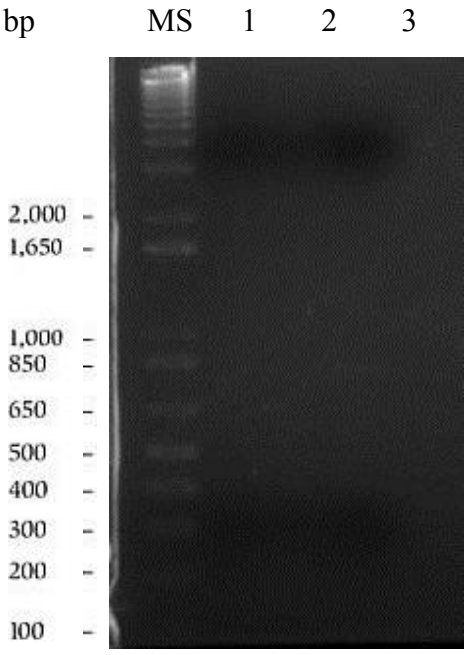

bp
MS
1
2
3
2,000
1,650
1,000
850
650
500
400
300
200
100

Figure 12. A 1% agarose gel electrophoresis for the purified PCR products from kanamycin resistant clones. Primers annealing to the 5' and 3' junctions of *hhbp* insert (Table 1) would be expected to generate a 1764 bp the *hhbp*:Kan gene. The *hhbp* gene is 907bp and the kanamycin cassette 857 bp. M1 is the M107O-1 ladder from Bio Basic Canada. (MS) is the Supercoiled DNA Ladder from Invitrogen. No amplicons of the anticipated size were detected in lanes 1, 2, and 3 from the kanamycin resistant clones shown in Fig. 7.

Several attempts to verify the nucleotide sequence of the *hhbp*::kanamycin insert using sequencing primers annealing to the 5' and 3' junctions of the insert produced no readable nucleotide sequence. Although the experimental evidence is lacking, the kanamycin cassette may have inexplicably relocated to another site within the vector. This explanation accounts for the isolation of the appropriate sized plasmid construct from kanamycin resistant clones and the inability to confirm the existence of the resistant gene within *hhbp*. The use of different *Taq* polymerases, *Pfx50* (Invitrogen, Carlsbad, CA), *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), Platinum®*Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA), and Phusion Flash High-Fidelity (Thermo Scientific, Affibody AB, Sweden), failed to generate a PCR product of the expected size to confirm that the kanamycin cassette had ligated within the *hhbp* gene. Restriction digest analysis using *Nsi*I of several kanamycin resistant transformants did not release the Kanamycin cassette from the construct, and nucleotide sequencing of the plasmids isolated from the same kanamycin resistant clones was unreadable despite the use of a buffer that relaxes the DNA and any formed hairpin loops. We speculate that the kanamycin cassette may have ligated elsewhere in the plasmid, or be integrated into the chromosome of the TOP 10 cells after the transformation.

To exclude the possibility that an intrinsic unknown property of the kanamycin cassette derived from pCMV6-Entry accounted for the above ligation failures, the kanamycin resistance gene was PCR amplified from a different plasmid, pET30a, using primers flanked with *Nsi*1 restriction sites (Table 1) generating the appropriate sized PCR amplicon. The size of the resulting DNA fragment corresponded to the known size of the

kanamycin cassette, 813 bp, when analyzed by agarose gel electrophoresis (Figure 13). After gel purification, the identity of the product was confirmed by nucleotide sequencing.

Once again, despite systematically changing the ligation protocol as described above for the pCMV-6-Entry kanamycin cassette, no appropriate kanamycin insertionally inactivated *hhbp* mutants resulted. Experiments typically produced ligation products larger than the expected 4720 bp size (Figure 14, lanes 1 to 4), in which the two most prominent bands represent a 5.2 kb construct of pBluHhbp ligated with two kanamycin cassettes and an approximate 12 kb construct of multiple pBluHhbp concatamers.

When ligation products of the correct size were introduced into TOP 10 cells, no kanamycin resistant colonies were isolated (data not shown). One of the ligations produced multiple bands of which two (Figure 15) were approximately the expected size of the pBluHhbp and the kanamycin cassette (4720 bp). The upper bands were larger than the expected size of the ligation products, and likely represent pBluHhbp ligated with two kanamycin cassettes (~5.5 kb). When kanamycin resistant transformants were isolated, PCR analysis of the plasmid constructs displayed either PCR products larger than the expected size or no amplicons. Once again, the use of different Taq polymerases, *Pfx50* (Invitrogen, Carlsbad, CA), *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), Platinum®*Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA), and Phusion Flash High-Fidelity (Thermo Scientific, Affibody AB, Sweden), failed to generate an amplicon of the expected size to confirm that the kanamycin cassette has ligated within the *hhbp* gene.

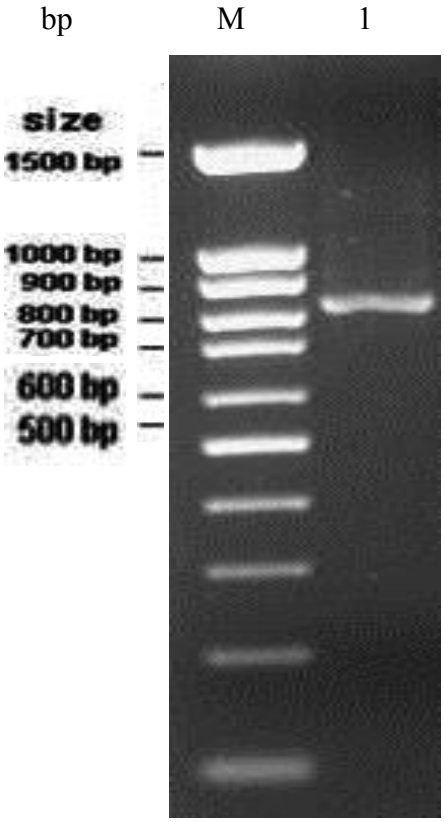

bp
M
1
size
1500 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp

Figure 13. A 1% agarose gel electrophoresis for the purified kanamycin cassette following PCR amplification from the pET30a. M is the M107O-1 ladder from Bio Basic Canada. The expected 813 bp PCR product is shown in lane 1.

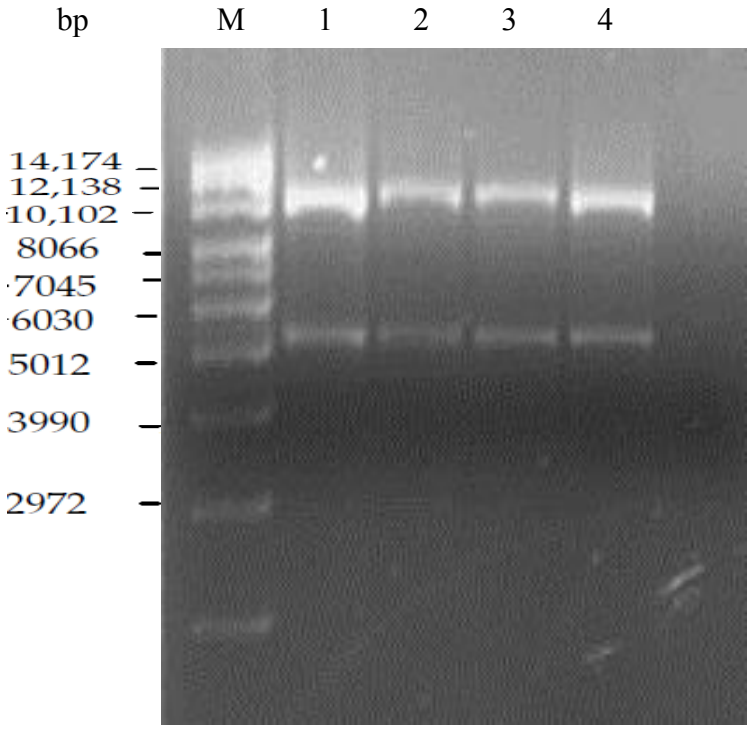

bp
M
1
2
3
4
14,174
12,138
10,102
8066
7045
6030
5012
3990
2972

Figure 14. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pET30a. Ligations were performed at 20 °C for 24h. The ratio between the kanamycin cassette and the pBluHhbp was 3:1. The expected size of the construct is 4720 bp. M is the Supercoiled DNA Ladder from Invitrogen. Ligation products from four separate reactions are shown in lanes 1, 2, 3, and 4.

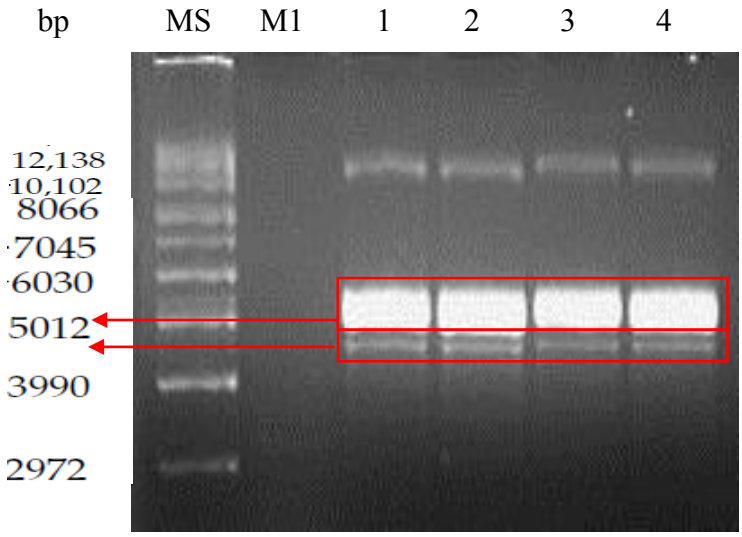

bp
MS
M1
1
2
3
4
12,138
10,102
8066
7045
6030
5012
3990
2972

Figure 15. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pET30a inserted into the *Nsi*1 restriction site. Ligations were incubated at 20 °C for 24 h. The ratio between the insert and the vector was 3:1. The expected size of the construct is 4720 bp. MS is the Supercoiled DNA Ladder from Invitrogen. M1 is the M107O-1 ladder from Bio Basic Inc. Lanes 1, 2, 3, and 4 show PCR products from four different kanamycin resistant isolates using primers bracketing the hhbp gene in the plasmid constructs. The bottom bands are around the expected size of the pBluHhbp + kanamycin cassette (4720 bp), while the upper bands are over the size of the expected ligation, which is around the size of the pBluHhbp with two kanamycin cassettes (~5.5 kb).

3.4 Construction of a *hhbp* mutant using the *Sty*1 restriction site

Because of difficulties encountered with using the *Nsi*1 site in *hhbp*, a second restriction site, *Sty*1, located at position near the middle of the *hhbp* gene was chosen for insertion of the kanamycin cassette.

In preparation for ligation of the kanamycin gene, *hhbp* was first digested with *Sty*1 according to the manufacturer's instructions. The kanamycin cassette was then PCR amplified from pCMV6-Entry using primers flanked with *Sty*1 restriction sites (Table 1) producing the expected 840 bp PCR product (Figure 16, lanes 5-8. The identity of the PCR product has not been confirmed by sequencing.

The initial ligation attempts using a molar ratio of insert to vector of 3:1 were unsuccessful (Figure 17, lanes 1 to 5) in producing a plasmid construct of the appropriate size. The 5.5 kb bands were the approximate size of pBluHhbp ligated with two copies of the kanamycin cassette. Subsequent experiments systematically changing the incubation time (Figure 18, lanes 5 to 8) and temperature (Figure 18, lanes 1 to 4) as previously described also failed to produce a ligation product of the anticipated size. The two prominant bands from these ligation reactions represent the 6.9 kb vector alone ligated end to end and a 16 kb construct comprising three copies of pBluHhbp::kanamycin. Several experiments were done using the products of ligation reactions that were not analyzed by agarose gel electrophoresis to transform TOP 10 cells. Although kanamycin resistant colonies were detected, plasmids extracted from these isolates were larger than the expected 4747 bp size (Figure 19, lanes 1 to 4). The identity of the 6.5 kb band is likely pBluHhbp ligated with three kanamycin cassettes.

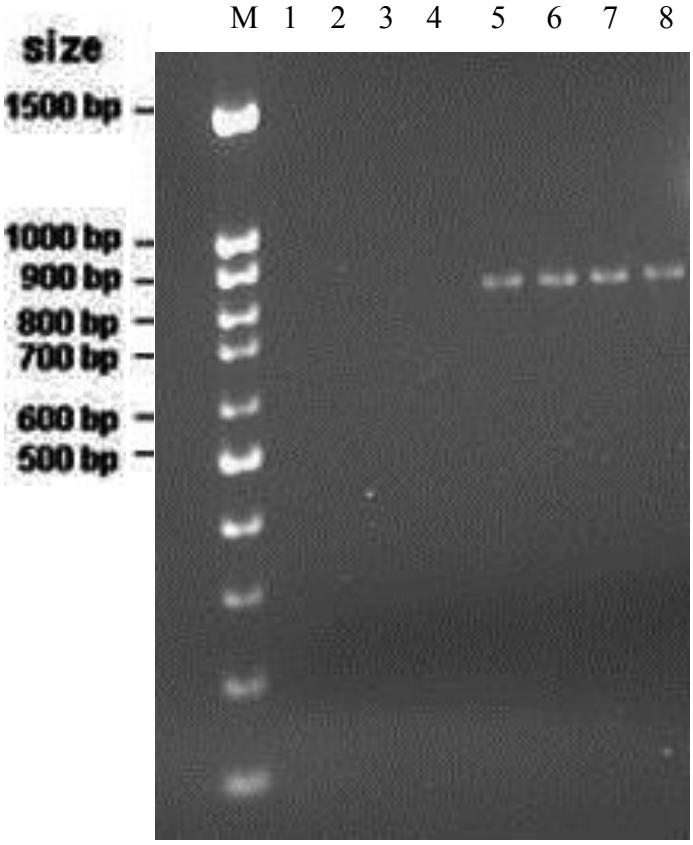

M 1 2 3 4 5 6 7 8
size
1500 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp

Figure 16. A 1% agarose gel electrophoresis for the purified kanamycin cassette PCR amplified from the pCMV6-Entry with primers incorporating the Sty1 restriction site. (M) is the M107O-1 ladder from Bio Basic Canada. The 840bp kanamycin gene is shown in lane 5, 6, 7, and 8. The lanes 1-4 are PCR samples that did not generate a DNA fragments.

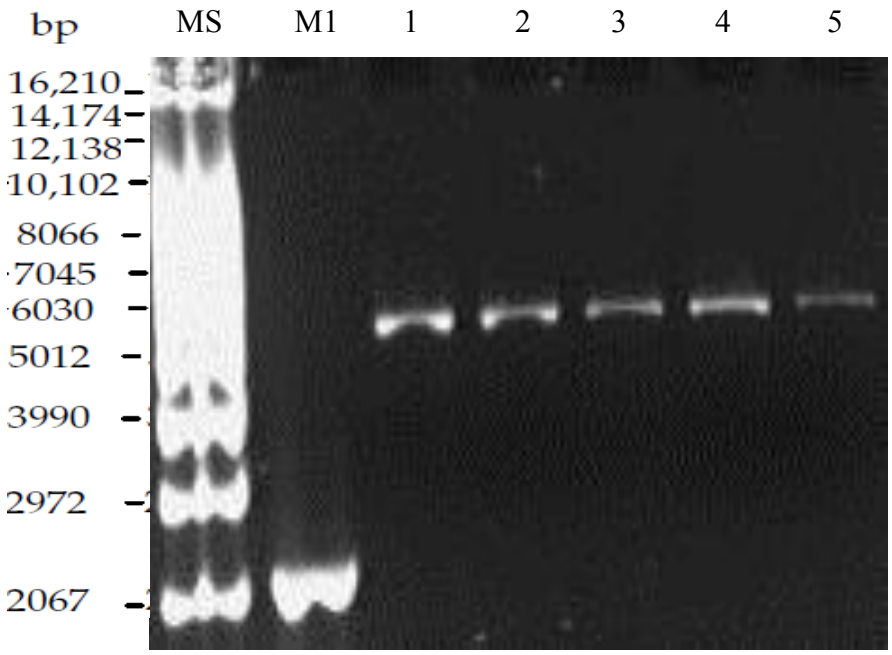

bp
MS
M1
1
2
3
4
5
16,210
14,174
12,138
10,102
8066
7045
6030
5012
3990
2972
2067

Figure 17. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. Ligations were performed at 20 °C for 24h using a ratio of insert to vector of 3:1. The expected size of the construct is 4747 bp. MS is the Supercoiled DNA Ladder from Invitrogen. M1 is the M107O-1 ladder from Bio Basic Canada. Lanes 1, 2, 3, 4, and 5 contain ligation products from five separate reactions.

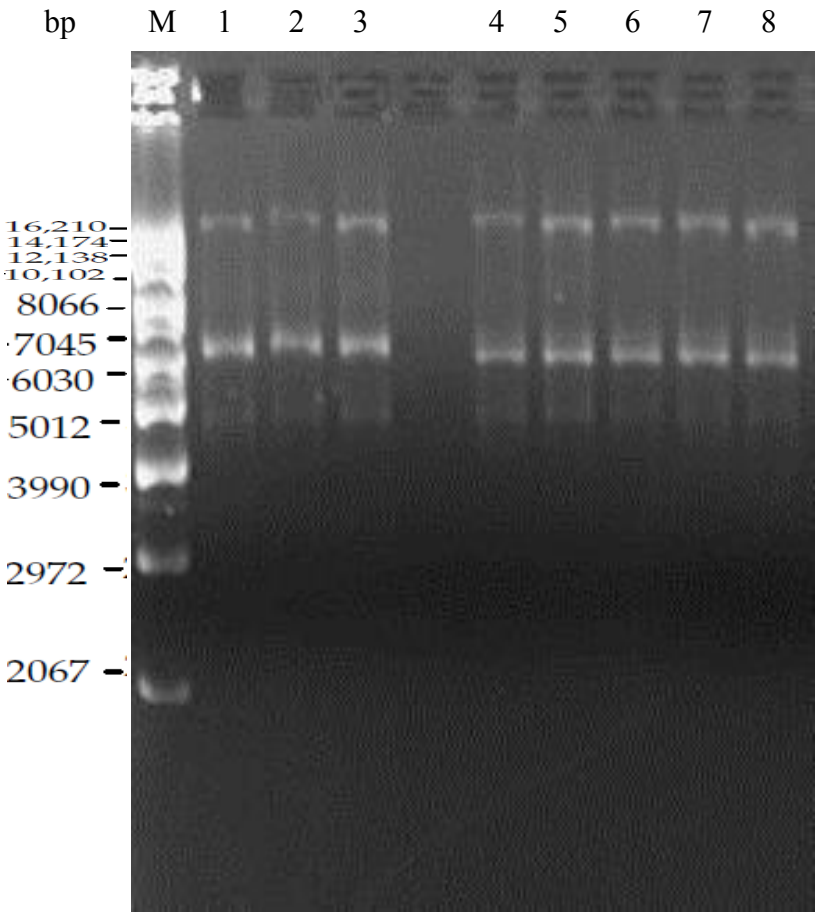

bp
M
1
2
3
4
5
6
7
8
16,210
14,174
12,138
10,102
8066
7045
6030
5012
3990
2972
2067

Figure 18. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pCMV6-Entry. The ratio of vector to insert was 3:1. M is the Supercoiled DNA Ladder from Invitrogen. The expected size of the construct is 4747 bp. Ligations were incubated for 1h at 37 °C in lane 1, at 14 °C in lane 2, at 4 °C in lane 3 and at 20 °C in lane 4. Ligations were performed at 20 °C for 2 h in lane 5, 4 h in lane 6, 16 h in lane 7 and 24 h in lane 8.

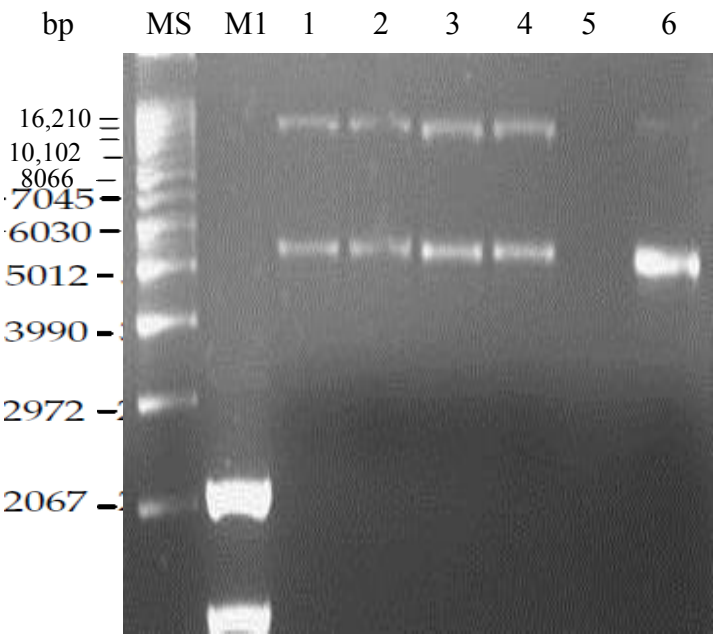

bp
MS
M1
1
2
3
4
5
6
16,210
10,102
8066
7045
6030
5012
3990
2972
2067

Figure 19. A 1% agarose gel electrophorisis for isolated pBluHhbp and the kanamycin cassette of transformants from TOP10 cells. The ratio of the ligation before the transformation was 3 molar of the kanamycin cassette to 1 molar of the pBluHhbp. The expected size of the construct is 4747 bp. The incubation temperature for the reactions were at room temperature and the ligation reaction incubated for 24 hours. (MS) is the Supercoiled DNA Ladder from invitrogen. (M1) is the M107O-1 ladder from Bio Basic Canada.

The 15.5 kb upper band represents a concatamer of four ligated pBluHhbps.

The kanamycin resistance cassette was PCR amplified from pET30a using primers flanked with *Sty*1 restriction sites (Table 1) generating the appropriate sized PCR amplicon. The size of the resulting DNA fragment corresponded to the known size of the kanamycin cassette, 857 bp, when analyzed by agarose gel electrophoresis (Figure 20). After gel purification, the identity of the product was confirmed by nucleotide sequencing. Once again, despite systematically changing the ligation protocol as described above for the pCMV-6-Entry kanamycin cassette and the insert to vector ratio as described for *Nsi*1, no kanamycin insertionally inactivated *hhbp* mutants resulted. Experiments produced either no ligation products (Figure 21), or no major ligation products of the expected size (Figure 22, lanes 1 to 6). The dominant 3.9 kb band is the size of pBluHhbp without the kanamycin cassette. The identity of the faint band corresponds to the expected 4747 bp construct. However, no kanamycin resistant tranformants were recovered when these products were introduced into TOP10 cells. Once again, to exclude the possibility that an intrinsic unknown property of the kanamycin cassette derived from pET30a accounted for the above ligation failures, a third kanamycin resistance gene was used. The kanamycin resistant gene was PCR amplified from pUC18K2 using primers flanked with *Sty*1 restriction sites (Table 1) generating the appropriate sized PCR amplicon. The size of the resulting DNA fragment corresponded to the known 853bp size of the kanamycin cassette when analyzed by agarose gel electrophoresis (Figure 23). After gel purification, the identity of the product was confirmed by nucleotide sequencing.

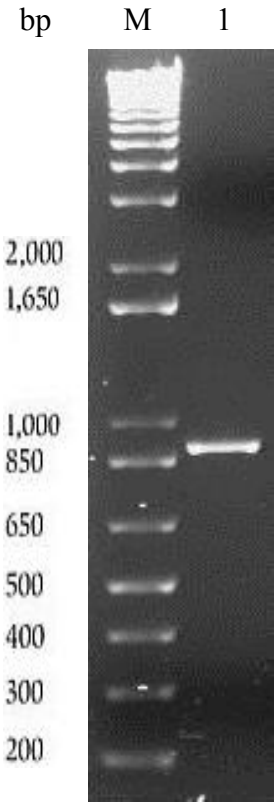

bp
M
1
2,000
1,650
1,000
850
650
500
400
300
200

Figure 20. A 1% agarose gel electrophoresis for the purified kanamycin cassette amplified from the pET30a with primers incorporating the Sty1 restriction site. M is the M107O-1 ladder from Bio Basic Canada. Lane 1 shows the expected 857 bp kanamycin gene.

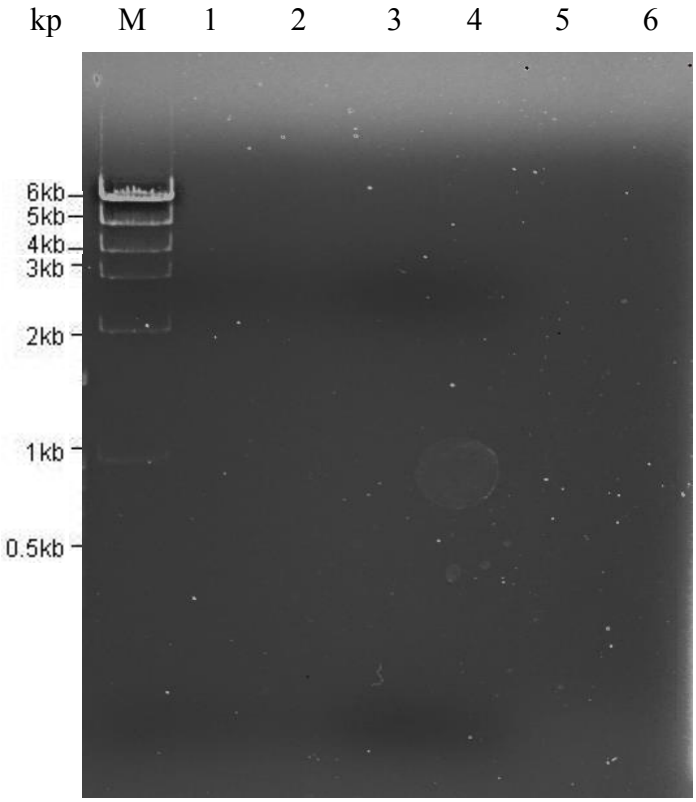

kp
M
1
2
3
4
5
6
6kb
5kb
4kb
3kb
2kb
1kb
0.5kb

Figure 21. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pET30a inserted into *Sty*1 restriction site. Ligations were incubated at 20 °C for 24h using a vector to insert ratio of 3:1. The expected size of the construct is 4747 bp. (M) is the Supercoiled DNA Ladder Bio Basic Inc. Lane 1, 2, 3, 4, 5, and 6 are ligation reactions from six separate experiments.

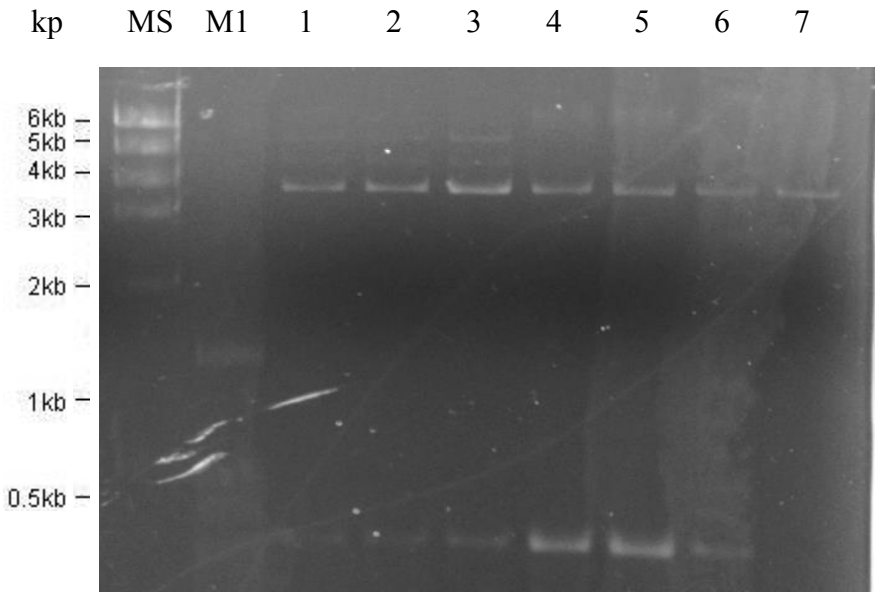

kp
MS
M1
1
2
3
4
5
6
7
6kb
5kb
4kb
3kb
2kb
1kb
0.5kb

Figure 22. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pET30a with restriction sites for *Sty*1. The ligation reactions conditions are altered. The expected size of the construct is 4747 bp. MS is the Supercoiled DNA Ladder Bio Basic Inc. Lanes 1, 2, 3, 4, 5, 6 and 7 are different ligation reactions of the pBluHhbp with different samples of the PCR products of the kanamycin cassette.

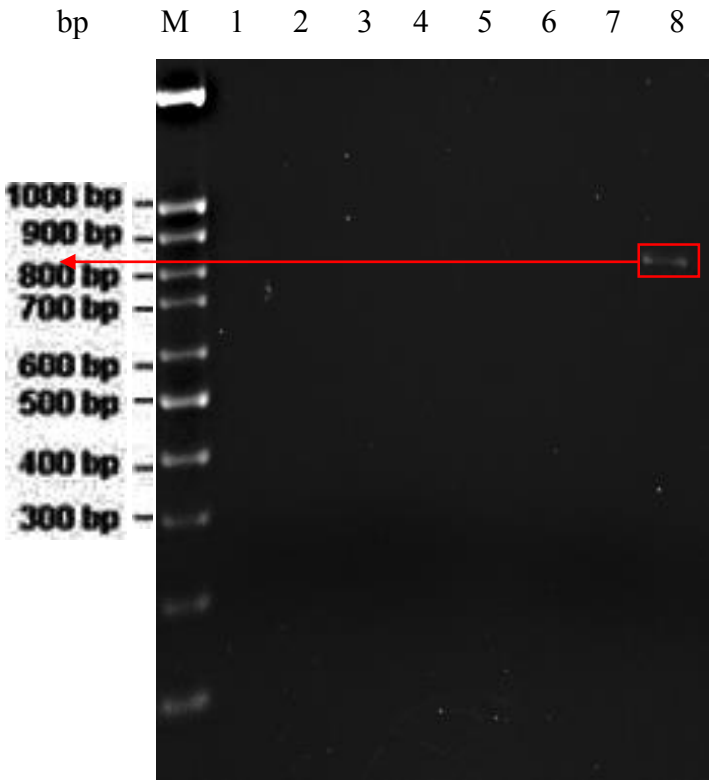

bp
M
1
2
3
4
5
6
7
8
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp

Figure 23. A 1% agarose gel electrophoresis for the purified kanamycin cassette PCR amplified from the pUC18K2. M is the M107O-1 ladder from Bio Basic Inc. The 853 bp kanamycin cassette is shown only in lane 8. Lanes1-7 are PCR reactions that did not generate a DNA fragment.

The initial ligation attempts were performed using an insert to vector molar ratio of 3:1 with reactions incubated at 20 °C for 24 h. The approximate 8 kb size of the ligation product was larger than the appropriate 4766 bp construct, and likely represented the ligation of two pBluHhbps (Figure 24, lanes 1 and 2).

When the incubation time was shortened to 1 h incubation time using the same insert to vector molar ratio of 3:1, experiments produced either no ligation products, or ligation products larger than the expected size. The identity of the faint 6.4 kb band likely represents either constructs containing one copy of pBluHhbp ligated with three copies of the kanamycin cassette, or constructs containing concatamers of three copies of pBluHhbps (Figure 25, lanes 1 and 2).

3.5 Constructing mutants in the IntPro and ATPase

After failing to construct an hhbp mutant with the previous approaches, attention was turned to two of the other genes in the locus that encodes the ABC transporter. The gene cluster is organized as an operon comprising an internal membrane protein (IntPro), a sulphate reductase gamma subunit (DsvC), a heme dependant periplasmic binding protein (hHBP) and an ATPase. A summer student in our lab had constructed mutants in both IntPro and the ATPase genes of the cluster by insertional inactivation using a kanamycin resistance cassette. The student performed the following experiments. Both the IntPro and the ATPase genes were PCR amplified using chromosomal H. ducreyi 35000 as template and ligated into EcoRV digested pBluescript KS II to form pBluIntPro and pBluATPase, respectively. The kanamycin cassette was PCR amplified from plasmid PCMV6-Entry using primers incorporating either StyI or BsgI restriction sites to facilitate the subsequent

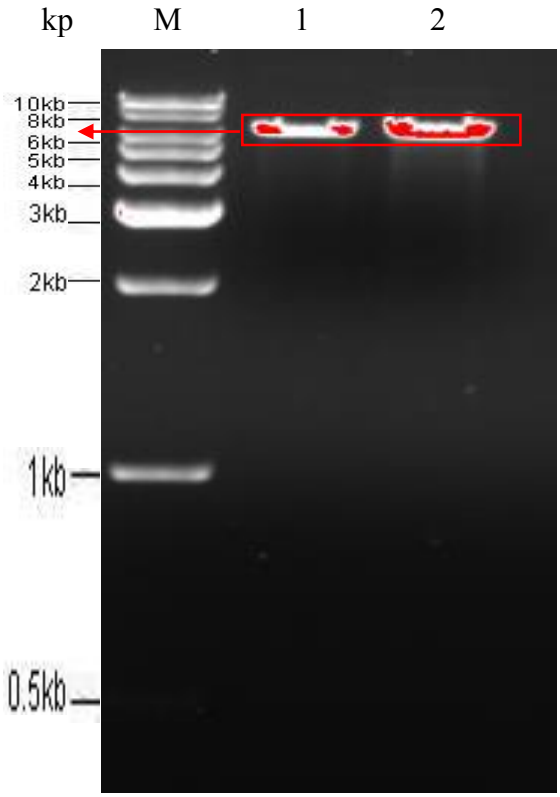

kp
M
1
2
10kb
8kb
6kb
5kb
4kb
3kb
2kb
1kb
0.5kb

Figure 24. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pUC18K2 with restriction sites for *Sty*1. Ligations were performed at 20 °C for 24h using a vector to insert ratio of 3:1. The expected size of the construct is 4766 bp. (M) is the Supercoiled DNA Ladder from Bio Basic Inc. Lanes 1 and 2 are ligation reactions from two different experiments.

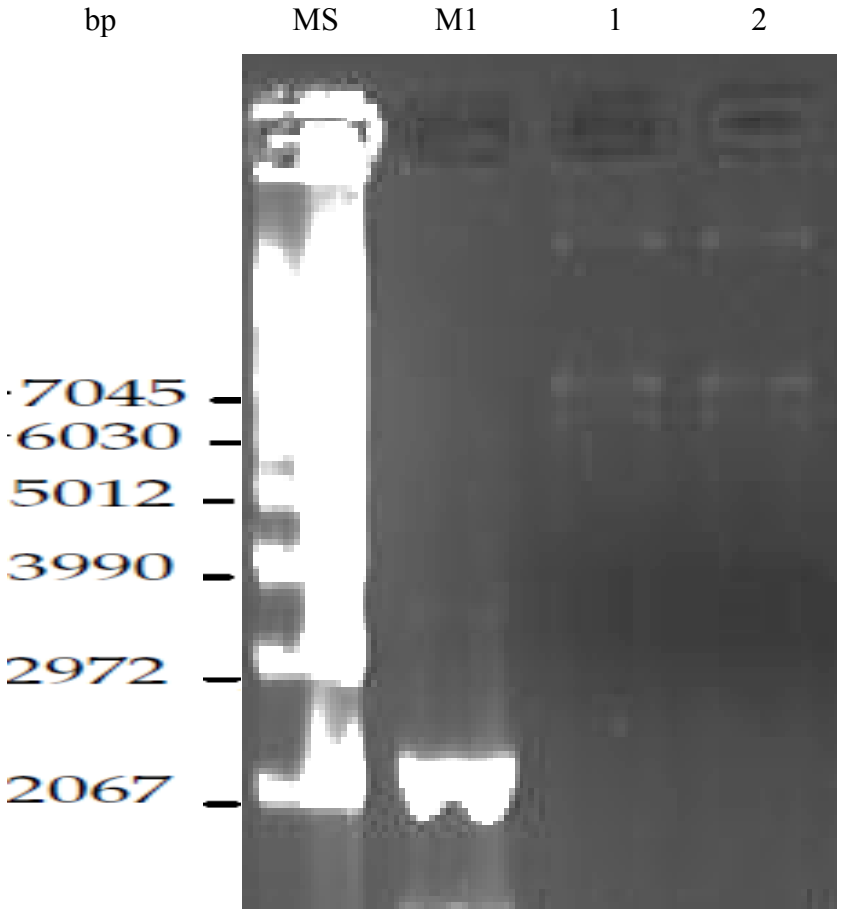

bp
MS
M1
1
2
7045
6030
5012
3990
2972
2067

Figure 25. A 1% agarose gel electrophorisis for ligation products of pBluHhbp and the kanamycin cassette from pUC18K2 with restriction sites for *Sty*1. The ligation reactions were incubated at 20 °C for 1h. The ratio between the kanamycin cassette and the pBluHhbp was 3:1. The expected size of the construct is 4766 bp. M is the Supercoiled DNA Ladder from Bio Basic Inc. Lanes 1, and 2 are ligation products from two separate reactions.

ligation into *Sty*I restricted pBluIntPro and *Bsg*I digested pBluATPase. The ligation products were chemically transformed into TOP10 cells. Transformants were plated onto LB agar (which served as positive control for cell viability), LB agar with ampicillin (which served as a positive control for the transformation protocol), and LB agar with kanamycin. Transformants were recovered on all three plates. However, the size of the 6 kb recombinant plasmids extracted and purified from kanamycin resistant clones were larger than the expected ~ 4.5 kb (Figure 26a, lanes 1 and 2).

3.5.1 Confirming the insertion of the kanamycin cassette into pBluIntPro and pBluATPase

Several experiments were conducted to resolve identity of these plasmid constructs. To determine whether the kanamycin cassette was present within each gene, each of the constructs served as template in a PCR amplification analysis using primers anneal to the 5' and 3' junctions of the inserted kanamycin gene, and a PCR amplification analysis using primers annealing to the 5' and 3' junctions of the inserted IntPro and ATPase gene, and a PCR amplification analysis using primers annealing to 120 bp downstream and 120 bp upstream from the junction where the IntPro and ATPase genes inserted into the pBluscript. No amplicons of the expected sizes were produced. The faint 5.5 kb band seen in lanes 1 and 2 in (Figure 27) represents pBluATPase ligated with three kanamycin genes and the faint 5 kb bands seen in lanes 3 and 4 in (Figure 27) represents amplicons containing a single copy of pBluIntPro ligated with two kanamycin cassettes. Sequencing of the constructed plasmids with primers annealing to the 5`of the IntPro and the ATPase produced

IntPro
a

ATPase
b

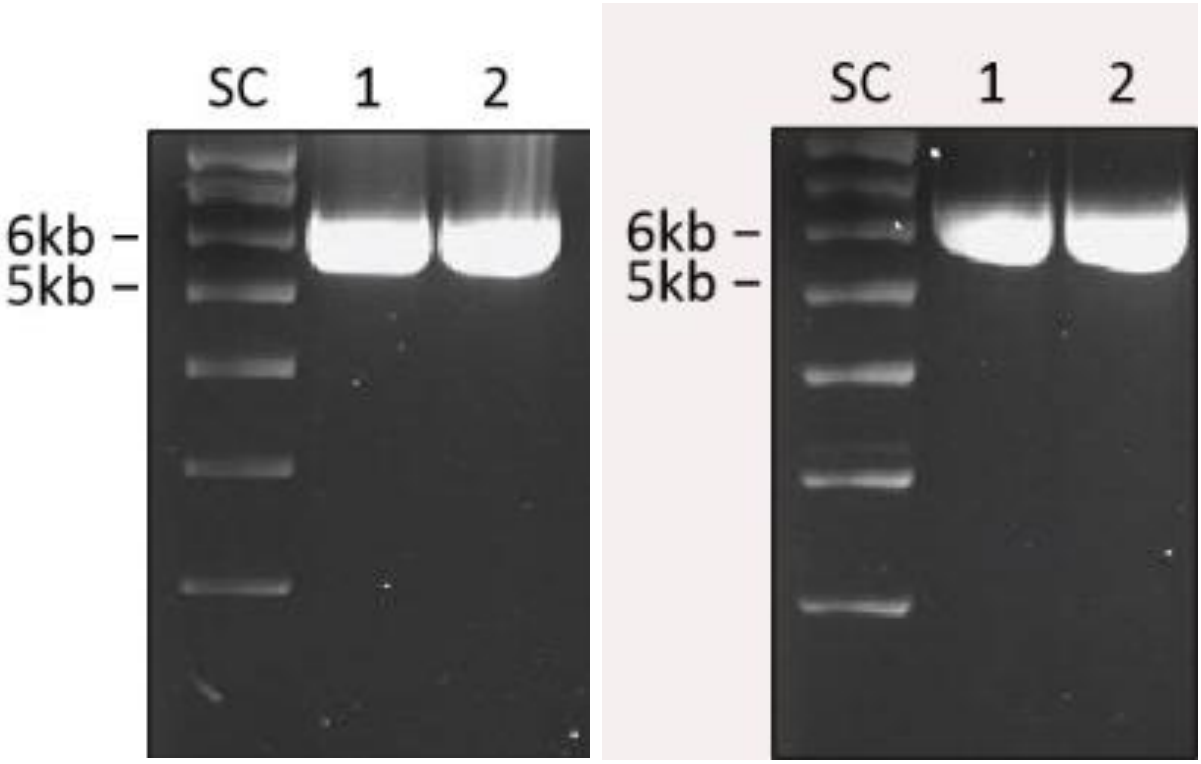


Figure 26. A 1% agarose gel electrophorisis of pBluIntPro and pBluATPase with the kanamycin cassette from pCMV6-Entry with Sty1 and Bsg1 restriction site, respectively. The ligation reaction was performed 20 °C for 24 h. The ratio between the insert and the vector was 3:1. The expected size of pBluIntPro is 4460 bp and for pBluATPase 4553 bp. SC is the Supercoiled DNA Ladder from Invitrogen. Lanes 1, and 2 in Fig 22a are different colonies from the LB plates with kanamycin which contain the pBluIntPro with the kanamycin cassette. Lanes 1, and 2 in Fig 22b are different colonies from the LB plates with kanamycin which contain the pBluATPase with the kanamycin cassette. The figure was taken by the summer student who performed the experiments.

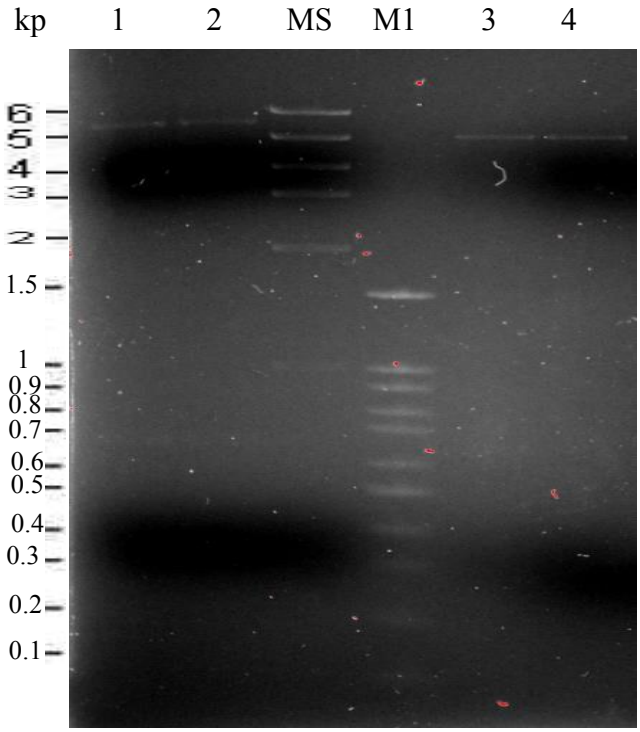

kp
1
2
MS
M1
3
4
2
1.5
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

Figure 27. A 1% agarose gel electrophorisis for PCR products of kanamycin cassette from pCMV6-Entry within the genes the pBluATPase and the pBluIntPro. The primers anneal to the genes. The PCR reactions performed as the manufacturer suggested. MS is the Supercoiled DNA Ladder from Bio Basic Inc. M1 is the M107O-1 ladder from Bio Basic Canada. Lanes 1, and 2 contain ligation products of pBluATPase and the kanamycin cassette. Lanes 3, and 4 contain ligation products of pBluIntPro and the kanamycin cassette. The expected sizes of the PCR products are 1460 bp for ATPase and 1553 bp for IntPro.

no readable nucleotide sequence and the PCR analysis did not reveal the presence of a ligated kanamycin cassette within either gene.

One final series of ligation experiments were performed using reagents purified by the summer student. Several attempts to ligate the kanamycin cassette into pBluATPase and pBluIntPro recapitulated the prior results seen in (Figure 26). In (Figure 28) the 2.3 kb band in lanes 1 to 5 represents the size of three kanamycin cassettes ligated together, the 5.3 kb band represents a construct of pBluATPase ligated with two kanamycin cassettes and the size of the upper bands, over 6 kb, are not precisely determined because a 6 kb DNA ladder was used, which did not allow for their precise identity. The faint 5.3 kb bands seen in lanes 7, 9, 10, 11, 12 represent pBluIntPro ligated with two kanamycin cassettes and the size and identity of the upper bands, over 6 kb, could not be determined.

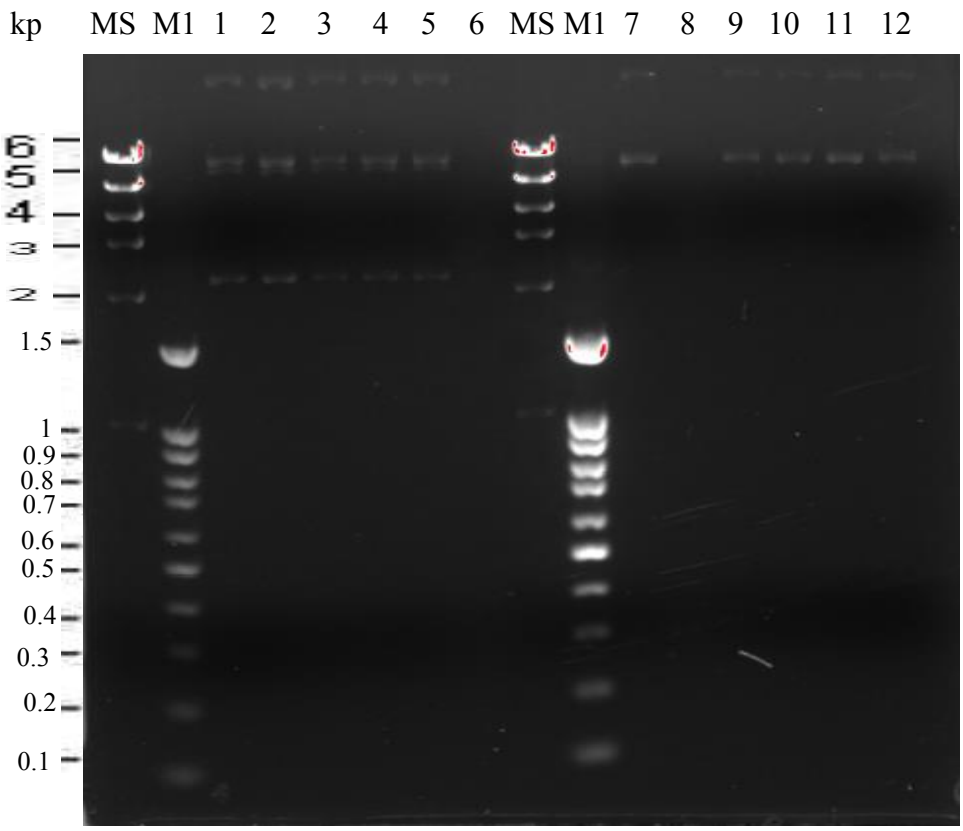

kp
MS
M1
1
2
3
4
5
6
MS
M1
7
8
9
10
11
12
3
2
1.5
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

Figure 28. A 1% agarose gel electrophorisis for ligation products of kanamycin cassette from pCMV6-Entry within the pBluATPase and the pBluIntPro. The ligation reactions were performed at room temperature for 1 h. The ratio between the insert to vector was 3:1. The expected size of the construct is 4460 bp for pBluATPase and 4553 bp for pBluIntPro. MS is the Supercoiled DNA Ladder from Bio Basic Inc. M1 is the M107O-1 ladder from Bio Basic Inc. Lanes 1, 2, 3, 4, 5, and 6 contain ligation products from 6 separate reactions between pBluATPase and the kanamycin cassette. Lanes 7, 8, 9, 10, 11, and 12 contain ligation products from 6 different reactions between pBluIntPro and the kanamycin cassette.

4 Conclusions

4.1 Disscusion

The explanations for the difficulty in constructing a mutation in the *H. ducreyi hhbp* gene are unclear. The strategy of gene interruption by insertional inactivation using a variety of antibiotic resistant cassettes has been shown to be successful in creating *H. ducreyi* mutants [128-130]. For example, Gangaiah et al. inserted a *cat* cartridge to construct an *H. ducreyi hfq* mutant to analyze the contribution of the protein Hfq to the stationary phase gene regulation and virulence in humans in *H. ducreyi* [128]. Inactivation of *hfq* diminished the capability of *H. ducreyi* to form microcolonies, reduced the *H. ducreyi* serum susceptibility and diminished the ability of *H. ducreyi* to resist phagocytosis. Moreover, kanamycin resistant genes derived from a variety of different plasmids have also been used to construct gene mutations in *H. ducreyi*. Bauer et al inserted a kanamycin cassette from pUC18K into *H. ducreyi fgbA*, a gene encoding a fibrinogen-binding lipoprotein, to examine the role of this gene to virulence. An isogenic *fgbA* mutant was significantly attenuated to cause infection in a human challenge model of chancroid [129]. Leduc et al demonstrated that the outer membrane protein of *H. ducreyi*, DltA, contributes to the serum resistance phenotype. Isogenic mutants, constructed in *H. ducreyi* 35000HP by insertion of a kanamycin cassette from pUC4K were more vulnerable to the bactericidal effects of normal human serum (NHS) than the parental strain [131]. Simlarly, to inspect the role of the Sap ABC transporter in conferring resistance against antimicrobial peptides

in *H. ducreyi*, Mount et al mutagenized SapA the periplamsic component of the transporter by insertion of a kanamycin cassette derived from pUC18K. Unlike the parental isolate, the survival of the *sapA* mutant was significantly decreased after exposure to the human antimicrobial peptide cathelicidin LL-37 [130]. Thus, gene interruption using either a chloramphenicol or kanamycin antibiotic resistant cartridge is a viable approach to construct gene mutations in *H. ducreyi*.

Sytematic alterations in the ligation temperature, incubation time, enzyme concentration and insert to vector molar ratio failed to identify a set of factors that would consistently result in the successful insertion of the various kanamycin cassettes within *hhbp*. Less obvious contributing factors such as adjusting the concentration of divalent cations, the empiric addition of other co-factors, were not explored.

The inability to verify the presence of the kanamycin cassette within *hhbp* in the few experiments in which kanamycin resistant transformants were isolated following the introduction of appropriate size plasmid constructs into *E. coli* TOP10 cells suggests that the kanamycin gene could not be stably maintained within *hhbp*. This supposition is supported by the observation that genotypic analysis of the purified plasmid constructs indicated that the kanamycin gene relocated to another site within the plasmid vector. However, none of the kanamycin cassettes possessed genetic elements encoding tansposases that would catalyze this reaction. Although pBluescript has routinely been used as a cloning vehicle to construct mutations in *H. ducreyi* genes [132-134] and *E. coli TOP10* cells have served as an intermediate bacterial host to stably maintain these plasmid constructs [135-137], the combination of kanamycin cartridges from pET30a and pCMVEntry-6 potentially harbouring unusual nucleotide sequences and uncommon *Nsi*1

restriction sites for ligation may have contributed for the instability of the kanamycin insert. Finally, an unknown intrinsic property of *hhbp* may have precluded the construction of an insertion mutant.

4.2 Future work

In the future, an insertion/deletion mutant using FLP recombinase technology is a potential strategy to study the *hhbp* gene in *H. ducreyi* as it has been used in *H. influenza* [138] and in *H. ducreyi* [139]. Using the *hhbp* as a template, primers will be used to include the 20 bp from the 5` of the gene (H1) and 20 bp from the 3`of the gene (H2). Primers will also be designed to amplify the antibiotic resistance marker, the spectinomycin resistance gene cassette (*spec-rpsL*) cassette, from plasmid pRSM2832 with addition of bps to form a flanking arm of 50 bp in the 5` and 3`of the cassette containing the FLP recognition target (FRT). These primers start with the sequence of (H1) and (H2) respectively to facilitate the replacing the *hhbp* gene. After replacing the *hhbp* gene with the *spec-rpsL* cassette, the expression of the FLP recombinase will result in the deletion of the *spec-rpsL* cassette. The mutant will then be characterized phenotypically and the wild type gene will be rescued to confirm the findings of the phenotypic characterization.

Another approach is the construction of a deletion mutant, as previously described [140], where 1 kb corresponding to the upstream region of the 5` of *hhbp* will be amplified using primers HH1 and HH2 and another fragment of the DNA, approximately 0.5 kb, will be amplified using primers HH3 and HH4 corresponding to the downstream of the 3`of the *hhbp* gene. A *cat* cassette is then amplified from pML122 with primers HC1 and HC2. HC1 will contain a 21 bp complimentary sequence of the 3`of the HH2 and HC2 has a 21

bp complimentary sequence of the 5` of HH3. The resulting three pieces of DNA are purified and used as template of overlapping extension PCR using HH1 and HH4. This resulting fragment will be electroporated into Hd35000 and then selected on chocolate plates containing chloramphenicol. The chloramphenicol resistant colonies will be subjected to phenotypic analysis and the *hhbp* mutant will be rescued by the wild type gene introduced on the shuttle plasmid to confirm the findings of the phenotypic analysis.

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