An Investigation of *Histophilus somni* Virulence Factors in Pathogenesis and Diagnosis

Yu Pan

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biomedical and Veterinary Sciences

Thomas J. Inzana, Chair

Stephen M. Boyle

Stephen B. Melville

Sharon G. Witonsky

9/24/2014

Blacksburg, Virginia

Keywords: *Histophilus somni*, ELISA, diagnosis, *luxS*, *uspE*, regulation, phagocytosis, *ibpA*, biofilm, monocytes

An Investigation of *Histophilus somni* Virulence Factors in Pathogenesis and Diagnosis

Yu Pan

ABSTRACT

H. somni is capable of forming a prominent biofilm, and *luxS* is known to play an important role in biofilm formation through quorum sensing, but has also been postulated to function in gene regulation. In order to further study the function of *H. somni* LuxS, mutants 2336::Tn*luxS* and 2336::Tn*uspE* were identified from a bank of mutants generated with EZ-Tn5 <KAN-2>Tnp transposome (EpiCentre). The 2336::Tn*luxS* and 2336::Tn*uspE* mutants were highly attenuated in mice, but only 2336::Tn*uspE* was deficient in biofilm formation. However, the electrophoretic profiles of the LOS and serum sensitivity of both mutants were substantially altered compared to the parent strain, but exopolysaccharide production during biofilm formation also only decreased in 2336::Tn*uspE*. The altered phenotypes were partially restored in complemented recombinant clones obtained using shuttle vector pHS649S. To clarify whether *luxS* regulates the expression of various virulence genes, mRNA from both the parent strain and 2336::Tn*luxS* was sequenced. It was determined that the transcription level of 53 genes in 2336::Tn*luxS* and 42 genes in 2336::Tn*uspE* in planktonic form were changed. In biofilm, 320 genes in 2336::Tn*luxS* and 230 genes in 2336::Tn*uspE* were differentially regulated compared to biofilm formed by strain 2336.

The immunogloblin binding protein A (IbpA) of *H. somni* is known to be cytotoxic to phagocytic cells. In this study, we found that strains with a mutation in *ibpA* were less capable of

early replication in monocytes. The IbpA protein concentrated from the culture supernatant of strain 2336 facilitated the intracellular survival of strain 129Pt, which lacks IbpA. However, the ability of several *ibpA* mutants to resist intracellular killing was not significantly impaired by 48 h post-infection. Two transposon mutants 2336::Tn*luxS* and 2336::Tn*uspE* replicated in monocytes in a similar manner as the *ibpA* mutants. Confocal microscopy revealed that the intracellular-replicable strains (2336, 64Vc, 2336::Tn*luxS*, 2336::Tn*uspE* and the *ibpA* mutants) prevented the acidification of the bacterial-containing phagosome and the expression of lysosome marker LAMP-2, which may facilitate survival of *H. somni* in monocytes.

An enzyme-linked immunosorbent assay was developed to detect bovine antibodies to the *H. somni* exopolysaccharide that is formed during biofilm formation. When an index value of 0.268 was used the sensitivity of the assay for experimentally- and naturally-infected calves was 90.5% at 3 weeks post-infection, and the specificity of the assay for healthy calves was 92.5%. The EPS ELISA may aid in identifying calves with diseases due to *H. somni*.

Dedication

For my family, who are the sources of unconditional support.

For my friends, who gave me a second family. May our future be bright!

Acknowledgement

I first wish to express my great appreciation and sincere gratitude to my advisor, Dr. Thomas J. Inzana. Dr. Inzana's instruction was indispensable for me to finish this work. His kindness and thoughtfulness inspired me throughout my doctoral study. It has been a great honor and a pleasure for me to be his student.

I would also like to thank the members of my committee, Dr. Stephen Boyle, Dr. Stephen Melville, and Dr. Sharon Witonsky. Dr. Boyle has been patient and detailed in his instruction, yet he always helped me look at the bigger picture of my project. Dr. Melville offered me more insightful ideas on the molecular aspects of my study. As the only immunologist on my committee, Dr. Witonsky offered great suggestions for the phagocytosis study.

My doctoral research has also greatly benefited from other Virginia Tech researchers. Abey, Anna and Indra were always a great help for technical issues. Dr. Lindsay provided me with the M-617 cell line, on which a large portion of my work depended. The support and help from Kathy, Kristi, and other university technicians, who helped me to carry out some experiments are also greatly appreciated.

Finally, and most importantly, I would like to thank my family and friends, without their support, both physical and mental, I could never possibly accomplish my work.

This research was supported by USDA-NIFA grants 2013-67015-21314 *and* 2007-35204-18338 from the United States Department of Agriculture-National Institute of Food and Agriculture, and the Virginia-Maryland Regional College of Veterinary Medicine.

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 2

Chapter 3

Chapter 4

Attribution

Several colleagues aided in the writing and research behind two of my chapters presented as part of this dissertation. A brief description of their contributions is included here.

Chapter 2: Functional and phenotypic characterization of *Histophilus somni luxS*

Abey Bandara, PhD (Dr. Thomas Inzana's lab, Biomedical Sciences & Pathobiology) is currently a research assistant professor at Virginia Tech. Dr. Bandara created the complemented mutants used in this study.

Indra Sandal, PhD (School of Medicine, University of Utah) is currently an adjunct assistant professor in internal medicin at the University of Utah. Dr. Sandal created the mutants 2336::Tn*luxS* and 2336::Tn*uspE* and conducted the animal challenge.

Allan Dickerman, PhD (Virginia Bioinformatics Institute, Virginia Tech) is currently a research assistant professor at Virginia Tech. Dr. Dickerman helped with the analysis of the RNA sequencing.

Chapter 4: Detection of antibodies to the biofilm exopolysaccharide of *Histophilus somni* following infection in cattle by enzyme-linked immunosorbent assay

Chapter 4 is published on Clinical and Vaccine Immunology.

Christina Olk (Dr. Thomas Inzana's lab, psychology department) was an undergraduate at Virginia Tech. Miss Olk was a co-author on this paper and helped with the ELISA test.

Taylor Fisher (Dr. Thomas Inzana's lab, department of animal and poultry sciences) was an undergraduate at Virginia Tech. Miss Fisher was a co-author on this paper and helped with the ELISA test.

Chapter 1: Introduction

1.1. General description and significance of *Histophilus somni*

Histophilus somni is a commensal and opportunistic Gram-negative bacterial pathogen associated with multisystemic diseases in cattle and sheep. Although it is a common genital tract commensal, it is capable of causing illness in several organs except the digestive tract. It was first described in 1960 as "*Haemophilus* like organism" causing infectious meningo-encephalitis of feedlot cattle in California, although similar syndromes have been reported before [1, 2]. One of the most frequently reported diseases is thrombotic meningoencephalitis (TME). Other diseases due to *H. somni* include shipping fever (upper respiratory infection), pneumonia, bacteremia, abortion, arthritis, myocarditis, mastitis, infertility and ovine epididymitis. The severity of disease and morbidity rates can vary across cattle herds. The reproductive tract and upper respiratory tract are usually considered a reservoir of *H. somni* and urine or secretions from carrier animals may contaminate the environment [3, 4]. However, *H. somni* cannot survive in the environment for prolonged periods of time [5].

H. somni infections are most prevalent in cattle in western and, Midwestern North America, parts of Western Europe, and Scandinavia, and can result in severe economic losses. Different diagnostic and treatment methods may be used, depending on the stage of the disease and manifestation presented. At early stages, treatment with antibiotics is usually effective. However, infected animals are usually left with chronic disabilities, even after treatment [5]. For example, in the case of TME, the infected animals are likely to remain recumbent, if treatment is given after the CNS symptoms have developed [6]. Vaccination is currently the only preventive measure. Increased surveillance during an outbreak is a standard procedure, but it is usually inadequate due to the rapid progress of the diseases [7]. Current bacterin vaccines do not provide

complete protection, although they may reduce morbidity and mortality [8-11]. Thus, further understanding of the virulence factors of *H. somni* will be useful in developing new and improved diagnostic tests and treatment, providing protective strategies and vaccines.

1.2. Classification

H. somni belongs to family *Pasteurellaceace*, genus *Histophilus*, and species *somni*. The bovine isolates were formerly known as *Haemophilus somnus* [12] and ovine isolates as *Histophilus ovis* [13] or *Haemophilus agni* [14]. It was first classified into the genus *Haemophilus* because of its requirement for blood factors [15]. It has been reclassified into the genus *Histophilus* based on evidence from phylogenetic analyses using 16S rRNA and RNA polymerase B (*rpoB*) gene sequences [16].

1.3. Characteristics

H. somni is a small Gram-negative, pleomorphic coccobacillary, facultatively anaerobic, non-spore forming, non-motile bacterium. It requires enriched medium (ie. Brain heart infusion agar), supplemented with 5-10% sheep blood under 5-20% carbon dioxide at 37°C to grow. Like other members in the family, it has host specificity, which is partly due to specificity for host iron transferrin [17].

Colonies of *H. somni* are usually circular, grayish or yellow colored, moist and glistening with smooth edges on plates. The yellow color can be better observed if the bacteria are picked up with a loop or swab. These characteristics make it relatively easy to identify *H. somni* in the laboratory. *H. somni* has weak or no hemolytic activity, and is catalase and urease negative [18-20]. However, it can ferment D-glucose and is oxidase and indole positive [20].

1.4. Virulence factors

H. somni virulence factors enable the bacteria to colonize host tissue and evade killing by the host immune system. Progress has been made toward understanding these virulence factors. However, there are still many aspects about its pathogenetic mechanisms that have not been fully understood. In studies of its virulence factors, *H. somni* is often compared with the related human pathogens *Haemophilus influenzae* and *Neisseria gonorrhoeae*.

1.4.1 Modification of lipooligosaccharide (LOS)

H. somni LOS consists of endotoxic lipid A, inner core glycoses, outer core glycoses, and various non-glycose modifications, such as sialylation and incorporation of phosphorylcholine (ChoP), but lacks O-antigen, which is present in enteric lipopolysaccharides (LPS) from enteric and other Gram-negative bacteria. The LOS can phase vary, which results in random, variable modification of the LOS outer core oligosaccharide structure, and is a well-characterized feature of other pathogenic bacteria related to *H. somni* that express an LOS. Phase variation occurs primarily through slip-strand mispairing (SSM) between the template and the nascent strands in microsatellites of repeating DNA motifs, during semi-conservative DNA replication [21, 22]. Mispairing in the repeating motifs results in gain or loss of one or more repeats. SSM acts as a molecular switch in genes involved in LOS biosynthesis, particularly glycosyltransferases [21-23]. Structural and antigenic phase variation commonly occurs in pathogenic strains, but rarely in serum-sensitive commensal strains [24].

H. somni can incorporate ChoP and N-acetyl-5-neuraminic (sialic) acid into its LOS [25, 26]. ChoP is associated with adherence of *H. somni* to epithelial cells of the bovine respiratory tract and is also phase variable [25, 27]. Sialic acid is nonimmunogenic, because it is a common

component of mammalian cells. Thus, it can camouflage the bacteria from recognition by the immune system. Sialylation of the LOS can inhibit antibody binding to *H. somni* and enhance serum resistance [26].

1.4.2 Host-parasite interaction

Induction of host cell apoptosis by *H. somni* is an important virulence factor and an effective way to evade killing by the host immune system. Furthermore, *H. somni* is cytotoxic to bovine endothelial cells (BECs), polymorphonuclear leukocytes (PMNs), and macrophages *in vitro* [28-30].

In addition to live bacteria, both culture filtrate and heat-killed bacteria can induce apoptosis in BECs [28]. Damage to BECs exposes their underlying basement membrane. The lesion activates the coagulation mechanism and formation of thrombi [31], which may block the blood supply to multiple organs, resulting in damage to the tissue and development of characteristic clinical syndromes. *H. somni* LOS is one component that can induce apoptosis of BECs through activation of caspase-3 [32, 33]. Morphological changes in PMNs infected with *H. somni* indicate that *H. somni* may also be able to induce apoptosis in PMNs, and direct contact of bacteria with host cells is required [30]. However, the specific mechanism by which *H. somni* induces apoptosis in PMNs is unknown.

H. somni can survive killing by bovine PMNs, monocytes, and macrophages [34, 35]. Thus, it is likely that the bacteria can interfere with some of the bactericidal functions of these cells. *H. somni* can interfere with the phagocytic activity of host cells, which has been supported by studies with bovine macrophages and PMNs [36, 37]. Phagocytic cells infected with live *H. somni* are also less capable of internalizing a secondary target, such as opsonized *Staphylococcus*

aureus and microspheres [36, 37]. Killed whole bacteria or supernatant from heat-killed bacteria have the same inhibitory effect on PMNs, but not on bovine macrophages [36, 37].

Immunoglobulin binding proteins (IbpA) are a series of surface proteins, encoded by the gene *ibpA* [38]. IbpA is involved in inducing cytotoxicity in bovine macrophages through its Fic motif [29, 39]. Exposure to the Fic motif results in rounding up of the cells, increased detachment of infected macrophages, and disruption of actin fibers. The interruption of actin filament assembling by the Fic domain of IbpA may contribute to the reduction of phagocytic activity by these cells [29].

The generation of oxygen radicals is one strategy used by phagocytic cells to eliminate pathogens. *H. somni* is a catalase-negative pathogen, but can inhibit H_2O_2 production by PMNs, although it cannot reduce H_2O_2 *in vitro* [19]. *H. somni* can also inhibit superoxide production by both PMNs and monocytes/macrohpages [40]. Only live bacteria have this inhibitory effect and bacterial contact with the cells is required. Commensal strains are less capable or incapable of inhibiting the oxidative burst of phagocytic cells [40]. However, the specific mechanism of this inhibition is still unknown. Inhibition of the oxidative burst may be related to the capability of *H. somni* to survive intracellularly.

There remains controversy over whether *H. somni* is capable of surviving inside phagocytic cells or is simply toxic for phagocytic cells. Several other bacteria can evade intracellular killing by interfering with phagosome maturation such as *Salmonella* and *Brucella* [41, 42]. Brucella-containing vacuole (BCV) obtains early endosomal markers within several munites after infection, but des not acquire late lysosome markers, which indicates alternation of the phagosome lysosome fusion pathway [42, 43]. Determing the sequential events that occur after *H. somni* is internalized would help to solve this question.

1.4.3 Production of exopolysaccharide (EPS) and biofilm formation

Bacterial biofilms are aggregations of bacteria that live in a highly structured and organized community. Biofilms may contribute to persistent and recurring infections because bacteria in a biofilm are more resistant to the host immune response and to antibiotics. *H. somni* pathogenic strain 2336 and commensal strain 129Pt have been extensively studied and there are clear differences in the architecture of their biofilms. The biofilm of strain 2336 is thicker, mound-shaped, encased in an amorphous extracellular matrix, and has profound water channels. However, the biofilm of strain 129Pt is thinner and the structure is less mature [44].

Quorum sensing is a mechanism of interbacterial communication that has been shown to be involved in the expresion of several complex phenotypes and in the virulence properties and biofilm formation of some pathogenic bacteria [45]. This type of communication occurs due to accumulation of soluble signaling mediators, called auto-inducers. Auto-inducer 2 (AI-2) is a molecule that has been identified as a quorum signaling molecule, which is produced by most Gram-negative bacteria and is involved in interspecies signaling. The *luxS* gene encodes for the protein S-ribosylhomocysteinase and is considered essential for synthesis of the AI-2 signal molecule in many bacteria [46]. Homologs have been found in *H. somni* (strains 2336 and 129Pt) and other related species, such as *H. influenzae* [47]. LuxS not only facilitates the maturation of biofilm in some bacteria, but may also regulate several virulence factors [48-51]. *H. influenzae luxS* mutants are more invasive and apparently more virulent than the wildtype [48]. In some pathogenic *E. coli* strains, LuxS acts as a global regulator that controls the expression of a number of genes [52], and many of these genes funcion in virulence, such as motility, adhesion, and toxin production. In a few bacteria, *luxS* mutants have been shown to be growth deficient [53,

54]. Therefore, further studies are needed to confirm the role of *luxS* and other virulence factors in biofilm formation by *H. somni*.

Although *H. somni* is non-capsulated, it can produce an exopolysaccharide (EPS) under stress conditions, such as low oxygen concentration [55]. EPS is also abundant during biofilm formation, and is an important component of the biofilm matrix. One locus has been identified in *H. somni* that may be responsible for EPS synthesis [55]. Although many of these genes have not been intensively studied, their homologs in other bacterial species are responsible for carbohydrate synthesis and export. By understanding the functions of these genes, we may obtain more insight into the biofilm of *H. somni*.

1.4.4 Surface Proteins

Several surface proteins of *H. somni* have been characterized. A 40-kDa outer membrane protein (OMP) induces a strong antibody response [56] and a 41-kDa major OMP (MOMP) has been identified as a porin [57]. In addition, a heat-modifiable OMP [58] and a 17.5 kDa OMP have also been characterized, but their roles in virulence have not been determined [59].

Immunoglobulin binding proteins (IgBP) are unique virulence factors related to other surface proteins and are important for the persistence of *H. somni* within the host. They can bind to the Fc region of IgG2 and form a surface fibrillar network composed of a series of high-molecular-weight (HMW) proteins of various sizes [60]. They are encoded by the *ibpA* gene [38]. IbpA has a toxic domain (Fic) in the C-terminal region and can induce cytotoxicity in bovine macrophages [29]. A functional IbpA protein is not expressed in some commensal strains, and these strains are not cytotoxic [60]. Besides inducing cytotoxicity, IbpA may also contribute to resistance to complement-mediated killing and adherence to host cells. Commensal strains

lacking IbpA are complement-sensitive and an *ibpA* mutant strain adheres less effectively to bovine endothelial cells [38].

References

1. Kennedy, P.C., Biberstein F. L., Howarth J. A., Frazier L. M., Dungworth D. L., *Infectious meningoencephalitis in cattle, caused by a Haemophilus-like organism.* Am J Vet Res, 1960. **21**: p. 403-409.
2. Griner, L.A., Jensen R., Brown B. B., *Infectious embolic meningoencephalitis in cattle.* J Am Vet Med Assoc, 1956. **129**: p. 417-421.
3. Stephens L. R., L.P.B., Humphrey J. D., Wilkie B. N., Barnum D. A., *Vaccination of cattle against experimentally induced thromboembolic meningoencephalitis with a Haemophilus somnus bacterin.* Am J Vet Res, 1982. **43**: p. 1339-1342.
4. Humphrey, J. and L. Stephens, *Haemophilus somnus: a review.* Veterinary Bulletin, 1983. **53**.
5. Harris, F.W. and E.D. Janzen, *The Haemophilus somnus disease complex (Hemophilosis): A review.* Can Vet J, 1989. **30**(10): p. 816-22.
6. Little, P. and D. Sorensen, *Bovine polioencephalomalacia, infectious embolic meningoencephalitis, and acute lead poisoning in feedlot cattle.* Journal of the American Veterinary Medical Association, 1969. **155**(12): p. 1892.
7. Harris, F.W. and E.D. Janzen, *The Haemophilus somnus disease complex (Hemophilosis): A review.* The Canadian Veterinary Journal, 1989. **30**(10): p. 816.
8. Saunders J. R., J.E.D., *Haemophilus somnus infections II. A Canadian field trial of a commercial bacterin: clinical and serological results.* Can Vet J, 1980. **21**: p. 219-224.

9. Humphrey J. D., L.P.B., Barnum D. A., Doig P. A., Stephens L. R., Thorsen J., *Occurrence of Haemophilus somnus in bovine semen and in the prepuce of bulls and steers.* Can j Comp Med, 1982. **46**: p. 215-217.

10. Ribble C. R., J.G.K., Janzen E. D., *Efficacy of immunization of feedlot calves with a commercial haemophilus somnus bacterin.* Can J Vet Res, 1988. **52**: p. 191-198.

11. Hall R. F., W.J.M., Smith G. L., *Field evaluation of Haemophilus somnus bacterin.* Vet Med Small Anim Clin, 1977. **72**(1368-1370).

12. Bailie, W.E., *Characterization of Haemophilus somnus, a new species, a microorganism isolated from infectious thromboembolic meningoencephalitis of cattel*. 1969, Kansas State University: PhD thesis.

13. Mitchell, C.A., *Haemophilus ovis (nov. spec.) as the cause of a specific disease in sheep.* J Am Vet Med Assoc, 1925. **68**: p. 8-18.

14. Kennedy, P.C., L.M. Frazier, G.H. Theilen, and E.L. Biberstein, *A septicemic disease of lambs caused by Hemophilus agni (new species).* Am J Vet Res, 1958. **19**(72): p. 645-54.

15. Inzana, T.J. and L.B. Corbeil, *Development of a defined medium for Haemophilus somnus isolated from cattle.* Am J Vet Res, 1987. **48**(3): p. 366-9.

16. Angen, O., P. Ahrens, P. Kuhnert, H. Christensen, and R. Mutters, *Proposal of Histophilus somni gen. nov., sp. nov. for the three species incertae sedis 'Haemophilus somnus', 'Haemophilus agni' and 'Histophilus ovis'.* Int J Syst Evol Microbiol, 2003. **53**(Pt 5): p. 1449-56.

17. Yu, R.H., S.D. Gray-Owen, J. Ogunnariwo, and A.B. Schryvers, *Interaction of ruminant transferrins with transferrin receptors in bovine isolates of Pasteurella haemolytica and Haemophilus somnus.* Infect. Immun., 1992. **60**(7): p. 2992-2994.

18. Kilian M., B.E.L., ed. *Genus II. Haemophilus*. Bergey's Manual of Systematic Bacteriology, ed. H.J.G. Krieg N. R. Vol. 1. 1984, Williams & Wilkins: Baltimore.

19. Sample, A.K. and C.J. Czuprynski, *Elimination of hydrogen peroxide by Haemophilus somnus, a catalase-negative pathogen of cattle.* Infect. Immun., 1991. **59**(7): p. 2239-2244.

20. Garcia-Delgado, G.A., P.B. Little, and D.A. Barnum, *A comparison of various Haemophilus somnus strains.* Can j Comp Med, 1977. **41**(4): p. 380-8.

21. Wu, Y., J.H. McQuiston, A. Cox, T.D. Pack, and T.J. Inzana, *Molecular cloning and mutagenesis of a DNA locus involved in lipooligosaccharide biosynthesis in Haemophilus somnus.* Infect Immun, 2000. **68**(1): p. 310-9.

22. McQuiston, J.H., J.R. McQuiston, A.D. Cox, Y. Wu, S.M. Boyle, and T.J. Inzana, *Characterization of a DNA region containing 5'-(CAAT)n-3' DNA sequences involved in lipooligosaccharide biosynthesis in Haemophilus somnus.* Microbial Pathogenesis, 2000. **28**(5): p. 301-312.

23. Inzana, T.J., J. Hensley, J. McQuiston, A.J. Lesse, A.A. Campagnari, S.M. Boyle, and M.A. Apicella, *Phase variation and conservation of lipooligosaccharide epitopes in Haemophilus somnus.* Infection and immunity, 1997. **65**(11): p. 4675-4681.

24. Inzana, T.J., R.P. Gogolewski, and L.B. Corbeil, *Phenotypic phase variation in Haemophilus somnus lipooligosaccharide during bovine pneumonia and after in vitro passage.* Infect Immun, 1992. **60**(7): p. 2943-51.

25. Howard, M.D., A.D. Cox, J.N. Weiser, G.G. Schurig, and T.J. Inzana, *Antigenic Diversity of Haemophilus somnus Lipooligosaccharide: Phase-Variable Accessibility of the Phosphorylcholine Epitope.* Journal of Clinical Microbiology, 2000. **38**(12): p. 4412-4419.

26. Inzana, T.J., G. Glindemann, A.D. Cox, W. Wakarchuk, and M.D. Howard, *Incorporation of N-Acetylneuraminic Acid into Haemophilus somnus Lipooligosaccharide (LOS): Enhancement of Resistance to Serum and Reduction of LOS Antibody Binding.* Infection and Immunity, 2002. **70**(9): p. 4870-4879.
27. Kuckleburg, C.J., S.F. Elswaifi, T.J. Inzana, and C.J. Czuprynski, *Expression of Phosphorylcholine by Histophilus somni Induces Bovine Platelet Aggregation.* Infection and Immunity, 2007. **75**(2): p. 1045-1049.
28. Sylte, M.J., L.B. Corbeil, T.J. Inzana, and C.J. Czuprynski, *Haemophilus somnus induces apoptosis in bovine endothelial cells in vitro.* Infect Immun, 2001. **69**(3): p. 1650-60.
29. Hoshinoo, K., K. Sasaki, A. Tanaka, L.B. Corbeil, and Y. Tagawa, *Virulence attributes of Histophilus somni with a deletion mutation in the ibpA gene.* Microbial Pathogenesis, 2009. **46**(5): p. 273-282.
30. Yang, Y.F., M.J. Sylte, and C.J. Czuprynski, *Apoptosis: a possible tactic of Haemophilus somnus for evasion of killing by bovine neutrophils?* Microb Pathog, 1998. **24**(6): p. 351-9.
31. Thompson K. G., L.P.B., *Effect of Haemohpilus somnus on bovine endothelial cells in organ culture.* Am J Vet Res, 1981. **42**: p. 748-754.
32. Sylte, M.J., F.P. Leite, C.J. Kuckleburg, T.J. Inzana, and C.J. Czuprynski, *Caspase activation during Haemophilus somnus lipooligosaccharide-mediated apoptosis of bovine endothelial cells.* Microb Pathog, 2003. **35**(6): p. 285-91.
33. Kuckleburg, C.J., M.J. Sylte, T.J. Inzana, L.B. Corbeil, B.J. Darien, and C.J. Czuprynski, *Bovine platelets activated by Haemophilus somnus and its LOS induce apoptosis in bovine endothelial cells.* Microbial Pathogenesis, 2005. **38**(1): p. 23-32.

34. Gomis, S.M., D.L. Godson, G.A. Wobeser, and A.A. Potter, *Intracellular survival of Haemophilus somnusin bovine blood monocytes and alveolar macrophages.* Microbial Pathogenesis, 1998. **25**(5): p. 227-235.

35. Czuprynski, C.J. and H.L. Hamilton, *Bovine neutrophils ingest but do not kill Haemophilus somnus in vitro.* Infection and Immunity, 1985. **50**(2): p. 431-436.

36. Hubbard, R.D., M.L. Kaeberle, J.A. Roth, and Y.W. Chiang, *Haemophilus somnus, Induced interference with bovine neutrophil functions.* Veterinary Microbiology, 1986. **12**(1): p. 77-85.

37. Gomis, S.M., D.L. Godson, T. Beskorwayne, G.A. Wobeser, and A.A. Potter, *Modulation of phagocytic function of bovine mononuclear phagocytes byHaemophilus somnus.* Microbial Pathogenesis, 1997. **22**(1): p. 13-21.

38. Tagawa, Y., J.D. Sanders, I. Uchida, F.D. Bastida-Corcuera, K. Kawashima, and L.B. Corbeil, *Genetic and functional analysis of Haemophilus somnus high molecular weight-immunoglobulin binding proteins.* Microbial Pathogenesis, 2005. **39**(5-6): p. 159-170.

39. Worby, C.A., S. Mattoo, R.P. Kruger, L.B. Corbeil, A. Koller, J.C. Mendez, B. Zekarias, C. Lazar, and J.E. Dixon, *The Fic Domain: Regulation of Cell Signaling by Adenylylation.* Molecular Cell, 2009. **34**(1): p. 93-103.

40. Howard, M.D., J.H. Boone, V. Buechner-Maxwell, G.G. Schurig, and T.J. Inzana, *Inhibition of bovine macrophage and polymorphonuclear leukocyte superoxide anion production by Haemophilus somnus.* Microbial Pathogenesis, 2004. **37**(5): p. 263-271.

41. Fields, P.I., R.V. Swanson, C.G. Haidaris, and F. Heffron, *Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent.* Proceedings of the National Academy of Sciences, 1986. **83**(14): p. 5189-5193.

42. Delrue, R.M., C. Deschamps, S. Léonard, C. Nijskens, I. Danese, J.M. Schaus, S. Bonnot, J. Ferooz, A. Tibor, and X. De Bolle, *A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of Brucella melitensis.* Cellular microbiology, 2005. **7**(8): p. 1151-1161.

43. Celli, J., C. de Chastellier, D.-M. Franchini, J. Pizarro-Cerda, E. Moreno, and J.-P. Gorvel, *Brucella evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum.* The Journal of experimental medicine, 2003. **198**(4): p. 545-556.

44. Sandal, I., W. Hong, W.E. Swords, and T.J. Inzana, *Characterization and Comparison of Biofilm Development by Pathogenic and Commensal Isolates of Histophilus somni.* Journal of Bacteriology, 2007. **189**(22): p. 8179-8185.

45. Parsek, M.R. and E.P. Greenberg, *Sociomicrobiology: the connections between quorum sensing and biofilms.* Trends in Microbiology, 2005. **13**(1): p. 27-33.

46. Schauder, S., K. Shokat, M.G. Surette, and B.L. Bassler, *The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule.* Molecular Microbiology, 2001. **41**(2): p. 463-476.

47. Siddaramappa, S.S. *Comparative and functional genomic studies of Histophilus somni (Haemophilus somnus)*. 2007; Available from: http://scholar.lib.vt.edu/theses/available/etd-05022007-150407.

48. Daines, D.A., M. Bothwell, J. Furrer, W. Unrath, K. Nelson, J. Jarisch, N. Melrose, L. Greiner, M. Apicella, and A.L. Smith, *Haemophilus influenzae luxS mutants form a biofilm and have increased virulence.* Microbial Pathogenesis, 2005. **39**(3): p. 87-96.

49. Labandeira-Rey, M., D.M. Janowicz, R.J. Blick, K.R. Fortney, B. Zwickl, B.P. Katz, S.M. Spinola, and E.J. Hansen, *Inactivation of the Haemophilus ducreyi luxS Gene Affects the*

Virulence of This Pathogen in Human Subjects. Journal of Infectious Diseases, 2009. **200**(3): p. 409-416.

50. Li, L., R. Zhou, T. Li, M. Kang, Y. Wan, Z. Xu, and H. Chen, *Enhanced biofilm formation and reduced virulence of Actinobacillus pleuropneumoniae luxS mutant.* Microbial Pathogenesis, 2008. **45**(3): p. 192-200.
51. Kim, S.Y., S.E. Lee, Y.R. Kim, C.M. Kim, P.Y. Ryu, H.E. Choy, S.S. Chung, and J.H. Rhee, *Regulation of Vibrio vulnificus virulence by the LuxS quorum-sensing system.* Molecular Microbiology, 2003. **48**(6): p. 1647-1664.
52. Wang, L., J. Li, J.C. March, J.J. Valdes, and W.E. Bentley, *luxS-Dependent Gene Regulation in Escherichia coli K-12 Revealed by Genomic Expression Profiling.* Journal of Bacteriology, 2005. **187**(24): p. 8350-8360.
53. Lyon, W.R., J.C. Madden, J.C. Levin, J.L. Stein, and M.G. Caparon, *Mutation of luxS affects growth and virulence factor expression in Streptococcus pyogenes.* Molecular Microbiology, 2001. **42**(1): p. 145-157.
54. Fong, K.P., L. Gao, and D.R. Demuth, *luxS and arcB Control Aerobic Growth of Actinobacillus actinomycetemcomitans under Iron Limitation.* Infection and Immunity, 2003. **71**(1): p. 298-308.
55. Sandal, I., T.J. Inzana, A. Molinaro, C. De Castro, J.Q. Shao, M.A. Apicella, A.D. Cox, F. St Michael, and G. Berg, *Identification, structure, and characterization of an exopolysaccharide produced by Histophilus somni during biofilm formation.* BMC Microbiol, 2011. **11**: p. 186.

56. Gogolewski, R.P., S.A. Kania, H.D. Liggitt, and L.B. Corbeil, *Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of Haemophilus somnus.* Infection and Immunity, 1988. **56**(9): p. 2307-2316.

57. Tagawa, Y., M. Haritani, H. Ishikawa, and N. Yuasa, *Antigenic analysis of the major outer membrane protein of Haemophilus somnus with monoclonal antibodies.* Infection and Immunity, 1993. **61**(5): p. 2257-2259.

58. Tagawa, Y., M. Haritani, H. Ishikawa, and N. Yuasa, *Characterization of a heat-modifiable outer membrane protein of Haemophilus somnus.* Infection and Immunity, 1993. **61**(5): p. 1750-1755.

59. Tagawa, Y., M. Haritani, and N. Yuasa, *Characterization of an immunoreactive 17.5-kilodalton outer membrane protein of Haemophilus somnus by using a monoclonal antibody.* Infection and Immunity, 1993. **61**(10): p. 4153-4157.

60. Corbeil, L.B., F.D. Bastida-Corcuera, and T.J. Beveridge, *Haemophilus somnus immunoglobulin binding proteins and surface fibrils.* Infection and Immunity, 1997. **65**(10): p. 4250-7.

Chapter 2

Functional and phenotypic characterization of *Histophilus somni luxS*

Pan, Yu, Abey Bandara, Indra Sandal, Allan Dickerman, and Thomas J. Inzana.

Abstract

LuxS is an S-ribosylhomocysteinase that is involved in the synthesis of quorum sensing molecule autoinducer-2 (AI-2) in many Gram-negative bacteria. The *luxS* gene is highly conserved in most members of the *Pasteurellaceae*. *H. somni* was found to contain a *luxS* gene, which complemented the biosynthesis of AI-2 signal molecules in *luxS*-deficient *Escherichia coli* DH5α. Although AI-2 has been proposed to be a 'universal signal' for quorum sensing, it was found that culture supernatant from *H. somni* could not induce luminescence in the AI-1 and AI-2 deficient *Vibrio harveyi* reporter strain BB170. *H. somni* is capable of forming a prominent biofilm, and *luxS* is known to play an important role in biofilm formation through quorum sensing, but has also been postulated to function in gene regulation. In order to further study the function of *H. somni luxS*, strain 2336::Tn*luxS* was identified from a bank of mutants generated with EZ::Tn5™<KAN-2>Tnp Transposome™. The 2336::Tn*luxS* mutant was highly attenuated in mice, but formed a normal biofilm. However, the electrophoretic profile of the lipooligosaccharide (LOS) and serum sensitivity of 2336::*TnluxS* were substantially altered compared to the parent strain, but exopolysaccharide (EPS) production and the electrophoretic profile of outer membrane proteins were not. Complementation of *luxS* in 2336::Tn*luxS* using shuttle vector pHS649S partially restored the altered phenotypes. A *uspE* (universal stress protein that is involved in regulation of stress responses in *E. coli*) Tn mutant was also found to be attenuated in mice, and altered in LOS electrophorictic profile and serum sensitive. However, mutant 2336::Tn*uspE* was also deficient in biofilm formation and EPS synthesis. RNA sequencing revealed that the transcription levels of some regulator genes in 2336::Tn*luxS* and a large number of ribosomal protein genes in 2336::Tn*uspE* were significantly different from that in strain 2336. However, the genes up-regulated or down-regulated are different

in 2336::Tn*luxS* and 2336::Tn*uspE*. Therefore, *luxS* and *uspE* may function in direct or indirect expression of *H. somni* genes.

2.1 Introduction

Quorum sensing is an evolutionarily conserved mechanism used by some bacteria to sense and respond not only to members of their own species, but also other bacterial species occupying the same ecological niche [1, 2]. Small molecules known as autoinducers (AI), which are often produced during quorum sensing by bacteria, have been shown to accumulate and regulate the expression of genes whose bacteria are at high cell densities. Most Gram-negative bacteria produce an AI known as AI-2, which is thought to be involved in inter-species communication [3], and some Gram-negative bacteria, such as the luminescent marine bacterium *Vibrio harveyi*, also produce AI-1, which is responsible for intra-species communication [4]. The product of the *luxS* gene (S-ribosylhomocysteinase) has been shown to be an intermediary enzyme in the biosynthesis of the furanosyl borate diester signal molecules for AI-2 [5]. In *V. harveyi*, LuxP, which is a periplasmic protein, acts as a receptor for AI-2 molecules and transmits the signal to LuxQ, which is a sensor histidine kinase. A transcriptional regulator (LuxR) mediates gene expression in response to the AI-2 type quorum sensing signals [1, 5]. Using *V. harveyi* AI-1 and AI-2 mutant strain BB170 as a reporter, it has been shown that culture supernatant from several *luxS* containing bacteria can induce luminescence via the AI-2 system [1]. However, the ability of members of the *Pasteruellaceae* family differ significantly in their ability to induce luminescence in strain BB170 [6].

Quorum sensing has been shown to regulate genes involved in swarming motility, biofilm formation, exopolysaccharide (EPS) production, and type III secretion [7, 8]. A biofilm can be defined briefly as “a community of microorganisms attached to a suitable surface” [9]. A vast majority of bacteria in their original niche are thought to exist as structured biofilm communities [10]. In nature, a bacterial biofilm is usually formed when cells transition from a

planktonic state to a sessile state and adhere to a surface. An intricate matrix, usually comprised of polysaccharide, protein, and DNA exuded by the bacteria, provides the necessary stability and structure to the biofilm [11]. In contrast to the planktonic state, bacterial biofilms are less susceptible to innate and acquired immune systems as well as the action of many antibiotics [11-13]. In view of their complex nature, bacterial biofilms are thought to be regulated by several genes and environmental cues [14].

Medically important members of the *Pasteurellaceae* have also been shown to form biofilms *in vivo* and/or *in vitro* [14, 15]. *Histophilus somni* is an important opportunistic pathogen of cattle and sometimes sheep, but can also persist on the mucosal surfaces of these animals. We have shown that *H. somni* forms a very prominent biofilm under most growth conditions except under conditions of high aeration (rapid shaking in broth) [16]. Furthermore, we have shown that *H. somni* forms a biofilm in systemic disease sites such as the lungs and myocardium [17]. Since attachment and colonization of *H. somni* to mucosal surfaces is essential to their survival in their obligate hosts, the ability to adhere and form a biofilm on the epithelial layers may provide this bacterium a selective advantage. It has been reported that several bacteria related to *H. somni* possess a *luxS* gene and that culture supernatants from these bacteria can induce AI-2 type signaling in *V. harveyi*. [18-20]. A homolog of the *luxS* gene in *H. somni* has been identified [6]. However, the function of the *luxS* gene has not been examined. Although *luxS* was initially found to be involved in quorum sensing, LuxS also may have non-quorum sensing functions due to the lack of AI-2 receptor genes in many bacteria with a functional *luxS* [21]. LuxS is also involved in the activated methyl cycle (AMC), which is responsible for the methylation of cellular components and the recycling of sulfur [22, 23].

The universal stress proteins are involved in protecting bacteria from environmental stress. In *Escherichia coli*, *uspE* promotes motility and negatively affects adhesion, as well as enhances stress resistance [24]. In *Pseudomonas aeruginosa* Usp proteins promote anaerobic survival and pyruvate fermentation [25]. The *E. coli* UspE homolog has been identified in *H. somni* as UspA. However, the function of the *H.somni uspE* has not been investigated. In this study, the mutagenesis and partial characterization of the *luxS* and *uspE* genes in *H. somni* were compared. Moreover, phenotypic characterization of strains 2336::Tn*luxS* and 2336::Tn*uspE* and RNA-sequencing were performed to determine the role of *luxS* and *uspE* in gene expression. The factors that modulate biofilm growth remain to be further characterized within the context of *H. somni*.

2.2 Materials and Methods

2.2.1 Bacteria and culture conditions

All *H. somni* strains used in this study (Table 2.1) were grown on Brain Heart Infusion (BHI) agar with 5% sheep blood under 5% CO_2 at 37 °C overnight. Colonies were transferred to BHI broth supplemented with 1% yeast extract, 0.1% Trizma base, and 0.01% thiamine monophosphate (TMP) [26]. The bacteria were grown at 37 °C with rapid shaking (200 rpm) to mid-log phase. When growing mutants 2336::Tn*luxS*, 2336::Tn*uspE* and the complementation mutants of 2336::Tn*luxS*, 50 ul/ml kanamycin was added to the agar or broth.

2.2.2 Construction of *luxS* and *uspE* knockout mutants

Mutant 2336::Tn*luxS* and 2336::Tn*uspE* were selected from a bank of mutants made with EZ::Tn5™ <KAN-2>Tnp Transposome™ (Epicentre, Chicago, IL) [17]. Strain 2336::Tn*uspE* was isolated from the bank of mutants in a screen for biofilm deficiency, while strain 2336::Tn*luxS* was selected randomly from those mutants that were not deficient in biofilm formation. The transposon insertion site was confirmed by sequencing the ends of the transposon and the flanking chromosomal region.

2.2.3 Complementation of mutant 2336::Tn*luxS*

Polymerase chain reactions (PCR), DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed following standard procedures [27]. The streptomycin resistance gene was amplified from vector pLS88 and inserted into a plasmid isolated from *H. somni* strain 649 (pHS649), resulting in plasmid pHS649S. The *luxS* gene with its own promoter was amplified from the genome of *H. somni* strain 2336. An uptake signal sequence (AAGTGCGGT)

was amplified from *H. influenzae* and ligated to to the 3' end of the cloned *luxS* gene. The *luxS* gene with the uptake sequence was then inserted into pHS649S at a AclI site. The resulting plasmid was introduced into strain 2336::Tn*luxS* by electroporation, as previously described [28].

2.2.4 mRNA isolation and sequencing

mRNA was isolated from cells in planktonic or biofilm modes of growth. For generation of biofilm cells, 5 ml of planktonic bacterial culture was inoculated into 45 ml of Columbia broth with 1% glucose, 0.1% Trizma base, and 0.01% TMP at 37 °C for 5 days without shaking. Bacteria harvested from the planktonic culture or biofilm were mixed with RNAprotect, as redommended by the manufacturer. The cell debris and matrix material in the biofilm were removed with QiaShredder (Qiagen, Valencia, CA). Total RNA was then extracted with the RNeasy mini kit (Qiagen, Valencia, CA), as described by the manufacturer. The integrity of RNA was determined with a Bioanalyzer of Virginia Bioinformatics Institute, Blacksburg, VA. The mRNA was enriched on a Wafergen 324 Apollo Robot using Ribo-Zero rRNA removal kit (Epicentre, Chicago, IL). The libraries were prepared on an Apollo 324 with the Wafergen Illumina compatible mRNA library Prep Kit with TruSeq Compatible Adapters. The libraries were then sequenced on the HiSeq 2500 on a 100 Paired End Sequencing Run.

The genome sequence of *H. somni* strain 2336 was used as the reference genome, which can be downloaded from Patric (http://wwww.patricbrc.org/portal/portal/patric/Genome?cType=genome&cId=93646). The reads were mapped to the reference genome using bowtie [29]. The significant differences in the pairwise tests were identified with Cuffdiff. The differences of gene expression in the pairewise test were

expressed as log ratio. Log_2 fold of change that was greater than 1 or less than -1 was considered to be significant.

2.2.5 Purification of LOS and electrophoretic analysis

LOS was extracted from strains 2336, 2336::Tn*uspE*, 2336::Tn*luxS,* and 4 complemented mutants of 2336::Tn*luxS* (P, Q, T, and V) using a hot phenol-water microextraction method, as previously described [30]. The electrophoretic profile of *H. somni* LOS was determined by sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described [31]. The LOS profiles were visualized by silver staining [31].

2.2.6 Serum sensitivity

The bactericidal assay was performed as previously described [32] with the following modifications. The bacterial culture was supplemented with 100 μg/ml of sialic acid to sialylate the LOS of *H. somni* [33]. Bacteria in mid-log phase were suspended in phosphate buffered saline, pH 7.4 (PBS) with 0.15 μM $CaCl_2$ and 0.5 μM $MgCl_2$. The mid-log bacterial suspension containing 10^9 colony forming units (CFU) was mixed with different concentrations of pre-colostral calf serum on ice. The mixtures were serially diluted. Twenty μl of each dilutions were placed onto BHI blood agar before and after 1 h incubation at 37 °C. After incubation overnight at 37 °C, the CFU per ml of bacterial mixture were calculated. The serum sensitivity was recorded as the percentage survival = (100 – [number of colonies at 60 min incubation/number of colonies at 0-time incubation x100]).

2.2.7 Biofilm formation, staining, and visualization

H. somni was cultured on Columbia blood agar (Becton Dickinson, Sparks, MD) plates containing 5% sheep blood for 24-36 hours at 37 ^{0}C and 5% CO_2. A loopful of fresh bacteria from agar plates was resuspended in BHI broth containing 0.1% Trizma base and 0.01% TMP. For biofilm growth in 50-ml plastic conical tubes, 3 ml of the bacterial suspension was added to each tube and then Terrific broth (Becton Dickinson, Sparks, MD) with 1% glucose, 0.1% Trizma base, and 0.01% TMP was added to the top of the tube. The 50-ml tubes were incubated at 37 ^{0}C for 4-5 days. Biofilm formation was tested by staining with 1% crystal violet for 15 minutes at room temperature (procedure modified from [34]). After washing three times with sterile distilled waterr, the crystal violet sustained by the biofilm was solubilized with 95% ethanol, and quantified by absorbance measurement at 630 nm.

2.2.8 Purification of exopolysaccharide (EPS) from biofilm

EPS was extracted as previously described [35]. The biofilm was grown in a 1-L bottle, the clear supernatant removed, and extracted with 45% aqueous phenol. The phenol was removed by dialysis, and EPS was precipitated with excess -20°C ethanol in the presence of 30 mM sodium acetate. The precipitate was collected by centrifugation (10,000 x *g* for 30 min), and digested with DNase (20 μg/ml), RNase (20 μg/ml), and Proteinase K (25 μg/ml). The enzymes were removed by phenol extraction. Any LOS in the mixture was removed after ultracentrifugation at 125,000 × g at 4°C for 4 hr., and precipitated with 30 mM sodium acetate and 5 volumes of cold (-20 °C) 95% ethanol. The EPS was resuspended in water and lyophilized.

2.2.9 Extraction of outer membrane proteins (OMP) and electrophoresis analysis

The OMPs were extracted as previously described [36]. Briefly, the bacterial cells were lysed in a French press cell at 8,000 lb/in^2 and after removal of the unlysed cells the total membranes were pelleted by ultracentrifugation at 60,000 rpm for 60 min in a Ti-60 rotor. The pellet was re-suspended in 0.01 M, pH 7.4, HEPES with 2 mM $MgCl_2$ and 2% Triton-X 100. The suspension was pushed through a 1-ml syringe with #18, #20, and #22 guages needles sequencially. The insoluble protein-enriched outer membranes were pelleted by centrifugation at 50,000 rpm for 90 min in a Ti-50 rotor. Extraction of the pellet was repeated, and the protein-enriched outer membranes were collected and resuspended in water. The electrophoretic profile of *H. somni* OMP was determined by sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the NuPAGE Gels (Life Technologies, Carlsbad, CA) and following the manufacturer's recommondations. The LOS profiles were visualized by silver staining [31] and blue staining .

2.2.10 Animal challenge

To evaluate the virulence of the transposon mutants, a mouse septicemia and mortality model was used as previously described [37]. *H. somni* colonies were scrapped off BHI blood agar and suspended in PBS to a concentration of 10^8/ml (75% light transmittance at 610 nm), and confirmed by viable plate counts. The bacterial suspension was injected into the peritoneal cavity of female, 7-8 weeks old, Swiss Webster mice. The survival rate of the mice was recorded daily.

2.3 Results

2.3.1 The impact of *luxS* on growth

The Klett units values measured every hour to assess the cell density of the culture. When grown planktonically, parent strain 2336 and 2336::Tn*luxS* grew at similar rate. However, the lag phage of 2336::Tn*uspE* was significantly shorter, although the growth rate during log phase was similar to that of the parent strain (Fig. 2.1).

2.3.2 Effect of *luxS* and *uspE* mutations on the electrophoretic profile of OMPs

Several OMPs have been identified and may be related to virulence, such as a 41-kDa porin [38] and IbpA [39]. Therefore we expected some changes in the OMP electrophoretic profiles of the two less virulent mutants. However, there was no difference between the 1-D electrophoretic profiles of the mutants and the parent strain, visualized by both blue staining and silver staining (Fig. 2.2).

2.3.3 Impact of the *luxS* and *uspE* genes on biofilm formation and EPS production

Mutant strains 2336::Tn*uspE* and 2336::Tn*luxS* were isolated from a bank of Tn mutants following screening of the Tn bank for biofilm formation. On the initial screen 2336::Tn*uspE* was deficient in biofilm formation, whereas 2336::Tn*luxS* was not. The amount of biofilm formed by parent strain 2336, and mutant strains 2336::Tn*luxS* and 2336::Tn*uspE* was quantified by crystal violet staining (Fig. 2.3). Complementation of 2336::Tn*luxS* had no impact on the amount of biofilm formed (Fig. 2.3). The amount of EPS, which is a major component of the *H. somni* biofilm matrix [35], produced by 2336::Tn*uspE* was also deficient, but there was not a substantial change in the amount of EPS produced by 2336::Tn*luxS* (Fig. 2.4).

2.3.4 Effect of *luxS* and *uspE* on LOS electrophoretic profile and serum sensitivity

The electrophoretic profile of *H. somni* strain 2336 LOS normally consists of four bands [40]. When *H. somni* is grown in the presence of sialic acid the terminal galactose residue on the oligosaccharide is sialyated. As a result, the two highest molecular size bands shift up in size [40]. The two largest upper molecular size bands were absent in both strains 2336::Tn*uspE* and 2336::Tn*luxS* (Fig. 2.5).The incorporation of sialic acid into *H. somni* LOS contributes to serum resistance [40]. As expected, strains 2336::Tn*uspE* and 2336::Tn*luxS* were more sensitive to killing by pre-colostral calf serum (antibody free source of complement) after 1 h incubation (Fig. 2.7).

The *luxS* gene was cloned into *H. somni* plasmid pHS649, and elecrotransformed into 2336::Tn*luxS*. Four complemented recominants were isolated and examined. The LOS electrophoretic profiles of the four complement mutants were partially restored (Fig. 2.6), as was partial restoration of serum resistance (Fig. 2.7). The success of the transformation and transcription of the transformed *luxS* was determined by reverse transcriptase PCR (RT-PCR). The results showed that *luxS* was expressed in parent strain 2336 and all four complemented mutants, but not in 2336::Tn*luxS* (Fig. 2.8).

2.3.5 Virulence of strains 2336::Tn*upsE* and 2336::Tn*luxS* in a mouse model

Although the mouse is not a natural host for *H. somni,* we and others have shown that virulent strains can be differentiated from avirulent strains by assessing bactermeia and mortality in a mouse model [37]. Mice infected with *H. somni* strain 2336 died within 24 h, but none of those infected with 2336::Tn*luxS* or 2336::Tn*uspE* died within 6 days (Table 2.1). Therefore, the

mutants were highly attenuated in comparison to the parent strain. Strains 2336::Tn*luxS* and 2336::Tn*uspE* also did not cause any disease following intramuscular inoculation of cattle with 10^{10} cells (data not shown).

2.3.6 Gene expression analysis

As expected, the transcription levels of a number of genes were changed in mutants 2336::Tn*luxS* and 2336::Tn*uspE* in comparison to strain 2336 in planktonic growth (Table 2.2 and 2.3). However, different genes were up-regulated or down-regulated in 2336::Tn*luxS* and 2336::Tn*uspE*. Although many of the differentially reguated genes were related to metabolism in mutant 2336::Tn*luxS*, several of genes with altered transcription level are regulators, such as MarR family transcriptional regulator (NC_010519:1981269-1981719) and heat shock protein 60 family co-chaperone GroES (NC_010519:884344-884635). Furthermore, a cell division protein (NC_010519:1334822-1335452) was also down-regulated (Table 2.2). In contrat to 2336::Tn*luxS*, which has more genes down-regulated (Fig. 2.9), more genes were up-regulated in 2336::Tn*uspE* (Fig. 2.10). While some metabolic proteins genes were also differentially regulated, most of the down-regulated genes encoded for ribosomal proteins or ribosomal RNA.

When grown in a biofilm, 1141 genes out of total 2123 genes in strain 2336 were differentially regulated in comparison to strain 2336 grown in planktonic form (data not shown). The genes expressed by the bacteria in planktonic and biofilm forms were compared for strains 2336, 2336::Tn*uspE*, and 2336::Tn*luxS*. The genes differentially expressed by the cells in a biofilm compared to in planktonic form were similar in all three strains (863 genes) (Fig. 2.11). There were 183 genes of 2336::Tn*uspE* and 364 genes of 2336::Tn*luxS* differentially regulated when the bacteria were grown in biofilm compared to strain 2336 in a biofilm. Among the

differentially expressed genes, 152 genes were up- or down-regulated in both 2336::Tn*uspE* and 2336::Tn*luxS* (Fig. 2.12A). Even though *luxS* did not have much impact on biofilm formation, it affected the expression of more genes than *uspE* when the bacteria were grown as a biofilm. Among the 152 genes differentially regulated in both mutants 2336::Tn*luxS* and 2336::Tn*uspE*, those up-regulated in 2336::Tn*luxS* were also up-regulated in 2336::Tn*uspE*; those down-regulated in 2336::Tn*luxS* were also down-regulated in 2336::Tn*uspE* (Fig. 2.12B).

Table 2. 1 Virulence of mutants 2336::Tn*uspE* and d2336::Tn*luxS* in a mouse model.

Strain	Challenge dose (CFU/mouse [10^8] ($5XLD_{50}$)	No. of mice dead/total (% mortality)	Duration (hrs/day) mice survived
2336 (parent)	2.51	5/5	24 h
2336::Tn*luxS*	2.88	0/5	6 d
2336::Tn*uspE*	2.53	0/5	6 d

Table 2. 2 Genes significantly up- or down-regulated in planktonic 2336::Tn*luxS* compare to *H. somni* strain 2336.

Locus	Log_2 Fold Change	Product
Down-regulated in 2336::Tn*luxS*		
NC_010519:63365-65936	-3.074	ClpB protein
NC_010519:2128090-2128971	-2.949	DNA transposition protein
NC_010519:410485-412393	-2.794	Chaperone protein DnaK
NC_010519:1334822-1335452	-2.702	Cell division protein FtsJ / Ribosomal RNA large subunit methyltransferase J (EC 2.1.1.-)
NC_010519:2126128-2128087	-2.193	Phage transposase
NC_010519:619912-622213	-1.882	Cholinephosphate cytidylyltransferase (EC 2.7.7.15)
NC_010519:1023240-1023864	-1.678	NinG recombination protein
NC_010519:492270-493119	-1.562	Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)
NC_010519:884344-884635	-1.440	Heat shock protein 60 family co-chaperone GroES
NC_010519:1635003-1637045	-1.378	Succinyl-CoA ligase [ADP-forming] beta chain (EC 6.2.1.5)
NC_010519:249498-250527	-1.359	Phage portal protein
NC_010519:903273-904080	-1.353	Oligopeptide transport ATP-binding

		protein OppF (TC 3.A.1.5.1)
		Haemophilus-specific protein%2C
NC_010519:1028685-1030863	-1.296	uncharacterized
		ATP-dependent protease HslV (EC
NC_010519:655581-656109	-1.291	3.4.25.-)
		Heat shock protein 60 family chaperone
NC_010519:884665-886309	-1.269	GroEL
		D-allose ABC transporter%2C substrate-
NC_010519:648198-649134	-1.150	binding component
		Dihydrolipoamide succinyltransferase
		component (E2) of 2-oxoglutarate
NC_010519:1637098-1638322	-1.150	dehydrogenase complex (EC 2.3.1.61)
		Ribose ABC transport system%2C
NC_010519:1394938-1395967	-1.108	permease protein RbsC (TC 3.A.1.2.1)
		ATP-dependent hsl protease ATP-binding
NC_010519:654232-655564	-1.094	subunit HslU
NC_010519:247700-249488	-1.087	Phage terminase%2C ATPase subunit
NC_010519:867910-869791	-1.081	Chaperone protein HtpG
		UDP-N-acetylmuramoylalanyl-D-
		glutamyl-2%2C 6-diaminopimelate--D-
NC_010519:723719-726190	-1.080	alanyl-D-alanine ligase (EC 6.3.2.10)
NC_010519:878275-878593	-1.037	Ribosome hibernation protein YfiA
NC_010519:1159196-1160621	-1.008	Dihydrolipoamide dehydrogenase of

		pyruvate dehydrogenase complex (EC 1.8.1.4)
NC_010519:484586-485024	-1.008	Histone acetyltransferase HPA2 and related acetyltransferases
NC_010519:131836-133015	-0.996	GTP-binding protein Obg
NC_010519:1233965-1235336	-0.992	Glutathione reductase (EC 1.8.1.7)
NC_010519:1033402-1036102	-0.954	hypothetical protein
NC_010519:1829255-1829972	-0.936	3-demethylubiquinol 3-O-methyltransferase (EC 2.1.1.64)
NC_010519:577973-579011	-0.936	Lipoprotein NlpD
NC_010519:2073213-2074687	-0.934	Sodium-dependent transporter
NC_010519:2025501-2027913	-0.898	ATP-dependent protease La (EC 3.4.21.53) Type I
Up-regulated in 2336::Tn*luxS*		
NC_010519:329204-329549	0.881	Preprotein translocase subunit SecG (TC 3.A.5.1.1)
NC_010519:1846732-1847038	0.891	YafQ toxin protein
NC_010519:1824677-1825763	0.895	ABC transporter%2C periplasmic spermidine putrescine-binding protein PotD (TC 3.A.1.11.1)
NC_010519:1840791-1841517	1.021	Lipopolysaccharide ABC transporter%2C ATP-binding protein LptB
NC_010519:1981741-1982056	1.044	Queuosine Biosynthesis QueE Radical

		SAM
NC_010519:1981269-1981719	1.067	Transcriptional regulator%2C MarR family
		Zn-dependent protease with chaperone
NC_010519:535811-536576	1.103	function
NC_010519:851719-851983	1.104	SSU ribosomal protein S20p
NC_010519:2107550-2108000	1.140	Flavoprotein MioC
NC_010519:1163469-1164321	1.146	Translation elongation factor Ts
NC_010519:1974486-1974684	1.182	hypothetical protein
NC_010519:217834-218533	1.268	Signal transduction histidine kinase
NC_010519:1972852-1974457	1.284	Multicopper oxidase
NC_010519:1596816-1597314	1.346	Nonheme iron-containing ferritin
NC_010519:28759-29449	1.405	LSU ribosomal protein L1p (L10Ae)
NC_010519:1022909-1023095	1.432	hypothetical protein
NC_010519:1969448-1970996	1.523	Multicopper oxidase
		Dihydroorotate dehydrogenase%2C
NC_010519:1444094-1445081	1.967	catalytic subunit (EC 1.3.3.1)
NC_010519:1971329-1971509	1.995	hypothetical protein
		Ribonuclease P protein component (EC
NC_010519:2261444-2263620	2.624	3.1.26.5)
NC_010519:815537-815657	inf	hypothetical protein

Table 2. 3 Genes significantly up- or down-regulated in planktonic 2336::Tn*uspE* compare to *H. somni* strain 2336.

Locus	Log2 Fold	Product
Down-regulated in 2336::Tn*uspE*		
NC_010519:629606-631087	-2.166	Small Subunit Ribosomal RNA%3B ssuRNA%3B SSU ribosomal RNA
NC_010519:513277-514758	-2.146	Small Subunit Ribosomal RNA%3B ssuRNA%3B SSU ribosomal RNA
NC_010519:985636-987117	-2.136	Small Subunit Ribosomal RNA%3B ssuRNA%3B SSU ribosomal RNA
NC_010519:672270-673751	-2.110	Small Subunit Ribosomal RNA%3B ssuRNA%3B SSU ribosomal RNA
NC_010519:2118532-2120013	-2.090	Small Subunit Ribosomal RNA%3B ssuRNA%3B SSU ribosomal RNA
NC_010519:2120410-2123399	-1.994	Large Subunit Ribosomal RNA%3B lsuRNA%3B LSU ribosomal RNA
NC_010519:674148-677137	-1.993	Large Subunit Ribosomal RNA%3B lsuRNA%3B LSU ribosomal RNA
NC_010519:987514-990503	-1.990	Large Subunit Ribosomal RNA%3B lsuRNA%3B LSU ribosomal RNA
NC_010519:515383-518372	-1.977	Large Subunit Ribosomal RNA%3B lsuRNA%3B LSU ribosomal RNA
NC_010519:631712-634701	-1.976	Large Subunit Ribosomal RNA%3B

		lsuRNA%3B LSU ribosomal RNA
NC_010519:63365-65936	-1.932	ClpB protein
NC_010519:1281321-1281540	-1.364	Translation initiation factor 1
		D-allose ABC transporter%2C substrate-
NC_010519:648198-649134	-1.057	binding component
NC_010519:410485-412393	-1.045	Chaperone protein DnaK
NC_010519:45433-46420	-0.994	Long-chain fatty acid transport protein
NC_010519:249498-250527	-0.883	Phage portal protein
NC_010519:1033402-1036102	-0.866	hypothetical protein
		Haemophilus-specific protein%2C
NC_010519:1028685-1030863	-0.860	uncharacterized
Up-regulated in 2336::Tn*uspE*		
NC_010519:2226420-2226807	0.888	LSU ribosomal protein L17p
NC_010519:1596816-1597314	0.893	Nonheme iron-containing ferritin
NC_010519:1981741-1982056	0.929	Queuosine Biosynthesis QueE Radical SAM
NC_010519:553397-553640	0.943	Long-chain fatty acid transport protein
		Preprotein translocase subunit SecG (TC
NC_010519:329204-329549	0.954	3.A.5.1.1)
NC_010519:2219095-2219524	0.958	LSU ribosomal protein L5p (L11e)
NC_010519:2060546-2061017	0.991	SSU ribosomal protein S7p (S5e)
NC_010519:1974486-1974684	0.993	hypothetical protein
NC_010519:1163469-1164321	0.997	Translation elongation factor Ts
NC_010519:2219535-2219841	1.054	SSU ribosomal protein S14p (S29e) @ SSU

		ribosomal protein S14p (S29e)%2C zinc-independent
NC_010519:851719-851983	1.093	SSU ribosomal protein S20p
NC_010519:1968978-1969434	1.168	CopG protein
NC_010519:28759-29449	1.215	LSU ribosomal protein L1p (L10Ae)
NC_010519:1969448-1970996	1.218	Multicopper oxidase
NC_010519:1444094-1445081	1.244	Dihydroorotate dehydrogenase%2C catalytic subunit (EC 1.3.3.1)
NC_010519:8209-8446	1.245	LSU ribosomal protein L28p
NC_010519:1974825-1976554	1.324	Lead%2C cadmium%2C zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5)%3B Copper-translocating P-type ATPase (EC 3.6.3.4)
NC_010519:889941-891417	1.329	3-polyprenyl-4-hydroxybenzoate carboxy-lyase (EC 4.1.1.-)
NC_010519:965227-965443	1.343	SSU ribosomal protein S21p
NC_010519:1602050-1603415	1.417	Diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)
NC_010519:1466316-1466793	1.422	Low molecular weight protein tyrosine phosphatase (EC 3.1.3.48)
NC_010519:1971329-1971509	1.450	hypothetical protein
NC_010519:1022909-1023095	1.871	hypothetical protein
NC_010519:784695-784974	2.856	hypothetical protein

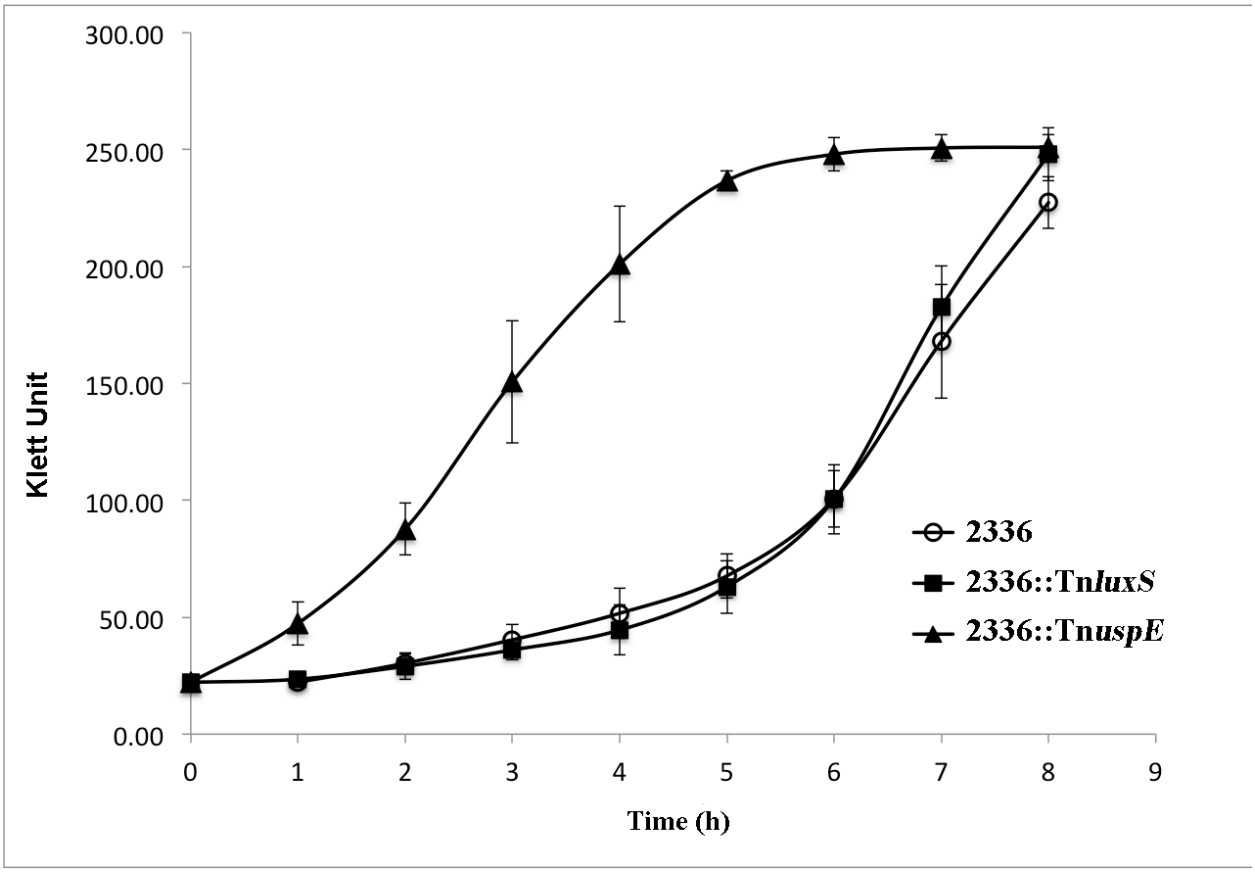

Figure 2. 1 The growth curves of strain 2336 and mutants 2336::Tn*luxS* and 2336::Tn*uspE*.

The bacteria were grown in BHITT with rapid shaking (200 rpm). The density of the culture was reflected by Klett Unit, which was recorded hourly. Mutant 2336::Tn*luxS* grew at the same rate as the parent strain 2336, but mutnat 2336::Tn*uspE* had shorter lag phase.

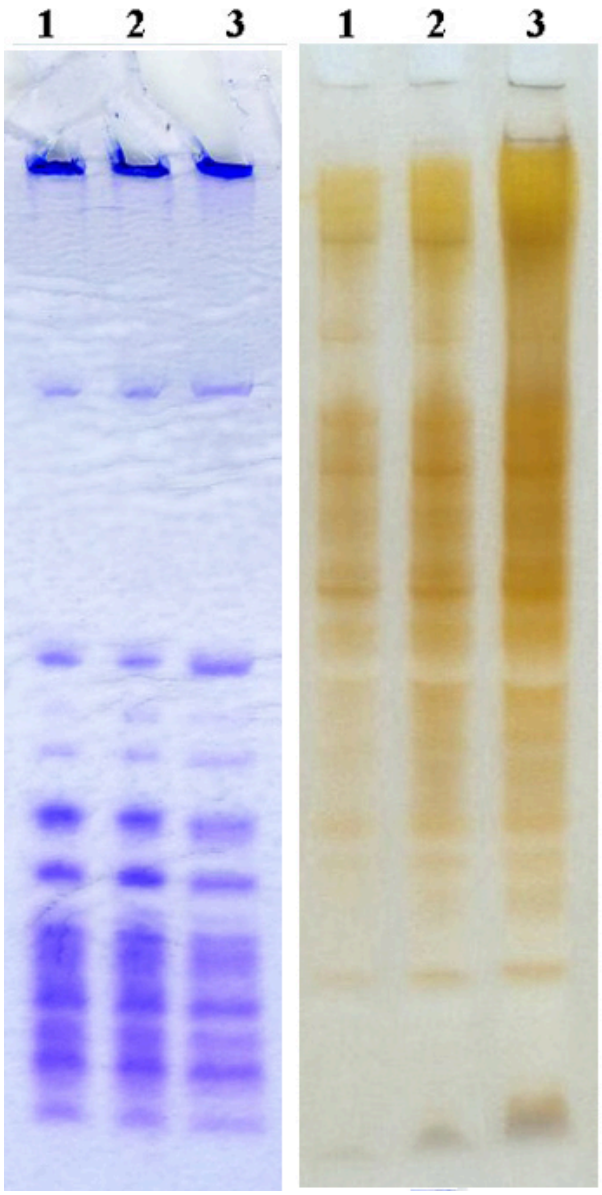

Figure 2. 2 Electrophoretic profile of OMPs from strains 2336, 2336::Tn*luxS* and 2336::Tn*uspE*.

The picture on the left was stained with blue stain and the one on the right was silver stain. Lanes: 1, strain 2336; 2, 2336::Tn*luxS*; 3, 2336::Tn*uspE*

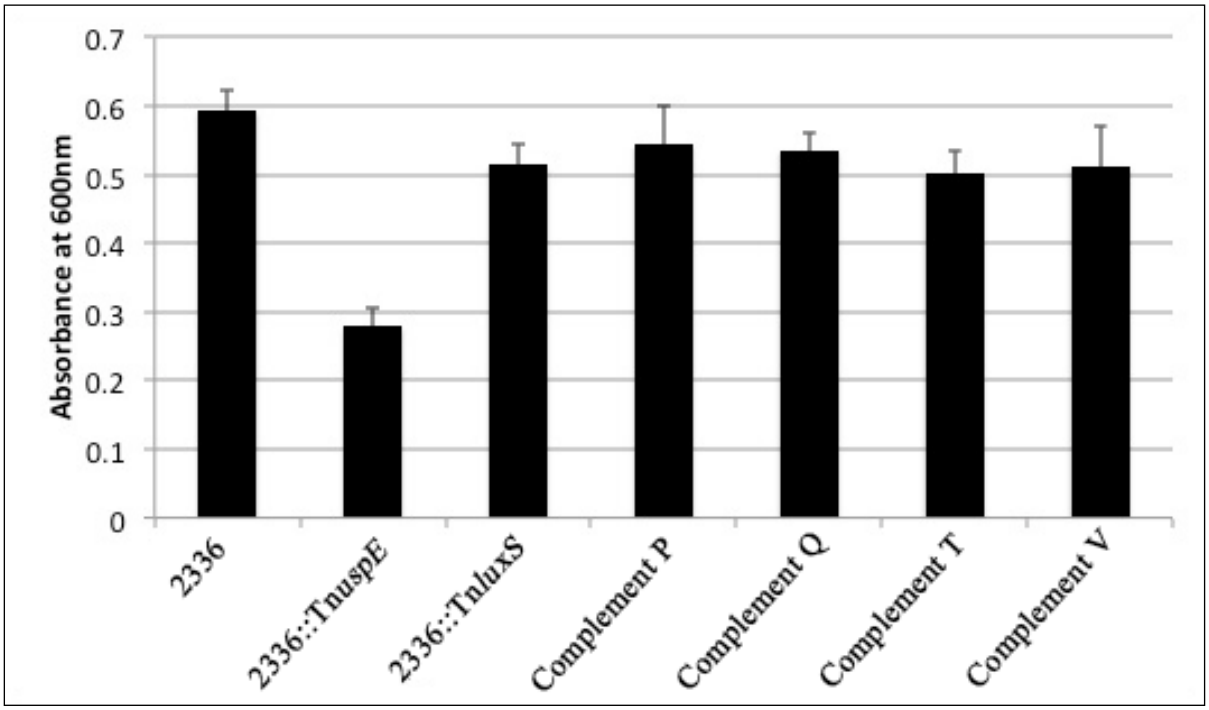

Figure 2. 3 The relative amount of biofilm formed by parent, mutants 2336::Tn*uspE*, and 2336::Tn*luxS*, and complemented clones of 2336::Tn*luxS*.

The amount of biofilm formed was quantified by crystal violet staining. Complement P, Q, T and V are complemented mutants of 2336::Tn*luxS*. Mutant 2336::Tn*uspE* formed less biofilm, compared to the parent strain. The amount of biofilms formed by mutant 2336::Tn*luxS* and all the complemented mutnats was not significantly different from that by strain 2336.

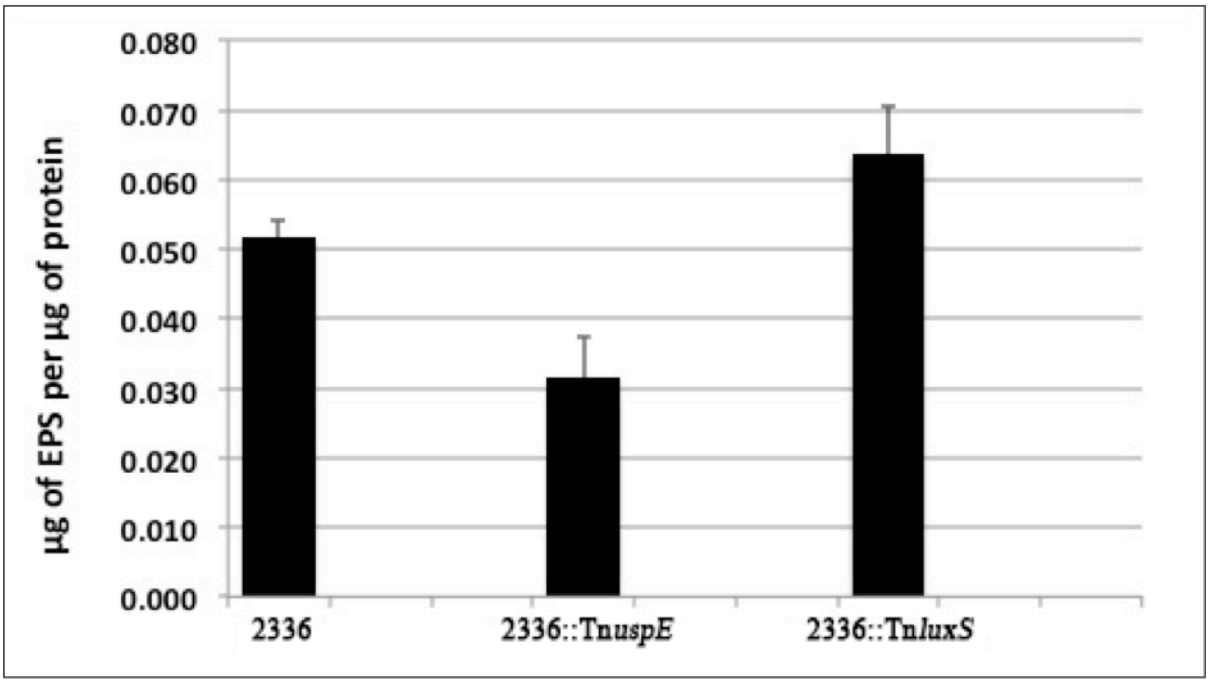

Figure 2. 4 Comparison of the amount of EPS produced by strains 2336, 2336::Tn*uspE*, and 2336::Tn*luxS*.

EPS was extracted from biofilm by phenol extraction, weighted and standardized against the amount of protein. The biofilm-deficient mutant 2336::Tn*uspE* produced less EPS compared to the parent strain, but not mutnat 2336::Tn*luxS*.

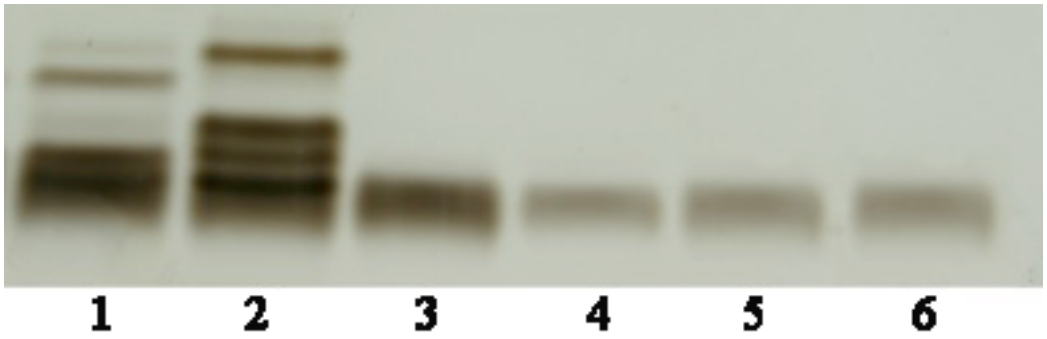

Figure 2. 5 LOS electrophoretic profiles of strain 2336 and mutants 2336::Tn*luxS* and 2336::Tn*uspE*.

The LOS electrophoretic profiles were determined by SDS-PAGE and visualized by silver staining. Lanes: 1, 3, and 5: LOS extracted from *H. somni* grown in broth lacking sialic acid; 2, 4, and 6: LOS from *H. somni* grown with 100 μg/ml sialic acid; 1 and 2, strain 2336; 3 and 4, 2336::Tn*luxS;* 5 and 6, 2336::Tn*uspE*. The LOS was extracted with phenol-water microextraction and the electrophoretic profiles were visualized by silver stain. The upper bands, which shifted up with the additon of sialic acid on the electrophoretic profiles of strain 2336, were absent on the profile of the two mutants.

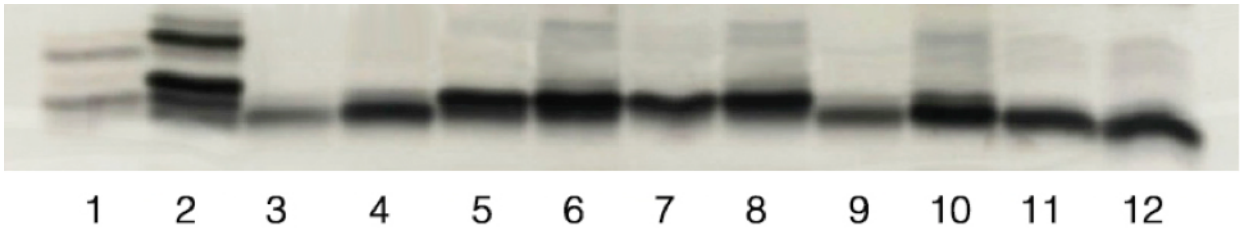

Figure 2. 6 Comparison of LOS electrophoretic profiles of strains 2336, 2336::Tn*luxS* and completment mutants of 2336::Tn*luxS*.

The LOS electrophoretic profiles were determined by SDS-PAGE and visualized by silver staining. Odd lanes: LOS extracted from *H. somni* grown in broth lacking sialic acid. Even lanes: LOS from bacteria grown with 100 μg/ml sialic acid. Lanes: 1 and 2, strain 2336; 3 and 4, 2336::Tn*luxS;* 5 and 6, complement T; 7 and 8, complement V; 9 and 10, complement P; 11 and 12, complement Q. The upper bands were restored on the LOS electrophoretic profiles of the complemented mutants and were more visible with the addition of sialic acid.

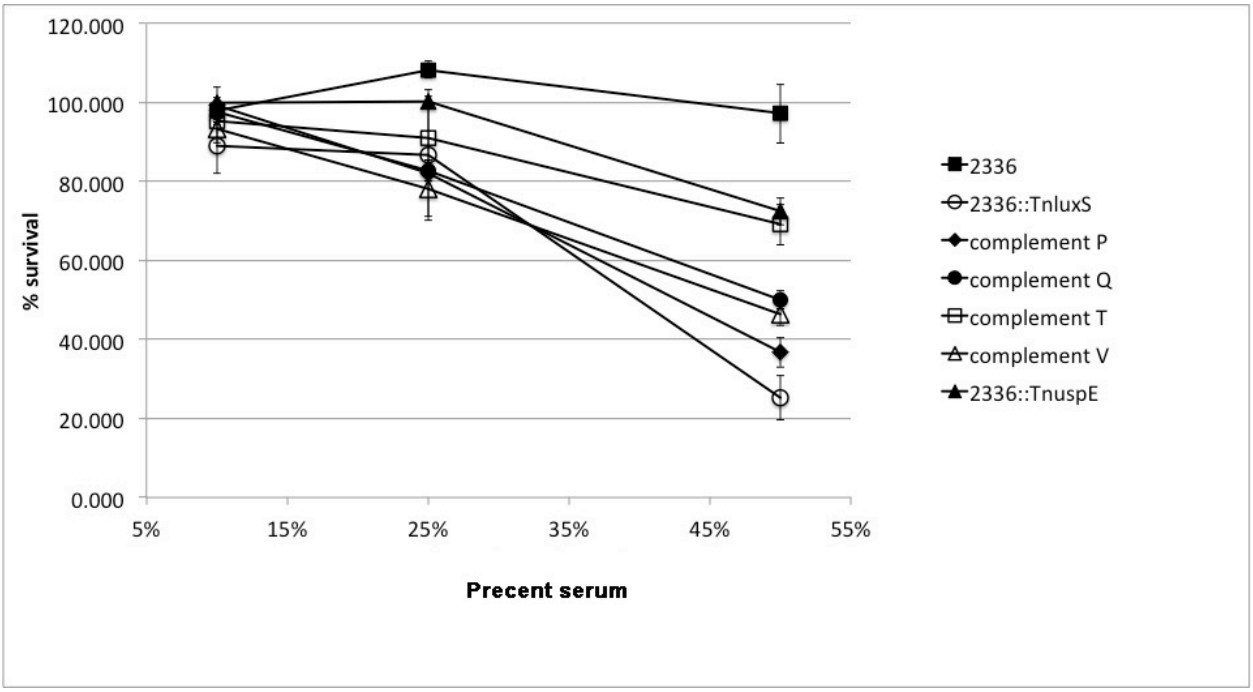

Figure 2. 7 Serum resistance of *H. somni* parent strain 2336, mutants 2336::Tn*luxS* and 2336::Tn*uspE*, and the complemented mutants of 2336::Tn*luxS*.

The percent survival = (100 – [number of colonies at 60 min incubation/number of colonies at 0-time incubation x100]). Strain 2336 was resistant to serum killing at serum concentration of up to 50%, while mutants 2336::Tn*uspE* and 2336::Tn*luxS* were sensitive. The serum resistance of the complemented mutants were partially restored.

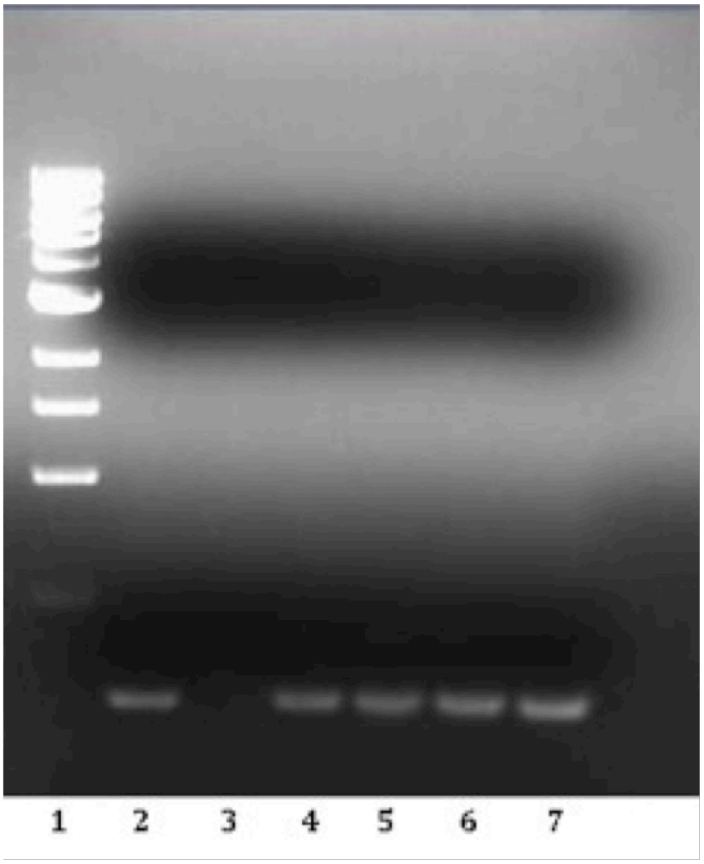

Figure 2. 8 The transcription of *luxS* in strains 2336 and 2336::Tn*luxS* and completmented mutants of 2336::Tn*luxS*.

The transcription of *luxS* in the strains was determined by RT-PCR. Lanes: 1, 1 kb ladder; 2, strain 2336; 3, 2336::Tn*luxS*; 4, complement P; 5, complement Q; 6, complement T; 7, complement V. The *luxS* gene was transcribed in parent strain 2336 and all the complemented mutants, but not in mutant 2336::Tn*luxS*.

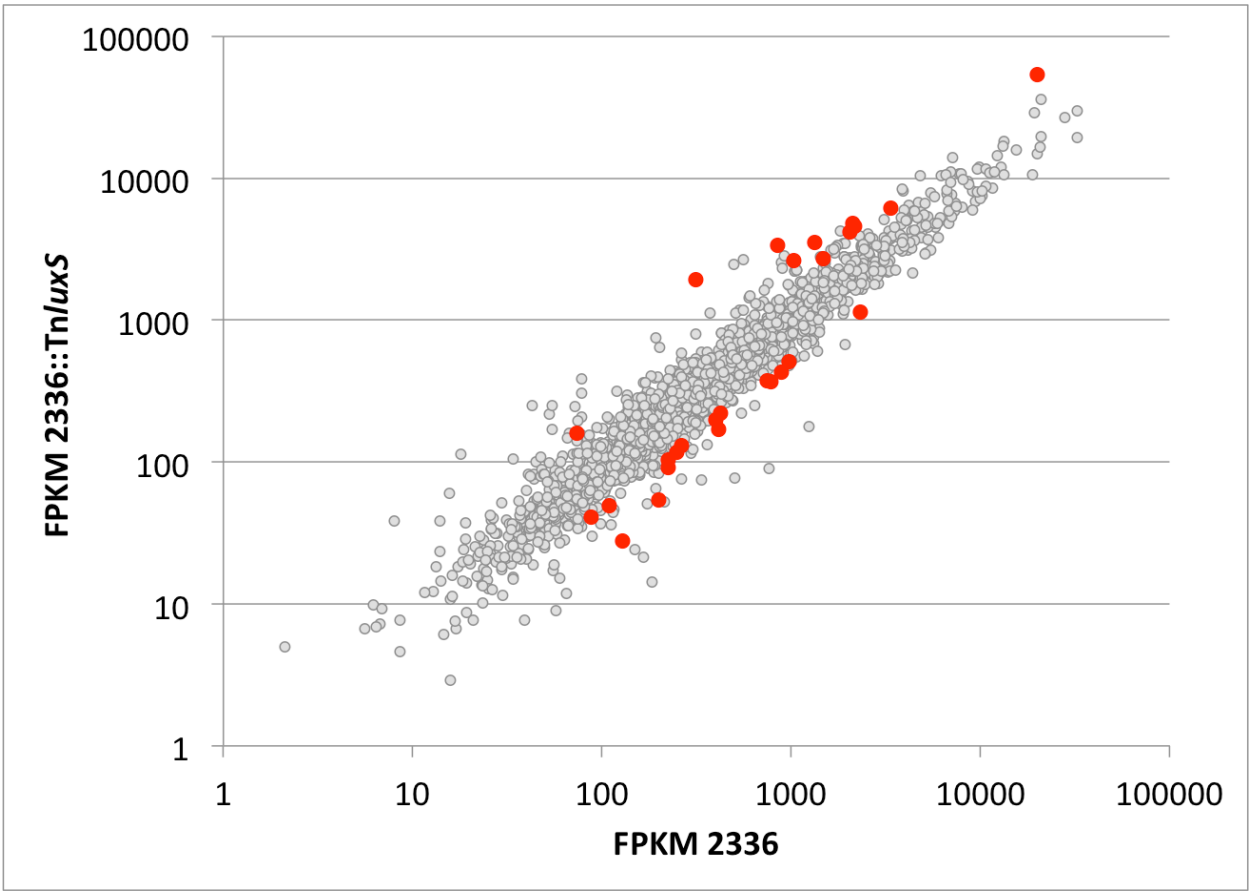

Figure 2. 9 The transcription level of all genes in 2336::Tn*luxS* in comparison to 2336.

The red dots are genes, of which transcription levels were significantly changed in 2336::Tn*luxS* compared to 2336. The gray dots are genes without singficant changes in transcription levels. The dots above the diagonal line were up-regulated, while the dots below diagonal represents the genes that were down-regulated. The difference of gene transcription level was expressed as log ratio. Log_2 fold of change that is greater than 1 or less than -1 was considered to be significant.

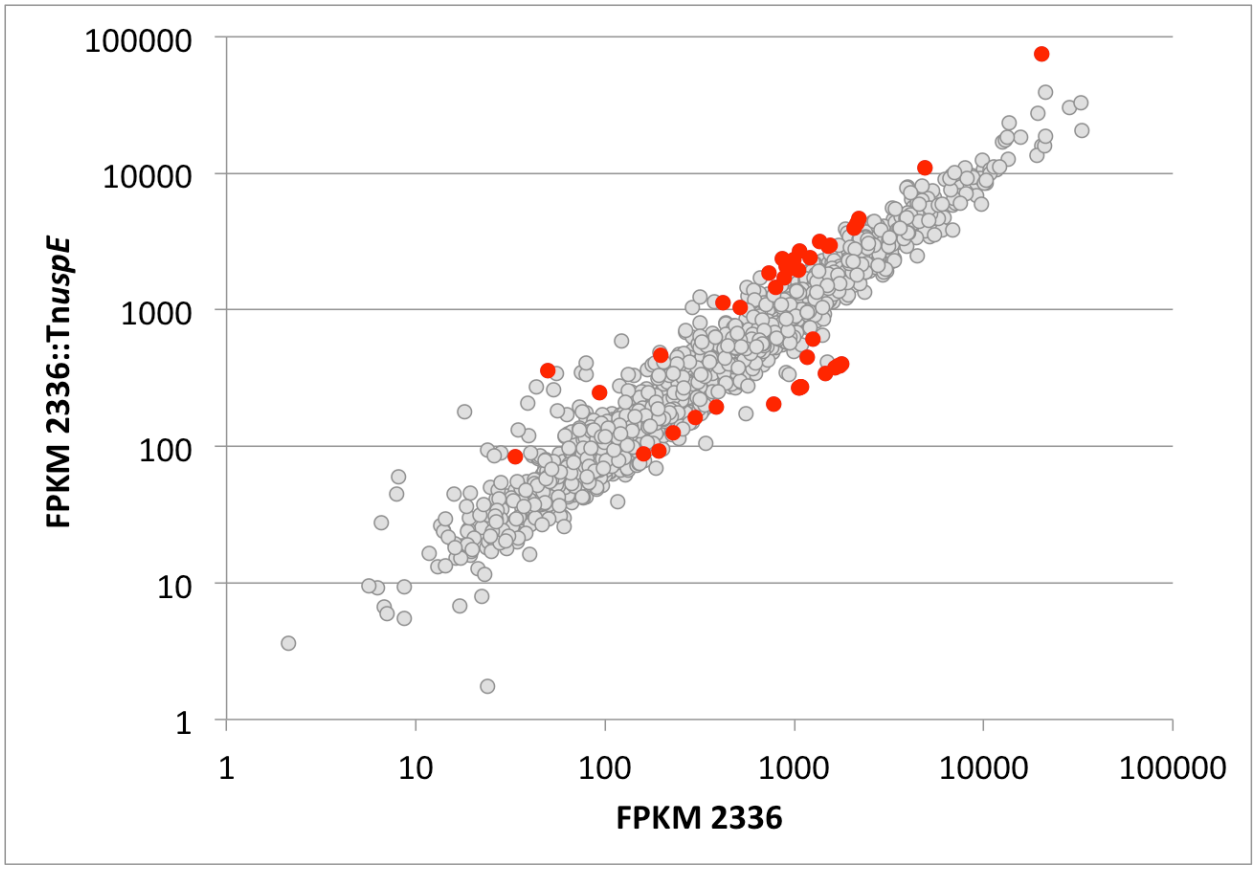

Figure 2. 10 The transcription level of all genes in 2336::Tn*uspE* in comparison to 2336.

The red dots are genes, of which transcription levels were significantly changed in 2336::Tn*uspE* compared to 2336. The gray dots are genes without singficant changes in transcription levels. The dots above the diagonal line were up-regulated, while the dots below diagonal represents the genes that were down-regulated. The difference of gene transcription level was expressed as log ratio. Log_2 fold of change that is greater than 1 or less than -1 was considered to be significant.

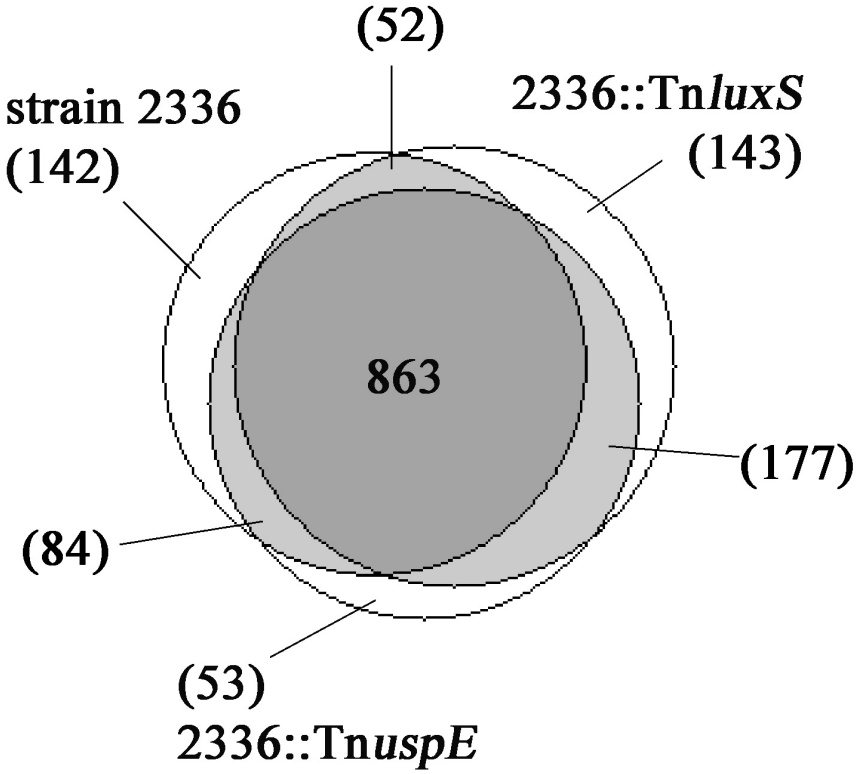

Figure 2. 11 The genes differentially expressed in planktonic and biofilm form in strain 2336, 2336::Tn*luxS* and strain 2336::Tn*uspE*.

The circles represent all the genes that were differentially expressed in biofilm compared to planktonic form for each strains. The numbers in brackets are number of genes differentially expressed in each segments. The dark gray segment includes genes that were differentially regulated in biofilms formed by all strains. The light gray segments include genes that were differentially regulated in biofilms of two of the three strains. The white segments includes genes that were differentially regulated in biofilms of one strain.

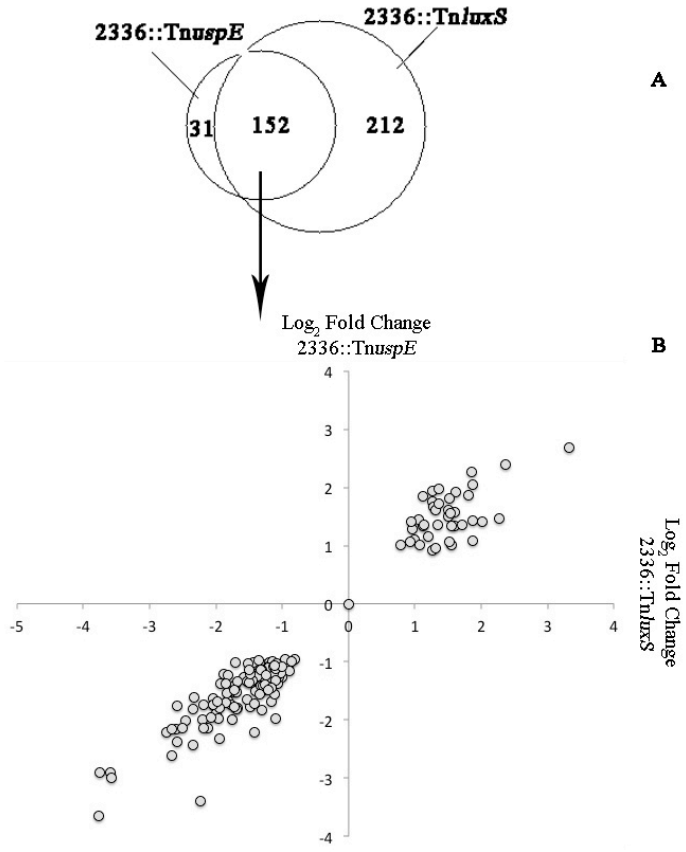

Figure 2. 12 Comparison of differentially expressed genes of 2336::Tn*uspE* and 2336::Tn*luxS* in a biofilm.

A: The circles represent all the genes that were differentially expressed in biofilm by 2336::Tn*uspE* and 2336::Tn*luxS* compared to that in biofilm by strain 2336. The numbers indicates number of genes differentially expressed in each segments. The middle segment includes genes that were differentially regulated in biofilms formed by both mutants, while the segments on both sides includes genes differentially regulated in biofilm by only one mutant. B: Comparison of the $\log_2$ fold changes of genes differentially regulated in both 2336::Tn*uspE* and 2336::Tn*luxS*.

2.4 Discussion

The *luxS* ORFs in *H. somni* strains 2336 and 129Pt are highly homologous [6]. The product of *luxS* catalyzes the conversion of S-ribosylhomocysteine to 4,5-dihidroxy-2,3-pentanedione (DPD), which is an intermedate step of AI-2 synthesis [3]. Unpublished research in our lab has determined that the *H. somni luxS* can complement *E. coli* DH5α, which lacks a functional *luxS*. The supernatant of the complemented *E. coli* strain was able to induce luminescence in the *V. harveyi* autoinducer mutant strain BB170 [6]. Although the *H. somni luxS* is functional, the supernatant of *H. somni* cannot induce luminescence in the *Vibrio harveyi* reporter strain [6]. Moreover, LuxS is associated with the activated methyl cycle (AMC), which is responsible for sulfur recycling. AMC is a central metabolic pathway that is essential for the methylation of cellular components [41]. A metabolite profiling study showed that a mutation in *luxS* resulted in the concentration of several AMC metabolites in *E. coli* [23], *Helicobacter pylori* [42], and *Neisseria meningitidis* [43]. The interruption of AMC may result in a growth deficiency for *E. coli* [23], *Helicobacter pylori* [42], and *Neisseria meningitidis* [43] mutants without a functional *luxS*. Deletion of *luxS* in *Lactobacillus rhamnosus* also showed pleiotropic physiological effects, including slow growth [44]. The growth deficiency in these bacteria can be partially reversed by adding cysteine [42-44]. The syhthesis of AI-2 and AMC are coupled by S-ribosylhomocysteine [23], which makes it difficult to determine the role of *luxS* solely in quorum sensing or the AMC metabolic pathway. However as described by our results, the growth of 2336::Tn*luxS* was not substantially changed, while the lag phase of mutant 2336::Tn*uspE* was shortened, which may indicate a minor impact of *luxS* on the AMC pathway. A mutation in *Haemophilus influenzae luxS* also has no impact on planktonic growth [45]. Therefore, it is possible that the changes of other phenotypes are not the result of the interruption of AMC

pathway. This hypothesis may also be supported by the results of RNA sequencing. Although several genes associated with sulfur metabolism were differentially regulated in biodilm compared to planktonic growth, such as serine acetyltransferase (NC_010519:16559-17357) and cystathionine gamma-synthase (NC_010519:298437-299547), their transcriptional level were not significantly different in strains 2336 and 2336::Tn*luxS* under the same growth conditions.

Biofilm formation is found to be regulated by quorum sensing in a number of bacteria, such as *Vibrio cholerae*, *Salmonella enterica* and *Pseudomonas aeruginosa* [46-48]. Culture supernatant from *Haemophilus influenzae* can induce AI-2 response in *V. harveyi* reporter strain, which indicates possible role of *H. influenzae luxS* in quorum sensing [45]. Although mutation in *luxS* results in deficiency of biofilm formation in many bacteria, including *Salmonella* and *Streptocuccus spp.* [48-50], a *H. influenzae luxS* mutant forms biofilm the same as the wildtype strain [45]. Similar to *H. influenzae*, *H. somni* mutant 2336::Tn*luxS* was also capable of forming mature biofilm as the parent strain. Biofilm is not only an aggreation of bacterial cells, but also involves significant changes in metabolism of the cells within biofilm. As shown in the RNA sequencing results, about half of the *H. somni* genes were differentially regulated in strain 2336 biofilm compared to planktonic growth. Most of the differentially regulated genes in biofilm were associated with change of metabolic status. Although most of the genes are not annotated, the majority of the annotated genes are involved in the metabolism of purine, pyrimidine, amino acid or saccharide.

In some bacteria with a functional *luxS,* but incapable of quorum sensing, an AI-2 receptor gene is absent in its genome [21]. Lsr (LuxS-regulated) genes are identified in *Salmonella typhimurium* and *E. coli*, associated with AI-2 quorum sensing [51, 52]. An LsrB homolog, which is the AI-2 recepter, was found in *H. somni* [21], and the functionality of the *H.*

somni AI-2 receptor homolog is still unknown. As shown by unpublished results from our lab, the supernatant from members of the *Pasteurellaceae* vary in their ability to induce luminescence in *V. harveyi* strain BB170 [6]. Detailed analyses of LuxP, LuxR, and LuxS homologs from different bacteria have revealed that although the AI-2 biosynthetic pathways are similar, the components of the AI-2 response pathway, such as the LuxP receptors, are different [53]. It's possible that the structural differences in the AI-2 receptors of *H. somni* and *V. harveyi* results in the incapability of *H. somni* supernatant to induce luminescence in the *V. harveyi* reporter strain. When AI-2 produced by a bacterium is structurally different from that of *V. harveyi*, the non-specific interaction could cause structural modifications of *V. harveyi* LuxP, ineffective signal transduction, and very little luminescence induction in the reporter strain [54]. Furthermore, the addition of boric acid has been reported to increase the production of AI-2 molecules as well as luminescence induction in *V. harveyi* [5], which indicates the incorporation of boric acid in *V. harveyi* AI-2. However, addition of boric acid at 0.1 mg/ml had no effect on luminescence induction by *H. somni* culture supernatants, indicating that the *H. somni* AI-2 may be different from that of *V. harveyi* and may not contain boron. Although AI-2 signaling has been considered universal, culture supernatants from several other bacterial species induced a range of responses in the *V. harveyi* luminescence system [55-57]. The AI-2 molecule from *Salmonella typhimurium* is also known to be structurally different from that of *V. harveyi* [58]. Thus, whether *H. somni* produces AI-2 that is structurally different from that produced by *V. harveyi* has yet to be determined. In case that *H. somni* conducts quorum sensing through a different AI-2, it's also possible that biofilm formation in *H. somni* is not regulated by quorum sening.

In addition to biofilm formation, a number of other virulence factors are under the regulation of quorum sensing [59-61]. *H. influenzae luxS* mutant has been found to be more virulent in the chinchilla model [45]. However, the *H. somni* mutant 2336::Tn*luxS* was attenuated in both mouse and calf models. *H. somni* is host specific, in which the utilization of hemoglobin and transferrin are thought to play a role [62, 63]. Although *H. somni* does not induce the same symptoms in mouse as in bovine, a high dose of bacterial challenge can induce septicemia in mice [37]. Therefore, the mouse septicemia model is used extensively to evaluation the virulence of *H. somni* strains [37, 64]. The attenuation of mutants 2336::Tn*luxS* may likely be the combined results of the change of several virulence factors.

The capability of *H. somni* to incorporate sialic acid into LOS is an important virulence factor [65]. The electrophoretic profile of *H. somni* strain 2336 LOS contains 4 bands. The sialic acid is added onto the Galβ-(1-3)-GlcNAc, which causes the upper bands to shift up on the LOS electrophoretic profile [40]. As shown in the results, the upper two bands were missing on the electrophoretic profile of mutants 2336::Tn*luxS* and 2336::Tn*uspE*, indicating that the LOS produced by the mutants were truncated. Therefore, the LOS of the mutants cannot be decorated by sialic acid. Sialic acid exists ubiquitously in animal tissues [66]. By incorporating sialic acid, strain 2336 can inhibit the binding of antibodies to its LOS and enhance its resistance to killing by antiserum [40]. Therefore, the two mutants were less resistant to serum killing, because of the lack of the site for sialic acid addition. The LOS electrophoretic profile and serum resistance were partially restored in the complemented mutants. Although the upper bands of the complemented mutants appeared faintly on the LOS electrophoretic profile, their visibility was enhanced by the incorporation of sialic acid. The complemented mutants were more resistant to serum killing, but their resistance was not restore to the same degree as the parent strain. Because

the transcription of the transformed *luxS* gene has been confirmed with RT-PCR, the partial restoration of phenotyes may be due to the downstream regulation of gene expression, such as the regulation of translation, and the interactions of genes affected by *luxS*.

In this study, mutant 2336::Tn*uspE* has a mutation in a gene known as a global regulator [67, 68]. The results showed that a number of changes in phenotypes and gene transcription were observed in *H. somni* mutant 2336::Tn*uspE*. Therefore, it is likely that *uspE* is also a global regulator in *H. somni* and mutant 2336::Tn*uspE* was also compared to the parent strain and mutant 2336::Tn*luxS*. Universal stress proteins (Usp) regulate the responses to environmental stress and resistantce to DNA damage in *E. coli.* [67]. In *Salmonella typhimurium*, UspA contributes to the stress resistance, virulence and persistance in host [69]. In this study, all the virulence factors affected by *luxS* were also affected by *uspE*, although planktonic growth rate, biofilm formation and EPS production were only affected by *uspE*, but not *luxS*.

RNA sequencing resultes revealed that numerous genes were differentially regulated in mutants 2336::Tn*luxS* and 2336::Tn*uspE*. Different genes were affected in mutants 2336::Tn*luxS* and 2336::Tn*uspE* in planktonic growth. In addition to several regulator genes, a cell division protein was also affected by *luxS*, which may be the reason why mutant 2336::Tn*luxS* elongated after 24-h co-incubation with monocytes (see chapter 3). However, most of the genes down-regulated in 2336::Tn*uspE* were ribosonal RNA genes. Therefore, the mechanisms of regulation by *luxS* and *uspE* may be different, although a number of virulence factors were affected by both mutations. most of the genes affected when the bacteria were in biofilm phase were the same.

The metabolic status of cells in biofilm is significantly different from the planktonic form, which is the result of a large number of genes differentially regualted [70-72]. Among the 1141 genes differentially regulated in the biofilm formed by strain 2336 compared to the planktonic

form, 863 genes were also differentially regulated in the biofilm of mutants 2336::Tn*luxS* and 2336::Tn*uspE*. Although most of these 863 genes were not annotated, a number of them may be related to the metabolic status in different growth phase, and not specific to biofilm. Gene expression of mutants 2336::Tn*luxS* and 2336::Tn*uspE* in biofilm was also compared to that of strain 2336 in biofilm. Among the 183 genes differentially regulated in mutant 2336::Tn*uspE*, 152 genes were also differentially regulated in mutant 2336::Tn*luxS*. However, 212 additional genes were only differentially regulated in 2336::Tn*luxS* biofilm, which indicates that *luxS* may have some impact on biofilm formation other than the biomass of biofilm.

In conclusion, this study has shown that *H. somni luxS* affected multiple virulence factors and the expression of a number of genes in both planktonic and biofilm form. However, the mechanism of the effect of *luxS* on the expression of other genes is still unknown. Additional investigation of the interaction of *luxS* with affected genes will be needed to clarify the regulatory role of *luxS*.

References

1. Federle, M.J. and B.L. Bassler, *Interspecies communication in bacteria.* Journal of Clinical Investigation, 2003. **112**(9): p. 1291-1299.
2. Fuqua, C. and E.P. Greenberg, *Self perception in bacteria: quorum sensing with acylated homoserine lactones.* Current opinion in microbiology, 1998. **1**(2): p. 183-189.
3. Xavier, K.B. and B.L. Bassler, *LuxS quorum sensing: more than just a numbers game.* Current opinion in microbiology, 2003. **6**(2): p. 191-197.
4. Henke, J.M. and B.L. Bassler, *Three parallel quorum-sensing systems regulate gene expression in Vibrio harveyi.* Journal of bacteriology, 2004. **186**(20): p. 6902-6914.
5. Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B.L. Bassler, and F.M. Hughson, *Structural identification of a bacterial quorum-sensing signal containing boron.* Nature, 2002. **415**(6871): p. 545-549.
6. Siddaramappa, S., *Comparative and Functional Genomic Studies of Histophilus somni (Haemophilus somnus)*. 2007, Virginia Polytechnic Institute and State University.
7. Keller, L. and M.G. Surette, *Communication in bacteria: an ecological and evolutionary perspective.* Nature Reviews Microbiology, 2006. **4**(4): p. 249-258.
8. Kendall, M.M. and V. Sperandio, *Quorum sensing by enteric pathogens.* Current opinion in gastroenterology, 2007. **23**(1): p. 10-15.
9. O'Toole, G., H.B. Kaplan, and R. Kolter, *Biofilm formation as microbial development.* Annual Reviews in Microbiology, 2000. **54**(1): p. 49-79.
10. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases.* Nature Reviews Microbiology, 2004. **2**(2): p. 95-108.

11. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clinical microbiology reviews, 2002. **15**(2): p. 167-193.

12. Stewart, P.S. and J. William Costerton, *Antibiotic resistance of bacteria in biofilms.* The Lancet, 2001. **358**(9276): p. 135-138.

13. Trampuz, A. and A.F. Widmer, *Infections associated with orthopedic implants.* Current opinion in infectious diseases, 2006. **19**(4): p. 349-356.

14. Haase, E.M., T. Bonstein, R.J. Palmer Jr, and F.A. Scannapieco, *Environmental influences on Actinobacillus actinomycetemcomitans biofilm formation.* Archives of oral biology, 2006. **51**(4): p. 299-314.

15. Gallaher, T.K., S. Wu, P. Webster, and R. Aguilera, *Identification of biofilm proteins in non-typeable Haemophilus influenzae.* BMC microbiology, 2006. **6**(1): p. 65.

16. Sandal, I., W. Hong, W.E. Swords, and T.J. Inzana, *Characterization and Comparison of Biofilm Development by Pathogenic and Commensal Isolates of Histophilus somni.* Journal of Bacteriology, 2007. **189**(22): p. 8179-8185.

17. Sandal, I., J.Q. Shao, S. Annadata, M.A. Apicella, M. Boye, T.K. Jensen, G.K. Saunders, and T.J. Inzana, *Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge.* Microbes and Infection, 2009. **11**(2): p. 254-263.

18. Daines, D.A., M. Bothwell, J. Furrer, W. Unrath, K. Nelson, J. Jarisch, N. Melrose, L. Greiner, M. Apicella, and A.L. Smith, *Haemophilus influenzae luxS mutants form a biofilm and have increased virulence.* Microbial pathogenesis, 2005. **39**(3): p. 87-96.

19. Fong, K.P., W.O. Chung, R.J. Lamont, and D.R. Demuth, *Intra-and Interspecies Regulation of Gene Expression by Actinobacillus actinomycetemcomitansLuxS.* Infection and immunity, 2001. **69**(12): p. 7625-7634.

20. Van der Vinne, A.N., R.Y. Lo, and P.E. Shewen, *Construction and analysis of a Mannheimia haemolytica A1 luxS mutant.* Veterinary microbiology, 2005. **110**(1): p. 53-66.

21. Rezzonico, F. and B. Duffy, *Lack of genomic evidence of AI-2 receptors suggests a non-quorum sensing role for luxS in most bacteria.* BMC microbiology, 2008. **8**(1): p. 154.

22. Winzer, K., K. HARDIE, and P. Williams, *LuxS and Autoinducer-2: Their Contribution to Quorum.* Advances in applied microbiology, 2003. **53**: p. 291.

23. Halliday, N., K. Hardie, P. Williams, K. Winzer, and D. Barrett, *Quantitative liquid chromatography–tandem mass spectrometry profiling of activated methyl cycle metabolites involved in LuxS-dependent quorum sensing in Escherichia coli.* Analytical biochemistry, 2010. **403**(1): p. 20-29.

24. Nachin, L., U. Nannmark, and T. Nyström, *Differential roles of the universal stress proteins of Escherichia coli in oxidative stress resistance, adhesion, and motility.* Journal of bacteriology, 2005. **187**(18): p. 6265-6272.

25. Schreiber, K., N. Boes, M. Eschbach, L. Jaensch, J. Wehland, T. Bjarnsholt, M. Givskov, M. Hentzer, and M. Schobert, *Anaerobic survival of Pseudomonas aeruginosa by pyruvate fermentation requires an Usp-type stress protein.* Journal of bacteriology, 2006. **188**(2): p. 659-668.

26. Inzana, T.J. and L.B. Corbeil, *Development of a defined medium for Haemophilus somnus isolated from cattle.* Am J Vet Res, 1987. **48**(3): p. 366-9.

27. Green, M.R. and J. Sambrook, *Molecular cloning: a laboratory manual*. 2012: Cold Spring Harbor Laboratory Press New York.

28. Sandal, I., M.N. Seleem, S.F. Elswaifi, N. Sriranganathan, and T.J. Inzana, *Construction of a high-efficiency shuttle vector for Histophilus somni.* Journal of microbiological methods, 2008. **74**(2): p. 106-109.

29. Langmead, B., C. Trapnell, M. Pop, and S.L. Salzberg, *Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.* Genome Biol, 2009. **10**(3): p. R25.

30. Cox, A.D., M.D. Howard, J.R. Brisson, M. van der Zwan, P. Thibault, M.B. Perry, and T.J. Inzana, *Structural analysis of the phase-variable lipooligosaccharide from Haemophilus somnus strain 738.* European journal of biochemistry, 1998. **253**(2): p. 507-516.

31. Inzana, T.J., *Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of Haemophilus influenzae.* J Infect Dis, 1983. **148**(3): p. 492-9.

32. Inzana, T.J. and P. Anderson, *Serum factor-dependent resistance of Haemophilus influenzae type b to antibody to lipopolysaccharide.* Journal of Infectious Diseases, 1985. **151**(5): p. 869-877.

33. Balyan, R., *Effect of Sialylation of Histophilus somni Lipooligosaccharide on Virulence and Resistance to Host Defenses*. 2007, Virginia Polytechnic Institute and State University.

34. O'Toole, G.A., L.A. Pratt, P.I. Watnick, D.K. Newman, V.B. Weaver, and R. Kolter, *[6] Genetic approaches to study of biofilms.* Methods in enzymology, 1999. **310**: p. 91-109.

35. Sandal, I., T.J. Inzana, A. Molinaro, C. De Castro, J.Q. Shao, M.A. Apicella, A.D. Cox, F. St Michael, and G. Berg, *Identification, structure, and characterization of an exopolysaccharide produced by Histophilus somni during biofilm formation.* BMC Microbiol, 2011. **11**: p. 186.

36. Loeb, M., A. Zachary, and D. Smith, *Isolation and partial characterization of outer and inner membranes from encapsulated Haemophilus influenzae type b.* Journal of bacteriology, 1981. **145**(1): p. 596-604.

37. Geertsema, R.S., R.A. Kimball, and L.B. Corbeil, *Bovine plasma proteins increase virulence of Haemophilus somnus in mice.* Microbial pathogenesis, 2007. **42**(1): p. 22-28.

38. Tagawa, Y., M. Haritani, H. Ishikawa, and N. Yuasa, *Antigenic analysis of the major outer membrane protein of Haemophilus somnus with monoclonal antibodies.* Infection and Immunity, 1993. **61**(5): p. 2257-2259.

39. Hoshinoo, K., K. Sasaki, A. Tanaka, L.B. Corbeil, and Y. Tagawa, *Virulence attributes of Histophilus somni with a deletion mutation in the ibpA gene.* Microbial Pathogenesis, 2009. **46**(5): p. 273-282.

40. Inzana, T.J., G. Glindemann, A.D. Cox, W. Wakarchuk, and M.D. Howard, *Incorporation of N-Acetylneuraminic Acid into Haemophilus somnus Lipooligosaccharide (LOS): Enhancement of Resistance to Serum and Reduction of LOS Antibody Binding.* Infection and Immunity, 2002. **70**(9): p. 4870-4879.

41. Chiang, P., R. Gordon, J. Tal, G. Zeng, B. Doctor, K. Pardhasaradhi, and P. McCann, *S-Adenosylmethionine and methylation.* The FASEB journal, 1996. **10**(4): p. 471-480.

42. Doherty, N.C., F. Shen, N.M. Halliday, D.A. Barrett, K.R. Hardie, K. Winzer, and J.C. Atherton, *In Helicobacter pylori, LuxS is a key enzyme in cysteine provision through a reverse transsulfuration pathway.* Journal of bacteriology, 2010. **192**(5): p. 1184-1192.

43. Heurlier, K., A. Vendeville, N. Halliday, A. Green, K. Winzer, C.M. Tang, and K.R. Hardie, *Growth deficiencies of Neisseria meningitidis pfs and luxS mutants are not due to inactivation of quorum sensing.* Journal of bacteriology, 2009. **191**(4): p. 1293-1302.

44. Lebeer, S., S.C. De Keersmaecker, T.L. Verhoeven, A.A. Fadda, K. Marchal, and J. Vanderleyden, *Functional analysis of luxS in the probiotic strain Lactobacillus rhamnosus GG reveals a central metabolic role important for growth and biofilm formation.* Journal of bacteriology, 2007. **189**(3): p. 860-871.

45. Daines, D.A., M. Bothwell, J. Furrer, W. Unrath, K. Nelson, J. Jarisch, N. Melrose, L. Greiner, M. Apicella, and A.L. Smith, *Haemophilus influenzae luxS mutants form a biofilm and have increased virulence.* Microbial Pathogenesis, 2005. **39**(3): p. 87-96.

46. Hammer, B.K. and B.L. Bassler, *Quorum sensing controls biofilm formation in Vibrio cholerae.* Molecular microbiology, 2003. **50**(1): p. 101-104.

47. Hentzer, M., K. Riedel, T.B. Rasmussen, A. Heydorn, J.B. Andersen, M.R. Parsek, S.A. Rice, L. Eberl, S. Molin, and N. Høiby, *Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound.* Microbiology, 2002. **148**(1): p. 87-102.

48. Parsek, M.R. and E.P. Greenberg, *Sociomicrobiology: the connections between quorum sensing and biofilms.* Trends in Microbiology, 2005. **13**(1): p. 27-33.

49. Prouty, A., W. Schwesinger, and J. Gunn, *Biofilm Formation and Interaction with the Surfaces of Gallstones by Salmonella spp.* Infection and immunity, 2002. **70**(5): p. 2640-2649.

50. Merritt, J., F. Qi, S.D. Goodman, M.H. Anderson, and W. Shi, *Mutation of luxS affects biofilm formation in Streptococcus mutans.* Infection and immunity, 2003. **71**(4): p. 1972-1979.

51. Taga, M.E., J.L. Semmelhack, and B.L. Bassler, *The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in Salmonella typhimurium.* Molecular microbiology, 2001. **42**(3): p. 777-793.

52. Xavier, K.B. and B.L. Bassler, *Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in Escherichia coli.* Journal of bacteriology, 2005. **187**(1): p. 238-248.

53. Schauder, S., K. Shokat, M.G. Surette, and B.L. Bassler, *The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule.* Molecular microbiology, 2001. **41**(2): p. 463-476.

54. Stock, A.M., *Transmembrane signaling by asymmetry.* Nature structural & molecular biology, 2006. **13**(10): p. 862-864.

55. Bassler, B.L., E.P. Greenberg, and A.M. Stevens, *Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi.* Journal of Bacteriology, 1997. **179**(12): p. 4043-4045.

56. Forsyth, M.H. and T.L. Cover, *Intercellular communication in Helicobacter pylori: luxS is essential for the production of an extracellular signaling molecule.* Infection and immunity, 2000. **68**(6): p. 3193-3199.

57. Greenberg, E., J. Hastings, and S. Ulitzur, *Induction of luciferase synthesis in Beneckea harveyi by other marine bacteria.* Archives of Microbiology, 1979. **120**(2): p. 87-91.

58. Miller, S.T., K.B. Xavier, S.R. Campagna, M.E. Taga, M.F. Semmelhack, B.L. Bassler, and F.M. Hughson, *Salmonella typhimurium Recognizes a Chemically Distinct Form of the Bacterial Quorum-Sensing Signal AI-2.* Molecular cell, 2004. **15**(5): p. 677-687.

59. Zhu, J., M.B. Miller, R.E. Vance, M. Dziejman, B.L. Bassler, and J.J. Mekalanos, *Quorum-sensing regulators control virulence gene expression in Vibrio cholerae.* Proceedings of the National Academy of Sciences, 2002. **99**(5): p. 3129-3134.

60. Smith, R.S. and B.H. Iglewski, *P. aeruginosa quorum-sensing systems and virulence.* Current opinion in microbiology, 2003. **6**(1): p. 56-60.

61. De Kievit, T.R. and B.H. Iglewski, *Bacterial quorum sensing in pathogenic relationships.* Infection and Immunity, 2000. **68**(9): p. 4839-4849.

62. Ekins, A. and D.F. Niven, *Production of transferrin receptors by Histophilus ovis: three of five strains require two signals.* Canadian journal of microbiology, 2001. **47**(5): p. 417-423.

63. Tremblay, Y.D., F. Bahrami, and D.F. Niven, *Acquisition of haemoglobin-bound iron by Histophilus somni.* Veterinary microbiology, 2006. **114**(1): p. 104-114.

64. Geertsema, R.S., C. Worby, R.P. Kruger, Y. Tagawa, R. Russo, D.S. Herdman, K. Lo, R.A. Kimball, J. Dixon, and L.B. Corbeil, *Protection of mice against H. somni septicemia by vaccination with recombinant immunoglobulin binding protein subunits.* Vaccine, 2008. **26**(35): p. 4506-4512.

65. Gyles, C.L., J.F. Prescott, G. Songer, and C.O. Thoen, *Pathogenesis of bacterial infections in animals*. 2008: John Wiley & Sons.

66. Severi, E., D.W. Hood, and G.H. Thomas, *Sialic acid utilization by bacterial pathogens.* Microbiology, 2007. **153**(9): p. 2817-2822.

67. Kvint, K., L. Nachin, A. Diez, and T. Nystrem, *The bacterial universal stress protein: function and regulation.* Current Opinion in Microbiology, 2003. **6**(2): p. 140-145.

68. Nyström, T. and F.C. Neidhardt, *Isolation and properties of a mutant of Escherichia coli with an insertional inactivation of the uspA gene, which encodes a universal stress protein.* Journal of bacteriology, 1993. **175**(13): p. 3949-3956.

69. Liu, W.-T., M.H. Karavolos, D.M. Bulmer, A. Allaoui, R.D.C.E. Hormaeche, J.J. Lee, and C. Anjam Khan, *Role of the universal stress protein UspA of Salmonella in growth arrest, stress and virulence.* Microbial pathogenesis, 2007. **42**(1): p. 2-10.

70. Dötsch, A., D. Eckweiler, M. Schniederjans, A. Zimmermann, V. Jensen, M. Scharfe, R. Geffers, and S. Häussler, *The Pseudomonas aeruginosa transcriptome in planktonic cultures and static biofilms using RNA sequencing.* PloS one, 2012. **7**(2): p. e31092.

71. Becker, P., W. Hufnagle, G. Peters, and M. Herrmann, *Detection of differential gene expression in biofilm-forming versus planktonic populations of Staphylococcus aureus using micro-representational-difference analysis.* Applied and environmental microbiology, 2001. **67**(7): p. 2958-2965.

72. Beloin, C., J. Valle, P. Latour-Lambert, P. Faure, M. Kzreminski, D. Balestrino, J.A. Haagensen, S. Molin, G. Prensier, and B. Arbeille, *Global impact of mature biofilm lifestyle on Escherichia coli K-12 gene expression.* Molecular microbiology, 2004. **51**(3): p. 659-674.

Chapter 3

Histophilus somni survives in macrophages by interfering with phagosome-lysosome fusion

Abstract

Histophilus somni is capable of survivig intracellularly in professional phagocytic cells. However, the mechanism that enables the bacteria to survive is not understood. The IbpA fibrillar network protein of *Histophilus somni* is cytotoxic to epithelial and phagocytic cells, which may interfere with the function of these cells and enable the bacteria to evade killing. To determine the role of IbpA in intracellular survival, a number of strains with or without the *ibpA* gene were tested for their ability to replicate inside monocytic cells. The strains without *ibpA* were less capable of replicating in monocytes. The IbpA protein, concentrated from the culture supernatant of *ibpA*-positive strain 2336, facilitated the survival of *ibpA*-negative strain 129Pt in monocytes, but was not as effective in enhancing survival of other *ibpA* negative strains. Examination of intracellular trafficking by *H. somni* revealed that early phagosomal marker EEA-1 colocalized with both pathogenic strain 2336 and commensal strain 129Pt. However, strain 2336 prevented the acidification of phagosomes containing the bacteria and the expression of lysosome marker LAMP-2, which may account for the survival of *H. somni* in monocytic cells. In contrast, strain 129Pt induced stronger expression of LAMP-2 and acidification of the phagosomes. Addition of IbpA did not change the intracellular trafficking and the expression markers induced by strain 129Pt. Moreover, several mutants with a transposon (Tn) insertion in *ibpA*, including 2336::Tn*luxS* and 2336::Tn*uspE* were not significantly impaired in their overall resistance to intracellular killing, although intracellular survival by these mutants was significantly decreased during the first 24 h [$P = 0.05$]. Therefore, although IbpA may interfere with the ability of monocytes to eliminate intracellular *H. somni*, other mechanisms may also be involved in changing the intracellular trafficking of the strains that are able to replicate in monocytes.

3.1 Introduction

The gram-negative bacterium *H. somni* is an opportunistic pathogen associated with multi-systemic diseases in cattle and sheep. Although it may be present as a common mucosal site commensal, it can be responsible for multi-systemic infections. The initial disease attributed to this organism is thrombotic meningoencephalitis (TME). Other diseases that can be caused by *H. somni* include pneumonia, bacteremia, abortion, arthritis, myocarditis, mastitis and infertility [1]. The severity of disease and morbidity rates can vary substantially. The reproductive tract is usually considered a reservoir of *H. somni* [2].

Immunoglobulin binding proteins (IbpA) are a series of surface proteins, encoded by the gene *ibpA* [3]. They form a fibrillar network on the surface of *H. somni*, and can also be shed into the culture supernatant [4]. The C-terminal end of *ibpA* is homologous with the region encoding YopT in *Yersinia spp* [3]. Structural studies of IbpA have revealed that there are two direct repeats (DR1 and DR2) upstream of the YopT homologous region, each of which contain a filamentation-induced by c-AMP (Fic) domain [3]. The Fic domain can be found in both bacterial and eukaryotic cells [5] and is proposed to be responsible for inducing cytotoxicity in bovine macrophages through its Fic domain [5, 6]. Rounding up of the cells, increased detachment of infected macrophages, and disruption of actin fibers support the conclusion [6].

It has been observed that *H. somni* can survive killing by bovine PMNs, monocytes, and macrophages [7, 8]. Phagocytic cells infected with live *H. somni* are less capable of internalizing a secondary target, such as opsonized *Staphylococcus aureus* and microspheres [9, 10]. Killed, whole bacteria or supernatant from heat-killed bacteria can also inhibit the internalization of *Staphylococcus aureus* by PMNs, but not bovine macrophages [9, 10]. Because Fic domain induces disruption of the actin filaments that are involved in phagocytosis, it is possible that *H.*

somni can survive intracellular killing through interfering with the function of phagocytotic cells with IbpA. In this study, we tried to identify the role of the IbpA Fic in protecting *H. somni* from intracellular killing.

3.2 Materials and methods

3.2.1 Cells and bacteria

All *H. somni* strains used in this study (Table 3.1) were grown on Brain Heart Infusion (BHI) agar with 5% sheep blood in 5% CO_2 overnight from frozen stocks. The colonies were transferred to BHI broth supplemented with 1% yeast extract, 0.1% Trizma base and 0.01% thiamine monophosphate (TMP) (BHIY-TT) [11], and shaken rapidly (200 rpm) at 37 °C to mid-log phase. M-617 bovine monocytic cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37 °C [12].

3.2.2 Isolation of bovine peripheral blood monocytes

Peripheral blood was collected from the jugular vein of Holstein cows into an EDTA-coated tube. The buffy coat layer was separated from the red blood cells and plasma by centrifugation at 1000 x *g* for 30 min at 15 °C. The buffy coat layer was diluted with HBSS (Life Technologies, Carlsbad, CA) and then laid over Ficoll-Paque (Pharmacia, Piscataway, NJ). The monocytes were recovered following centrifugation, as per the manufacturer's instructions. The viability of the isolated peripheral blood monocytes (PBMC) was greater than 95%, as determined by trypan blue staining, and were then cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum.

3.2.3 Construction of *ibpA* insertion mutants

Seven Tn insertion mutant sof *ibpA* #9, #3, #13, #91, #27, #23 and #137 were selected from a bank of mutants made with EZ::Tn5™<KAN-2>Tnp Transposome™ (Epicentre, Chicago, IL) [13]. Electrocompetent cells were prepared as previously described [14]. Briefly, the bacteria

were grown to mid-log phase in BHI broth supplemented with 0.01% TMP and 10% Levinthal base, chilled on ice for 30 min, and washed twice with 272 mM sucrose. The bacteria were suspended in 272 mM sucrose to 1% of its original volume. Forty µl of the electrocompetent cells were mixed with 1 µl of EZ::Tn5™<KAN-2>Tnp Transposome™ in a 1 mm gap-electroporation cuvette. After briefly chilling on ice, electroporation was performed with a BTX ECM 600 electroporator (BTX, Inc., San Diego, CA) at 50 µF, 1.6 kV, and 200 ohms, as previously described [14]. The cells were diluted with 1 ml BHI broth, chilled on ice for 10 min, and the bacteria were allowed to recover at 37°C for 3 h. The bacteria were then plated on BHI blood agar and screened for kanamycin resistance. Genomic DNA from the kanamycin-resistant isolates was isolated with the Genomic DNA Purification Kit (Qiagen). The Tn insertion site was confirmed by sequencing the ends of the Tn and the flanking chromosomal region. The expression of the *ibpA* gene in the mutants was determined by reverse transcription polymerase chain reactions.

3.2.4 Concentration of IbpA

The IbpA protein secreted into the culture medium was concentrated as previously described with minor modifications [15]. Briefly, *H. somni* strain 2336 was grown in 300 ml of BHIY-TT broth to mid-log phase. The bacteria were removed by centrifugation at 8000 × *g* for 10 min. The supernatant was filtered through a 0.2-µM filter to remove any residual bacteria and then concentrated to 4 ml with a 10,000 MW centriprep centrifugation filter unit (Millipore, Billerica, MA) by centrifugation at 4,000 × *g* for 30min at 4°C. The supernatant was used as a source of enriched IbpA in the phagocytosis assay.

3.2.5 Phagocytosis assay

Bacteria in mid-log phase were co-incubated with M-617 monocytic cells for 1 h at a 100:1 multiplicity of infection (bacteria:monocytes). The monocytes were incubated with gentamicin for 30 min to kill extracellular bacteria, washed three times with PBS, then incubated at 37 °C. After incubation of 1 h, 24 h, 48 h, or 72 h, the monocytes were lysed with distilled water and the lysate was cultured on BHI blood agar to determine the number of viable intracellular bacteria. To determine the cytotoxicity of IbpA, the monocytes were co-incubated with concentrated IbpA-containing culture supernatant for 2 h before co-incubation with bacteria.

To determine the uptake of *H. somni* by the monocytes, colonies were counted before and after gentamicin was added to the cells. The uptake rate = (number of colonies with gentamicin/number of colonies without gentamicin x100).

3.2.6 Confocal Microscopy

To determine the intracellular trafficking of the bacteria in M-617 cells, the monocytes were fixed with 3% paraformaldehyde for 10 min or 3 h after co-incubation for 30 min at room temperature. The cells were then permeabilized with 0.1% saponin solution with 1% BSA and 5% goat serum for 30 min. The intracellular *H. somni* and phgosomal/lysosomal markers were labeled with rabbit antibodies and visualized with goat anti-rabbit antibodies conjugated with Alexa Fluor® 488 or Alexa Fluor® 546 (Life Technologies, Carlsbad, CA) by confocal microscopy. The nucleus of the monocytes were visualized with DAPI (Life Technologies, Carlsbad, CA). To determine the acidification of the phagosome, the monocytes infected with *H. somni* were incubated with Lysotracker (Molecular Probes, Eugene, OR, USA) for 1h, following the instruction of the manufacturer. The cells were then fixed as described above. To determine

the percentage of *H. somni* co-localized with the markers, the markers and intracellular *H. somni* were picked by SpotFinder Z and counted manually in 20 monocytes [16, 17].

3.3 Results

3.3.1 Comparison of the intracellular survival of *H. somni* in M-617 cells and PBMC

Several bovine macrophage or monocyte cell lines were examined for the capability to survive intracellularly, as shown for freshly collected phagocytic cells [6, 18]. The M-617 bovine monocyte cell line was found to be most suitable for these experiments. *H. somni* pathogenic strain 2336 can survive in PBMC, whereas commensal strain 129Pt cannot (Fig. 3.1). Strain 2336 was also capable of replicating in the M-617 cell line, but strain 129Pt was killed by these cells within 24 h, which is similar to the results obtained with PMBC (Fig. 3.1). Mouse macrophage cell line J774.1 and human leukemic cell line THP-1 were also tested, but found to be unable to eliminate commensal strain 129Pt (data not shown). Therefore, M-617 cells were used for all subsequent studies.

3.3.2 Confirming intracellular survival of *H. somni* strains

Several pathogenic and commensal *H. somni* strains were tested for their ability to survive inside M-617 bovine monocytes following phagocytosis. The intracellular number of all pathogenic strains and some commensal strains increased after 24 h co-incubation with the monocytes (Fig. 3.2). The Fic domain of IbpA has been shown to be cytotoxic to host cells [6], and the presence of the *ibpA* gene in all *H. somni* strains used in this study has been previously examined (Table 3.1) [19]. All the pathogenic strains and most of the vaginal isolates tested were able to replicate intracellularly, but most of the peputial isolates did not (Table 3.1 and Fig. 3.2). Preputial isolates 1P, 129Pt, 130Pf, and 133P were unable to produce IbpA and were unable to replicate intracellularly after 24 h co-incubation (Table 3.1 and Fig. 3.2). However, a few *ibpA*-positive strains (24P, 124P, and 20P) were also killed in monocytes.

3.3.3 The role of IbpA in intracellular survival of *H. somni*

To further evaluate the effect of IbpA on intracellular survival, the monocytes were incubated with concentrated IbpA at different concentration for 2 h, then infected with *H. somni* strain 129Pt. After 24 h incubation with monocytes the intracellular number of strain 129Pt cells was significantly ($p = 0.05$) greater than for monocytes not incubated with IbpA (Fig. 3.3). Furthermore, the effect of IbpA on the intracellular survival of strain 129Pt was dose-dependent (Fig. 3.3). IbpA significantly ($p = 0.05$) enhanced the intracellular survival of the other *H. somni* strains that also lacked the *ibpA* gene (24P, 1P, 133P, and 133Pf), altough not to the extent as in strain 129Pt (Fig. 3.4). However, the addition of IbpA did not further enhance the intracellular survival of strains 124P and 20P (Fig. 3.4).

3.3.4 Survival of *H. somni ibpA* mutants in M-617 cells

Several mutants with insertion mutations in *ibpA* were selected from a bank of Tn mutants. The capability of the mutants to replicate intracellularly in M-617 cells after 48 h co-incubation was not significantly changed ($P=0.05$), although the intracellular number of mutants was lower than that of the parent strain during the first 24 h (Fig. 3.5). However, none of these mutants contained the tranposon within the Fic domain.

A 2 kbp region at the C-terminus (region 10-12kbp) of *ibpA* contains the Fic domain, which is responsible for the cytotoxicity of IbpA. The transcription of every other 1 kbp region of *ibpA* was examined by reverse transcription polymerase chain reaction (RT-PCR) to determine if the Fic domain was transcribed. As shown in Fig. 3.6, the full length of the *ibpA* gene was transcribed from parent strain 2336. In *ibpA* mutants, the *ibpA* gene was transcribed at the 5' and 3' ends, but not within the area of the Tn insertion (Fig. 3.6).

3.3.5 *H. somni* strains capable of surviving intracellularly can alter intracellular trafficking by M-617 cells

To further clarify the role of IbpA in the capability of *H. somni* to survive intracellularly, two additional Tn mutants, 2336::Tn*luxS* and 2336::Tn*uspE*, which are attenuated and have multiple changes in phenotype (see chapter 2), were also tested for their capability to survive inside monocytes. The survival of mutant 2336::Tn*uspE* intracellularly in M-617 cells was not significantly different from that of the parent strain (P = 0.05). However, the intracellular number of 2336::Tn*luxS* cells was significantly lower than the number of parent strain 2336 cells at 72 h post co-inculation, although 2336::Tn*luxS* was also able to replicate intracellularly. Furthermore, both 2336::Tn*luxS* and 2336::Tn*uspE* were more sensitive to killing by the phagocytic cells during the first 24 h of incubation compared to strain 2336 (Fig. 3.7).

To elucidate the mechanism of the intracellular survival of *H. somni*, we examined the intracellular trafficking of pathogenic strains 2336, commensal strain 129Pt with or without addition of concentrated IbpA in culture media, and the two mutants 2336::Tn*luxS* and 2336::Tn*uspE* by confocal microscopy. As shown in Fig. 3.8, early phagosome marker EEA-1 and late lysosome marker LAMP-2 were both expressed in M-617 and co-localized with strain 129Pt with and without the addition of IbpA (Fig. 3.8). Strain 129Pt-containing phagosomes were also acidified (Fig. 3.8 and 3.11). Therefore, the intracellular trafficking of strain 129Pt in M-617 was not affected by the addition of IbpA. The acidification and expression of LAMP-2 in cells infected with pathogenic strain 2336 were lower and LAMP-2 was not co-localized with the bacteria (Fig. 3.9 and 3.11).

The vaginal isolates used in this study were also capable of surviving in M-617, although many of them are commensal strains. To compare the intracellular trafficking of the commensal strains to pathogenic strain 2336, strain 64Vc were examined with confocal microscopy. Strain 64Vc co-localized with early phagosomal marker EEA-1 10 min after infection, but also inhibited the expression of LAMP-2 and the acidification of the phagosomes (Fig. 3.10).

Like parent strain 2336, mutants 2336::Tn*luxS* and 2336::Tn*uspE* also induced the expression of EEA-1, but inhibited the acidification of the phagosome and the expression of LAMP-2, which is an indication of interference with phago-lysosome maturation (Fig. 3.9). Although mutant 2336::Tn*uspE* was able to replicate intracellularly and interfere with phagosome maturation, its uptake rate after 1 h co-incubation was higher than that of the parent strain (Fig. 3.12). However, the intracellular number of 2336::Tn*uspE* was similar to that of strain 2336 and 2336::Tn*luxS* (data not shown). The change of uptake rate was not observed in mutant 2336::Tn*luxS*. In addition, both mutants changed from cocco-bacilli to long rods or filaments after co-incubated with monocytes for 24 h (Fig. 3.13).

Table 3. 1 *H. somni* trains used in this study.

Strain	Source of isolation	Contains normal ibpA[a]	Serum resistance
2336	pneumonia	+	+
2336::Tn*fhaB* #3	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*fhaB* #13	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*fhaB* #91	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*fhaB* #9	*ibpA* mutant of strain 2336	+	N/A[b]
2336::Tn*fhaB* #27	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*fhaB* #23	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*fhaB* #137	*ibpA* mutant of strain 2336	+	N/A
2336::Tn*luxS*	*luxS* mutant of strain 2336	+	-
2336::Tn*uspE*	*uspE* mutant of strain 2336	+	-
129Pt	prepuce	-	-
1p	prepuce	-	-
130pf	prepuce	-	-
133P	prepuce	-	-
124p	prepuce	+	-
20P	prepuce	+	+
24p	prepuce	+	-
221V	vaginal	+	-
22P	prepuce	+	+

1225	prepuce	N/A	N/A
80	vaginal	+	+
1297	pneumonia	+	+
5166	pneumonia	N/A	N/A
738	pneumonia	+	+
649	abortion	+	+
29Vb	vaginal	+	-
318	phase variation of strain 738	+	+
8025	TME	+	+
2089	abortion	+	+
41Vc	vaginal	+	-
208V	vaginal	+	-
202V	vaginal	+	-
570	abortion	+	-
64Vc	vaginal	+	+

[a]The presence of normal *ibpA* was determined by PCR and the production of IbpA by Western Blotting of the bacteria culture supernatant [19].

N/A – unknown or not tested.

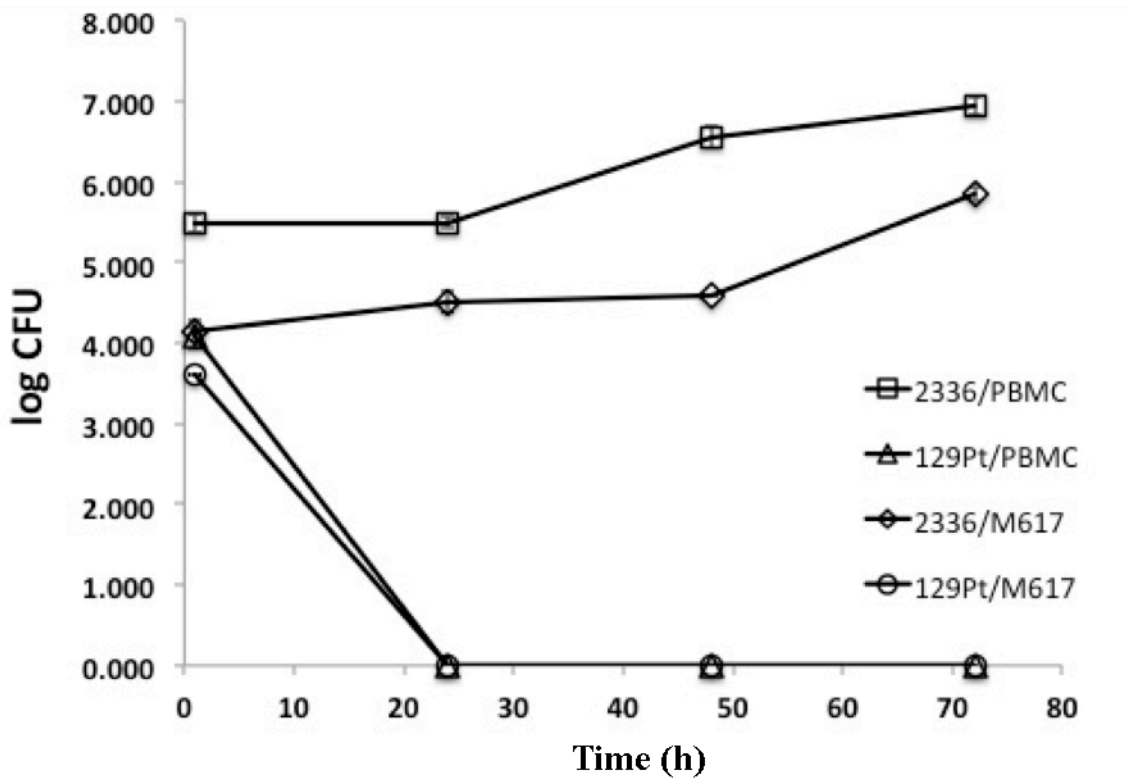

Figure 3. 1 Survival of *H. somni* strain 2336 and 129Pt in PBMC and M-617 monocyte cell line.

* The log CFU of strain 129Pt in both PBMC and M617 were not accurate since 24 h after infection, because concentration of the bacteria was below the countable concentration by colony counting on agar.

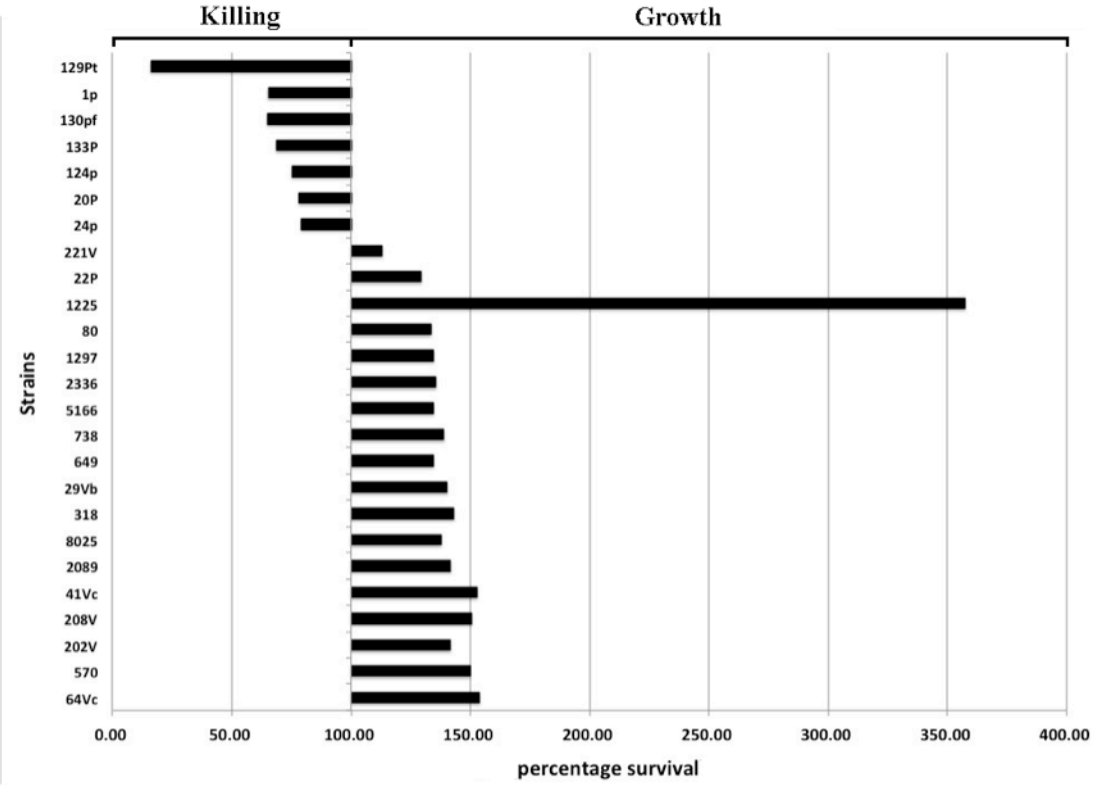

Figure 3. 2 Survival of *H. somni* strains in M-617 monocytes after 24 h co-incubation. The strains on the left of 100% were killed in monocytes; the strains on the right of 100% replicated in the monocytes. Percent survival of *H. somni* was determined by lysis of monocytes after 1 h and 24 h post co-incubation, and culture. The number of colonies recovered after 24 h incubation was divided by the number of colonies after 1 h of incubation.

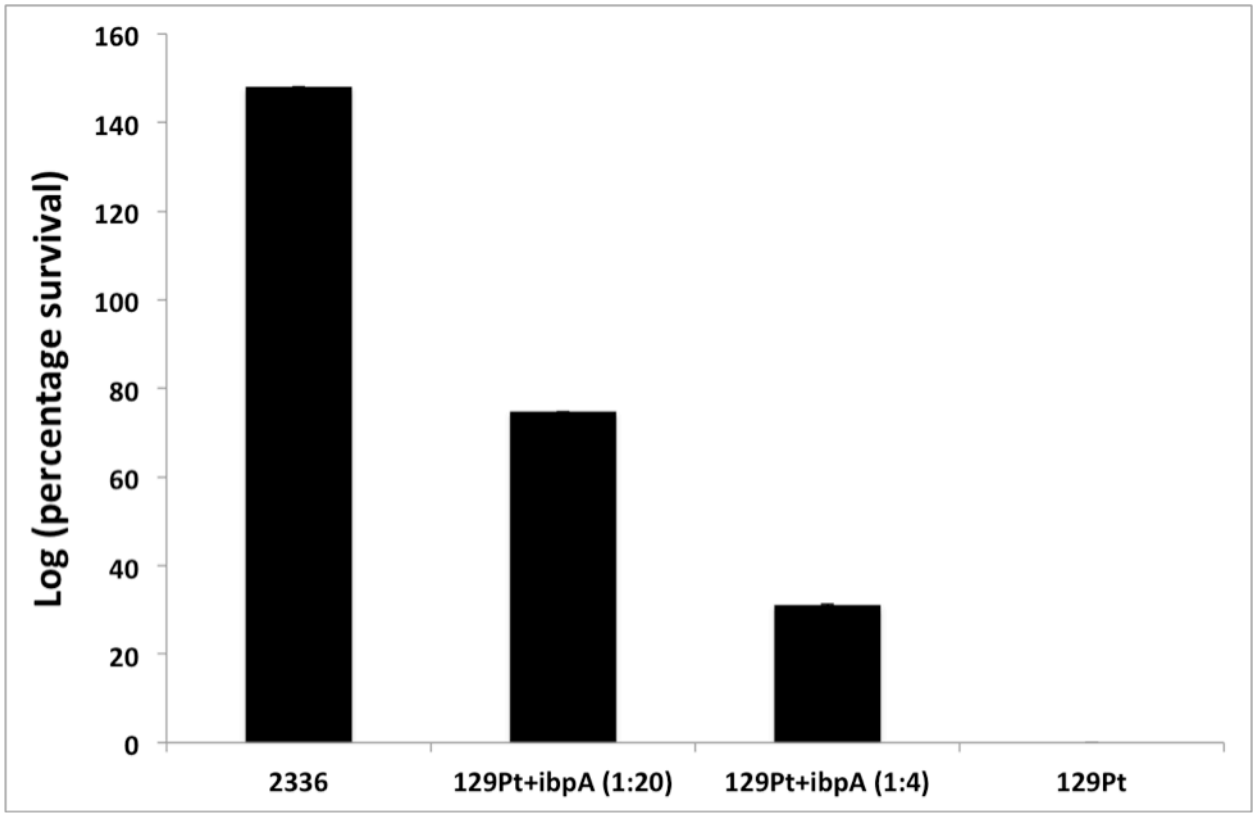

Figure 3. 3 Intracellular survival of *H. somni* 129Pt with and without incubation with IbpA. IbpA was concentrated 20-fold or 4-fold by filtering bacterial culture supernatant.

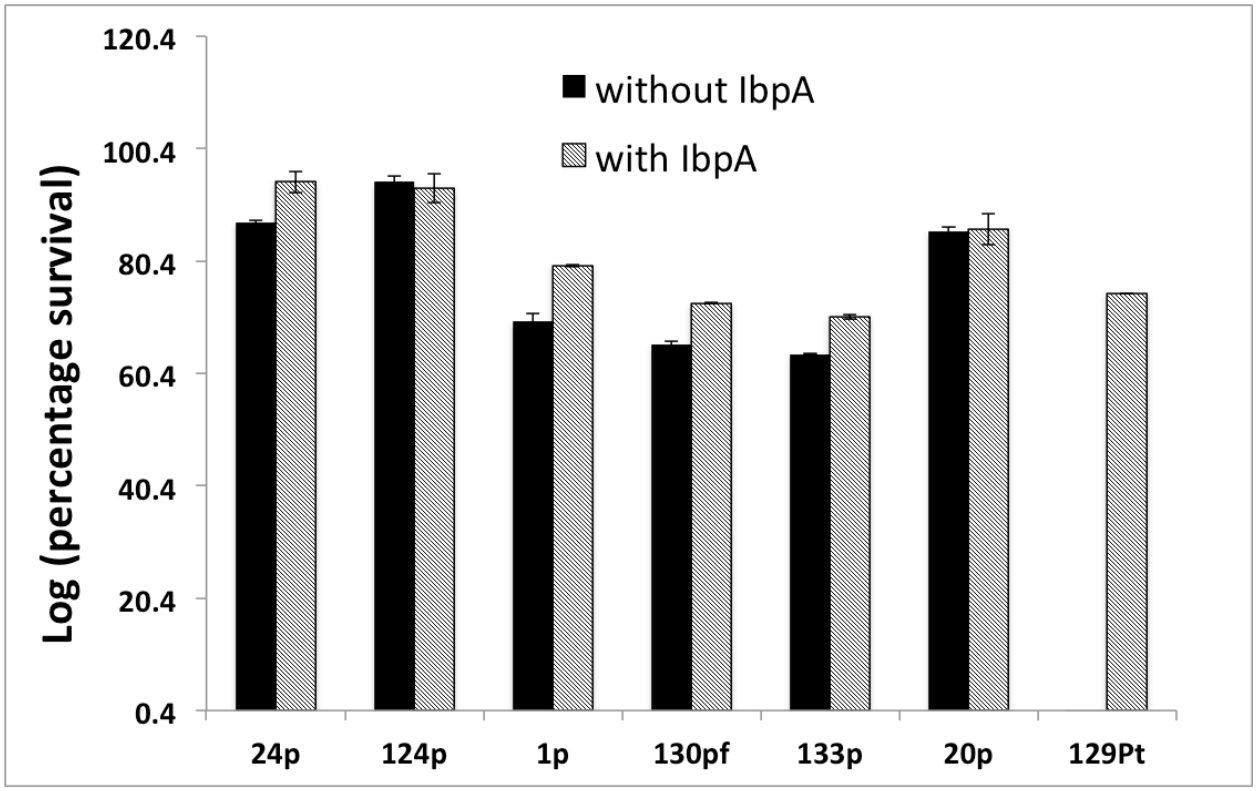

Figure 3. 4 Effect of IbpA on strains sensitive to intracellular killing by M-617 monocytes.

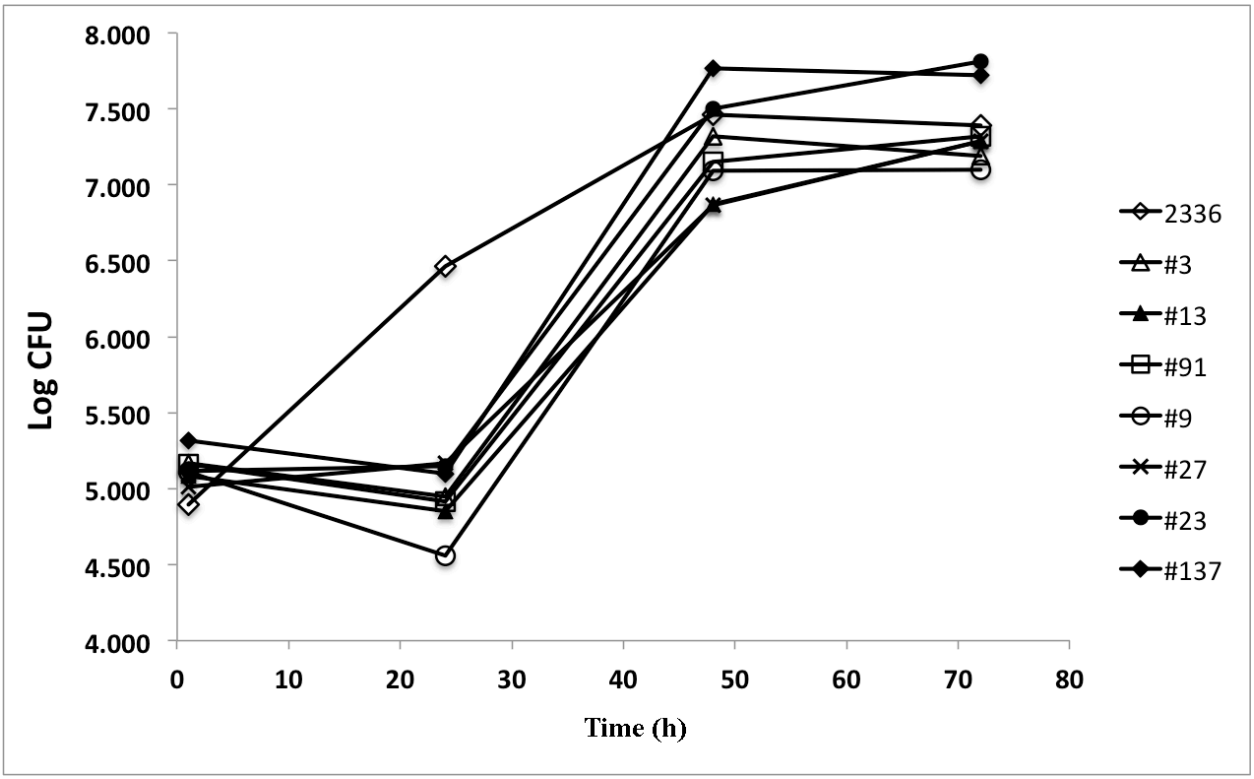

Figure 3. 5 Intracellular survival of *H. somni ibpA* mutants in M-617 monocytes.

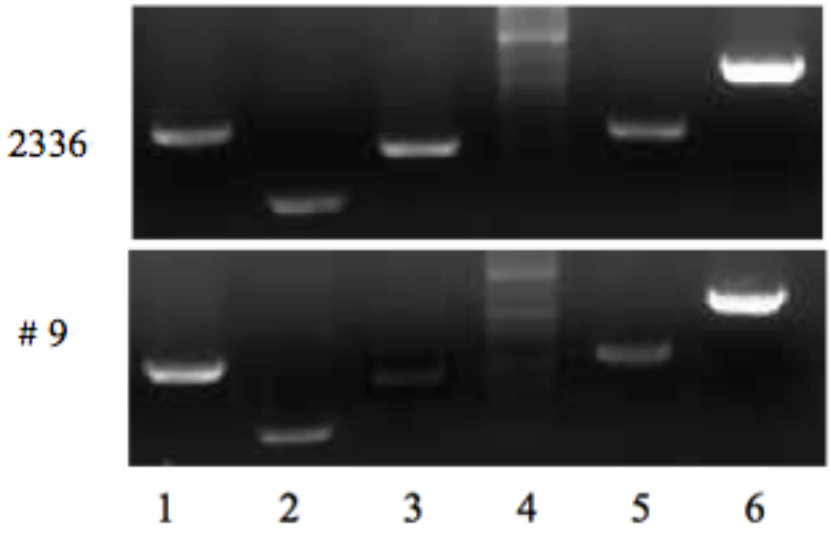

Figure 3. 6 Transcription of different regions of *ibpA* from strain 2336 and #9 *ibpA* mutant by RT-PCR.

Lanes: 1, 1-1000 bp; 2, 2-3 kbp; 3, 4-5 kbp; 4, 6-7 kbp; 5, 8-9 kbp; 6, 10-11 kbp.

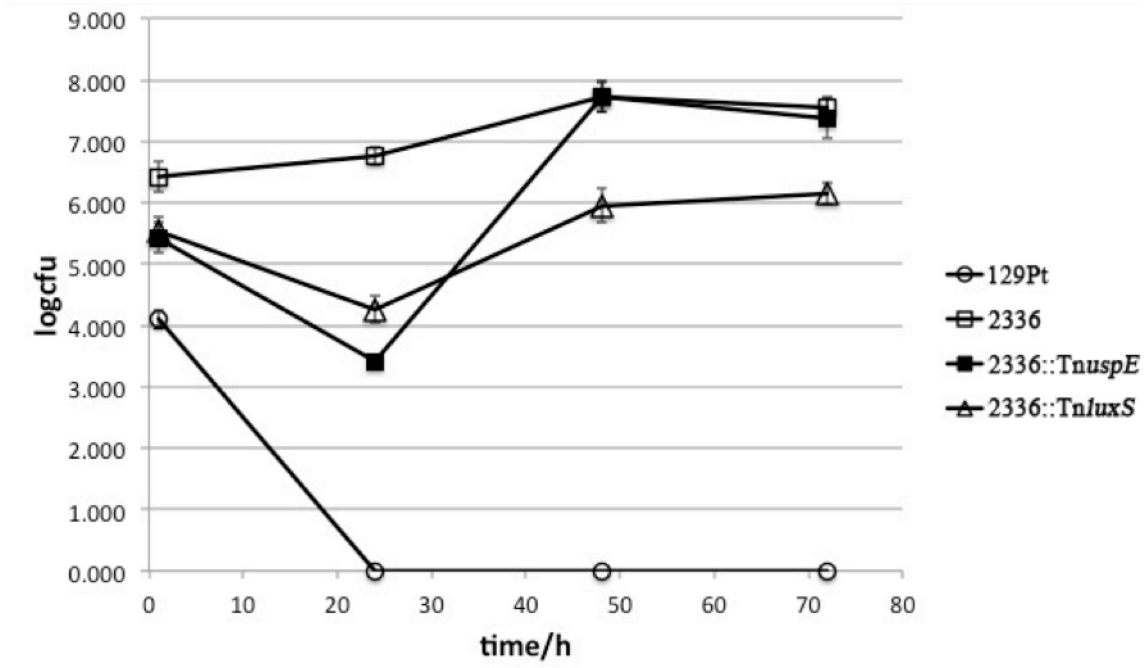

Figure 3. 7 Growth of *H. somni* strains 2336, 129Pt, 2336::Tn*uspE* and 2336::Tn*luxS* in M-617 bovine monocytes.

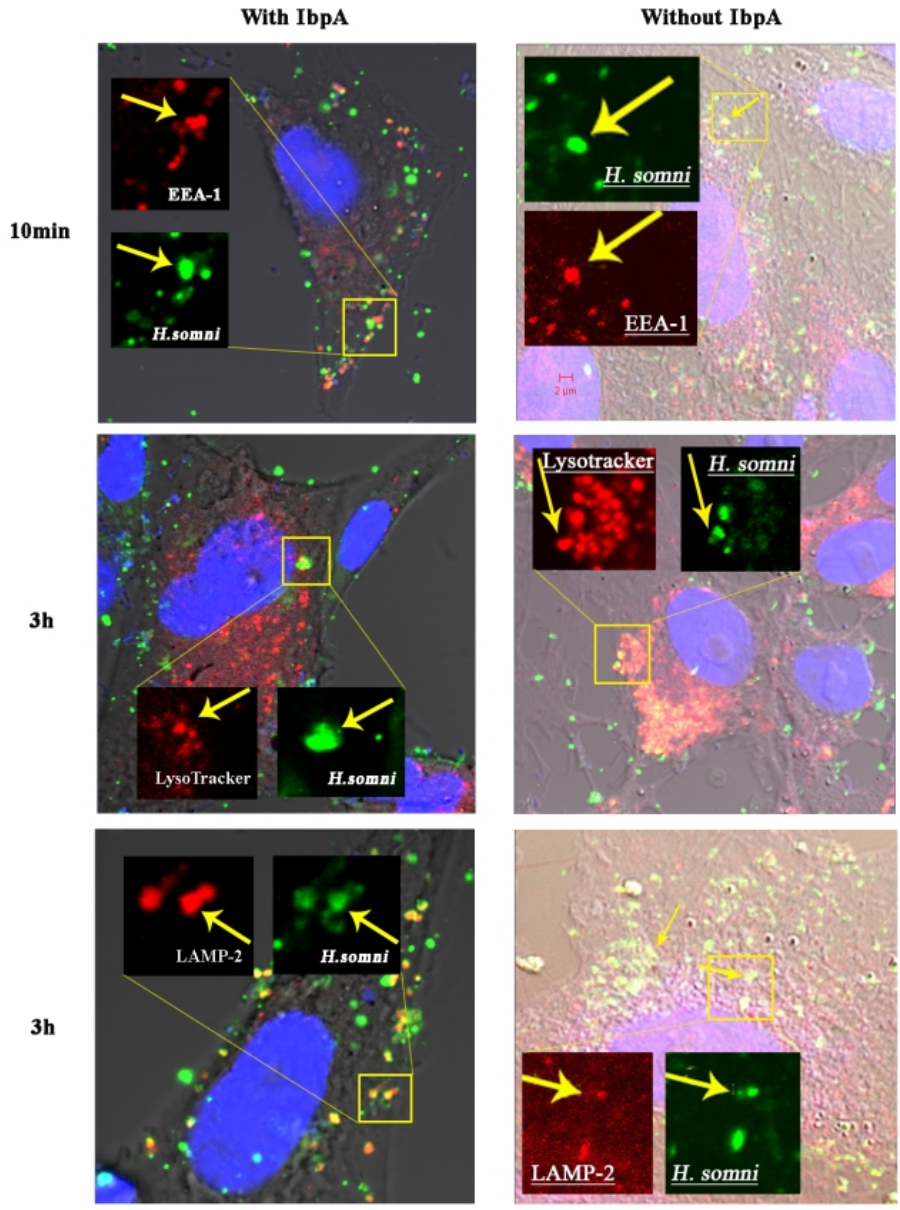

Figure 3. 8 Confocal microscopy of *H. somni* strain 129Pt following phagocytosis with and without addition of IbpA.

The markers were stained red and *H. somni* were stained green. Red arrowheads indicate the markers; the green arrowheads indicate *H. somni*; the yellow arrowheads indicate the colocalization of the markers and *H. somni*.

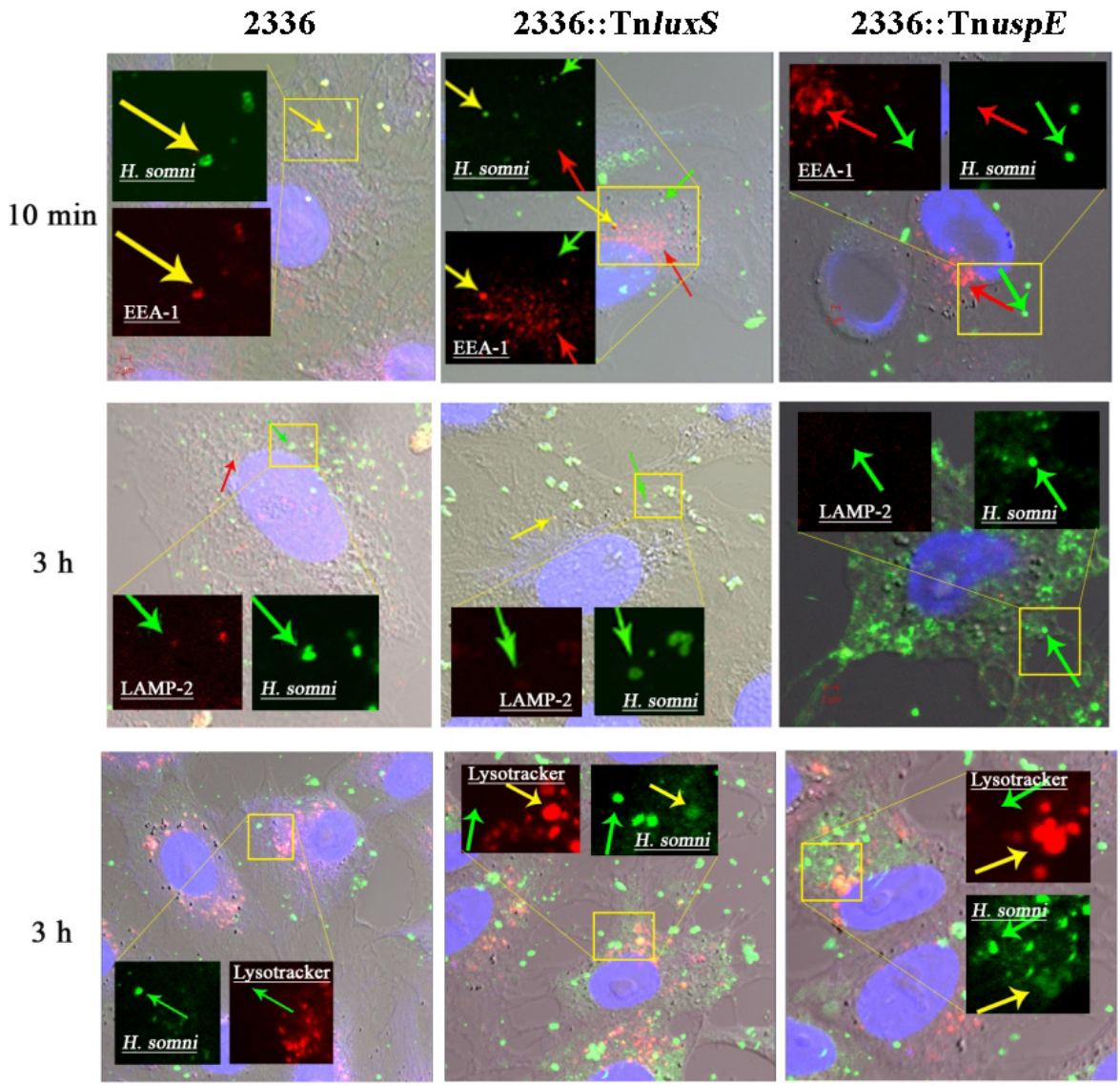

Figure 3. 9 Confocal microscopy of *H. somni* strains 2336, 2336::Tn*uspE*, and 2336::Tn*luxS* following phagocytosis.

The markers were stained red and *H. somni* were stained green. Red arrowheads indicate the markers; the green arrowheads indicate *H. somni*; the yellow arrowheads indicate the colocalization of the markers and *H. somni*.

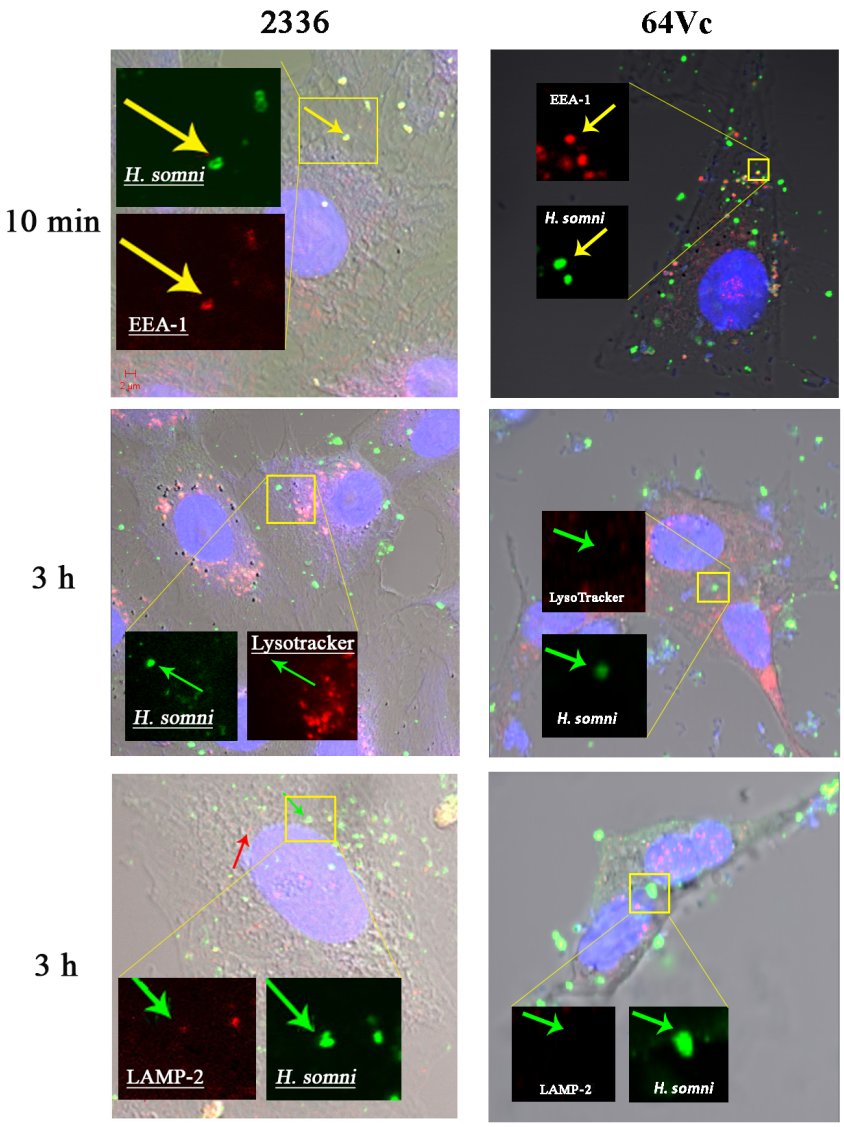

Figure 3. 10 Confocal microscopy of *H. somni* strain 64Vc following phagocytosis.

The markers were stained red and *H. somni* were stained green. Red arrowheads indicate the markers; the green arrowheads indicate *H. somni*; the yellow arrowheads indicate the colocalization of the markers and *H. somni*.

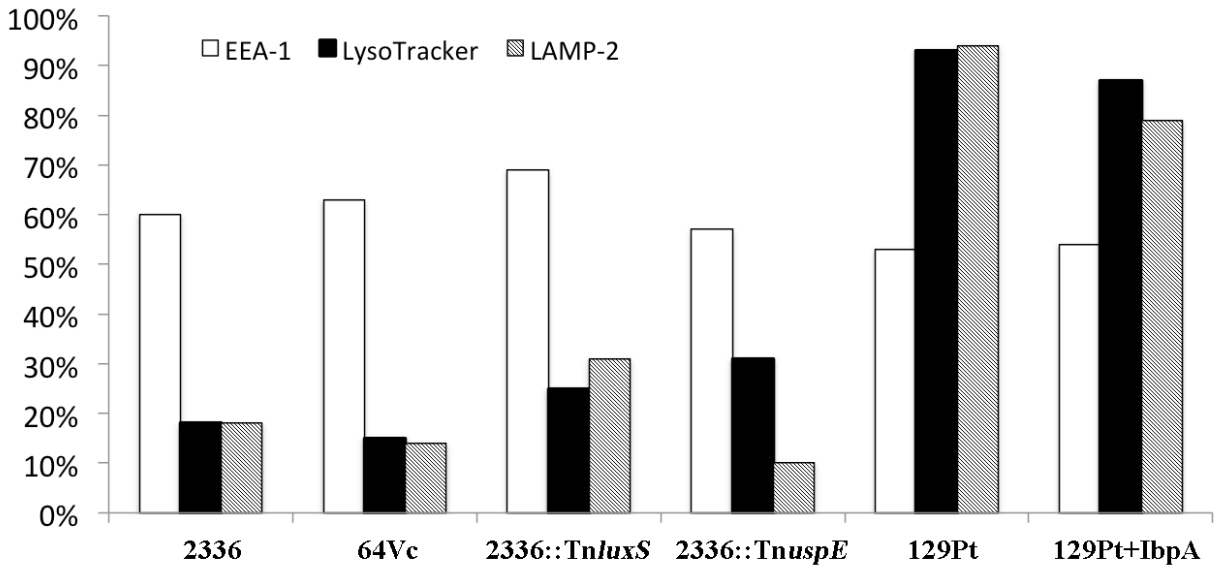

Figure 3. 11 Quantification of *H. somni* strains co-localized with the markers.

The colocalization of *H. somni* with each markers were quantified at different time point: EEA-1 (10 min), LysoTracker/acidification and LAMP-2 (3 h).

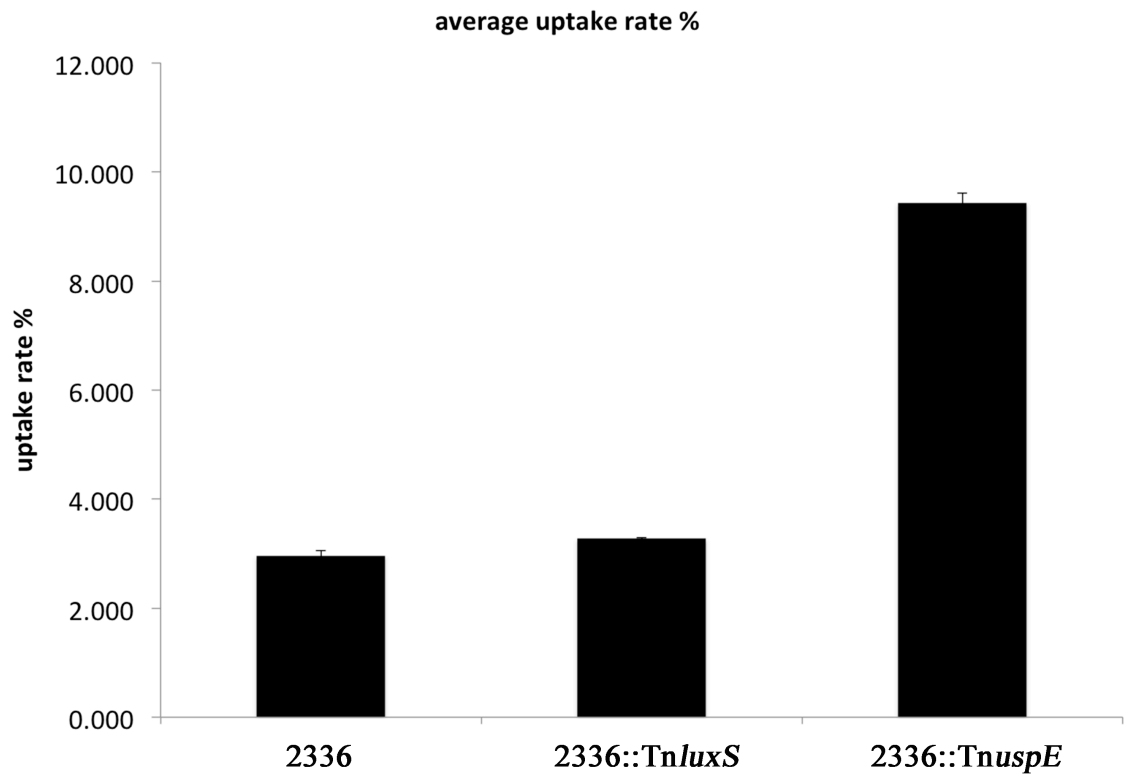

Figure 3. 12 The uptake rate of strains 2336, 2336::Tn*luxS*, and 2336::Tn*uspE* after 1 h co-incubation with M-617 monocytes.

The uptake rate = (number of colonies with gentamicin/number of colonies without gentamicin x100).

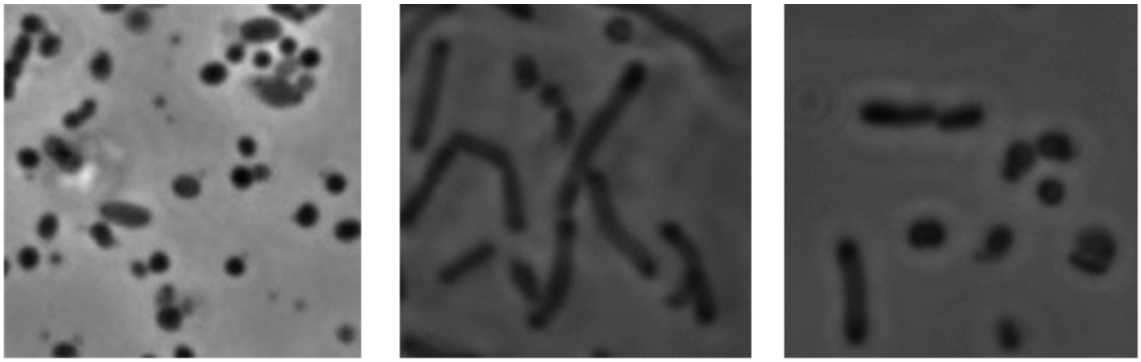

Figure 3. 13 Morphology of strains 2336, 2336::Tn*luxS* and 2336::Tn*uspE* after co-incubation with M-617 monocytes for 24 h.

3.4 Discussion

H. somni can be phagocytosed, but not eliminated by neutrophils, macrophages, and monocytic cells [7, 8]. However, the mechanism of intracellular survival of *H. somni* is not clear. Although *H. somni* can be internalized by monocytes, log phase *H. somni* can inhibit the uptake of opsonized *Straphylococcus aureus* by the host cells. Stationary phase and killed *H. somni* does not have the same inhibitory effect on the phagocytic function of monocytes [10]. However both live and heat-killed *H. somni* can inhibite the uptake of *Straphylococcus aureus* by PMNs, which may be attributed to a surface component of >300,000 MW [9]. Virulent strains of *H. somni* are also capable of inhibiting the oxidative burst of phagocytic cells, but avirulent strain 129Pt cannot [20, 21]. Reactive oxygen intermediates are important components of host immunity against bacteria [22]. Therefore, inhibition of the oxidative burst may be one mechanism that contributes to the resistance of *H. somni* to killing by phagocytic cells. In this study, pathogenic strains and many commensal strains were found capable of replicating intracellularly. However, the preputial isolates were less capable of replicating in monocytes than vaginal isolates. Thus, resistance of strains to killing by phagocytic cells correlates with serum resistance that was previously determined for these strains [19, 23]. Both pathogenic and commensal strains that replicated intracellularly evaded killing by monocytes by interrupting phagosome maturation which may be another mechanism used by *H. somni* to resist elimination by host cells.

Studies on the interaction between *H. somni* and phagocytic host cells have relied on the isolation of primary culture cells. In order to facilitate this research, several immortal cell lines were examined for their ability to kill or not kill susceptible and resistant strains of *H. somni*, respectively. The most commonly used phagocytic cell lines, such as J-774A.1 (mouse) and THP-1 (human), did not efficiently eliminate intracellular *H. somni* strain 129Pt, which is a

commensal strain readily killed by primary isolated PBMC. However, M-617 monocytes killed susceptible *H. somni*, and strain 2336 grew in M-617 monocytes in a manner similar to that of fresh PBMC. M-617 is an established monocyte cell line from blood monocytes of a 6-year-old Guernsey cow. The M-617 cells are esterase-positive and phagocytic, have a normal karyotype, and do not express class II antigens of the major histocompatibility complex [12]. This cell line has been used often in research on bovine parasites [24].

IbpA was initially identified in serum-resistant strains of *H. somni* as a high molecular size outer membrane proteins that bind immunoglobins in a non-antigen specific manner [4]. IbpA has been shown to be cytotoxic to bovine epithelial cells and macrophages [6, 25]. Following incubation with IbpA or strains expressing IbpA, bovine alveolar epithelial cells and macrophges round up, and their actin filaments are disrupted [6, 25]. We sought to determine how *H. somni* survives intracellularly, and if IbpA contributed to bacterial survival through host cell toxicity. The addition of IbpA to monocyte cultures did enhance the intracellular survival of strain 129Pt, and to a lesser extent some other commensal strains of *H. somni*, and did so in a dose-dependent manner. The Fic domain contributes to the cytotoxicity of IbpA [6, 19]. Although several *ibpA* Tn mutants were found to be capable of replicating in M-617 cells, the Tn insertion in these mutants was located at the 5' end of the *ibpA* gene. In contrast, the Fic motif is located at the C-terminus of the protein and was still transcribed. The *ibpA* gene is 12 kbp in length, which is much larger than most bacterial genes. The Tn insertion may have caused a frame shift that created a new start codon in the middle of *ibpA*, or the gene remained in frame enabling Fic to be transcribed. Although the capability of the *ibpA* mutants to suvive intracellularly was not significantly impaired, they are deficient in biofilm formation [13], which may due to insertion of the Tn close to the 5' end of *ibpA*. The N-terminus of IbpA is

homologous with the *Bordetella pertussis* filamentous hemagglutinin (Fha), which contributes to adherence [5, 15], and is the first step in biofilm formation.

Although the addtion of IbpA to culture medium increased the intracellular number of some IbpA-negative strans 24 h after co-incubation, intracellular killing of these strains was not completely abrogated. Moreover, the addition of IbpA to the monocytes before co-incubation with strain 129Pt did not alter the intracellular trafficking of strain 129Pt. Therefore, IbpA may interrupt some essential functions of the phagocytic cells, such as the rearrangement of actin through cytotoxicity [6], but other factors appear to be essential to enable *H. somni* to replicate intracellularly.

Although the Tn mutants 2336::Tn*luxS* and 2336::Tn*uspE* were able to replicate in M-617 monocytes for up to 72 h after infection, the cells of these mutants were elongated after 24 h co-incubation with the monocytes. Elongation of bacterial cells has also been observed with uropathogenic *Escherichia coli* (UPEC), when the bacteria is ready to exit the exfoliating UPEC-containing superficial bladder cells [26]. It is believed that UPEC elongates and is released from the epithelial cell in order to colonize adjacent epithelial cells before its current host epithelial cell exfoliates. Septa can also be seen within the elongated bacteria by electron microscopy, indicating that cell division is involved in elongation [26]. *Streptococcus pneumoniae* can also change its size to resist killing by neutrophils. Instead of elongating, *S. pneumoniae* reduce its chain length to decrease opsonization by antibody and uptake by neutrophils [27]. Agglutination of bacteria by antibodies to promote phagocytosis is a strategy applied by the host when minimization of bacterial size contributes to virulence [28]. Although the contribution of agglutination by antibody in phagocytosis of *H. somni* has not been evaluated, opsonization is known to increase the uptake of *H. somni* by neutrophilis [29]. Therefore, it is possible that both

bacterial size and antibodies may contribute to the intracellular survival of *H. somni*. Further study will be needed to determine if cell elongation contributes to *H. somni* virulence.

The role of intracellular survival in *H. somni* virulence and pathogenesis remains to be determined. It is possible that the cytotoxicity of *H. somni* primarily aids the bacteria to interrupt the epithelial lining of the blood vessel walls or alveolus, enabling them to translocate from the blood stream [25, 30], while other mechanisms, such as inhibition of the oxidative bursts and interruption of phagosome maturation, primarily contribute to intracellular survival.

References

1. Sandal, I., L. Corbeil, and T. Inzana, *Haemophilus.* Pathogenesis of Bacterial Infections in Animals, Fourth Edition, 2010: p. 387-409.
2. Stephens L. R., L.P.B., Humphrey J. D., Wilkie B. N., Barnum D. A., *Vaccination of cattle against experimentally induced thromboembolic meningoencephalitis with a Haemophilus somnus bacterin.* Am J Vet Res, 1982. **43**: p. 1339-1342.
3. Tagawa, Y., J.D. Sanders, I. Uchida, F.D. Bastida-Corcuera, K. Kawashima, and L.B. Corbeil, *Genetic and functional analysis of Haemophilus somnus high molecular weight-immunoglobulin binding proteins.* Microbial Pathogenesis, 2005. **39**(5-6): p. 159-170.
4. Corbeil, L.B., F.D. Bastida-Corcuera, and T.J. Beveridge, *Haemophilus somnus immunoglobulin binding proteins and surface fibrils.* Infection and Immunity, 1997. **65**(10): p. 4250-7.
5. Worby, C.A., S. Mattoo, R.P. Kruger, L.B. Corbeil, A. Koller, J.C. Mendez, B. Zekarias, C. Lazar, and J.E. Dixon, *The Fic Domain: Regulation of Cell Signaling by Adenylylation.* Molecular Cell, 2009. **34**(1): p. 93-103.
6. Hoshinoo, K., K. Sasaki, A. Tanaka, L.B. Corbeil, and Y. Tagawa, *Virulence attributes of Histophilus somni with a deletion mutation in the ibpA gene.* Microbial Pathogenesis, 2009. **46**(5): p. 273-282.
7. Gomis, S.M., D.L. Godson, G.A. Wobeser, and A.A. Potter, *Intracellular survival of Haemophilus somnus in bovine blood monocytes and alveolar macrophages.* Microbial Pathogenesis, 1998. **25**(5): p. 227-235.

8. Czuprynski, C.J. and H.L. Hamilton, *Bovine neutrophils ingest but do not kill Haemophilus somnus in vitro.* Infection and Immunity, 1985. **50**(2): p. 431-436.

9. Hubbard, R.D., M.L. Kaeberle, J.A. Roth, and Y.W. Chiang, *Haemophilus somnus, Induced interference with bovine neutrophil functions.* Veterinary Microbiology, 1986. **12**(1): p. 77-85.

10. Gomis, S.M., D.L. Godson, T. Beskorwayne, G.A. Wobeser, and A.A. Potter, *Modulation of phagocytic function of bovine mononuclear phagocytes byHaemophilus somnus.* Microbial Pathogenesis, 1997. **22**(1): p. 13-21.

11. Inzana, T.J. and L.B. Corbeil, *Development of a defined medium for Haemophilus somnus isolated from cattle.* Am J Vet Res, 1987. **48**(3): p. 366-9.

12. Speer, C.A., D.W. Reduker, D.E. Burgess, W.M. Whitmire, and G.A. Splitter, *Lymphokine-induced inhibition of growth of Eimeria bovis and Eimeria papillata (Apicomplexa) in cultured bovine monocytes.* Infect. Immun., 1985. **50**(2): p. 566-571.

13. Sandal, I., J.Q. Shao, S. Annadata, M.A. Apicella, M. Boye, T.K. Jensen, G.K. Saunders, and T.J. Inzana, *Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge.* Microbes and Infection, 2009. **11**(2): p. 254-263.

14. Sandal, I., M.N. Seleem, S.F. Elswaifi, N. Sriranganathan, and T.J. Inzana, *Construction of a high-efficiency shuttle vector for Histophilus somni.* Journal of microbiological methods, 2008. **74**(2): p. 106-109.

15. Geertsema, R., B. Zekarias, L. Scheuch, C. Worby, R. Russo, L. Gershwin, D. Herdman, K. Lo, and L. Corbeil, *IbpA DR2 subunit immunization protects calves against Histophilus somni pneumonia.* Vaccine, 2011. **29**(29): p. 4805-4812.

16. Guberman, J.M., A. Fay, J. Dworkin, N.S. Wingreen, and Z. Gitai, *PSICIC: noise and asymmetry in bacterial division revealed by computational image analysis at sub-pixel resolution.* PLoS computational biology, 2008. **4**(11): p. e1000233.

17. Delrue, R.M., C. Deschamps, S. Léonard, C. Nijskens, I. Danese, J.M. Schaus, S. Bonnot, J. Ferooz, A. Tibor, and X. De Bolle, *A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of Brucella melitensis.* Cellular microbiology, 2005. **7**(8): p. 1151-1161.

18. Woo, S.-R., J. Sotos, A.P. Hart, R.G. Barletta, and C.J. Czuprynski, *Bovine monocytes and a macrophage cell line differ in their ability to phagocytose and support the intracellular survival of Mycobacterium avium subsp. paratuberculosis.* Veterinary immunology and immunopathology, 2006. **110**(1): p. 109-120.

19. Zekarias, B., D. O'Toole, J. Lehmann, and L. Corbeil, *Histophilus somni IbpA Fic cytotoxin is conserved in disease strains and most carrier strains from cattle, sheep and bison.* Veterinary microbiology, 2011. **149**(1): p. 177-185.

20. Gomis, S.M., D.L. Godson, G.A. Wobeser, and A.A. Potter, *Effect of Haemophilus somnus on nitric oxide production and chemiluminescence response of bovine blood monocytes and alveolar macrophages.* Microbial pathogenesis, 1997. **23**(6): p. 327-333.

21. Howard, M.D., J.H. Boone, V. Buechner-Maxwell, G.G. Schurig, and T.J. Inzana, *Inhibition of bovine macrophage and polymorphonuclear leukocyte superoxide anion production by Haemophilus somnus.* Microbial Pathogenesis, 2004. **37**(5): p. 263-271.

22. Beaman, L. and B.L. Beaman, *The role of oxygen and its derivatives in microbial pathogenesis and host defense.* Annual Reviews in Microbiology, 1984. **38**(1): p. 27-48.

23. Corbeil, L.B., K. Blau, D.J. Prieur, and A. Ward, *Serum susceptibility of Haemophilus somnus from bovine clinical cases and carriers.* Journal of clinical microbiology, 1985. **22**(2): p. 192-198.

24. Tunev, S., M. McAllister, R. Anderson-Sprecher, and L. Weiss, *Neospora caninum in vitro: evidence that the destiny of a parasitophorous vacuole depends on the phenotype of the progenitor zoite.* Journal of Parasitology, 2002. **88**(6): p. 1095-1099.

25. Zekarias, B., S. Mattoo, C. Worby, J. Lehmann, R.F. Rosenbusch, and L.B. Corbeil, *Histophilus somni IbpA DR2/Fic in virulence and immunoprotection at the natural host alveolar epithelial barrier.* Infection and immunity, 2010. **78**(5): p. 1850-1858.

26. Mulvey, M.A., J.D. Schilling, and S.J. Hultgren, *Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection.* Infection and immunity, 2001. **69**(7): p. 4572-4579.

27. Dalia, A.B. and J.N. Weiser, *Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody.* Cell host & microbe, 2011. **10**(5): p. 486-496.

28. Ax, W., E.-J. Kanzy, and F. Seiler, *In-vivo phagocytosis: enhancement of bacterial clearance by native and enzyme-treated immunoglobulins.* Immunobiology, 1981. **159**(4): p. 349-365.

29. Siddaramppa, S. and T.J. Inzana, *Haemophilus somnus virulence factors and resistance to host immunity.* Animal Health Research Reviews, 2004. **5**(01): p. 79-93.

30. Patrick, D., J. Betts, E.A. Frey, R. Prameya, K. Dorovini-Zis, and B.B. Finlay, *Haemophilus influenzae lipopolysaccharide disrupts confluent monolayers of bovine brain endothelial*

cells via a serum-dependent cytotoxic pathway. Journal of Infectious Diseases, 1992. **165**(5): p. 865-872.

Chapter 4

Detection of antibodies to the biofilm exopolysaccharide of *Histophilus somni* following infection in cattle by enzyme-linked immunosorbent assay

Pan, Yu, Taylor Fisher, Christina Olk, and Thomas J. Inzana. "Detection of antibodies to the biofilm exopolysaccharide of *Histophilus somni* following infection in cattle by enzyme-linked immunosorbent assay." *Clinical and Vaccine Immunology* 21, no. 10 (2014): 1463-1467.

Abstract

An enzyme-linked immunosorbent assay was developed to detect bovine antibodies to the *Histophilus somni* exopolysaccharide that is formed during biofilm formation. When an index value of 0.268 was used the sensitivity of the assay for experimentally- and naturally-infected calves was 90.5% at 3 weeks post-infection, but the number of positive animals increased by week 4 and later as the infection persisted. The specificity of the assay for healthy calves was 92.5%. The EPS ELISA may aid in identifying calves with diseases due to *H. somni*.

4.1 Introduction

Histophilus somni is associated with respiratory and systemic diseases in cattle and sheep. Although *H. somni* is commonly present in the genital and respiratory tracts, it is capable of causing diseases such as thrombotic meningoencephalitis, pneumonia, bacteremia, abortion, arthritis, myocarditis, mastitis, and infertility [1] . Serum samples from healthy cattle may have positive titers to *H. somni* because this opportunistic pathogen may be part of the normal flora, because of previous subclinical or clinical infection, or because of cross-reactive antibodies with other bacteria. Cross reactivity is especially a problem with agglutination tests because cross-reactive IgM plays a major role in this assay [2, 3]. Thus, currently used serological assays are not adequately sensitive or specific for *H. somni* diagnosis [4, 5]. Therefore, definitive diagnosis of *H. somni* infection relies primarily on isolation of the bacterium. Although multiplex polymerase chain reaction (PCR) assays have been developed for diagnosis of *H. somni* [6, 7], serological diagnosis would still be useful, as most laboratories can accommodate antibody detection formats that are rapid and inexpensive, such as ELISA.

Exopolysaccharide (EPS) is a major component of the *H. somni* biofilm matrix, but is also produced under growth-restricting stress conditions [8], which are likely to occur during the disease process as well as colonization of the mucosal epithelia such as in the vagina and prepuce. Furthermore, *H. somni* is known to form a biofilm and produce abundant EPS in cardio-pulmonary tissue during the disease process [9]. Whether biofilms are formed by *H. somni* in the carrier state on preputial, vaginal, or upper respiratory epithelium has not been determined. We have purified the EPS from *in vitro* biofilm growth, and the purified EPS can induce production of antigen-specific antibodies [8]. Therefore, we sought to identify the presence of antibodies to EPS in healthy cattle and in experimentally- and naturally-infected cattle, and if the EPS could

be used in a direct enzyme-linked immunosorbent assay (ELISA) for serological differentiation of animals with *H. somni* disease from healthy animals that may carry this opportunistic pathogen.

4.2 Materials and Methods

H. somni pathogenic strains 738 or 7735 were grown on Brain Heart Infusion (BHI) agar with 5% sheep blood in 5% CO_2 overnight. The colonies were transferred to BHI broth supplemented with 1% yeast extract, 0.1% Trizma base and 0.01% thiamine monophosphate (TMP) [10]. The bacteria were grown at 37 °C with rapid shaking (200 rpm) to mid-log phase. To grow the cells as a biofilm, an aliquot of bacteria at mid-log phase was transferred to a 1-L bottle filled with Columbia broth supplemented with 1% glucose, 0.1% Trizma base, and 0.01% TMP, and grown at 37 °C with slow shaking (50 rpm) for 5 days [11]. EPS was purified from the 1-L culture of *H. somni* strain 738 grown as a biofilm as previously described [8]. Briefly, the top ~900 mls of medium was removed and the EPS was extracted from the biofilm sediment with 45% phenol, dialyzed, digested with DNase, RNase, and proteinase K, and any lipooligosaccharide (LOS) removed by ultracentrifugation at 125,000 × *g*. The EPS was confirmed to be free of LOS by chemical analysis [8] and silver staining following electrophoresis [1]. The EPS in the supernatant was precipitated with excess 95% ethanol containing 30 mM sodium acetate and incubated at -20 °C overnight. Insoluble material was recovered by centrifugation, and the pellet rinsed with 70% ethanol, suspended in distilled water, and lyophilized.

Blood samples were obtained from all healthy cattle available at the time (forty-nine) with no history of respiratory disease, and cattle experimentally- (fifteen) and naturally-infected (four) with *H. somni*. Some experimentally-infected 8-12 week old calves were infected with 10^7 colony forming units (CFU) of *H. somni* intrabronchially, as previously described [12]. The passive transfer status of challenged calves was not determined, but all had been weaned at least two weeks prior to challenge. Most healthy cattle were 6 months of age or older. Weekly serum

samples were obtained from five challenged calves weekly until bronchoalveolar lavage (BAL) specimens were culture-negative or until the experiment was terminated at 10 weeks post-infection. Normal respiratory tract bacteria were not isolated from BAL specimens, indicating *H. somni* isolates recovered from BAL were not part of the normal upper respiratory tract flora. Random serum samples were also obtained from ten additional ~12-week-old male Holstein calves that were experimentally-challenged with an attenuated strain of bovine herpes virus 1 three days before challenge with 3 x 10^9 to 10^{10} CFU of *H. somni* strain 7735 intranasally and intrabronchially. The prechallenge was used to stress the animals and to suppress the innate immune response. Without prior pre-challenge, the animals become colonized, but do not develop disease [13]. These pre-challenged, stresseed animals developed clinical symptoms of pneumonia. Post-mortem examination revealed that the calves had varying degrees of purulent bronchopneumonia with abscesses and multifocal purulent myocarditis. A more complete description of the pathology of each calf has been described [13]. Some calves (11, 13, 161, and 565) were also treated with 0.1 mg/kg/day of dexamethazone 4 days prior to challenge to further suppress innate immunity. All procedures that involve experimental use of animals in research at Virginia Tech are reviewed by The University Animal Care and Use Committee to assure humane care and treatment of animals. The University Animal Welfare Assurance # on file with the Public Health Service Office of Laboratory Animal Welfare is A-3208-01, expiration date 7-31-17. Sera were also obtained from four naturally-infected cattle from a local farm experiencing an outbreak of *H. somni* disease. The animals were clinically ill with pneumonia due to *H. somni*, which was confirmed by culture of lung specimens from animals that had died. Serum prepared from the blood was stored at -20 °C until tested. The sources of all sera are shown in Table 1.

Microtiter plates (Immulon 4 HBX-Extra High Binding plates, Thermo Fisher Scientific Inc., Waltham, MA) were coated overnight with 10 μg/ml of purified EPS (0.1 ml per well) at room temperature in carbonate-bicarbonate buffer. The ELISA was modified from a previously described protocol [5]. Briefly, the microtiter plates were washed with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (washing buffer) three times, blocked with 5% nonfat dry milk and 5% goat serum in PBS at 37 °C for 1 h, and incubated with the serum samples diluted 1:100 at 4 °C overnight. After washing the plates 3 times with washing buffer, goat anti-bovine IgG, H&L chain, (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) conjugated to horseradish peroxidase (diluted 1:1000) was added and the plates incubated at 37 °C for 1 h. The color was developed with TMB Substrate Reagent (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), as described by the manufacturer. Color development was stopped by adding 100 μl of 2 N sulfuric acid, and the optical density (OD) at 450 nm (A_{450}) was determined from 3 replicates of each sample. Sera from cattle immunized with purified EPS were used as a positive control. Sera from healthy calves with no evidence or history of disease due to *H. somni* were used as negative controls. All serum samples included a control lacking antigen to control for binding to nonspecific serum components, such as IgM, or to skim milk in the blocking buffer. A control lacking the enzyme conjugate was also included with the positive control serum to EPS. Control background absorbances were subtracted from test results, and were generally 0.1 A_{450} or less.

The OD of all samples was transformed into a sample index by the formula: Sample index = (Sample OD – N)/(P – N), where N is the average of three optical density (OD) values for normal calf sera (negative controls), and P is the average of three OD values of anti-strain 738 EPS calf serum (positive control). The optimal cutoff value was determined by the two-

graph receiver operating characteristic (TG-ROC) [14]. Briefly, the sample indexes (ranging from -0.086 to 1.091, and increments of 0.07) were plotted against the OD from sera of known positive animals (challenged) and negative animals (healthy; no history of *H. somni* disease) in a TG-ROC graph. The point at which the sensitivity and specificity curves crossed was considered the optimal cutoff value. Normal probability plots showed that sample indexes followed an approximately normal distribution. Subsequently, a student t test was used to compare confirmed positive samples with confirmed negative samples irrespective of clinical group at 3 weeks of follow-up. A p value <0.05 was considered significant. The sensitivity and specificity of the assay at the sample index cutoff value was calculated as: sensitivity = (number of true positive samples/number of true positive samples + number of false negative samples) x 100, and specificity = (number of true negative samples/ number of true negative samples + number of false positive samples) x 100.

4.3 Results

As shown in Fig. 1, sera from pre-infected healthy calves (week 0) had low reactivity to EPS. After experimental challenge the level of reactivity to *H. somni* EPS gradually increased during the first few weeks of infection, but then decreased. However, IgG antibody levels to whole cells remained elevated through at least 10 weeks postchallenge (data not shown). There was variation between individual animals in the production of antibody to EPS. For calves E5 and E7, the infection was cleared by 6 weeks post-challenge, and antibody levels to EPS were at their maximum during weeks 3-4. The infection persisted until 10 weeks post-challenge for calf 93, but calves 94 and 95 remained culture-positive at 10 weeks when the experiment was terminated. The anti-EPS titer of calves 93, 94, and 95 also peaked later than for the 2 animals that cleared the infection earlier. The titer of antibody produced in response to the infection also varied. The antibody response of calves E5, E7, and 95 were similar, except that antibody titers peaked a few weeks later in calf 95, compared to E5 and E7. Calf 93 responded with a stronger antibody response to EPS that peaked later than that of the other calves. The antibody response by calf 94 was lower than that of the other calves, and was not clearly positive until 7 weeks post-infection. These differences are likely due to variation between individual animals, and/or the course or severity of the infection. Although weekly serum samples were not obtained from the other 10 challenged animals in the study, the time-variable but consistent EPS responses of the individual animals shown in Fig. 1 appear to be representative of the other challenged animals. For each of these five calves, a peak index was determined as the highest index obained during follow up. The peak index was 0.495 at week 3 for E5, 0.363 at week 3 for E7, 0.748 at week 8 for calf 93, 0.289 at week 7 for calf 94 and 0.513 at week 6 for calf 95. Differences between the peak index values and that of the pre-inoculation sera also followed an

approximately normal distribution and were significantly different from zero (paired t test $p<0.05$). The results indicated that antibodies to *H. somni* EPS increased after clinically symptomatic infection, but remain at low levels in healthy animals.

To evaluate the ELISA test as a diagnostic tool, additional serum samples from healthy, and naturally- and experimentally-infected calves were tested for all weeks available. The sample index value determined by TG-ROC analysis to distinguish healthy from infected cattle was 0.268 at 3 weeks post-infection (Fig. 2 and Table 2). An infectious disease diagnosis can be made if a documented 4-fold or greater rise in specific antibody titer is determined between acute and convalescent serum samples. Such a rise in titer normally takes 10-14 days. Serum samples obtained at clinical evaluation (acute) may actually be at least 1 week after initial infection, and convalescent serum obtained at least 2 weeks later would be at least 3-4-weeks post-infection. Therefore, the antibody response in the experimentally-infected animals in this study at 3-4 weeks post-infection would be similar in timing to naturally infected animals when convalescent serum is taken at least 2-weeks after initial evaluation. At 3 weeks post-infection (the only time for which all samples were available) the sensitivity and specificity of the assay was 90.5% and 92.5%, respectively. However, of the two calves that were ELISA-negative at 3 weeks post-challenge, 1 became positive at 4 weeks post-challenge. The area under the ROC curve was 93.7% at 3 weeks post-infection, which indicates a high degree of diagnostic accuracy. Sera from naturally-infected, clinically ill animals were collected about 2 weeks after culture confirmation (likely at least 3-4 weeks post-infection), and all were strongly positive.

Table 4. 1 Reactivity of antibodies to *H. somni* EPS in sera from uninfected, naturally-infected, and experimentally-infected calves by ELISA compared to culture.

Infection	Calf #	Culture Confirmation[a]	ELISA Reactivity[b]	Sample Index
Uninfected or Prechallenged[c]				
	9-14	-	-	0.159
	118	-	+	0.286
	617	-	-	0.008
	6-24	-	-	0.073
	112	-	-	0.115
	10-21	-	-	0.200
	113	-	-	0.098
	09-11	-	-	0.048
	05-1	-	-	0.257
	08-2	-	-	0.174
	106	-	-	0.198
	100	-	-	0.040
	08-22	-	-	0.202
	105	-	-	0.020
	01-03	-	-	-0.046
	107	-	-	-0.035
	09-23	-	-	0.183
	117	-	-	0.199

598	-	-	0.152
957	-	-	0.031
674	-	-	0.253
627	-	-	0.201
293	-	+	0.673
4600	-	-	0.248
4645	-	-	0.119
680	-	-	0.025
623	-	-	0.186
629	-	-	0.160
921	-	-	0.064
4766	-	-	-0.027
494	-	-	0.282
736	-	-	0.052
4771	-	+	0.433
475	-	+	0.510
E5 R85/89 (0 weeks)[d]	-	-	-0.023
E7 R85/89 (0 weeks)	-	-	0.030
R86-93 (0 weeks)	-	-	0.148
R86-94 (0 weeks)	-	-	-0.086
R86-95 (0 weeks)	-	-	-0.053
565 (0 weeks)	-	-	0.003
670 (0 weeks)	-	-	0.246

104 (0 weeks)	-	-	0.091
13 (0 weeks)	-	-	0.129
12 (0 weeks)	-	-	0.268
494-1 (0 weeks)	-	-	0.189
11 (0 weeks)	-	-	0.178
161 (0 weeks)	-	-	0.172
10 (0 weeks)	-	-	0.028
167 (0 weeks)	-	-	0.024
Experimentally- Infected[e]			
R86-94 (3 wks)	+	-	0.047
R86-94 (4 wks)	+	-	0.122
R86-94 (7 wks)	+	+	0.289
R86-93 (3 wks)	+	+	0.278
R86-93 (4 wks)	+	+	0.346
R86-93 (7 wks)	+	+	0.617
E7 R85/90 (3 wks)	+	+	0.363
E7 R85/90 (4 wks)	+	-	0.250
E5 R85/89 (3 wks)	+	+	0.495
E5 R85/89 (4 wks)	+	+	0.482
R86-95 (3 wks)	+	-	0.242
R86-95 (4 wks)	+	+	0.317
R86-95 (7 wks)	+	+	0.469
565 (3 wks)	+	+	0.636

670 (3 wks)	+	+	0.828
104 (3 wks)	+	+	0.316
13 (3 wks)	+	+	1.031
12 (3 wks)	+	+	0.764
494 (3 wks)	+	+	0.980
11 (3 wks)	+	+	0.705
161 (3 wks)	+	+	0.780
10 (3 wks)	+	+	0.812
167 (3 wks)	+	+	0.846
Naturally Infected			
580	+	+	0.734
155	+	+	0.586
563	+	+	1.091
588	+	+	0.748

[a]Confirmed diagnosis is based on clinical symptoms and isolation of *H. somni* from tissues.

[b]Result from this ELISA.

[c]Uninfected cattle were healthy, but asymptomatic carriage of *H. somni* was not determined. Prechallenged cattle were culture-free of *H. somni* in the upper respiratory tract.

[d]Serum collected at 0 weeks was obtained just prior to experimental challenge.

[e]The number of weeks post-infection when the sera were collected is shown in parentheses.

Table 4. 2 Sensitivity, specificity, and the TG-ROC analysis results

ELISA	Definitive Diagnosis		Youden Index		Area Under the	Sensitivity	Specificity
Diagnosis[a]	Positive	Negative	Youden Index J	Optimal Cut-off	ROC Curve	95% CI	95% CI
Positive	17	4					
Negative	2	45	88.61%	> 0.268	93.75%	66.9 -98.7%	77.8 – 96.6%

[a]Data included in Table 1 from 3 weeks post-infection

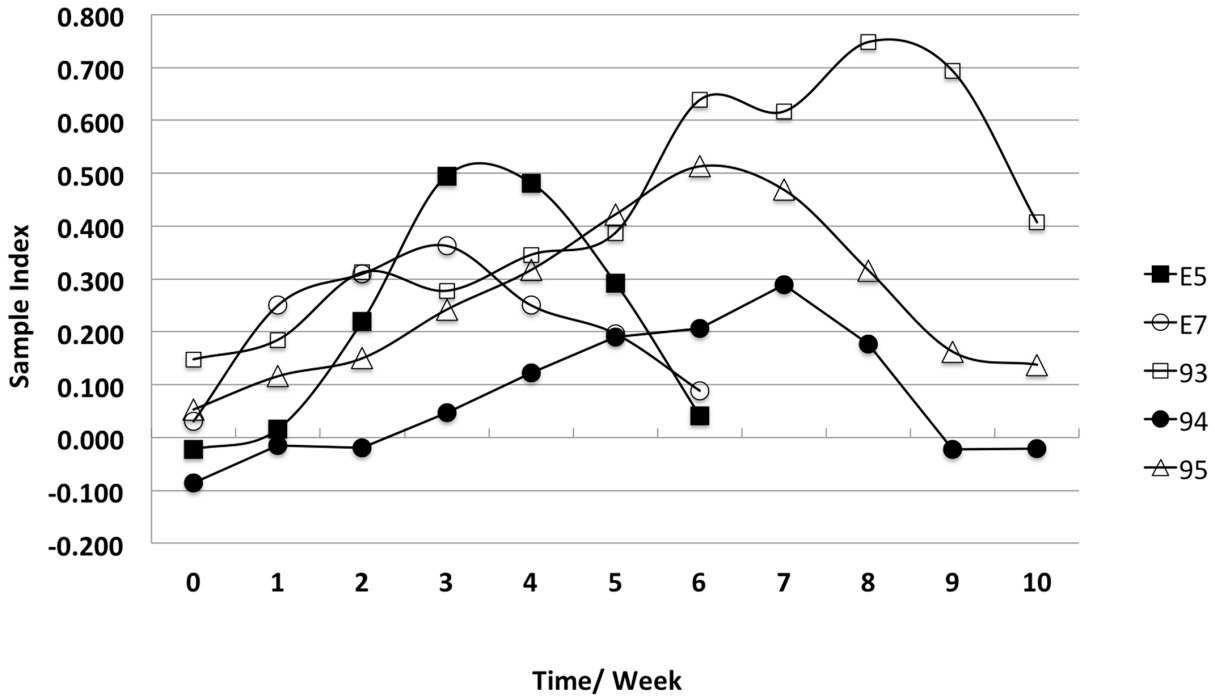

Figure 4. 1 Antibody responses to EPS from 5 experimenally-infected calves (E5, E7, 93, 94, and 95).

Sera were initially collected at week 0, just prior to challenge. Blood samples were obtained weekly until bronchial lavage cultures were negative or until the experiment was terminated (10 weeks post-infection). In calves E5 and E7 the infection was cleared by 6 post-challenge, and therefore no additional serum samples were collected after 6 or 7 weeks, respectively. In calves 94 and 95 the animals were still culture-positive at 10 weeks post-challenge, but calf 93 had cleared the infection at week 10 post-challenge. The IgG (H&L chain) response to EPS was measured.

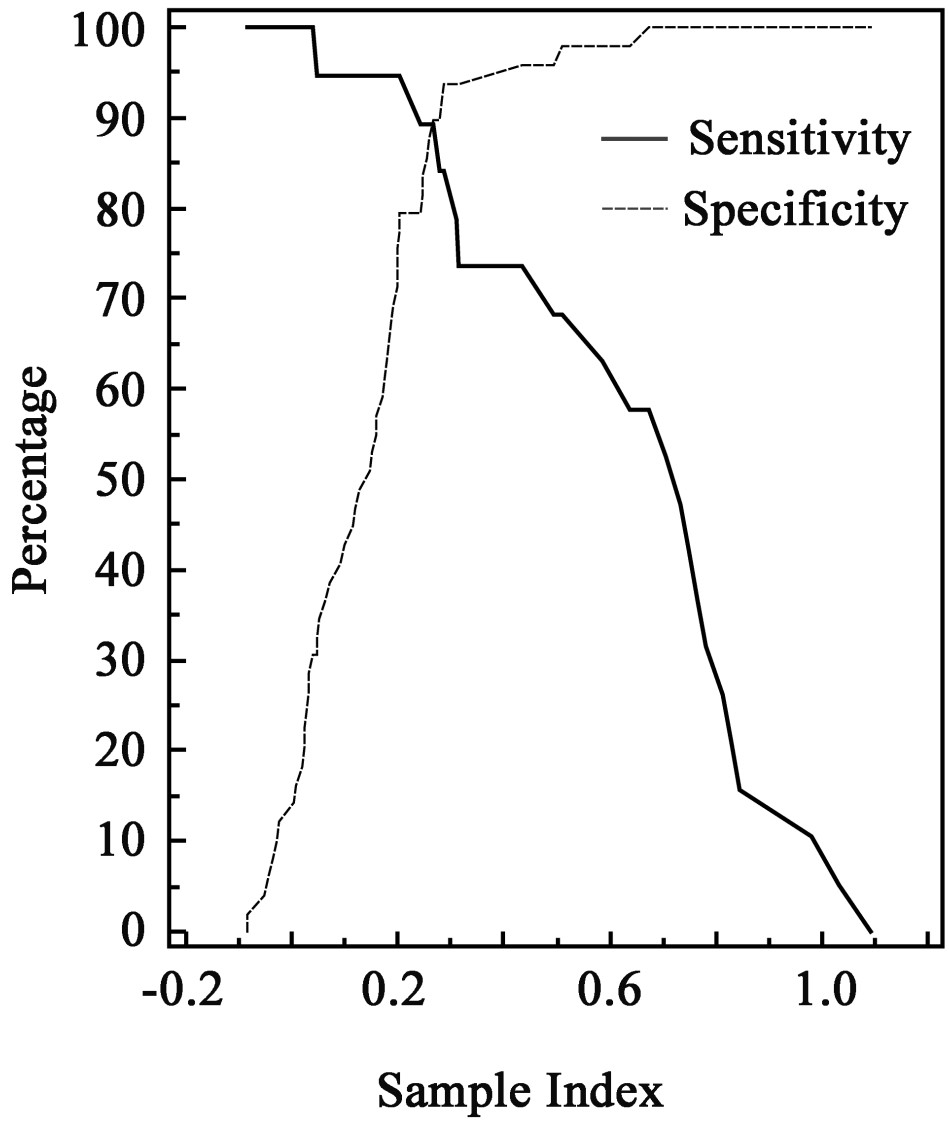

Figure 4. 2 Two-graph receiver operating characteristic (TG-ROC) analysis of the ELISA test. The sensitivity (—) and specificity (---) at different cutoff sample indexes were plotted. At 3 weeks post-infection for the animals tested the two curves intercross at sample index 0.268, which is the proposed optimal cutoff sample index.

4.4 Discussion

Diagnosis by bacterial culture is time-consuming and identification of *H. somni* may be difficult due to overgrowth of contaminants, normal flora, or other pathogens. Real-time PCR, though highly useful, may be unavailable to many diagnostic laboratories. In this study, *H. somni* EPS was used to develop an ELISA to differentiate *H. somni*-infected from healthy animals. The number of culture-confirmed samples available was limited because infections due to *H. somni* predominately occur in feedlots, where environment, weaning, feed provided, stress, and other pathogens commonly predispose the animals to systemic *H. somni* infection [15-17]. Feedlots are not present in the Eastern United States, where this study was done. Nonetheless, *H. somni* commonly inhabits bovine mucosal sites. As a result, cattle may at some time develop cross-reactive antibodies to *H. somni* [4] making serology using whole cells as antigen in serological assays unreliable. Although agglutination assays predominately detect IgM antibodies, which are highly cross-reactive, several outer membrane proteins of *H. somni* have also been shown to cross- react with antigens of other Gram-negative bovine pathogens [18, 19]. Therefore, an assay that measures the antibody response to an antigen that may be predominately expressed during active disease may enhance serological sensitivity and specificity. Earlier studies from our laboratory showed that detection of IgG2 antibodies to the high molecular size (approximately 270 K) fibrillar immunoglobulin binding proteins of *H. somni*, now called IbpA, by immunoblotting with peroxidase-conjugated Protein A can also differentiate most animals with *H. somni* disease from asymptomatic carriers and culture-negative animals by 5-6 weeks post-challenge [20]. However, an advantage to the use of EPS as an antigen is that it is only produced during biofilm growth or stress conditions, and therefore healthy carrier animals and animals vaccinated with a killed *H. somni* vaccine would remain serologically negative. A

disadvantage is that polysaccharide antigens are not strong immunogens and a substantial antibody response to EPS may be weaker than to a protein. This is reflected by the fact that the sera were used at a 1:100 dilution for the EPS ELISA. At least 3 weeks post-infection was required to obtain an adequate immune response in enough animals to obtain >90% sensitivity with the EPS ELISA, but this was also true of the IbpA immunoblot assay [20]. Therefore, if infection due to *H. somni* is suspected, the EPS ELISA is negative at the initial visit, and culture or real-time PCR is inconclusive or unavailable, testing of a repeat serum sample by EPS ELISA 2 weeks later may support the diagnosis.

H. somni produces an EPS in the biofilm state *in vitro* and the chemical structure of the EPS has been characterized [8]. Previous research from our lab has established that *H. somni* forms a biofilm in the cardiopulmonary tissue of infected calves [9]. A comparison of biofilm formation between a pathogenic and a serum-sensitive commensal strain of *H. somni*, which also lacks IbpA, indicated that the pathogenic strain produced more biofilm and EPS than the commensal strain. In addition to being produced abundantly under conditions that favor biofilm formation, EPS is also produced by *H. somni* under stress conditions, including anaerobiosis, stationary phase, and high salt concentration [8]. Although biofilms and their matrix components (including EPS and likely IbpA, other surface proteins, DNA, and lipids) may be produced at mucosal sites during colonization, a strong antibody response may not be made to some of these antigens except during pulmonary or systemic infection. Phagocytosis by neutrophils, macrophages, dendritic cells, etc., and presentation to T and B cells may be required to induce an adequate antibody response. Nonetheless, an association between an antibody response to EPS and bovine disease was apparent, and we propose that antibodies to the EPS have potential for use in assays to identify disease due to *H. somni* in cattle.

References

1. Sandal, I., C.L. B, and T.J. Inzana, *Haemophilus*, in *Pathogenesis of Bacterial Infections in Animals*, C.L. Gyles, et al., Editors. 2010, Wiley-Blackwell: Ames, IA. p. 387-409.
2. Corbeil, L.B., *Histophilus somni host-parasite relationships.* Anim Health Res Rev, 2007. **8**(2): p. 151-60.
3. Harris, F.W. and E.D. Janzen, *The Haemophilus somnus disease complex (Hemophilosis): A review.* Can Vet J, 1989. **30**(10): p. 816-822.
4. Corbeil, L.B., P.R. Widders, R. Gogolewski, J. Arthur, T.J. Inzana, and A.C. Ward, *Haemophilus somnus: bovine reproductive and respiratory disease.* Can Vet J, 1986. **27**(2): p. 90-93.
5. Widders, P.R., L.G. Paisley, R.P. Gogolewski, J.F. Evermann, J.W. Smith, and L.B. Corbeil, *Experimental abortion and the systemic immune response to "Haemophilus somnus" in cattle.* Infect Immun, 1986. **54**(2): p. 555-60.
6. Moustacas, V.S., T.M. Silva, L.F. Costa, M.N. Xavier, C.A. Carvalho, Jr, E.A. Costa, T.A. Paixao, and R.L. Santos, *Species-specific multiplex PCR for the diagnosis of Brucella ovis, Actinobacillus seminis, and Histophilus somni infection in rams.* BMC Vet Res, 2013. **9**: p. 51.
7. Saunders, V.F., L.A. Reddacliff, T. Berg, and M. Hornitzky, *Multiplex PCR for the detection of Brucella ovis, Actinobacillus seminis and Histophilus somni in ram semen.* Aust Vet J, 2007. **85**(1-2): p. 72-7; quiz 85.
8. Sandal, I., T.J. Inzana, A. Molinaro, C. De Castro, J.Q. Shao, M.A. Apicella, A.D. Cox, F. St Michael, and G. Berg, *Identification, structure, and characterization of an*

exopolysaccharide produced by Histophilus somni during biofilm formation. BMC Microbiol, 2011. **11**: p. 186.

9. Sandal, I., J.Q. Shao, S. Annadata, M.A. Apicella, M. Boye, T.K. Jensen, G.K. Saunders, and T.J. Inzana, *Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge.* Microbes Infect., 2009. **11**(2): p. 254-263.
10. Inzana, T.J. and L.B. Corbeil, *Development of a defined medium for Haemophilus somnus isolated from cattle.* Am J Vet Res, 1987. **48**(3): p. 366-9.
11. Sandal, I., W. Hong, W.E. Swords, and T.J. Inzana, *Characterization and comparison of biofilm development by pathogenic and commensal isolates of Histophilus somni.* J Bacteriol, 2007. **189**(22): p. 8179-8185.
12. Gogolewski, R.P., D.C. Schaefer, S.K. Wasson, R.R. Corbeil, and L.B. Corbeil, *Pulmonary persistence of Haemophilus somnus in the presence of specific antibody.* J Clin Microbiol, 1989. **27**(8): p. 1767-74.
13. Elswaifi, S.F., W.K. Scarratt, and T.J. Inzana, *The role of lipooligosaccharide phosphorylcholine in colonization and pathogenesis of Histophilus somni in cattle.* Vet. Res., 2012. **43**: p. 49. doi: 10.1186/1297-9716-43-49.
14. Greiner, M., *Two-graph receiver operating characteristic (TG-ROC): update version supports optimisation of cut-off values that minimise overall misclassification costs.* J Immunol Methods, 1996. **191**(1): p. 93-4.
15. Jensen, R., R.E. Pierson, P.M. Braddy, D.A. Saari, L.H. Lauerman, J.J. England, H. Keyvanfar, J.R. Collier, D.P. Horton, A.E. McChesney, A. Benitez, and R.M. Christie, *Shipping fever pneumonia in yearling feedlot cattle.* J Am Vet Med Assoc, 1976. **169**(5): p. 500-6.

16. Martin, S.W., A.H. Meek, D.G. Davis, J.A. Johnson, and R.A. Curtis, *Factors associated with mortality and treatment costs in feedlot calves: the Bruce County Beef Project, years 1978, 1979, 1980.* Can J Comp Med, 1982. **46**(4): p. 341-9.
17. Ribble, C.S., A.H. Meek, G.K. Jim, and P.T. Guichon, *The pattern of fatal fibrinous pneumonia (shipping fever) affecting calves in a large feedlot in Alberta (1985-1988).* Can Vet J, 1995. **36**(12): p. 753-7.
18. Corbeil, L.B., S.A. Kania, and R.P. Gogolewski, *Characterization of immunodominant surface antigens of Haemophilus somnus.* Infect Immun, 1991. **59**(12): p. 4295-301.
19. Kania, S.A., R.P. Gogolewski, and L.B. Corbeil, *Characterization of a 78-kilodalton outer membrane protein of Haemophilus somnus.* Infect Immun, 1990. **58**(1): p. 237-44.
20. Yarnall, M. and L.B. Corbeil, *Antibody response to Haemophilus somnus Fc receptor.* J Clin Microbiol, 1989. **27**(1): p. 111-7.

Chapter 5

Conclusions

Histophilus somni is a gram-negative bacterial pathogen of bovines and ovines. Commensal strains usually inhabit the reproductive and upper respiratory tracts [1, 2]. Pathogenic strains of *H. somni* are opportunistic, but can cause systemic diseases when the animal is under stress or has a previous infection. The diseases caused by *H. somni* include thrombotic meningoencephalitis (TME), bronchopneumonia, bacteremia, abortion, arthritis, myocarditis, mastitis, infertility, ovine epididymitis, and others [3].

H. somni can resist host immunity through the following virulence factors:

1. Formation of biofilm *in vivo* [4, 5].
2. LOS phase variation and decoration with phosphorylcholine (ChoP) and sialic acid [6-11].
3. Resistance to serum killing [12, 13].
4. Survival in phagocytic cells [14, 15].
5. Inhibition of oxygen radical production [16, 17].
6. Production of immunoglobulin-binding proteins [18, 19].
7. Cytotoxicity to host cells [20-23].

This study investigated three aspects of *H. somni* virulence factors:

1. The role of *luxS* and *uspE* in regulating virulence factors.
2. Insight into the mechanism of *H. somni* survival in bovine phagocytic cells.
3. Development of an enzyme-linked immunosorbent assay using EPS for diagnosis of *H. somni* infection.

Quorum sensing is one common mechanism that bacteria use to regulate biofilm formation [24]. The *luxS* gene encodes S-ribosylhomocysteinase, which is involved in the synthesis of autoinducer-2 [25]. *luxS* has been identified in the genome of *H. somni* and related strains within the family *Pasteurellaceae* [26, 27]. The *H. somni luxS* gene was functional when cloned into an *E. coli* strain that lacks a functional *luxS*. However, *luxS* did not participate in or regulate biofilm formation through quorum sensing in *H. somni*. A transposon mutant, 2336::Tn*luxS*, formed a biofilm as thick and structurally mature as the parent strain, as determined by cystal violet staining and SEM.

The universal stress proteins have been shown to be involved in responses to environmental stress in several bacterial species [28]. An *E. coli uspE* homolog was also identified in *H. somni*. Tn insertion mutants of both *luxS* and *uspE* were selected from a bank of transposon mutants and both were attenuated in a mouse model of bacteremia and mortality. Although the *luxS* mutant was not affected in biofilm formation, mutations in both *luxS* and *uspE* had profound effects on numerous other genes. Several phenotypic changes in mutants 2336::Tn*luxS* and 2336::Tn*uspE* were observed, which include:

1. The lag phase of 2336::Tn*uspE* was shortened when grown in broth.
2. Strain 2336::Tn*uspE* was deficient in biofilm formation.
3. The LOSs of both 2336::Tn*luxS* and 2336::Tn*uspE* were truncated, and not decorated by sialic acid.
4. Both mutants were more sensitive to serum killing than the parent strain.
5. Both mutants still replicated inside monocytes, but did not survival as well as the parent strain during the first 24 h after infection.
6. Both mutants elongated after 24 h co-incubation with monocytes.

Complementation of the 2336::Tn*luxS* mutant *in trans* partially restored the original phenotypes. RNA-sequence analysis revealed differential expression of numerous genes resonsible for metabolism, stress response, and cell division.

Several pathogenic strains of *H. somni* have been studied that are capable of survival in macrophages, monocytes, neutrophils, and epithelial cells [14, 15]. However, the mechanism of this resistance remains unclear. In this dissertation, confocal microscopy clearly showed that *H. somni* strain 2336 interferes with phagosome maturation, which would contribute to intracellular survival. The phagosome containing commensal strain 129Pt merged with lysosomal particles and was acidified. The bacteria were then cleared. However, strain 2336 prevented the acidification and maturation of the phagosome.

The immunoglobolin binding protein IbpA has a Fic domain, which is cytotoxic [21] and may interfere with phagocytosis and the phagocytic cell's ability to eliminate intracellular bacteria. We found that strains that express IbpA are more resistant to intracellular killing. Mutants with a Tn insertion in the 5' end of *ibpA* can replicate as well as the parent strain inside monocytes. However, none of the mutants were compromised in expression of the Fic domain, which is at the C-terminus end of the protein and is responsible for cytotoxicity. However, concentrated bacterial culture supernatant containing IbpA did not enable *ibpA*-negative strains to replicate intracellularly. Therefore, cytotoxicity of the IbpA protein may interfere with the functions of phagocytic cells, but may not be the primary mechanism responsible for intracellular survival of *H. somni*.

H. somni EPS is produced under stress conditions and is a major component of the biofilm matrix [29]. Pathogenic strains of *H. somni* also form a biofilm *in vivo* [4, 5]. The struture of and the immunological response to the EPS has also been characterized [29]. Based

on this information, an enzyme-linked immunosorbent assay was developed to identify cattle with active disease due to *H. somni* using purified EPS as the antigen. The assay was highly sensitive and specific when tested with sera from naturally-infected calves and in animals challenged 4 -7 weeks previously. At a cutoff sample index value of 0.268, the sensitivity of the assay was 90.5% at 3 weeks post-infection, and 100% at 7 weeks post-infection. The specificity of the assay for healthy calves was 92.5%. Serology using EPS as an antigen may be a useful alternative to culture or PCR for diagnosis of active disease due to *H. somni*.

Future direction for this research are:

1. The components of supernatant may be separated by mass spectrometry in order to identify potential AI-2. If a signal molecule is identified, its structure and interaction with *H. somni* AI-2 receptor may be determined.
2. A mutant with deletion of the Fic domain has been created in another lab. Determining the intracellular survival of this mutant will help to clarify the role of *ibpA* in phagocytosis and intracellular survival of *H. somni*.
3. The immunosorbent assay explored the possibility to use EPS for diagnosis of *H. somni* infection. However, a clinic trail with larger number of samples will give a better evaluation of this assay.

References

1. Humphrey J. D., L.P.B., Barnum D. A., Doig P. A., Stephens L. R., Thorsen J., *Occurrence of Haemophilus somnus in bovine semen and in the prepuce of bulls and steers.* Can j Comp Med, 1982. **46**: p. 215-217.
2. Humphrey, J. and L. Stephens, *Haemophilus somnus: a review.* Veterinary Bulletin, 1983. **53**.
3. Sandal, I., L. Corbeil, and T. Inzana, *Haemophilus.* Pathogenesis of Bacterial Infections in Animals, Fourth Edition, 2010: p. 387-409.
4. Sandal, I., J.Q. Shao, S. Annadata, M.A. Apicella, M. Boye, T.K. Jensen, G.K. Saunders, and T.J. Inzana, *Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge.* Microbes and Infection, 2009. **11**(2): p. 254-263.
5. Sandal, I., W. Hong, W.E. Swords, and T.J. Inzana, *Characterization and Comparison of Biofilm Development by Pathogenic and Commensal Isolates of Histophilus somni.* Journal of Bacteriology, 2007. **189**(22): p. 8179-8185.
6. St Michael, F., J. Li, M.D. Howard, A.J. Duncan, T.J. Inzana, and A.D. Cox, *Structural analysis of the oligosaccharide of Histophilus somni(Haemophilus somnus) strain 2336 and identification of several lipooligosaccharide biosynthesis gene homologues.* Carbohydrate research, 2005. **340**(4): p. 665-672.
7. Wu, Y., J.H. McQuiston, A. Cox, T.D. Pack, and T.J. Inzana, *Molecular cloning and mutagenesis of a DNA locus involved in lipooligosaccharide biosynthesis in Haemophilus somnus.* Infect Immun, 2000. **68**(1): p. 310-9.

8. McQuiston, J.H., J.R. McQuiston, A.D. Cox, Y. Wu, S.M. Boyle, and T.J. Inzana, *Characterization of a DNA region containing 5'-(CAAT)n-3' DNA sequences involved in lipooligosaccharide biosynthesis in Haemophilus somnus.* Microbial Pathogenesis, 2000. **28**(5): p. 301-312.
9. Howard, M.D., A.D. Cox, J.N. Weiser, G.G. Schurig, and T.J. Inzana, *Antigenic Diversity of Haemophilus somnus Lipooligosaccharide: Phase-Variable Accessibility of the Phosphorylcholine Epitope.* Journal of Clinical Microbiology, 2000. **38**(12): p. 4412-4419.
10. Inzana, T.J., G. Glindemann, A.D. Cox, W. Wakarchuk, and M.D. Howard, *Incorporation of N-Acetylneuraminic Acid into Haemophilus somnus Lipooligosaccharide (LOS): Enhancement of Resistance to Serum and Reduction of LOS Antibody Binding.* Infection and Immunity, 2002. **70**(9): p. 4870-4879.
11. Kuckleburg, C.J., S.F. Elswaifi, T.J. Inzana, and C.J. Czuprynski, *Expression of Phosphorylcholine by Histophilus somni Induces Bovine Platelet Aggregation.* Infection and Immunity, 2007. **75**(2): p. 1045-1049.
12. Corbeil, L.B., K. Blau, D.J. Prieur, and A. Ward, *Serum susceptibility of Haemophilus somnus from bovine clinical cases and carriers.* Journal of clinical microbiology, 1985. **22**(2): p. 192-198.
13. Corbeil, L., R. Gogolewski, L. Stephens, and T. Inzana, *Haemophilus somnus: antigen analysis and immune responses*, in *Haemophilus, Actinobacillus, and Pasteurella*. 1995, Springer. p. 63-73.
14. Gomis, S.M., D.L. Godson, G.A. Wobeser, and A.A. Potter, *Intracellular survival of Haemophilus somnusin bovine blood monocytes and alveolar macrophages.* Microbial Pathogenesis, 1998. **25**(5): p. 227-235.

15. Czuprynski, C.J. and H.L. Hamilton, *Bovine neutrophils ingest but do not kill Haemophilus somnus in vitro.* Infection and Immunity, 1985. **50**(2): p. 431-436.

16. Sample, A.K. and C.J. Czuprynski, *Elimination of hydrogen peroxide by Haemophilus somnus, a catalase-negative pathogen of cattle.* Infect. Immun., 1991. **59**(7): p. 2239-2244.

17. Howard, M.D., J.H. Boone, V. Buechner-Maxwell, G.G. Schurig, and T.J. Inzana, *Inhibition of bovine macrophage and polymorphonuclear leukocyte superoxide anion production by Haemophilus somnus.* Microbial Pathogenesis, 2004. **37**(5): p. 263-271.

18. Corbeil, L.B., F.D. Bastida-Corcuera, and T.J. Beveridge, *Haemophilus somnus immunoglobulin binding proteins and surface fibrils.* Infection and Immunity, 1997. **65**(10): p. 4250-7.

19. Tagawa, Y., J.D. Sanders, I. Uchida, F.D. Bastida-Corcuera, K. Kawashima, and L.B. Corbeil, *Genetic and functional analysis of Haemophilus somnus high molecular weight-immunoglobulin binding proteins.* Microbial Pathogenesis, 2005. **39**(5-6): p. 159-170.

20. Sylte, M.J., L.B. Corbeil, T.J. Inzana, and C.J. Czuprynski, *Haemophilus somnus induces apoptosis in bovine endothelial cells in vitro.* Infect Immun, 2001. **69**(3): p. 1650-60.

21. Hoshinoo, K., K. Sasaki, A. Tanaka, L.B. Corbeil, and Y. Tagawa, *Virulence attributes of Histophilus somni with a deletion mutation in the ibpA gene.* Microbial Pathogenesis, 2009. **46**(5): p. 273-282.

22. Yang, Y.F., M.J. Sylte, and C.J. Czuprynski, *Apoptosis: a possible tactic of Haemophilus somnus for evasion of killing by bovine neutrophils?* Microb Pathog, 1998. **24**(6): p. 351-9.

23. Worby, C.A., S. Mattoo, R.P. Kruger, L.B. Corbeil, A. Koller, J.C. Mendez, B. Zekarias, C. Lazar, and J.E. Dixon, *The Fic Domain: Regulation of Cell Signaling by Adenylylation.* Molecular Cell, 2009. **34**(1): p. 93-103.

24. Keller, L. and M.G. Surette, *Communication in bacteria: an ecological and evolutionary perspective.* Nature Reviews Microbiology, 2006. **4**(4): p. 249-258.

25. Pei, D. and J. Zhu, *Mechanism of action of S-ribosylhomocysteinase (LuxS).* Current opinion in chemical biology, 2004. **8**(5): p. 492-497.

26. Daines, D.A., M. Bothwell, J. Furrer, W. Unrath, K. Nelson, J. Jarisch, N. Melrose, L. Greiner, M. Apicella, and A.L. Smith, *Haemophilus influenzae luxS mutants form a biofilm and have increased virulence.* Microbial Pathogenesis, 2005. **39**(3): p. 87-96.

27. Malott, R.J. and R.Y. Lo, *Studies on the production of quorum-sensing signal molecules in Mannheimia haemolytica A1 and other Pasteurellaceae species.* FEMS microbiology letters, 2002. **206**(1): p. 25-30.

28. Kvint, K., L. Nachin, A. Diez, and T. Nystr√∂m, *The bacterial universal stress protein: function and regulation.* Current Opinion in Microbiology, 2003. **6**(2): p. 140-145.

29. Sandal, I., T.J. Inzana, A. Molinaro, C. De Castro, J.Q. Shao, M.A. Apicella, A.D. Cox, F. St Michael, and G. Berg, *Identification, structure, and characterization of an exopolysaccharide produced by Histophilus somni during biofilm formation.* BMC Microbiol, 2011. **11**: p. 186.