CHAPTER VI

Summary and Conclusions

Summary

A. *Histophilus somni*

Histophilus somni is a commensal of the mucosal surfaces of the respiratory and reproductive tracts of cattle and sheep. As an opportunistic pathogen of cattle and sheep, *H. somni* has been demonstrated to cause diseases such as pneumonia, myocarditis, abortion, arthritis, and meningo-encephalitis. *H. somni* is one of the agents implicated in bovine respiratory disease and it is estimated that the cattle industry in North America annually loses about $1 billion due to this disease alone. The virulence potential of this bacterium has only been partially understood in the pre-genomic era using traditional genetic, biochemical, and immunologic approaches. Some of the virulence factors that may enable *H. somni* to cause disease include, but are not limited to, the phase variable lipooligosaccharide, induction of apoptosis of host cells, intraphagocytic survival, and immunoglobulin Fc binding proteins.

Previous studies have also shown that some strains of *H. somni* isolated from the urogenital tract of cattle are biochemically and serologically similar to the pathogenic strains, but are relatively less pathogenic. This classic dichotomy of *H. somni* strains is a matter of fundamental biological curiosity and several studies utilizing these two types of strains have identified the genetic traits that are responsible for the phenotypic dissimilarities. Based on these studies, it has been hypothesized that the two types of *H. somni* strains originated from a common ancestor and evolved independently within the bovine host.

A comprehensive understanding of the biological properties of *H. somni* and their interactions with the host are required to develop effective control measures against *H. somni*.

B. Genome Characterization

As a first step toward achieving a comprehensive understanding of the biological properties of *H. somni*, the genomes of pneumonia strain 2336 and preputial strain 129Pt were sequenced. The chromosomes of the two *H. somni* strains contain several interesting features in terms of overall size and total gene content. The chromosome of strain 129Pt is ~250,000 bp smaller than that of strain 2336 and the two genomes provided an opportunity for studying loss and gain of putative virulence genes and evolution. Several of the genes/loci common to the two strains were found in different regions of the chromosomes, indicating genome rearrangement and a lack of colinearity.

The most conspicuous feature was the overabundance of prophage-like sequences in the chromosomes of strains 2336 and 129Pt. These prophage sequences were randomly distributed in the two chromosomes and appeared to have enriched the genetic potential of each strain, since they constitute a large proportion of strain-specific DNA. For example, in the pneumonia strain 2336, putative virulence genes encoding hemagglutinins and a subtilase have been acquired via bacteriophages. Based on gene order, orientation of genes (forward/reverse), GC content, and homology of proteins encoded by the genes, two prophage regions from strains 2336 and 129Pt were inferred to have had a common ancestry. Although comparison of the prophage regions confirmed the ability of *H. somni* to acquire diverse genes using bacteriophages and/or transposons, the general lack of antibiotic resistance in this species is intriguing.

Continued analyses of the genomes of *H. somni* strains and functional characterization of strain-specific genes for their possible roles in colonization, survival, and pathogenesis are required to fully realize the genomic potentials of *H. somni*. In addition, a ‘comprehensive genome phylogeny’ of members of the *Pasteurellaceae* will need to be constructed.

C. Plasmid Characterization

Plasmids constitute an important class of mobile genetic elements and plasmid-borne traits are known to either enhance or reduce the fitness of host bacteria in their natural environments. Some plasmid-borne attributes can also be used to gain insights into the evolution of related plasmids and their hosts. In order to complement the knowledge obtained by genome sequencing and comparative genomics, the nucleotide sequences of plasmids from *H. somni* strains 649 and 129Pt were deciphered and compared.

The plasmid from strain 649 (designated pHS649) has a size of 1,347 bp and contains only two open reading frames (ORFs). One of these ORFs encodes a putative RepA protein that has 48% amino acid homology to the RepA protein of *Escherichia coli* plasmid pKL1. Based on the homology of pHS649 RepA to Rep proteins from plasmids that replicate by the rolling circle (RC) mode, and the presence of typical sequence features that resemble those found in the '*ori*' regions of plasmids that replicate by the RC mode, pHS649 was proposed to replicate by the RC mode. A modified version of pHS649 confers streptomycin resistance, is suitable for alpha complementation of LacZ in *E. coli*, and can be used as a shuttle vector.

The plasmid from strain 129 (designated pHS129) has a size of 5,178 bp and contains six open reading frames (ORFs). Two of these ORFs encode putative RepB proteins that are 87% similar (73% identity within 323 amino acids) to each other and are related to Rep proteins from plasmids that replicate by the theta mode. This plasmid contains two origins of replication that resemble the '*ori*' regions of plasmids that replicate by the theta mode. Based on these features, pHS129 was inferred to be a dimer and was proposed to replicate by the theta mode. It was also proposed that pHS649 and pHS129 originated by horizontal transfer from distinct sources during the evolution of *H. somni*.

D. LuxS and Biofilm Characterization

A biofilm is defined as “a community of microorganisms attached to a suitable surface”. Quorum sensing (QS) is a mechanism of interbacterial communication that has been shown to regulate biofilm formation in some pathogenic bacteria. The product of *luxS* (S-ribosylhomocysteinase) is an intermediary enzyme in the biosynthesis of AI-2 QS signal molecules. Since the chromosomes of *H. somni* strains contained *luxS*, the ability of this gene to complement the biosynthesis of AI-2 QS signal molecules in *E. coli* DH5α and influence biofilm formation was investigated.

Culture supernatants from *E. coli* DH5α containing *H. somni luxS* cloned on a multicopy plasmid were found to induce light production in *V. harveyi* strain BB170, confirming that *H. somni luxS* was functional and encodes S- ribosylhomocysteinase. However, it was also found that culture supernatants from different bacteria containing *luxS* differ significantly in inducing bioluminescence in *V. harveyi* strain BB170. Based on this evidence, and *in silico* analyses of LuxP proteins, it is proposed that AI-2 is not an ‘universal signal’.

E. coli DH5α containing *H. somni luxS* was also found to form a biofilm in comparison to *E. coli* DH5α without *luxS*. *H. somni* pneumonia strain 2336 was found to form a prominent biofilm in comparison to preputial strain 129Pt. *H. somni* pneumonia strain 93 overexpressing the phosphorylcholine moiety on its lipooligosaccharide was also found to form a prominent biofilm. Preliminary results indicated that *luxS* may influence biofilm formation by *H. somni*. The roles of *luxS* and phosphorylcholine in biofilm formation by *H. somni* will need to be investigated further by constructing isogenic mutants that lack one or both of these functions. Although a furanone is shown to inhibit biofilm formation by *H. somni* strains 2336 and 738, the mechanism of its antibacterial action remains to be characterized.

E. Restriction-Modification Characterization

Type I and Type II Restriction-Modification (RM) systems are the most frequently encountered DNA modifying enzymes among eubacteria. One of the proposed functions of RM systems is to provide defense against bacteriophage attack and maintain the integrity of the bacterial genome (the "*cellular defense hypothesis*"). The very low efficiency of transformation of pathogenic isolates of *H. somni* in the laboratory has been attributed to the presence of a tight RM system in these strains. A Type II restriction enzyme (R.HsoI), which recognizes the sequence 5'-GCGC-3' and cleaves it asymmetrically to leave a 5´-CG extension, has previously been purified from *H. somni* strain 2336. However, very little was known about the genetic basis of RM in *H. somni*. In view of this, the genomes of *H. somni* strains were examined for putative genes encoding the Type I and Type II RM enzymes.

The chromosome of *H. somni* strain 2336 contained putative genes encoding Type II RM enzymes. Based on homology modeling and DNA methylation assays, the Type II RM system was predicted to recognize the sequence 5'-GCGC-3'. The chromosome of *H. somni* strain 2336 also contained putative genes encoding Type I RM enzymes. The Type I RM system was predicted to be acquired via bacteriophage-mediated gene transfer and the DNA recognition specificity of this system remains to be identified.

The chromosome of *H. somni* strain 129Pt lacked genes encoding Type II RM enzymes. The chromosome of *H. somni* strain 129Pt also lacked two of the three genes that constitute the Type I RM system in strain 2336. However, this strain was found to contain a prophage-associated, strain-specific, RM system whose DNA recognition specificity remains to be identified. It was predicted that the relative ease with which strain 129Pt can be transformed in the laboratory was due to the absence of functional Type I and Type II RM systems.

Conclusions

The following conclusions were drawn from the comparative and functional genomic studies of *H. somni*:

1. *H. somni* is a versatile commensal and an opportunistic pathogen that can elaborate a variety of virulence factors in response to host defense mechanisms (chapter I).
2. The chromosomes of *H. somni* pneumonia strain 2336 and preputial strain 129Pt have undergone deletions, duplications, and inversions. They contain multiple bacteriophage insertions that have partially contributed to the genetic diversity of the two strains (chapter II).
3. *H. somni* pneumonia strain 2336 contains 11 homologs of filamentous hemagglutinins whereas preputial strain 129Pt lacks them. These genes may partially contribute to the serum resistance and pathogenicity of strain 2336 (chapter II).
4. *H. somni* strains contain different types of plasmids. These plasmids do not appear to have caused the loss or gain of genes potentially involved in virulence (chapter III).
5. *H. somni* strain 649 plasmid is compatible with *H. somni* strain 129 plasmid (chapter III).
6. *H. somni luxS* can complement the biosynthesis of AI-2 quorum sensing signal molecules in *E. coli* DH5α. *H. somni luxS* can also influence biofilm formation by *E. coli* DH5α (chapter IV).
7. A synthetic furanone can inhibit planktonic and sessile growth of *H. somni* (chapter IV).
8. *H. somni* strains contain a variety of RM systems. The Type II RM system of strain 2336 closely resembles the HinP1I RM system of *H. influenzae* P1 (chapter V).

APPENDIX A: PERMISSION LETTER

Date: Tue, 27 Mar 2007 12:22:52 +0100
From: "Moss, Marion (ELS-OXF)" <m.moss@elsevier.co.uk>
To: ssiddara@vt.edu
Subject: **RE: Obtain Permission**

Dear Mr Siddaramappa

We hereby grant you permission to reproduce the material detailed below at no charge in your thesis subject to the following conditions:

Title:	PLASMID
Author(s):	Siddaramappa, Duncan, Brettin, Inzana
Volume:	55
Issue:	3
Year:	2006
Pages:	227 - 234
Article title:	Comparative analyses of two cryptic plasmids

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier".

3. Your thesis may be submitted to your institution in either print or electronic form.

4. Reproduction of this material is confined to the purpose for which permission is hereby given.

5. This permission is granted for non-exclusive English rights only. For other languages please reapply separately for each one required.

6. This includes permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Marion Moss
Senior Rights Assistant
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford
OX5 1GB

Tel : +44 1865 843280
Fax : +44 1865 853333
E-mail : m.moss@elsevier.com

VITA

Shivakumara Swamy Siddaramappa
Ganeshana Beedhi, Shivani 577549, Karnataka, India
Phone: (+91) 826.124.6755
e-mail: ssiddara@vt.edu, sswamy57@rediffmail.com

❖ Education:

- **Doctor of Philosophy**

  [Biomedical and Veterinary Sciences (BMVS) with Molecular Cell Biology and Biotechnology (MCBB) option]
  Graduation: Spring 2007
  Virginia-Maryland Regional College of Veterinary Medicine
  Virginia Tech, Blacksburg, Virginia 24061, U.S.A.
  Dissertation title: 'Comparative and Functional Genomic Studies of *Histophilus somni* (*Haemophilus somnus*)'

- **Master of Science**

  [Veterinary Microbiology with Virology and Vaccine Science options]
  Graduation: November 1999
  The Royal Veterinary College, University of London, London NW1 0TU, U.K.
  Thesis title: 'An Investigation of the Efficacy of Possible Routes for DNA Vaccination of Chickens'

- **Bachelor of Veterinary Science**

  [Veterinary Medicine with Animal Husbandry option]
  Graduation: September 1997
  Veterinary College, University of Agricultural Sciences, Bangalore 560024, India.

- **Pre-University Course**

  [Physics, Chemistry, Mathematics, and Biology]
  Graduation: June 1992
  Desheeya Vidyashala Pre-University (Independent) College, Shimoga 577201, India.

- **Secondary School Leaving Certificate**

  [Science, Mathematics, and Social Studies]
  Graduation: May 1990
  Sri Taralabalu Jagadguru High School, Anubhava Mantapa, Davangere 577004, India.

❖ Professional Training/Experience:

- **Graduate Research and Teaching Assistant** (January 2002 to May 2007)
  Center for Molecular Medicine and Infectious Diseases
  Virginia-Maryland Regional College of Veterinary Medicine
  Virginia Tech, Blacksburg, Virginia 24061, U.S.A.

- **Research Associate** (June 2001 to December 2001)
  Project Directorate on Animal Disease Monitoring and Surveillance
  Indian Council of Agricultural Research, Bangalore 560024, India.

- **Undergraduate Instructor** (October 2000 to May 2001)
  Departments of Biochemistry and Livestock Production Management
  Veterinary College, University of Agricultural Sciences, Bangalore 560024, India.

- **Research Associate** (March 2000 to August 2000)
  Division of Biological Standardisation
  Indian Veterinary Research Institute, Izatnagar 243122, India.

❖ Awards/Honors:

- **Graduate Man of the Year 2005-2006**, Virginia Polytechnic Institute and State University.
- **Outstanding Graduate Student of the Year 2005-2006**, Virginia-Maryland Regional College of Veterinary Medicine.
- **Who's Who Among Students** in American Universities and Colleges in 2005-2006.
- **Graduate Research and Teaching Assistantship** of the Virginia-Maryland Regional College of Veterinary Medicine for Doctoral studies (2002-2007).
- **Commonwealth Scholarship** of the Government of United Kingdom for Master of Science studies (1998-1999).
- **Resident Merit Scholarship** of the University of Agricultural Sciences, Bangalore, India, for Graduate studies (1997-98).
- **National Merit Scholarship** of the Government of India for Pre-University and Undergraduate studies (1990-1997).

❖ Membership in Professional Organizations:

- Indian Veterinary Council, Karnataka State Chapter
- American Society for Microbiology
- American Association for the Advancement of Science
- Virginia Tech Graduate Scholars Society