

Towards Novel Methods of Mutagenesis for *Histophilus somni*

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Abstract

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Histophilus somni is an etiologic agent of shipping fever pneumonia, myocarditis, and other systemic diseases in bovines, although nonpathogenic commensal strains also exist. Virulence factors that have been identified in *H. somni* include biofilm formation, lipooligosaccharide phase variation, immunoglobulin binding proteins, survival in phagocytic cells, and many others. To identify genes responsible for virulence, an efficient mutagenesis system is needed. Mutagenesis of *H. somni* using allelic exchange is difficult due to its tight restriction modification system. Mutagenesis by natural transformation in *Haemophilus influenzae* is well established and may be enhanced by the presence of uptake signal sequences (USS) within the genome. We hypothesized that natural transformation occurs in *H. somni* because its genome is over-represented with USS and contains all the necessary genes for competence, except that ComD and ComE are mutated. For natural transformation, *H. somni* was grown to exponential phase, and then transferred to a non-growth defined medium to induce competence. *H. somni* strain 2336 was successfully transformed with homologous linear DNA (*lob2A*) containing an antibiotic marker gene, but at low efficiency. Shuttle vector pNS3K was also naturally transformed into *H. somni* at low efficiency. To attempt to improve transformation efficiency, *comD* and *comE* from *H. influenzae* were cloned into shuttle vector pNS3K to generate the plasmid pSScomDE. Although introduction of pSScomDE into *H. somni* was expected to increase the number and breadth of mutants generated by natural transformation, multiple attempts to electroporate pSScomDE into *H. somni* were unsuccessful. A native plasmid (pHS649) from *H. somni* strain 649 may prove to be a more efficient shuttle vector. Due to inefficiency in generating mutants by allelic exchange, transposon (Tn) mutagenesis with EZ::Tn5™<KAN-2>Tnp Transposome™ (Epicentre) was used to generate a bank of mutants, but the mutation efficiency was low. Therefore the *mariner* Tn element is being tested as a more efficient method for random mutagenesis of *H. somni*. The transposase, which is required for excision of the Tn, was over-expressed in *Escherichia coli*, and then purified using amylose resin. *H. somni* was then naturally transformed after in-vitro transposition using pMarStrep, which contains the *mariner* Tn with StrepR antibiotic gene marker, and a series of transposition and ligation components. However, *mariner* Tn mutants were not generated. Nonetheless, natural transformation and/or *mariner* Tn mutagenesis may still prove to be efficient methods for mutagenesis of *H. somni*. Through the use of more effective mutagenesis systems, genes responsible for the expression of virulence factors can be identified, and improved vaccine candidates can be developed.

Dedications

I would like to dedicate this thesis to my parents, Rajendra Shah and Damini Shah. Without their constant support and guidance, I would not have been able to endure the hardships of research. They have helped keep me grounded and of sound mind throughout the entire process. I would also like to dedicate this thesis to David Mishkel. I strive to be the kind of researcher he was, and will always think of him performing experiments by my side.

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Literature Review

1.1 Taxonomy and Physiology

Histophilus somni (*H. somni*), which was known as *Haemophilus somnus* until 2003, is a Gram-negative coccobacillus, facultative anaerobe (Siddaramappa, 2007). It is an obligate inhabitant of bovine and ovine mucosal surfaces within the family of *Pasteurellaceae* (Sandal et al., 2010). Revision of the genus name to *Histophilus* was based on genetic analysis of 16S rDNA gene sequences and *rpoB* gene sequences (Angen et al., 2003). Characteristics of *Haemophilus* spp. include a requirement for blood factors and the need for establishing a commensal relationship with their hosts. They require the blood factors nicotinamide adenine dinucleotide (NAD) and/or protoheme compounds such as hemin. *H. somni*, does not require NAD or hemin, but instead requires a complex, enriched medium for growth (Asmussen and Baugh, 1981; Inzana and Corbeil, 1987). While on agar plates, growth requirements include blood and incubation in 5-10% CO₂ (Inzana, 1999). However, blood can be replaced by complex medium such as Brain Heart infusion, 0.1 % Trizma base, and 0.01% thiamine monophosphate (TMP) (Asmussen and Baugh, 1981; Inzana and Corbeil, 1987). Growth in complex broth media requires supplementation with Trizma base and TMP or 5% bovine serum. *H. somni* occurs as either a commensal or an opportunistic pathogen. The bacterium is found on the prepuce of bulls and rams, as well as within the upper respiratory tract (Sandal et al., 2010). *H. somni* can attach to bovine turbinate cells, vaginal epithelial cells, and endothelial cells (Sylte et al., 2001). Interactions with the normal bacterial flora of hosts may enhance the growth of *H. somni in-vitro*, which was shown through *in vitro* co-culture of *H. somni* with normal flora isolates from nasal and preputial sites. However, most normal flora isolates that enhanced growth originated from preputial areas rather than the respiratory tract, and were Gram-positive (Corbeil et al., 1985b).

1.2 Diseases

In 1960, thrombotic meningoencephalitis (TME) was identified as the first disease to be associated with *H. somni*, which is now recognized worldwide as a bovine pathogen (Kennedy et al., 1960). Pathogenic isolates can disseminate from the genital or respiratory tracts and not only cause TME (Harris and Janzen, 1989; Miller et al., 1983; Stephens et al., 1981), but also, pneumonia (Andrews et al., 1985; Gogolewski et al., 1987a; Gogolewski et al., 1987b; Groom et al., 1988; Jackson et al., 1987), upper respiratory infection, bacteremia, abortion (Corbeil et al., 1985a; Widders et al., 1986), septicemia, myocarditis (Harris and Janzen, 1989), and arthritis. Disease due to *H. somni* can result from the bacterium acting as a sole pathogen or through concurrent or prior infection with other bacteria or viruses (Sandal and Inzana, 2010). Disease development can also be influenced by environmental conditions, including crowding, poor weather, and shipping (Gagea et al., 2006). Poor overall health increases the animal's vulnerability to developing disease, which often occurs in cattle within weeks of arrival at the feedlot (Saunders et al., 1980).

1.3 Virulence Factors

There are numerous genes that encode for virulence factors enabling *H. somni* to colonize its host. Some colonization factors include autotransporter adhesins, filamentous hemagglutinin homologs (Challacombe and Inzana, 2008), specific components of lipooligosaccharide (LOS) and some surface proteins (Sandal et al., 2010). Many of the virulence mechanisms *H. somni* uses to escape the host immune system are similar to those in the related bacteria *Haemophilus influenzae* (*H. influenzae*), *Neisseria meningitidis*, and *N. gonorrhoeae* (Howard et al., 2000). However, it is likely that many virulence factors are yet to be identified.

The endotoxin of *H. somni*, which is referred to as LOS, is a major component of the outer leaflet of the outer membrane. It is referred to as LOS rather than lipopolysaccharide (LPS) due to the lack of O-antigen side chains. It consists of two components: a complex core of oligosaccharide and Lipid A

(Hitchcock et al., 1986). LOS plays an important role in pathogenesis by inducing inflammation and protecting the bacterium from host defenses such as phagocytosis and complement-mediated killing (Inzana et al., 2008). LOS is capable of phase variation both *in vitro* and *in vivo*, which enable the bacteria to evade or delay a host's immune response (Inzana et al., 1992). Phase variation predominantly occurs in virulent isolates. Commensal isolates have been isolated from the normal prepuce that are unable to undergo phase variation. LOS phase variation in *H. somni* results from microsatellites of repeating DNA motifs, such as 5' – CAAT – 3' or 5' – GA – 3', located in open reading frames (ORF) (Inzana et al., 1992; Weiser et al., 1989). Phase variation occurs during replication, where slipped-strand mispairing results in either the loss or gain of one or more repeats (Kahler and Stephens, 1998). Genes *lob1* and *lob2A* have been identified as phase variable putative galactose and N-acetylglucosamine transferases respectively (McQuiston et al., 2000; Wu et al., 2000). *lob1* has 59% homology to LOS biosynthesis gene *lex2B* in *H. influenzae* (Inzana et al., 1997), while *lob2A* shows homology to several LOS biosynthesis proteins found in *H. influenzae* type B, such as Lex1/Lic2A and Lic2B (Cope et al., 1991; High et al., 1993; High et al., 1996). *Lob1* contains a microsatellite DNA repeating motif of 5' – CAAT – 3' and 59% DNA sequence and 46 % amino acid homology relative to the LOS biosynthesis gene *lex2B* found on *H. influenzae* (Inzana et al., 1997; Jarosik and Hansen, 1994; McQuiston et al., 2000). *lob2A* contains a repeating motif of 5' – GA – 3' and is located near the 3' end of the ORF (Sandal et al., 2010). Mutagenesis of *lob2A* resulted in a decrease of *H. somni* virulence and a reduced rate of phase variation in a mouse model (Sandal et al., 2010).

Phosphorylcholine (ChoP) is another component of *H. somni* LOS that is capable of phase variation, which can also be found on *H. influenzae* and a few other bacterial species (Sandal and Inzana, 2010). In *H. influenzae*, expression of ChoP enhances adherence to epithelial cells through interaction with the platelet activating factor (PAF) receptor and increases persistence in the respiratory tract (Swords et al., 2000; Weiser et al., 1998). Consequently, it activates the complement cascade by

binding to C-reactive protein and enhances serum susceptibility (Swords et al., 2000). Expression of ChOP on *H. somni* induces aggregation of bovine platelets through interaction either directly or indirectly with the PAF receptor (Kuckleburg et al., 2007). Activated platelets induce endothelial cell apoptosis (Kuckleburg et al., 2008), which leads to a pro-inflammatory response that could result in vascular inflammation and endothelial cell damage (Sandal et al., 2010), which can lead to vasculitis (Sylte et al., 2001).

LOS can also activate caspase-3 and caspase-8 and induce apoptosis of bovine pulmonary and brain vascular endothelial cells. This apoptosis is caspase-8 dependent and occurs via a death receptor-dependent mechanism (Sylte et al., 2001; Sylte et al., 2003; Sylte et al., 2006). Endothelial cells treated with exogenous recombinant bovine IL-1 β showed diminished rates of apoptosis (Sylte et al., 2005).

Immunoglobulin binding proteins (IgBPs), are homologous to filamentous hemagglutinins associated with many Gram-negative bacteria (Sandal et al., 2010; Siddaramappa, 2007). Twenty-nine serum-resistant isolates of *H. somni* express IgBPs on their surface whereas many serum-sensitive isolates are IgBP-negative (Widders et al., 1989). The IgBPs consist of a peripheral membrane protein, p76 (Corbeil et al., 1997), and a series of high-molecular-weight (HMW) proteins of varying sizes (Yarnall et al., 1988). IgBPs primarily bind to the Fc portion of bovine IgG2 or a major outer membrane protein (OMP) (Corbeil et al., 1997). Both p76 and HMW IgBPs are encoded by one ORF, *ibpA*, which encodes for IgBP A. *ibpA*-positive strains are resistant to complement mediated killing, and *ibpA* plays a critical role in enhancing bacterial viability in the blood (Sandal et al., 2010). Some avirulent strains lack the *ibpA* coding region and are sensitive to complement mediated killing (Cole et al., 1992). Mutant strains that expressed a truncated IgBP A adhered less efficiently to wild-type bovine pulmonary artery endothelial cells and decreased virulence in mouse models (Sanders et al., 2003).

1.4 Restriction-Modification Systems

Mutagenesis and expression of foreign DNA in *H. somni* is difficult due to the bacterium's apparently tight restriction-modification systems. Restriction modification systems consist of two components: a cognate modification enzyme that methylates a sequence, thereby protecting it from cleavage, and a restriction endonuclease that recognizes and cleaves specific sequences (Siddaramappa, 2007). According to Kobayashi (2004), there are three hypotheses for the maintenance of restriction-modification systems: "cellular defense hypothesis", "the selfish gene hypothesis", and "variation hypothesis".

The "cellular defense hypothesis" can be described as a protection system for cells from infection by foreign DNA, such as viral or plasmid origin. DNA that has not been modified by an appropriate methyltransferase will be cleaved by the restriction endonuclease. At the same time, self DNA will be recognized by the cognate modification enzyme as properly methylated, and protected from restriction enzymes.

The "selfish gene hypothesis" is associated with the theory that plasmids carrying a post-segregational killing gene complex are more likely to survive than other competitive plasmids. This theory can be linked back to the restriction-modification system. Different evolutionary analyses have found close homologues to restriction-modification genes in distantly related species, which suggests these genes have undergone extensive horizontal gene transfer. One explanation is that these gene complexes reside on a variety of mobile elements such as plasmids, transposons, conjugative transposons, genomic islands, and integrons. Once a restriction-modification element has integrated itself into a host, it is difficult to replace it with a competitor's genetic element. If replacement were to occur through homologous recombination, natural transformation, or bacteriophage-mediated transduction, cells experiencing replacement would not survive. The "selfish-gene hypothesis" is further explained through the dilution model. When a restriction modification gene complex is deleted through

homologous recombination the methyltransferase enzyme and the restriction enzyme will be diluted in subsequent replicates. Through dilution, the methyltransferase enzyme's ability to protect newly replicated chromosomal DNA will be lost and exposed sites of the chromosomal DNA will be cleaved. The restriction enzyme will also be diluted, but there is an imbalance of roles for the methyltransferase and the restriction enzyme. A methyltransferase must methylate hundreds of sites in order to protect chromosomal DNA. However, a single cleavage on the chromosome made by a restriction enzyme can cause cell death. This leads to post-segregational killing by loss of the restriction modification gene complex, hence the reason it is called the "selfish-gene".

The "variation hypothesis" is based on the assumption that restriction-modification systems assist in the generation of diversity. The generation of diversity can be coupled with the selfish gene theory. Restriction systems are lethal to hosts that lose the restriction-modification gene complex, but can protect the hosts from foreign substances that damage DNA. The variation of genomes can be induced by the actions of restriction-modification systems by allowing certain mutagenesis and recombination events to occur. This process will directly affect the evolution and variation of genomes (Kobayashi, 2004; Siddaramappa, 2007).

There are three different types of restriction modification systems: Type I, Type II, and Type III. Type I and Type II systems are the most frequently encountered systems in Eubacteria (Murray, 2000). Both Type I and Type II restriction modification systems are present in *H. somni* strain 2336 (Siddaramappa et al., 2011; Siddaramappa, 2007). Type I modification systems consist of three closely linked subunits: *hsdR*, *hsdM*, and *hsdS*. These multi-subunit proteins function as a single protein complex. *hsdR* encodes for a site-specific deoxyribonuclease, *hsdM* encodes for a DNA methylation subunit, and *hsdS* encodes for a DNA site-specificity unit. When encountered by unmethylated DNA as a substrate, Type I systems cleave the chromosomal DNA. Conversely, when encountered by

hemimethylated DNA as a substrate, S-adenosyl-L-methionine (SAM) is used as a methyl donor using the methyltransferase subunit (Roberts et al., 2003).

In contrast to Type I system's subunits, Type II subunits act independently. The endonucleases recognize specific DNA sequences that are cleaved to produce 5'- phosphates and 3'- hydroxyls, and can act as monomers, dimers, or tetramers. The methyltransferases act as monomers and transfer a methyl group from donor SAM to target DNA (Roberts et al., 2003). Type II systems in *H. somni* consist of M.hsol and R.hsol, which are a DNA-cytosine methyltransferase and a restriction endonuclease. (Siddaramappa et al., 2011).

1.5 Conventional Mutagenesis of *H. somni*

In order to identify genes involved in virulence, knockout strains must be generated. Due to its apparently tight restriction modification system, mutagenesis of *H. somni* has been very problematic. There have been only three genes that have been disrupted by site-specific mutagenesis: *lob2A* (Wu et al., 2000), *p76* (Sanders et al., 2003), and *ibpA* (Hoshino et al., 2009; Sandal et al., 2010).

lob2A encodes for a N-acetylglucosamine (GlcNAc) transferase, which transfers GlcNAc to the LOS oligosaccharide core (Wu et al., 2000). *lob2A* was mutated in strain 738, a clonal isolate of strain 2336 that was isolated after passage once on Colombia blood agar and in a calf (Inzana et al., 1997). Mutagenesis was performed by constructing a suicide vector, pCAAT Δ *lob2A*, derived from pCAAT. After *in-vitro* methylation using *HhaI* methylase, suicide vector pCAAT Δ *lob2A* was transformed into *H. somni* strain 738 through electroporation and mutants were selected for media containing kanamycin. Mutagenesis of *lob2A* in strain 738 significantly increased susceptibility to the bactericidal activity of normal bovine serum. The virulence of *H. somni* 738 Δ *lob2A* was also significantly decreased in a mouse model, causing both lower mortality and a lower degree of bacteremia (Wu et al., 2000).

p76 is a 76 kDa surface antigen functionally related to the HMW IgBP group (Cole et al., 1992), but is surface exposed rather than being shed (Corbeil et al., 1987). It is expressed by serum-resistant

strains and primarily binds to the Fc portion of bovine IgG2 (Corbeil et al., 1997). Mutagenesis of IgBP was performed using recombinant plasmid pJDS162. Plasmid pJDS162 was derived from plasmid pHS119, which is a subclone of pHS111. *H. somni* strain 8025 was used, rather than strain 2336, because 8025 did not exhibit a high level of spontaneous resistance to kanamycin or streptomycin, and did not carry detectable native plasmids (Sanders J.D., Cole S.P., and Corbeil L.B., unpublished data). A kanamycin gene derived from cloning vector pLS88 was flanked by sequences of the gene encoding for IgBP and used for homologous allelic recombination. The purpose of the study was to demonstrate the relationship of surface antigen p76 with HMW IgBP. Recombinant plasmid pJDS162 was treated by *in-vivo* methylation in *H. influenzae* strain DB117 and *in-vitro* methylation using *HhaI* methylase. Plasmid pJDS162 was introduced through electroporation, and recombinant mutants were screened by Southern Blotting. Studies showed that strain 8025 with a truncated HMW IgBP retained the ability to bind the Fc portion of bovine IgG2, indicating that the deletion was not required for the secretion or binding of the HMW IgBP, but was required for the downstream expression of gene *p76*. The mutant strain 8025 Kan^R was as equally serum resistant to the wild type strain, but less virulent. Furthermore the mutant strain did not adhere to bovine pulmonary endothelial cells as efficiently as the wild type parent strain (Sanders et al., 2003).

The gene encoding for HMW IgBPs is comprised of a single ORF of 12,285-bp, known as *ibpA*. This gene shows homology to large virulence factor exoproteins and their transporter proteins (Tagawa et al., 2005). Mutagenesis of *ibpA* was performed using suicide vector pKOH5106, resulting in four kanamycin-resistant clones. *H. somni* strain 2336 was used to generate the *ibpA* mutants. Fragments of gene *ibpA* and its flanking regions from strain 2336 were cloned into pBluescript II SK(+) (Stratagene, La Jolla, CA). A kanamycin resistance gene cassette (Kan^R), derived from pUC4K, was inserted between flanking regions of the *ibpA* gene to induce homologous allelic exchange. The suicide vector was treated *in vitro* with *HhaI* methylase and electroporated into strain 2336. PCR analysis was performed on both

internal portions of *ibpA* to ensure deletion of the fragment and the presence of Kan^R. Southern blot analysis confirmed the presence of Kan^R in the genome and that allelic exchange had occurred. Mutant and wild type strains both displayed similar adherence levels to bovine turbinate cells, endometrial epithelial cells and macrophage-like FBM-17 cells. Parent strains were able to evade phagocytosis by macrophage-like cells, while mutant strains were susceptible to phagocytosis (Hoshinoo et al., 2009).

Broad host range shuttle vector pLS88, derived from *Haemophilus ducreyi*, is the only known shuttle vector capable of transforming *H. somni* (Dixon et al., 1994). This vector was inefficiently transformed into disease isolates of *H. somni* due to its size (4.8-kb). Therefore, a more efficient shuttle vector was needed. Vector pNS3K was derived from pLS88 to transform *H. somni* at a higher efficiency than its predecessor due to its smaller size of 2.1-kb. Several steps were involved in construction of pNS3K. A kanamycin resistant gene promoter (KanPm) was amplified using pUC4K and cloned into promoterless vector pNS upstream of the multiple cloning site (MCS). Six histidine and one glycine residues were introduced downstream of the KanPm to “facilitate epitope tagging at the amino terminus” (Sandal et al., 2008; Seleem et al., 2004). The KanPm with the MCS was excised out of pNS using *Sall* and *EcoRI* restriction enzymes. Kan^R was amplified from pUC4K with its own promoter using primers that contained *EcoRI* and *SpeI* restriction sites. Additional *Scal* and *XbaI* sites were designed in the forward kanamycin primer to add to the MCS (Sandal et al., 2008), followed by a strong transcriptional stop signal (UAAU) (Collier et al., 2002; Tate and Mannering, 1996). The origin of replication was amplified from pLS88 using primers containing *Sall* and *XbaI* restriction sites. A three-way ligation was set up after each piece was amplified and the *XbaI* site for the origin of replication and *SpeI* site for the Kan^R were compatible for ligation and deleted once ligated together. This plasmid transforms into *H. somni* 100-fold more efficiently than pLS88 through electroporation. Efficiency can be further increased when isolated from *H. somni* and then retransformed into a wild type strain. This is a useful vector for potential genetic manipulation (Sandal et al., 2008).

Transposon mutagenesis is a random form of mutagenesis that has been used on *H. somni* strain 2336. Transposon mutants were generated using EZ::Tn5™<KAN-2>Tnp Transposome™ system (Epicentre) (Sandal et al., 2009). Cells were made electrocompetent through a series of washes using 272 mM sucrose after being grown to exponential phase (Sandal et al., 2008) and then electroporated with EZ::Tn5™<KAN-2>Tnp Transposome™ (Epicentre). Transposon mutants were screened, and some of these mutants were either deficient or enhanced in biofilm formation (Sandal et al., 2009). Some mutations include, but were not limited to *luxS*, *uspE*, *tolC*, *fhaB*, and others. However, this system did not provide a high number of mutants per experiment, and therefore was not efficient.

1.6 Novel Mutagenesis Procedures

Natural competence is “the ability of intact, living cells to actively take up DNA from their extracellular environment. If the sequences are sufficiently similar, this DNA may recombine with the host genome” (Maughan et al., 2008). Mutagenesis using natural transformation is well established in the model bacterium *H. influenzae* and we have recently determined that transformation can be applied to *H. somni*. Competence is generally induced through cell starvation via carbon depletion and a lack of energy sources (Macfadyen et al., 1996). Bacteria that are grown in nutrient-rich media do not express competence genes and DNA uptake does not occur. As nutrients begin to decrease and cells reach a stationary phase, cell competence begins to increase (Redfield, 1991; Redfield et al., 2005). However, this “spontaneous competence” occurs in only of 1% of cells in the culture, though cell competence can be further increased when bacteria are placed in a starvation medium (MIV). MIV media lacks purines, pyrimidines, sugars, and other required cofactors for DNA replication and cell division without inhibiting protein synthesis (Poje and Redfield, 2003). Competence activator protein (Sxy) and cAMP receptor protein (CRP) induce expression of genes in the competence regulon (Maughan et al., 2008). Once competent, genes homologous to components of the Type IV pilus machinery and of the ComEC/Rec2 membrane channel (Chen and Dubnau, 2004) are expressed and are responsible for binding and

bringing extracellular DNA across the outer membrane into the periplasm. Uptake of extracellular DNA shows a strong bias for fragments containing a specific uptake signal sequence (USS) motif, which has been identified as a 9-bp region for *H. influenzae*. USS motifs have been found to be highly overrepresented in the genome of *H. somni*. Further studies have shown that *H. somni* contains all of the competence genes required for DNA uptake and natural transformation with the exception of *comD* and *comE*, which contains only the 5' portion of *comD* that is fused to the 3' end of *comE*. The *comE* gene contains a frameshift, and is only 67% of the entire gene. (Redfield et al., 2006). Therefore, natural transformation may prove to be an efficient means of mutagenesis in *H. somni* (Sandal and Inzana, 2010; Sandal et al., 2010).

Another form of transposon mutagenesis involves the use of the *Himar1 mariner* transposon system. A purified *mariner* transposase from *Haematobia irritans*, the horn fly, is able to support all the activities that are necessary for transposition *in vitro* (Lampe et al., 1996). *Himar1* is a small, DNA mediated (Class II) transposable element, which is flanked by inverted terminal repeats (ITR), that are 28-bp in length. The transposable element encodes a single transposase protein, originally 1.3-kb in length. The ITR are recognized by the transposase, which excises the transposable element, and then inserts in random spots of the target DNA. Only TA dinucleotide site-specificity is required (Akerley and Lampe, 2002; Lampe et al., 1996). Plasmid pMMOrf was constructed as a transposon donor by cloning a mini*Himar1* transposon from pMiniMariner (Lampe et al., 1998) into the *Bam*HI site of pCDNAII (Invitrogen). There is a unique *Bgl*III site in the middle of the transposon element, and it contains no antibiotic marker for the transposon (Lampe et al., 1999). Transposition frequency can be altered by the amount of transposase used, and the size of the transposon. Increasing the size of the transposon, and using more than ~10 nM of transposase decreases transposition frequency (Lampe et al., 1998). The *Himar1* transposon system is an excellent prokaryotic genetic tool due to lack of specificity and the fact that there is no requirement for species-specific host factors. It has been successfully used in both *H.*

influenzae (Lampe et al., 1999) and *Neisseria gonorrhoeae* (Balthazar et al., 2011; Pelicic et al., 2000).

Transposon mutagenesis, using *in vitro* transposition, may be another efficient means of mutagenesis when combined with natural transformation. When combined, these two methods can help generate a bank of *H. somni* mutants that will enable the identification of genes involved in virulence.

Proposal

Bovine respiratory disease complex, has been prominent in feedlot cattle, where *H. somni* is a major pathogen that accounts for an estimated loss of \$1 billion each year (McVey, 2009). In order to develop an effective vaccine, the genes involved in virulence must be identified, but no effective mutagenesis system exists for *H. somni*. By developing either an effective mutagenesis system that will enable the generation of a bank of *H. somni* mutants that represent the entire genome, or a reliable method of site-specific mutagenesis, virulence genes can be identified. This would represent the first step in developing an effective vaccine against disease caused by *H. somni*.

Materials and Methods

3.1 Bacterial Strains and Growth Conditions

Bacterial strains that were used in this study are listed in Table 1. *E. coli* strains were grown in Luria Broth (LB) or on LB agar plates at 37°C. When using antibiotics as selection markers, the following concentrations were used: kanamycin 50 µg/ml, streptomycin 80 µg/ml, and ampicillin 50 µg/ml. *H. somni* strains were grown on Columbia Blood Agar (CBA) plates or BBL Brain Heart Infusion (BHI) agar plates, both supplemented with 5% sheep blood. Plates were incubated overnight at 37°C, 5% CO₂. For natural transformation broth cultures were grown in Bacto BHI broth supplemented with 0.1% Trizma Base and 0.01% thiamine monophosphate (TMP); 10% Levinthal's Base was added for electroporation. Broth cultures were shaken at 200 rpm and incubated at 37°C. The antibiotics kanamycin and streptomycin were used for selection at 80 µg/ml for natural transformation, and 100 µg/ml for electroporation. Table 1 describes the bacterial strains used in this study.

3.2 Bacterial Strains

Table 1: Different Bacterial Strains used in this Study

<u>Bacterial Strain</u>	<u>Description</u>	<u>Source</u>
<i>H. somni</i>		
2336	Pneumonia isolate	Animal Dis. Diagnostic Lab. Washington State
8025	Meningoencephalitis isolate	Dr. Lynette B. Corbeil UCSD Medical Center
649	Abortion isolate	Animal Dis. Diagnostic Lab. Washington State
738	Clonal isolate of stain 2336 after passage on Colombia Agar and in a calf	Animal Dis. Diagnostic Lab. Washington State
129pt	Isolate from the prepuce of healthy bull	Animal Dis. Diagnostic Lab. Washington State

E. coli

DH5 α

ElectroMAX™ DH5 α -E™
Competent Cells

Invitrogen, Carlsbad, CA

3.3 Genomic DNA, Plasmid Extraction and Gel Extraction

Genomic DNA was extracted using commercial methods using the DNeasy® Blood and Tissue Kit (Qiagen). Plasmids were isolated using the Miniprep Spin Kit (Qiagen). Desalting of plasmids following ligations was performed using commercial methods from the QIAx II Gel Extraction Kit (Qiagen). DNA products were excised and purified from 0.8% agarose gels using the Gel Extraction Kit (Qiagen).

3.4 Plasmids

Table 2: Plasmids used in this Study

<u>Plasmid</u>	<u>Description</u>	<u>Source or Reference</u>
pNS3K	High-efficiency shuttle vector for <i>H. somni</i>	Sandal et al. (2008)
pLS88	Broad host-range shuttle vector	Wilson et al. (1989)
pMALC 9.3	Plasmid encoding for <i>Himar1</i> transposase	Dr. David Lampe (Duquesne University) lampe@duq.edu
pMMOrf	Plasmid containing transposable element donor but no antibiotic marker	Dr. David Lampe (Duquesne University) lampe@duq.edu
RR1262	Plasmid containing <i>com</i> operon from <i>H. influenzae</i> Rd KW20	Dr. Sunita Singh (University of British Columbia) sinha@zoology.ubc.ca
pSScomDE	pNS3K with <i>comD</i> and <i>comE</i> genes	This study
pMarStrep	Plasmid containing transposable element donor (pMMOrf) with Streptomycin antibiotic marker	This study

3.5 Polymerase Chain Reaction (PCR) Parameters

Each PCR reaction was in 25 μ l, and consisted of 2.5 μ l of *Taq* Reaction Buffer (NEB), 200 nM of primers, 1 mM MgCl₂, and 60 ng of plasmid template DNA or 100 ng of chromosomal template DNA. Reactions were performed for 32 cycles with denaturation at 94°C for 1 minute. The annealing temperature was at different temperatures based on the primers used, and was for 2 minutes (annealing temperature listed at each step), extension was at 72°C for 1 minute and a final extension step was at 72°C for 10 minutes.

3.6 Enzymes and Reagents

DNA Taq Polymerase, T4 DNA Polymerase, T4 DNA ligase, amylose resin and restriction enzymes were from New England Biolabs (NEB, Beverly, MA), with their respected buffers. Recombinant shrimp alkaline phosphatase was purchased from Affymetrix (Santa Clara, CA). TypeOne™ Restriction Inhibitor was purchased through Epicentre (Madison, WI). Plasmid that encodes for *Himar1* transposase (pMALC9.3) was a kind gift from Dr. David Lampe from Duquesne University. The *Himar1* transposase purification reagents included: column buffer (20 mM Tris-HCl at pH 7.4, 200 mM NaCl, 1 mM EDTA), transposase wash buffer (20 mM Tris-HCl at pH 7.4, 200 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 10 v/v glycerol), and transposase elution buffer (20 mM Tris-HCl at pH 7.4, 200 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol, 10 mM maltose). Plasmid containing the transposable element between inverted terminal repeats (pMMOrf) was a gift from Dr. David Lampe from Duquesne University. The dNTPs were purchased from Promega Corp. (Madison, WI). The Bradford reagent was made by first taking 10 mg of Coomassie Brilliant Blue G-250 and dissolving it in 5 ml of 95% ethanol. Then 10 ml of 85% (v/v) phosphoric acid was added carefully to the solution, and diluted to 100 ml with water. It was then filtered through Whatman 185 mm Filter paper and stored at 4°C (Bradford, 1976). Table 3 describes the ingredients to create MIV media for natural transformation.

3.7 MIV Media Reagents

Components of MIV media used for *H. influenzae* and *H. somni* (Poje and Redfield, 2003)

Table 3: Components of MIV media

Solution 21	
L-Aspartic acid	4.0 g
L-Glutamic acid	0.2 g
Fumaric acid	1.0 g
NaCl	4.7 g
K ₂ HPO ₄	0.87 g
KH ₂ PO ₄	0.67 g
Tween-80	0.2 ml
Distilled water	850 ml
<i>Adjust pH to 7.4 with 4 N NaOH. Add distilled water to 1 L. Dispense in 100 ml per bottle and autoclave. Store at room temperature</i>	
Solution 22	
L- Cystine	0.04 g
L- Tyrosine	0.1 g
<i>Dissolve in 10 ml of 1 N HCl at 37°C. Bring up to 100 ml with distilled water then add:</i>	
L Citruline	0.06 g
L- Phenylalanine	0.2 g
L- Serine	0.3 g
L- Alanine	0.2 g
<i>Filter sterilize and store at 4°C</i>	
Solution 23	
CaCl ₂	0.1 M solution
<i>Autoclave and store at room temperature</i>	
Solution 24	
MgSO ₄	0.1 M Solution
<i>Autoclave and store at room temperature</i>	
Solution 40	
5 % (w/v) vitamin-free casamino acids (Difco) in distilled water	
<i>Filter sterilize and store at 4°C</i>	

3.8 Natural Transformation

Induction of Competence

The protocol for natural transformation was modified from Poje and Redfield (2003). *H. somni* was grown to early log phase of growth with $\sim 5 \times 10^8$ cfu. The cells were harvested at $4350 \times g$ for 10 minutes at room temperature and washed two times in MIV media and then incubated in MIV media for 100 minutes at 100 rpm at 37°C, to induce competence.

Transformation

One ml of competent cells were incubated with 1 μ g of homologous DNA, or circular plasmid. For circular plasmids, 300 μ l of 80% glycerol was added. The cells were incubated at 37°C for 30 minutes, sedimented at $9000 \times g$ for 1 min, and 850 μ l of supernatant was removed. The cells were resuspended and inoculated onto medium with kanamycin (80 μ g/ml), and then cultured onto media without antibiotic. Natural transformation was performed on *H. somni* using genomic DNA isolated from a *lob2A* mutant created by allelic exchange (Wu et al. 1999) or with the EZ::Tn5™<KAN-2>Tnp Transposome™ (Epicentre) system. The following muted genes were used for transformation: *lob2A* (Δ *lob2A* gDNA), *uspE* (Δ *uspE* gDNA), or *luxS* (Δ *luxS* gDNA). Approximately ~ 1000 - 2000μ g of genomic DNA was used for transformation and plated on kanamycin antibiotic plates to screen for transformants.

3.9 Cell Extracts

A small number of colonies were suspended in 100 μ l of water, boiled at 100°C for 10 minutes, and then centrifuged at $9,300 \times g$ for 10 minutes at 4°C. The supernatant was removed and used as a template for PCR. Table 4 indicates the primers used for mutant recognition.

Table 4: Primers used for Detection of Mutants Following Natural Transformation

<u>Primer</u>	<u>Description</u>	<u>Sequence</u>
<i>lob2A</i> F	<i>lob2A</i> forward primer	5` - TGATTGGCATTATCTAACATA – 3'

<i>lob2A</i> R	<i>lob2A</i> reverse primer	5' - TTAAAGCTGGGACATAGTG – 3'
<i>uspE</i> F	<i>uspE</i> forward primer	5' – ATGCAATTTAATAATACTTGTCTGTTTT – 3'
<i>uspE</i> R	<i>uspE</i> reverse primer	5' – TTATTTTTTACTCGGTTTAATTGCCAAC – 3'
<i>luxS</i> F	<i>luxS</i> forward primer	5' - ACAATGTCATGACCTGCTCGAT – 3'
<i>luxS</i> R	<i>luxS</i> reverse primer	5' – CACAAGGAATGCCAAGGTTTTTC – 3'

3.10 Primers used for *comD* and *comE* Detection

Table 5: Primers Designed from *H. influenzae* for *comD* and *comE* Genes in *H. somni*

<u>Primer</u>	<u>Sequence</u>
<i>comD</i> Forward	5' - ATGAAACATTGGTTTTTCCTGATT - 3'
<i>comD</i> Reverse	5' - CCATTCACTACTATCACATTG - 3'
<i>comE</i> Forward	5' - ATTTTTTAGTATGTTTTTGTTTGCC - 3'
<i>comE</i> Reverse	5' - TTATTTTTTACCCTCACTTTTTTGTTT - 3'

3.11 *comD* and *comE* Detection

PCR was performed on *H. somni* strains to determine the presence of *comD* and *comE*. The annealing temperature was set at 60°C for the *comD* and *comE* primers, followed by analysis of PCR products by gel electrophoresis. The primers are described in Table 5.

3.12 Primers used for Amplification of Genes

Table 6: Primers for Plasmid Construction

<u>Primer</u>	<u>Description</u>	<u>Sequence</u>
<i>comD</i> F	<i>comD</i> Forward primer with <i>EcoRI</i> site	5'GGGCCgaattcATGAAACATTGGTTTTTCCTGATT 3'
<i>comE</i> R	<i>comE</i> Reverse primer with <i>XbaI</i> site	5' GGCCtctagaTTATTTTTTACCCTCACTTTTTTGTTT 3'
Strep F	Streptomycin antibiotic marker forward primer with <i>BglII</i> site	5' CGGCGGagatctGGTATTGGCGGCGCTGAAAGAAAC 3'

Strep R Streptomycin antibiotic 5' CGGCGG**gagatct**CAAACGAGGCTGGAAAAGGTGTC 3'
marker reverse primer with
BglII site

3.13 Construction of pSScomDE and pMarStrep Plasmid

Plasmid pNS3K was sequentially digested with enzymes *EcoRI* and *XbaI*. Following two sequential digestions, pNS3k was treated with recombinant shrimp alkaline phosphatase (SIAP) to prevent self-ligation in case one of the enzymes did not efficiently cut the plasmid. PCR was performed to amplify *comD* and *comE* at an annealing temperature of 60°C using RR1262 as a template. *EcoRI* and *XbaI* sites were designed in the primers for directional cloning (Table 6). PCR products were sequentially digested with *EcoRI* and *XbaI*. The digested pNS3K and *comDE* PCR product was separated by gel electrophoresis and extracted using the Gel Extraction Kit (Qiagen). The following formula was used to set up a ligation overnight at 16°C:

$$\frac{\text{Length of Insert}}{\text{Length of Vector}} * \text{Molar ratio of Insert:Vector} * \text{ng of Vector} = \text{ng of Insert}$$

Ligation products were purified using QIAX II Gel Extraction Kit (Qiagen) and electroporated into ElectroMAX™ DH5α-E™ Competent Cells and cultured on LB kanamycin agar plates. Electroporation was performed using the Invitrogen protocol. Plasmid pSScomDE was digested with *EcoRI* and *XbaI* and sequenced to ensure proper construction.

Plasmid pMMOrf was digested with *BglII* and treated with recombinant SIAP to ensure no self-ligation. PCR was performed to amplify the streptomycin resistant gene (Strep^R) using pLS88 as plasmid template DNA with an annealing temperature at 55°C (Table 6). The PCR product was digested using *BglII*, and ligated between digested pMMOrf at 16°C overnight using the same formula as for pSScomDE.

Ligations were purified using QIAXII Gel Extraction Kit (Qiagen) and electroporated into ElectroMAX™ DH5α-E™ Competent Cells. Cells were cultured onto media containing streptomycin for screening, digested by *Bam*HI and *Bgl*II individually, and sequenced to ensure proper insertion of Strep^R.

3.14 Sequencing of Plasmids

Sanger sequencing was performed on each plasmid to produce plasmid maps. Table 7 contains the primers that were used for sequencing the plasmids. Primers 9-16 were used for sequencing pNS3K. Primers 1-16 were used to sequence pSS*comDE*. Primers 17-24 were used for sequencing pMMOrf, and primers 17-28 were used for sequencing pMarStrep.

Table 7: Primers for Plasmid Sequencing

<u>#</u>	<u>Primer</u>	<u>Description</u>	<u>Sequence</u>
1	<i>comD</i> For1	<i>comD</i> sequencing forward primer, beginning at base pair (bp) 104	5' - TGAGCCACATCAACGCCCTCTAAA - 3'
2	<i>comD</i> For2	<i>comD</i> sequencing forward primer, beginning at bp 213	5' - TCAAAGGAATTTTCAGCCGCTTGGC - 3'
3	<i>comD</i> Rev1	<i>comD</i> sequencing reverse primer beginning at bp 127 of kanamycin promoter	5' - TTAGAGGGCGTTGATGTGGCTCA - 3'
4	<i>comE</i> For1	<i>comE</i> sequencing forward primer, began sequencing Kan ^R at bp 1003	5' - ATTTCTGCAACCTGACCTTTGCC - 3'
5	<i>comE</i> For2	<i>comE</i> sequencing forward primer, beginning at bp 30	5' - ACGCCTCTAACGTTTCTCCTGCTT - 3'
6	<i>comE</i> Rev1	<i>comE</i> sequencing reverse primer, beginning at bp 1026	5' - GGCAAAGGTCAGGTTGCAGGAAAT - 3'

7	<i>comE</i> Rev2	<i>comE</i> sequencing reverse primer, might began sequencing <i>comD</i> , beginning at bp 503	5' - TTGCAAGCCCTCGCTTACTCACTA - 3'
8	<i>comE</i> Rev3	<i>comE</i> sequencing reverse primer, sequencing beginning of <i>comE</i> and all of <i>comD</i> , beginning at bp 53	5' - AAGCAGGAGAAACGTTAGAGGCGT - 3'
9	Ori-F	Original origin of replication primer from Sandal et al. (2008)	5' - CGAACGCCTTGCCTTCTATCTGCG - 3'
10	Ori-R	Original origin of replication primer from Sandal et al. (2008)	5' - CATAATGGTGTCCGTTCTTCTATT - 3'
11	<i>Ori</i> For1	Origin of replication forward sequencing primer, beginning at bp 298	5' - TCGTGCTACGGTTAGAAAGGCGAA - 3'
12	<i>Ori</i> Rev1	Origin of replication reverse sequencing primer, beginning of origin of replication and end of Kan ^R from pUC4K, beginning at bp 166	5' - TTGGTAGTAGGGACGCTTCGCAAT - 3'
13	<i>Kan</i> For1	Kanamycin forward sequencing primer, might began sequencing origin of replication as well, beginning at bp 346	5' - ATTCATTCGTGATTGCGCCTGAGC - 3'

14	<i>Kan For2</i>	Kanamycin forward sequencing primer, sequencing origin of replication for overlap, beginning at bp 560	5' - TGCTTGATGGTCGGAAGAGGCATA - 3'
15	<i>Kan Rev1</i>	Kanamycin reverse sequencing primer, sequencing comE or MCS, beginning at bp 369	5' - GCTCAGGCGCAATCACGAATGAAT - 3'
16	<i>Kan Rev2</i>	Kanamycin reverse sequencing primer, beginning at bp 583	5' - TATGCCTCTTCCGACCATCAAGCA - 3'
17	T7	Original primer from Lampe et al. (1999)	5' - TAATACGACTCACTATAGGG - 3'
18	SP6	Original primer from Lampe et al. (1999)	5' - CGATTTAGGTGACACTATAG - 3'
19	CDNAII For1	pCDNA II sequencing starting at bp 2919	5' - TGTGCTGCAAGGCGATTAAGTTGG - 3'
20	CDNAII For2	pCDNA II sequencing starting at bp 1072	5' - GCTCTTGATCCGGCAAACAAACCA - 3'
21	CDNAII For3	pCDNA II sequencing starting at bp 1633	5' - TTGTTGGCATTGCTACAGGCATCG - 3'
22	CDNAII Rev1	pCDNA II sequencing starting at bp 1095	5' - TGGTTTGTGGCCGGATCAAGAGC - 3'
23	CDNAII Rev2	pCDNA II sequencing starting at bp 982	5' - TTCAAGAACTCTGTAGCACCGCCT - 3'
24	CDNAII Rev3	pCDNA II sequencing starting at bp 2942	5' - CCAACTTAATCGCCTTGCAGCACA - 3'
25	Streseq For1	Forward primer starting at bp 773	5' - ATCTGCGATTGGACCCTCTGACTT - 3'

26	Streseq For2	Forward primer starting at bp 29	5' - AATCGCATTCTGACTGGTTGCCTG - 3'
27	Streseq Rev1	Reverse primer starting at 611 bp	5' - TCGATCAGACCCGTGCATTGAAGA - 3'
28	Streseq Rev2	Reverse primer starting at 258 bp	5' - CGGAATTGCCGTTATCACCAAGCA - 3'

3.15 Levinthal's Base

Defibrinated horse blood (50 ml) (Remel Lenexa, KS) warmed to 37°C, mixed by inversion, was aseptically added to 110 ml of sterilized BHI broth with 0.1% Trizma Base with continuous stirring. The broth-blood mixture was stirred for 30 minutes at 100°C and then transferred to an ice bath with continuous stirring for 30 minutes. Sterile NAD (0.075 w/v %) was added and the stirring continued for 10 minutes. The broth-blood-NAD mixture was then placed at 0°C and centrifuged at 16,000 x *g* for 30 minutes and the supernatant was aliquoted and stored at -20°C.

3.16 Electroporation of *H. somni*

Electroporation was performed as described (Sandal et al. 2008) with some modifications. *H. somni* was grown to a density of 120 Klett units (10⁹ colony forming units), chilled on ice for 30 minutes to inhibit growth, and harvested by centrifugation at 4350 x *g* for 10 minutes at 4°C. The cells were washed 4 times with 272 mM sucrose and sedimented after each wash at 4350 x *g* for 10 minutes at 4°C. The bacteria were suspended in 272 mM sucrose to 1% of the original volume, and aliquots of 40 µl were used for electroporation after addition of 500-1000 µg of plasmid DNA. Plasmid pNS3K was electroporated after purification, but pSScomDE was incubated with HhaI methylase (NEB), and purified using QIAX II Gel Extraction Kit (Qiagen). pSScomDE was also transformed with the addition of TypeOne™ Restriction Inhibitor (Epicentre). Electroporation was performed using a BTX ECM600 electroporator (BTX, Inc., San Diego, Calif.) at 50 µF, 1.6 kV, and 200 ohms with a cuvette gap of 1 mm.

Following electroporation the cells were diluted to 1 ml with BHI broth containing 0.1% TMP and chilled for 10 minutes on ice, shaken at 200 rpm at 37°C, and spread on antibiotic media for selection of transformants.

3.17 Plasmid Rapid Screening

Plasmid rapid screening was performed by resuspending ~1 loopful of the bacteria in 50 µl of water. Then 50 µl of phenol:chloroform:isoamyl alcohol was added and the mixture was vortexed for 10 seconds. The cells were centrifuged at 9000 x *g* at 4°C and 30 µl of the aqueous phase was analyzed by gel electrophoresis.

3.18 Purification of *Himar1* Transposase

Transposase purification was as described by Akerley and Lampe (2002). *E. coli* DB1 cells containing pMALC9.3 were cultured on LB plates with ampicillin (100 µg/ml) and grown overnight at 37°C. The colonies were inoculated into 80 ml of LB broth with ampicillin (100 µg/ml) and shaken at 37°C until the OD₆₀₀ reached ~0.5. Protein production was induced by adding isopropyl β-D-1 thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM, and incubation continued with shaking for 2 hours. The cells were harvested by centrifugation at 4000 x *g* for 10 minutes. The supernatant was discarded, and the cell pellet resuspended in 10 ml of cold column buffer (defined in enzymes and reagents section) and frozen at -20°C overnight. The cells were lysed by passage through a French Press 3-4 times at 8000-9000 lb/in². Insoluble material was removed by centrifugation at maximum speed in for 10 minutes at 4°C, and the supernatant aliquoted on ice. Amylose resin was prepared by thoroughly resuspending the resin stock and aliquoting 500 µl into 1.5-ml tube. The resin was pelleted by centrifugation at 15,700 x *g* for 1 min. at 4°C, and the supernatant was removed carefully. The resin was resuspended in 1.0 ml of transposase wash buffer (defined in enzymes and reagents section), pelleted and resuspended a total of 3 times. The final resuspended resin was transferred to the cell lysate in a 15-ml tube and placed on a rocking platform at 4°C for 1 hour. The resin was then pelleted at 15,700 x *g*

at 4°C for 10 min. The supernatant was carefully removed, and the resin resuspended in 2.0 ml of transposase wash buffer and split into two 1.5-ml tubes. The resin was pelleted by centrifugation at 15,700 x *g* for 1 min. at 4°C, and the pellet resuspended in an equal volume of transposase wash buffer a total of 4 times. After the 4th wash, the two tubes of resin were combined and resuspended in 400 µl of transposase elution buffer, and placed on ice for 5 min. The resin was then pelleted by centrifugation at 15,700 x *g* for 1 min. at 4°C, the supernatant was carefully removed, and 10 µl aliquots stored at -80°C.

3.19 Electrophoretic Analysis

Ten µl of supernatant from the transposase wash and purified transposase enzyme were placed into 1.5 mL tubes containing 2 µl of denaturing sample buffer with DTT. Samples were boiled for 10 minutes at 100°C then electrophoresed using NuPAGE® 4-12% Bis-Tris Gel (Life Technologies). Electrophoresis was performed at 200 V for 35 minutes to ensure full separation of bands and then placed in distilled water.

3.20 Silver Staining

The SilverSNAP Stain Kit II (ThermoFischer Scientific) was used for silver staining of polyacrylamide gels.

3.21 Bradford Analysis

Standards of bovine serum albumin (BSA) were prepared in the following concentrations: 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0 µg/ml in a 96-well micro-titer plate. Ten µl of each standard was added in triplicate, along with purified transposase. Two hundred µl of Bradford reagent was added to each well, and Micro-titer plate incubated at 37°C for 5 minutes, and the absorbance reading was taken at 595 nm. Microsoft excel was used to make a standard curve using the BSA standards and used to calculate the total concentration of protein.

3.22 *In-vitro* Transposition

In-vitro transposition was done as described from Akerley and Lampe (2002) and Pelicic, et al. (2000). Briefly, transposition reactions were carried out in 10% glycerol (v/v), 25 mM HEPES pH 7.9, 250 µg/ml (BSA), 2 mM DTT, 100 mM NaCl, and 5 mM MgCl₂. At least 500 ng of donor and target DNA and ~10 nM of transposase were mixed in a final volume of 20 µl. The mixtures were placed at 30°C for a total of 6 hours, the reactions inactivated at 75°C for 10 min., and the reaction products directly purified using the QIAx II Gel Extraction Kit (Qiagen). Single-stranded gaps were filled with T4 DNA Polymerase in a final volume of 20 µl for 30 minutes at 16°C. Reactions were carried out using 1X Buffer 2 (NEB) containing 50 ng of BSA (NEB) per µl, and 25 µM dNTPs (Promega). The polymerase was inactivated by exposure for 10 min. at 75°C and the incubation continued at 16°C overnight after addition of 2 µl of ligation mix (22 mM ATP, 5 U of T4 DNA Ligase). The reaction mix was then used in natural transformation with *H. somni*.

Results

4.1 Natural Transformation on *lob2A*, *luxS*, *uspE*, and pNS3K

Natural transformation was performed using *H. somni* strain 2336. Genomic DNA from 2336 Δ *lob2A*, 2336 *luxS*, or 2336 *uspE* was transformed into *H. somni*. The majority of over 700 kanamycin-resistant isolates were the result of spontaneous antibiotic-resistance, but a few contained an insertion of Kan^R into *lob2A*. Double crossover allelic exchange of *lob2A* was confirmed in these colonies. Transformation of *luxS* and *uspE* containing Kan^R resulted in only spontaneous antibiotic-resistance. Higher concentrations of antibiotic decreased spontaneous resistance, but did not result in the isolation of any potential mutants. Natural transformation was performed on *H. somni* strain 2336 with isolated pNS3K. Phenol:chloroform:isoamyl alcohol screening and plasmid isolation confirmed the presence of pNS3K into *H. somni*, but transformation occurred at very low efficiency (only a few colonies underwent successful natural transformation with pNS3K). Most colonies screened (>1000) arose through spontaneous kanamycin resistance. Natural transformation performed on *H. somni* strain 129pt was unsuccessful for all genes and pNS3K.

4.2 Detection of *com* genes in *H. somni* and *H. influenzae*

A BLAST search was performed comparing *comD* and *comE* genes from *H. influenzae* to both sequenced genomes of *H. somni* 2336 and 129pt. The *comD* gene had a 73% query coverage when comparing the *H. influenzae* gene to the *H. somni* 2336 genome (E-value 0.21), but only 66% coverage when comparing the gene in *H. influenzae* to the *H. somni* 129pt genome (E-value 0.19). However, when comparing the *comE* gene of *H. influenzae* to both *H. somni* 2336 and 129pt, both contained 86% query coverage (E-values of 9×10^{-152} and 5×10^{-148} respectively). The E-values are parameters which decrease exponentially as the score of the match increases. The closer the E-value score is to 0, the more “significant” the match is. PCR was performed to analyze *com* genes from different *H. somni* strains (2336, 8025, 738, 649 and 129pt) using *H. influenzae* as the positive control. PCR analysis confirmed the

presence of all *com* genes with the exceptions of *comD* and *comE*. *H. influenzae* contains functional *comD* and *comE* that are 414 bp and 1,338 bp respectively. Each primer set used for *comD* and *comE* were designed to amplify the entire gene from *H. influenzae*. The *H. somni* strain 2336 *comD* product was smaller than that of *H. influenzae*. All other strains of *H. somni* examined did not contain *comD* through PCR analysis. *comE* was found only in *H. influenzae* and was not amplified from any *H. somni* strains examined (Figure 1).

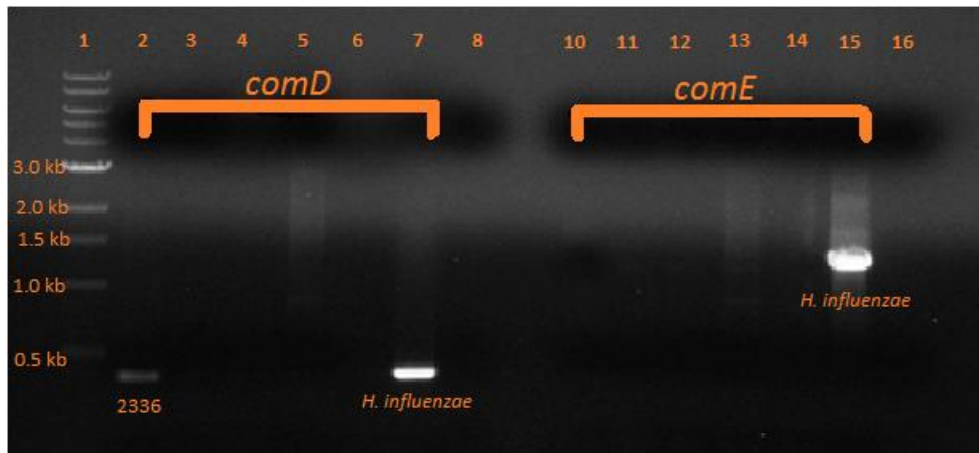


Figure 1: PCR of *comD* and *comE* in *H. somni* and *H. influenzae*

Lanes: 1, 1 Kb Ladder (NEB); 2-8, products of *comD* primers from *H. somni* strains 2336, 8025, 738, 649, 129pt, *H. influenzae*, and blank, respectively; 10-16, PCR products of *comE* in the same order as *comD*.

4.3 Introduction of *comD* and *comE* to *H. somni*

To increase natural transformation efficiency, shuttle vector pNS3K was used to introduce *comD* and *comE* from RR1262. RR1262 contains the entire *com* operon from *H. influenzae* but was not transformed into *H. somni* because of its size (7.362 kb). Size was the main problem with transforming pLS88 (4.8 kb) into *H. somni* and pLS88 was much smaller than RR1262. Multiple restriction digestions were performed to determine ligation sites for directional cloning of *comD* and *comE* into pNS3K (Figure 2).

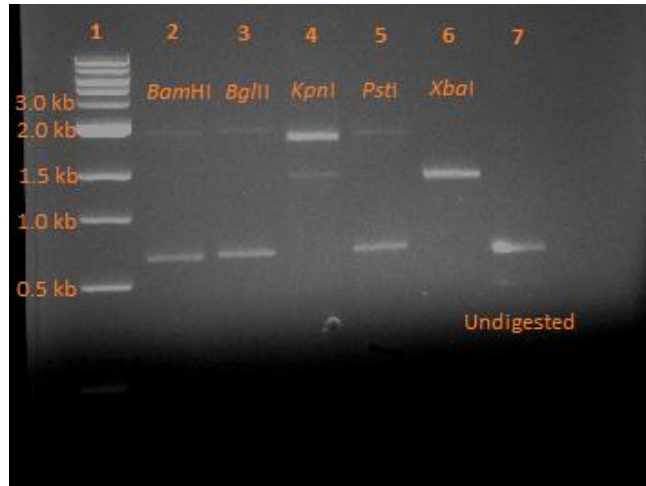


Figure 2 pNS3K Digestion with Different Restriction Enzymes

Lanes: 1, 1 Kb Ladder (NEB); 2-6, digestions of pNS3K with the restriction enzymes: *Bam*HI, *Bgl*II, *Kpn*I, *Pst*I, and *Xba*I; 7, undigested pNS3K.

Cloning of *comD* and *comE* into pNS3K proved difficult, as restriction sites published in the plasmid map of pNS3K were no longer functional (Sandal et al., 2008). After designing multiple primers, pNS3K was sent for Sanger sequencing, and a plasmid map was constructed using LaserGene10 SeqBuilder (Figure 3).

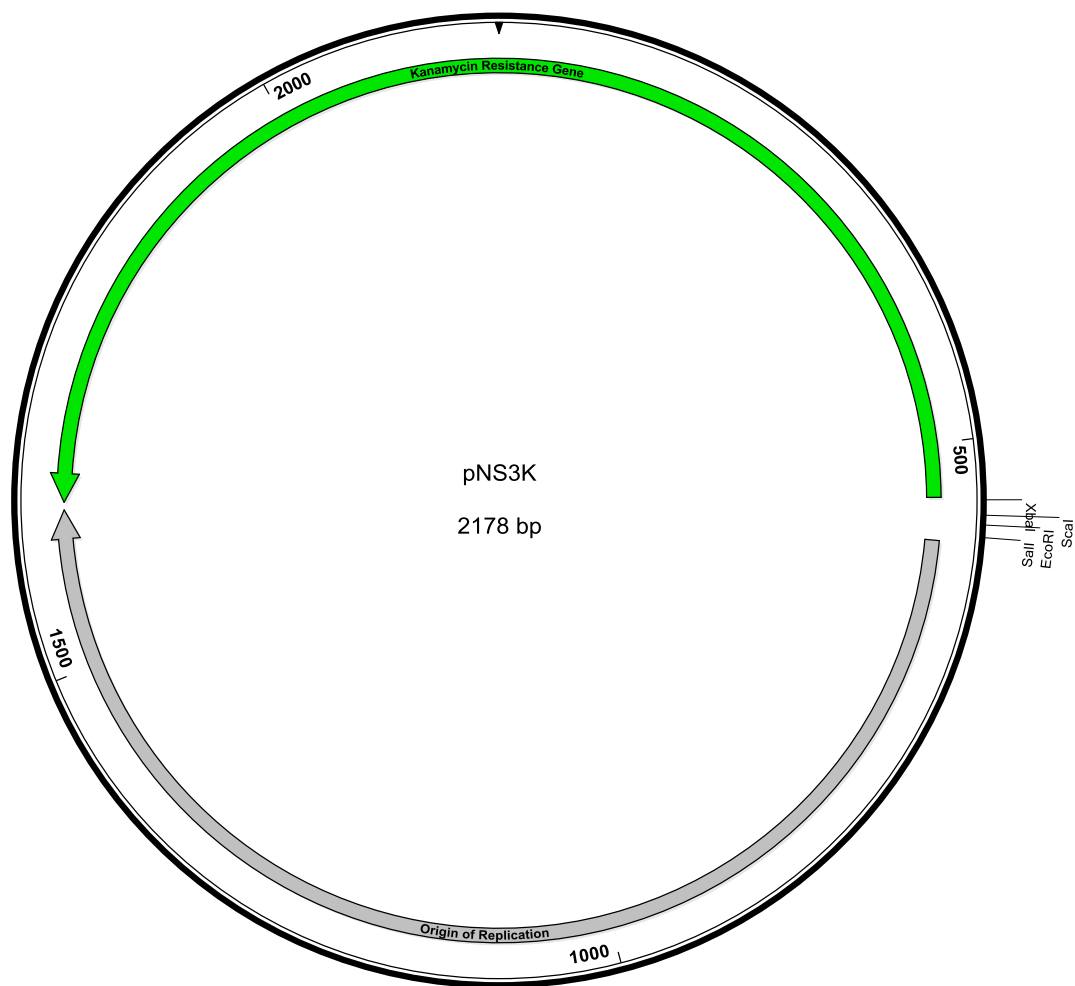


Figure 3: pNS3K Plasmid Map

The new plasmid map confirms restriction sites chosen for cloning were missing. Therefore, the restriction enzymes *EcoRI* and *XbaI* were chosen for directional cloning of *comD* and *comE*. Plasmid pNS3K was renamed pSS*comDE* after the insertion of *comD* and *comE*. The *comD* and *comE* were amplified using RR1262 as a template, with addition of *EcoRI* and *XbaI* sites on the primers as described in Methods (Figure 4). pSS*comDE* digestion with *EcoRI* and *XbaI* was performed (Figure 5) to confirm proper insertion of *comD* and *comE* into pNS3K. Primers were designed and pSS*comDE* was sent for

Sanger sequencing. Using LaserGene 10 SeqBuilder, a plasmid map was constructed for pSScomDE (Figure 6).

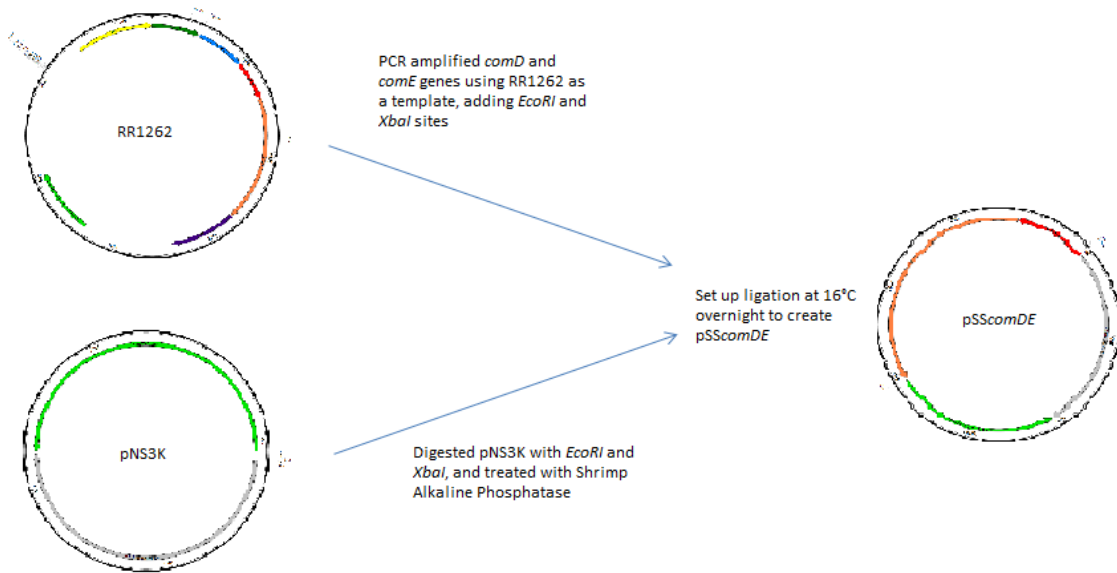


Figure 4: pSScomDE Plasmid Construction

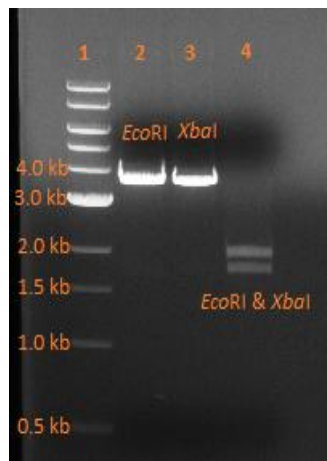


Figure 5: pSScomDE Digestion

Lanes: 1, 1 kb ladder (NEB); 2, pSScomDE digested with *EcoRI*; 3, pSScomDE digested with *XbaI*; 4, sequentially digested with both *EcoRI* and *XbaI*.

Electroporation of pSScomDE into *H. somni* strains 2336 and 8025 was unsuccessful after multiple attempts. Isolated plasmid from ElectroMAX™ DH5α-E™ Competent Cells was initially electroporated

into *H. somni*, but subsequent attempts were unsuccessful. A second attempt to electroporate pSScomDE with the addition of TypeOne™ Restriction Inhibitor (Epicentre) to the electroporation mix was also unsuccessful. A third unsuccessful attempt to electroporate pSScomDE was made after *in-vitro* methylation of the plasmid with HhaI methylase (NEB). Finally, pSScomDE was methylated *in-vitro* with HhaI methylase and electroporated with the addition of TypeOne Restriction Inhibitor to the electroporation mix, but was still unsuccessful.

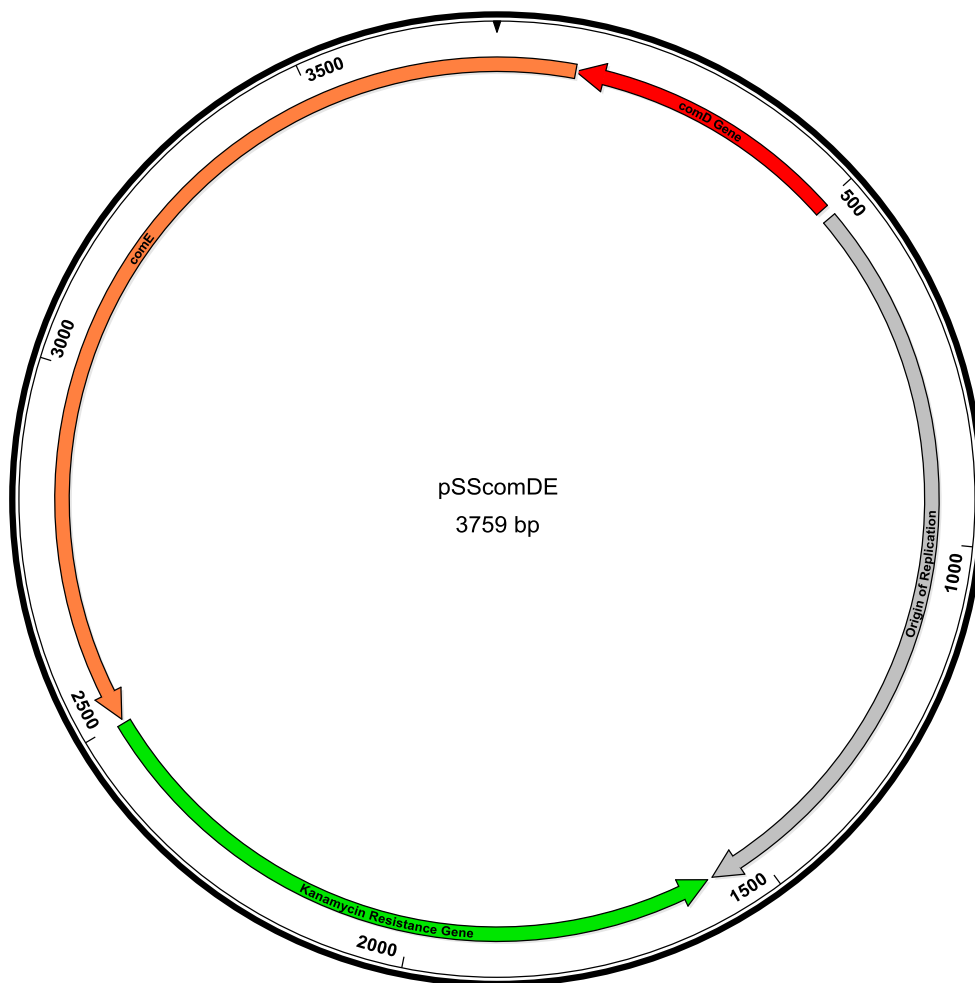


Figure 6: pSScomDE Plasmid Map

4.4 Transposon Mutagenesis

Transposon mutagenesis using the EZ::Tn5™<KAN-2>Tnp Transposome™ system (Epicentre) generated numerous mutants, but was inefficient at producing a bank of mutants that spanned the entire genome. *Himar1* transposon mutagenesis is an efficient method of transposon mutagenesis that has been successfully used with many bacteria that have been recalcitrant to other methods of mutagenesis (Lampe et al., 1999; Pelicic et al., 2000). This method of *in-vitro* transposition is performed by purifying *Himar1* transposase, and incubating it with a donor plasmid for the transposable element (pMMOrf) and target DNA (*H. somni* strain 2336 chromosomal DNA) in an optimal buffer. Plasmid pMMOrf contains a transposable *mariner* element, but the *mariner* element does not contain an antibiotic marker gene. pMMOrf was first digested with *Bam*HI and *Bgl*II to confirm the presence of these sites in the donor plasmid (Figure 7).



Figure 7: Digestion of pMMOrf

Lanes: 1, 1 Kb Ladder (NEB); 2 and 3, pMMOrf digested with restriction enzymes *Bam*HI and *Bgl*II.

Primers were constructed for sequencing of pMMOrf by Sanger sequencing, and LaserGene 10 SeqBuilder was used to construct a plasmid map (Figure 8). The Strep^R (1.0 kb) gene from plasmid pLS88 was amplified and inserted between the *mariner* element as an antibiotic marker. Three methods were

used to ensure correct insertion of the Strep^R gene of the newly constructed plasmid, renamed pMarStrep. First ElectroMAX™ DH5α-E™ Competent Cells containing pMarStrep were cultured on LB media containing streptomycin (80 µg/ml). In addition, pMarStrep was digested with *Bam*HI or *Bg*II (Figure 9). Finally, primers were designed for Sanger sequencing of pMarStrep. A plasmid map was constructed using LaserGene10 SeqBuilder (Figure 10).

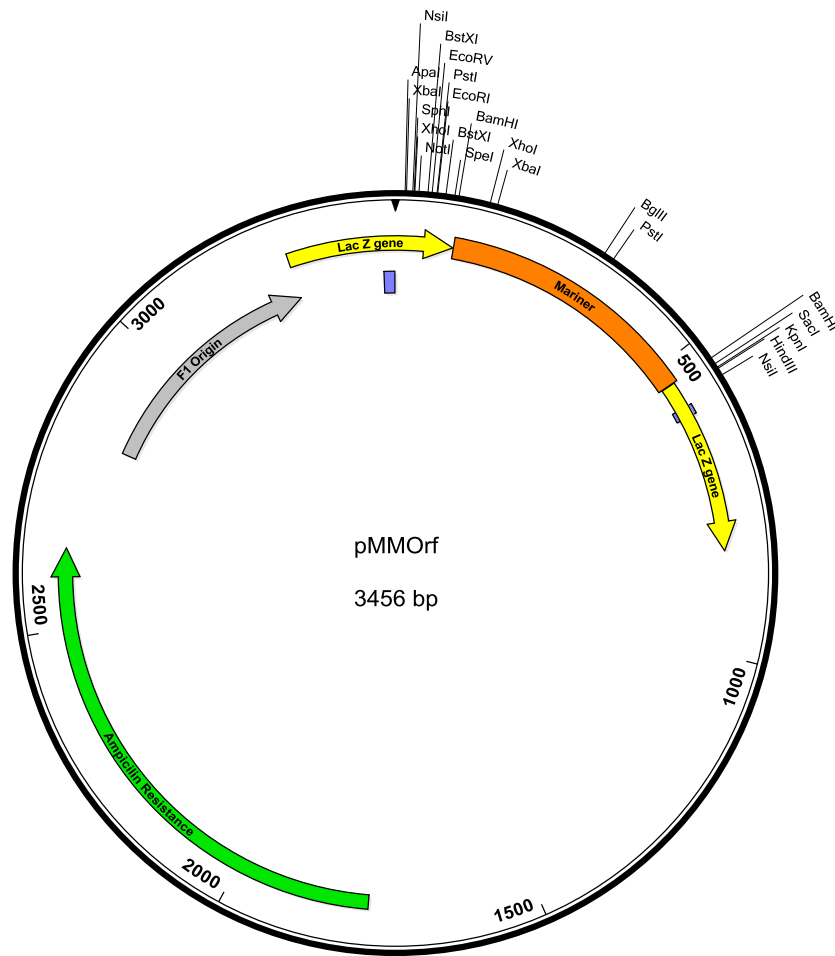


Figure 8: pMMOrf Plasmid Map

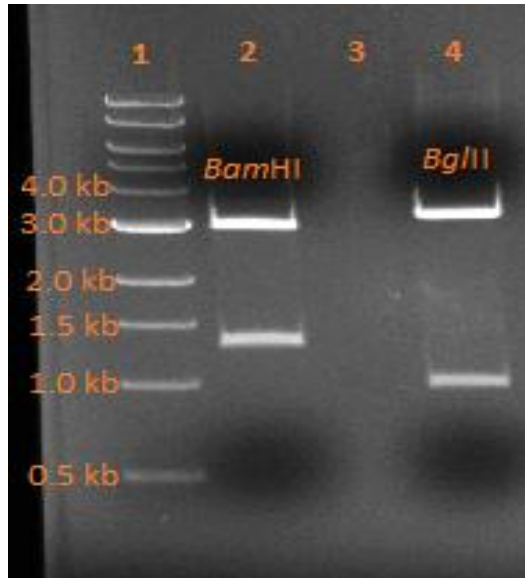


Figure 9: Digestion of pMarStrep

Lanes: 1, 1 Kb ladder (NEB); 2 and 4, Digestions of pMarStrep using *Bam*HI and *Bg*II respectively; 3, Blank

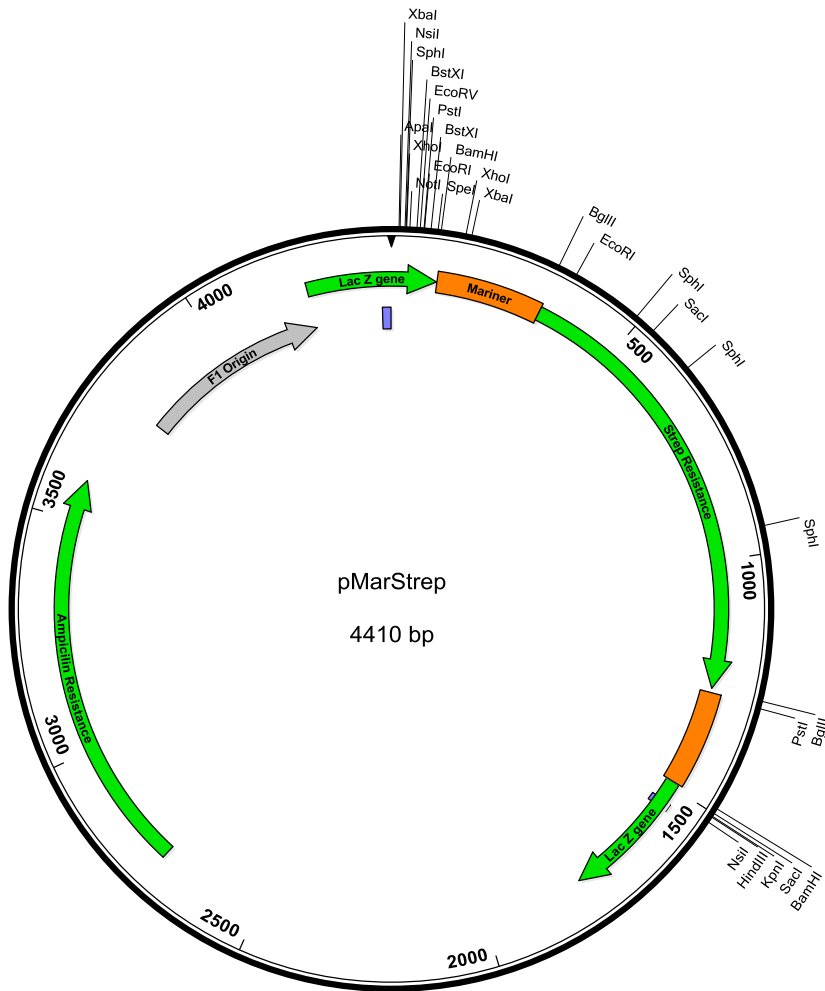


Figure 10: pMarStrep Plasmid Map

Himar1 transposase was expressed using pMALC 9.3 and purified for *in-vitro* transposition. For quality control, a 4-12% Bis-Tris SDS-Page analysis (Figure 11) and Bradford analysis were performed on the purified transposase. The final concentration of the purified transposase was confirmed to be 228 µg/ml.

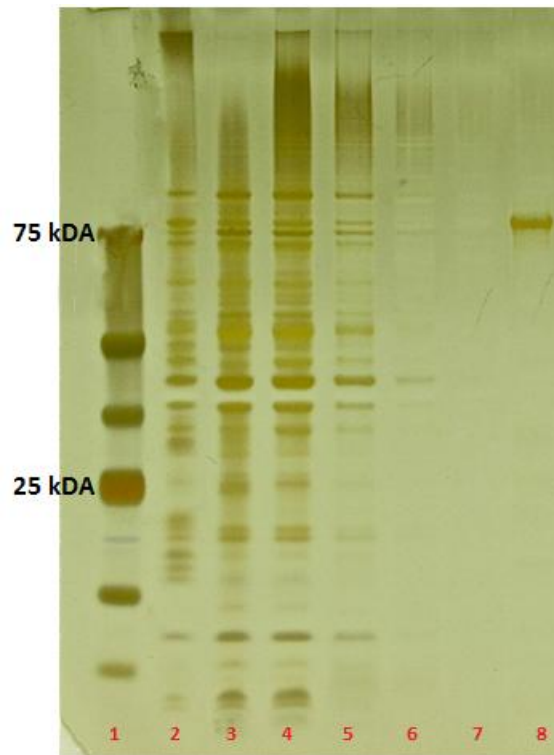


Figure 11: 4-12% Bis-Tris SDS Page Gel on *Himar1* transposase

Lanes: 1, dual precision ladder; 2, *E. coli* containing pMALC 9.3 (~10 µg); 3-7: supernatants of each wash step; 8: purified *Himar1* transposase (~2 µg).

In-vitro transposition was performed on *H. somni* strain 2336. Different concentrations of transposase ranging from 5 nM to 20 nM, and different concentrations of donor plasmid and target DNA were used. Donor plasmid and target chromosomal DNA concentrations ranged from 500 ng- 2 µg. Different combinations of DNA concentrations with transposase concentrations were attempted for *in-vitro* transposition. Following *in-vitro* transposition, the reaction products were purified and transformed naturally into *H. somni* strains 2336 and 8025. Potential mutants were screened by culture on media containing streptomycin (80 µg/ml). After multiple attempts at *in-vitro* transposition, no transposon mutants were generated with *H. somni*. *In-vitro* transposition on *H. influenzae* as a control was not performed.

Discussion

Natural transformation is a method in which bacteria gain the ability to take up DNA from their environment and incorporate new genes into their genetic code. *H. influenzae* is able to undergo natural transformation by the induction of competence genes (*com* operon, *dprA*, *pil* operon, *rec2*, and *sxy*) (Redfield et al., 2006; Redfield et al., 2005; VanWagoner et al., 2004), and recognition of USS 5' – AAGTGCGGT – 3' (Redfield et al., 2006). Using the protocol from Redfield and Poje (2003) with some modifications, natural transformation was attempted on *H. somni* using genomic DNA that contained a Kan^R gene insertion in either *lob2A*, *luxS*, or *uspE*. Double crossover allelic exchange of *lob2A* was confirmed in only a few colonies from each transformation attempt, indicating the transformation efficiency very low. Natural transformation was unsuccessful for *luxS* and *uspE* after multiple attempts, but should be attainable. For transformation to be efficient in *H. influenzae*, the presence of most of the *com* genes and the recognition of USS are required (Redfield et al., 2006). *H. somni lob2A* contains the same USS as *H. influenzae*. However, neither *luxS* nor *uspE* contain any of the USS sequences. The lack of USS in both *luxS* and *uspE* might have been the cause of unsuccessful transformation into *H. somni*. Competent cells will preferentially uptake DNA fragments containing USS. However, the absence of USS does not inhibit natural transformation, but the presence of USS could increase natural transformation efficiency by 100-fold. Cells will readily uptake fragments of DNA as long as there are not competing fragments within the chromosome (Poje and Redfield, 2003).

When performing transformation using plasmids optimization procedures must be applied. The DNA uptake machinery is unable to transport circular molecules through the inner membrane unless circumvented by osmotic shock through the addition of glycerol (Poje and Redfield, 2003). When transforming pNS3K, the addition of 300 µl of 80% glycerol to the transformation mix promoted osmotic glycerol shock. This facilitated the passage of intact pNS3K into the cytoplasm of *H. somni*. Nonetheless, transformation efficiency for pNS3K was very low, and could have been the result of pNS3K not

containing USS. Transformation most likely occurred for pNS3K, and not *luxS* and *uspE* because pNS3K lacked competitive DNA fragments. *H. somni* contains *luxS* and *uspE* in its genome, compared to the lack of homologous DNA in pNS3K.

Redfield et al. (2006) reported that *H. somni* strain 129pt contains defects in *comD* and *comE*. The 5' portion of the *comD* gene is fused to the 3' portion of *comE*. The *comE* gene contains a frameshift resulting in only 67% of the gene being in frame. The PCR results confirm the presence of *comD* in strain 2336 but amplified a smaller fragment of the gene when compared to the same gene from *H. influenzae*. PCR did not amplify *comE* from *H. somni*, as it did for *H. influenzae*. These results do not confirm a lack of *comE* in *H. somni*, but rather that 100% of the gene is not present in *H. somni* strains and/or the primers did not match the corresponding region in *H. somni*. The overall function of ComD is not fully known, but defects might be responsible for the inability to naturally transform DNA (Maughan and Redfield, 2009). The ComE protein is part of the type four pilus system, and contributes to the formation of the membrane channel protein. The two defective genes could be one of the causes of the low transformation efficiency with *lob2A*, *luxS*, *uspE*, or pNS3K.

It is possible that transformation could be increased by introducing the fully intact *comD* and *comE* genes from *H. influenzae* into *H. somni*. The *comD* and *comE* were successfully amplified and cloned into the high efficiency *H. somni* shuttle vector, pNS3K, which was then renamed pSS*comDE*. However, all attempts to introduce pSS*comDE* into *H. somni* through electroporation were unsuccessful. *H. somni* contains both Type I and Type II restriction medication systems. The addition of TypeOne™ Restriction Inhibitor (Epicentre) has been shown to increase transformation efficiency by acting as a molecular decoy and blocking binding sites for Type I restriction systems *in-vivo* (Sandal et al., 2010; Walkinshaw et al., 2002). The addition of TypeOne™ Restriction Inhibitor did not help the overall electroporation efficiency, and was unsuccessful.

H. somni contains the restriction endonuclease R. HsoI, which is part of the Type II restriction modification system (Siddaramappa, 2007). R. Hha, which originates from *Haemophilus haemolyticus* (Siddaramappa, 2007), is an isoschizomer of R. Hso, and *in-vitro* methylation from HhaI methylase protects DNA from cleavage by restriction endonuclease HhaI and HsoI (Sanders et al., 1997). *In-vitro* methylation by HhaI methylase has been shown to greatly increase transformation efficiency into *H. somni* (Briggs and Tatum, 2005), but efficiency drops as the size of the plasmid increases (Sanders et al., 1997). Nonetheless, unsuccessful transformation may be linked to mutations, size, and/or the construction method. Following Sanger sequencing pNS3K was found to lack most of the reported restriction sites and had no promoter upstream of the multiple cloning site. This could be the consequence of DNA damage due to multiple freeze/thaw cycles. Another reason for unsuccessful electroporation of the plasmid could be linked to the addition of a 1.7 kb fragment resulting in the overall increase in the size of the newly constructed plasmid.

Plasmid pNS3K was derived from pLS88, a broad host range shuttle vector. Introduction of pLS88 into *H. somni* through electroporation proved problematic due to its large size, excessive number of antibiotic markers, and limited number of cloning sites. pNS3K was constructed to increase efficiency of electroporation and to circumvent the drawbacks present in pLS88. The origin of replication that was added to pNS3K originated from *H. ducreyi*. A higher efficiency shuttle vector might be possible by using an origin of replication originating from *H. somni*. *H. somni* strain 649 contains a native plasmid (pHS649) that is 1.347 kb in size and contains an ORF encoding for RepA, which is possibly involved in plasmid replication (Siddaramappa et al., 2006). By inserting a MCS with a strong *H. somni* promoter upstream and an antibiotic marker, pHS649 may be a more effective shuttle vector compared to pNS3K. This newly developed vector may be useful for introducing *comD* and *comE* from *H. influenzae* into *H. somni*, which may increase overall natural transformation efficiency. Another method to increase transformation efficiency is through *in-vivo* methylation by passage through *H. influenzae* (Sanders et

al., 2003). Passage of pSScomDE through *H. influenzae* may help in introducing the plasmid into *H. somni*.

Transposon mutagenesis using the EZ::Tn5™<KAN-2>Tnp Transposome™ system (Epicentre) has been successful at generating mutations in *H. somni*, but was inefficient at producing a large bank of mutants that spanned the entire genome. The *Himar1 mariner* transposon system is another form of transposon mutagenesis that does not require species-specific host factors, and only requires the recognition of a TA dinucleotide for incorporation of the mobile element (Akerley and Lampe, 2002; Lampe et al., 1996). This method has been used successfully in similar bacteria, such as *H. influenzae* (Lampe et al., 1999), and should be an effective form of mutagenesis in *H. somni*. The donor plasmid for the *mariner* mobile element, pMMOrf, did not contain any antibiotic marker to identify successful transposition, so the Strep^R gene (1.0 kb) was chosen for insertion, and after successful insertion, the newly constructed plasmid was renamed pMarStrep. Strep^R was chosen as an antibiotic marker so *in-vitro* transposition could be combined with the vector pSScomDE. pSScomDE contained the Kan^R gene, and by using a different antibiotic for *in-vitro* transposition, both projects could be combined to ensure generation of a bank of mutants that saturated the genome. pMarStrep was used as the transposon element donor. The *Himar1* transposase is ~40.7 kDA, but is fused with a maltose binding protein to simplify purification using amylose resin.

After purification of the *Himar1* transposase and construction of the *mariner* transposon donor plasmid, *in-vitro* transposition reactions and mutagenesis of *H. somni* was attempted. The transposase concentration was optimized to ~10 nM per reaction because the transposase was found to have regulatory consequences (Lampe et al., 1998). During transposition reactions, the *mariner* must find its way into the genome, through horizontal transfer, and must create copies of itself to ensure its persistence. Every copy that is inserted ensures its persistence, but causes damage to the host

simultaneously by insertion within genes. Therefore, the damage must be limited to not kill the host, and higher concentrations of transposase inhibit the overall transposition reactions (Lampe et al., 1998). Different concentrations of transposase, ranging from 5 nM to 20 nM were tested. These variables were mixed with a combination of different concentrations of donor plasmid and target chromosomal DNA. Overall *in-vitro* transposition reactions were unsuccessful, which may have been due to a non-functional transposase, or an inability to naturally transform *H. somni*. A control to assess the problems of *in-vitro* transposition could have been done using *H. influenzae*, but this control method was not tested. Nonetheless, *Himar1 in-vitro* transposon mutagenesis may still have the potential to be an effective means of generating a bank of *H. somni* mutants, and help assess the role of genes putatively involved in virulence.

Conclusions

Millions of dollars are lost each year as cattle succumb to infections cause by *H. somni*. Previous vaccinations or pre-challenged bovines have shown some resistance to *H. somni* infection or disease. The efficacy of previous vaccines has been variable, but a live-attenuated vaccine derived from virulent isolates may be more effective (Siddaramppa and Inzana, 2004). Identification of genes involved in virulence is a first step in developing a more effective vaccine against *H. somni*. In order to identify such virulence genes, an efficient mutagenesis system is needed, which has been problematic due to the tight restriction modification system of *H. somni*. Natural transformation is a successful method for mutagenesis in *H. influenzae*, and is confirmed to be functional in *H. somni*, but at very low efficiency. Introduction of *comD* and *comE* genes from *H. influenzae* into *H. somni* may increase the natural transformation efficiency of *H. somni*. The plasmid pNS3K has proved to be an inefficient shuttle vector, but the plasmid pHS649 could potentially serve as a more effective, homologous shuttle vector. After reconstructing pHS649, this new vector may be used to introduce *comD* and *comE* from *H. influenzae* into *H. somni*. Furthermore, combination of *Himar1* transposon mutagenesis with introduction of *comD* and *comE* may generate a large enough bank of mutants to saturate the entire genome. Such a bank of mutants would identify currently unknown genes involved in virulence, and improved vaccines and diagnostic tests could be developed to improve control of diseases caused by *H. somni*.

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Vitae

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