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Thomas J. Inzana *Editor*

Histophilus somni

Biology, Molecular Basis of Pathogenesis, and Host Immunity

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Preface

Histophilus somni (formerly known as *Haemophilus somnus*) was first identified as a bovine pathogen and cause of thrombotic meningoencephalitis (TME) only 55 years ago, and *Haemophilus agni* (now also recognized as *H. somni*) a cause of septicemic disease in lambs two years earlier. The only known habitats of *H. somni* are the mucosal surfaces of ruminants, making this bacterium an opportunistic pathogen. *H. somni* is responsible for a wide variety of systemic diseases in addition to TME, including respiratory disease syndromes, myocarditis, reproductive disease syndromes, polyarthritis, mastitis, ocular disease, and septicemia. Nonetheless, some *H. somni* isolates from genital sites may not be pathogenic at all. Although this bacterium is capable of causing inflammation at systemic sites (vasculitis is a hallmark of infection due to *H. somni*) and is toxic to epithelial and phagocytic cells, the bacterium's wide array of virulence factors act primarily as a defense against, or to escape recognition from, host innate and adaptive immunity. Although no longer recognized as a member of the genus *Haemophilus* due to genetic dissimilarity, many genes responsible for virulence attributes in *H. somni* are conserved in *Haemophilus influenzae*, the type species of the genus, and homologs are common in other members of the *Pasteurellaceae* and *Neisseria* spp. Although antibodies to certain membrane or surface proteins have been shown to be protective, particularly of the IgG2 allotype, correlates of protective immunity are incomplete. The role of cellular immunity in protection against histophilosis has not been adequately examined, and therefore the specificity and nature of the broader host immune response required to provide optimal protection against systemic diseases due to *H. somni* remains unclear. Further complicating matters is that *H. somni* is capable of forming a biofilm in host tissues. The biofilm likely interferes with the susceptibility of the bacterium to host immunity and complicates measuring protective immunity in model systems that utilize planktonic cells. Furthermore, the genomes of only two strains of *H. somni*, a pathogenic pneumonia isolate and a nonpathogenic preputial genital isolate, have been sequenced. Therefore, unlike most other bacterial species of disease and economic importance, there is a woeful deficiency in the number of isolates whose genomes have been

sequenced. Essential information regarding whether isolates from various geographic regions share or lack genes responsible for virulence factors, or may have important virulence traits not yet recognized, is lacking.

In this volume experts who have contributed to the *H. somni* literature have reviewed what is currently known regarding the taxonomy, disease syndromes, genetics, biology, and pathogenic factors of *H. somni*, and the host immune response to this pathogen. We gratefully recognize that much of this work could not have been accomplished without the financial support of the United States Department of Agriculture-National Institute of Food and Agriculture, and other federal agencies worldwide.

Thomas J. Inzana

Contents

Taxonomy of *Histophilus somni*

Øystein Angen

Abstract *Histophilus somni* was proposed in 2003 as a common name for bacteria that earlier had been called "*Haemophilus somni*", "*Haemophilus agni*", "*Haemophilus somnifer*", and "*Histophilus ovis*". The species is clearly separated from other species and genera within the family *Pasteurellaceae*. The species is phenotypically variable, but highly uniform regarding the 16S rDNA sequence. Whole-genome sequencing has revealed distinct genetic differences between a commensal and a pathogenic strain, particularly in regard to putative virulence factors. However, broad generalizations regarding the genetics of *H. somni* cannot be applied to the entire species until the genomes of additional strains are sequenced.

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1 Historical Background

A disease condition in feedlot cattle called infectious thrombotic meningoencephalitis was described in 1956 (Griner et al. 1956). A disease with similar manifestations was observed a few years later, but a Gram-negative rod was then isolated from the cerebral lesions, and the disease was reproduced by intravenous injection of the isolated agent (Kennedy et al. 1960). The bacterium was labelled "*Haemophilus*-like". Although the bacterium did not require X- or V-factors for growth, it did require blood for growth. In 1969, the name "*Haemophilus somnus*" was proposed for this bacterium (Bailie 1969). However, phenotypically similar bacteria had previously been isolated from sheep. An organism named "*Histophilus ovis*" was isolated in 1925 (Mitchell 1925), but this isolate is no longer available. In 1956, a bacterium named "*Histophilus ovis*" was isolated from mastitis in sheep (Roberts 1956) and has since been isolated from ovine septicaemia, synovitis, epididymitis and ovine vaginas (Rahaley 1978; Rahaley and White 1977). In 1958, a bacterium was isolated in Australia from lambs with septicaemia and designated "*Haemophilus agni*" (Kennedy et al. 1958).

DNA–DNA hybridization was used to show that "*Histophilus ovis*", "*Haemophilus somnus*" and "*Haemophilus agni*" should be regarded as the same species (Piechulla et al. 1986; Walker et al. 1985) supporting earlier investigations of the antigenic and cytochemical relationships among these taxa (Stephens et al. 1983). However, the taxonomic position of these *Haemophilus*-like isolates was uncertain as they were not affiliated to the genus *Haemophilus* by DNA–DNA hybridization (Piechulla et al. 1986; Walker et al. 1985), DNA–rRNA hybridization (de Ley et al. 1990) or 16S rDNA sequencing (Dewhirst et al. 1993). Therefore, these isolates apparently represented a new genus within the family *Pasteurellaceae* (Piechulla et al. 1986; Bisgaard 1995). In the second edition of Bergey's Manual of Systematic Bacteriology, "*H. somnus*", "*H. agni*" and "*H. ovis*" were listed under the genus *Haemophilus* as "Other organisms" (Kilian 2005). "*Haemophilus somnus*" was for a long time used as a convenient, although incorrect name for these taxa. Nevertheless, "*Histophilus ovis*" was often preferred, e.g. by Australian authors. Furthermore, as the name "*Haemophilus somnus*" was grammatically incorrect, the name "*Haemophilus somnifer*" had also been suggested (Miles et al. 1972).

The taxonomic uncertainty was resolved with the publication by Angen et al. (2003), in which the name *Histophilus somni* was proposed for these organisms. This proposal was partly based on earlier investigations, which had shown that "*Haemophilus somnus*", "*Haemophilus agni*" and "*Histophilus ovis*" represented the same species. The taxonomic position of this species was further investigated by sequencing the 16S rDNA and *rpoB* genes of strains earlier investigated by DNA–DNA hybridization. These results clearly supported the allocation of this species to a new genus within the family *Pasteurellaceae*. The phenotypic separation of *Histophilus somni* from other genera of the family could for most strains be based on capnophilia, yellowish pigmentation and indole production. However, due to

phenotypic variation, the use of a species-specific PCR test based on the 16S rDNA gene (Angen et al. 1998) was included in the species description. This was justified by the high similarity of the 16S rDNA gene found within the species (Fig. 1) and the fact that the highest similarity of 16S rDNA found to any other taxa within the

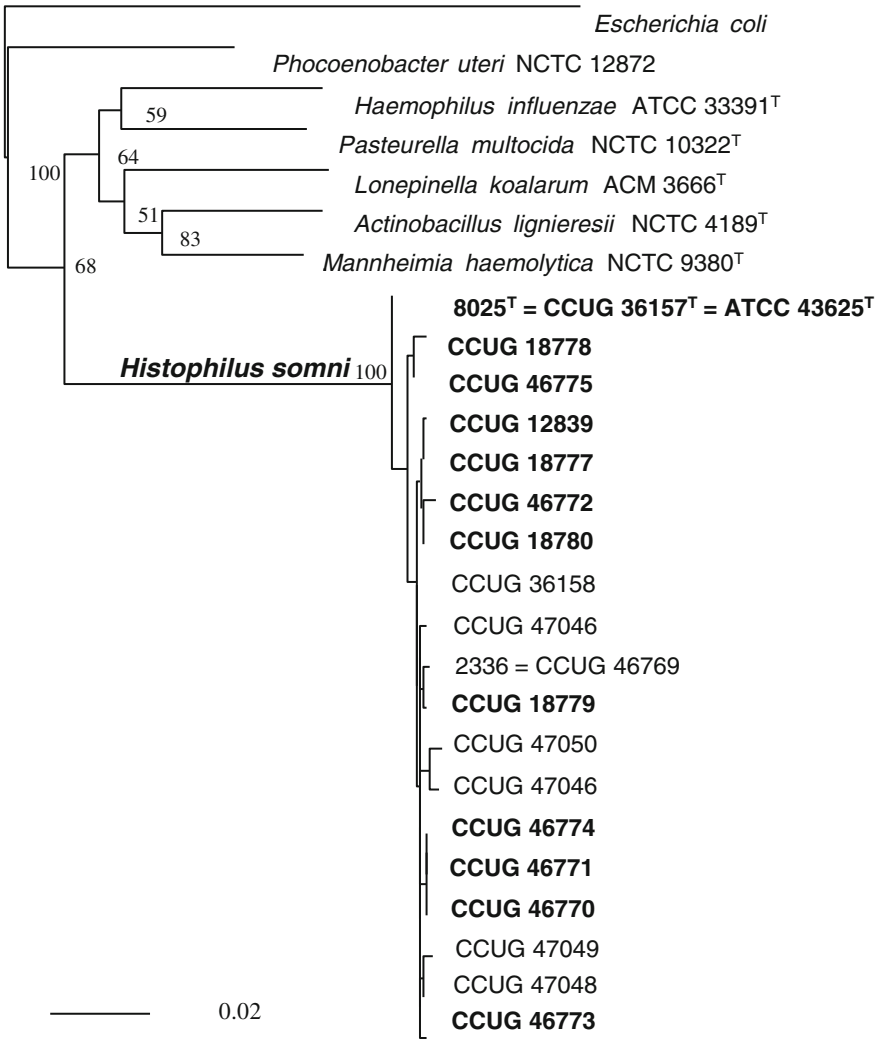

Fig. 1 Phylogenetic analysis based on 16S rDNA sequences of *Histophilus somni* isolates and type species of the genera in the family *Pasteurellaceae*. The tree was built with the neighbour-joining method based on Jukes–Cantor corrected distances. Bootstrap values of 500 repetitions are indicated as numbers out of 100 at the major branching points. Strains in bold type have been investigated by DNA–DNA hybridization. Copied with permission from Angen et al. (2003)

family is 93.4 %. Table 1 shows phenotypic characters separating *H. somni* from the other genera of the family.

The type strain is 8025^T = ATCC 43625^T = CCUG 36157^T isolated in the USA from a bovine brain with lesions of thrombotic meningoencephalitis (Brown et al. 1972). The G + C content of the type strain is 37.5 mol% (Piechulla et al. 1986). The origin of the Latin name is the following: *Histophilus* (his.to'phi.lus Gr. n. histos *tissue*; Gr. adj. philos *friendly*, latinized as philus; N.L.masc.n. Histophilus, thus meaning *the tissue friend*). *Histophilus somni* (som'ni. L. somnus *sleep*, gen. sing. somni, referring to one of the disease conditions associated with the bacterium).

2 Detection and Identification

H. somni grows on enriched media, e.g. blood agar plates, and most isolates need increased concentrations of CO_2 to grow. The bacterium is quite feeble-growing and reaches a colony size of 1–2 mm after 24–48-h incubation at 37 °C. Isolates of *H. somni* can have different appearances on blood agar as they can be either beta-haemolytic, alpha-haemolytic or non-haemolytic. The bacterium can easily be overgrown by the resident microflora in a clinical sample.

The 16S rDNA was used as basis for developing a species-specific PCR test (Angen et al. 1998). The appropriateness of targeting the 16S rDNA gene using PCR amplification is further based on the clear separation observed from all other taxa in the phylogenetic analysis (Fig. 2) and the high similarity of the 16S rDNA sequences within the species (Fig. 1). The inclusion of a PCR test among the criteria used in the species description provides a quick and reliable identification method for diagnostic laboratories. The PCR test has been applied to clinical samples, whereby the detection rate of *H. somni* in connection with calf pneumonia has been increased (Tegtmeier et al. 2000).

A clear separation of *H. somni* from other species within the family can also be obtained by using MALDI-TOF (Kuhnert et al. 2012), which can therefore be used for the identification of pure colonies of the bacterium in the clinical or diagnostic laboratory.

3 Phenotypic Characterization

Phenotypic separation of *H. somni* from other genera in the family is possible using the criteria listed in Table 1. In this table, the phenotypical data are restricted to taxa located in the 16S rDNA clusters containing the type strains of the different genera (Olsen et al. 2005). *H. somni* can normally be differentiated from other genera by isolation from a ruminant host (bovine, ovine, etc.) by capnophilia, yellowish pigmentation and indole production. However, capnophilia has also been reported for other taxa within the genus *Haemophilus* (Kilian 2005), and although the

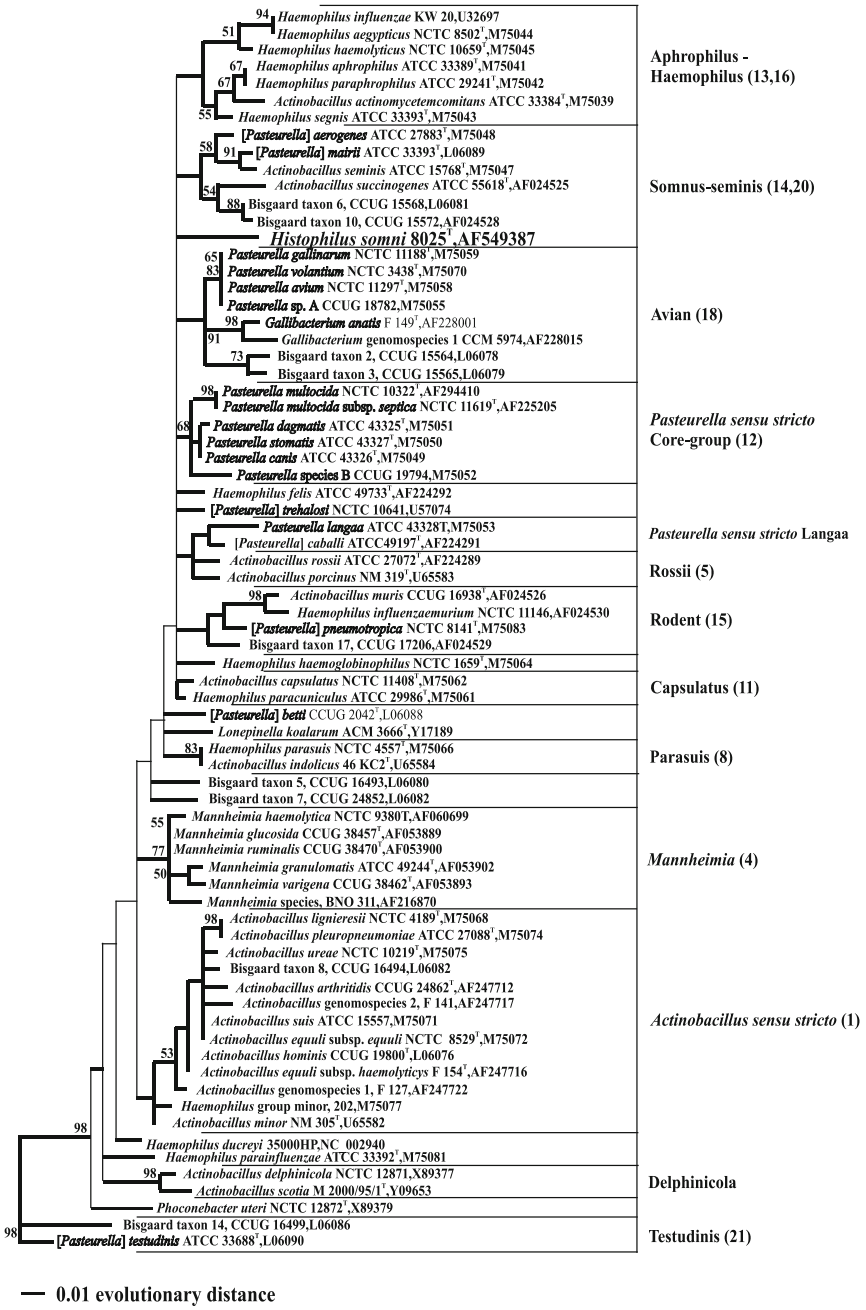

Fig. 2 Phylogenetic relationships between taxa of *Pasteurellaceae* based on maximum-likelihood analysis of 16S rDNA sequences. Strain designation and GenBank accession numbers are indicated. Monophyletic groups supported by "variable outgroup method" are indicated with bold lines. Support for monophyletic groups by "nonparametric bootstrap" analysis is indicated as numbers out of 100. Clusters correspond to designations given by Olsen et al. (2005). Copied with permission from Angen et al. (2003)

Table 1 Phenotypic characters separating *Histophilus* from the other genera of *Pasteurellaceae*

Test	*Histophilus*	*Haemophilus*	*Actinobacillus*	*Pasteurella*	*Mannheimia*	*Lonepinella*	*Phocoenobacter*	*Gallibacterium*
Haemolysis	d	d	d	−	d	−	−	d
Capnophilia	+	−	−	−	−	−	−	−
Yellowish pigmentation	+	−	−	−	−	−	−	−
V-factor dependency	−	+	d	−	−	−	−	−
X-factor dependency	−	+	−	−	−	−	−	−
Catalase	−	−	+	+	+	−	−	+
Oxidase	+	+	d	d	+*	−	+	+
Urease	−	d	+	d	−	−	−	−
VP 37 °C	−	−	−	−	−	+	+	−
Indole	+*	d	−	+	−*	−	−	−
Ornithine decarboxylase	−	d	−	d	d	−	−	−
Sucrose	−	−	+	+	+	d	−	+
D-Galactose	−	d	d	+	+	+	−	+
D-Fructose	−	d	+	+	+	+	Not known	+
Maltose	−	+	+	d	d	+	−	d
Trehalose	−	−	d	d	−	−	−	d

* Deviating strains occur; + indicates only positive reactions, − indicates only negative reactions, d indicates + or −

Phenotypical data are restricted to taxa located in the 16S rDNA clusters containing the type species of the different genera (according to Olsen et al. 2005). *Histophilus* data from the present investigation. *Haemophilus* data based on: H. *influenzae*, H. *aegyptius*, and H. *haemolyticus* (Kilian, 2005). Actinobacillus data based on the type strains of A. equuli subsp. equuli, A. equuli subsp. haemolyticus, A. suis, A. ureae, A. pleuropneumoniae, A. hominis, A. lignieresii, A. arthritidis and Bisgaard taxon 8 (Angen et al., 1999). Pasteurella data based on: P. multocida, P. dagmatis, P. stomatis, P. canis, and Pasteurella sp. B (Mutters et al., 2005). Mannheimia data according to Angen et al. (1999). Lonepinella data according to Osawa and Stackebrandt (2003). Phocoenobacter data according to Foster et al. (2000). Gallibacterium data according to Christensen et al. (2003)

growth of newly isolated strains of *H. somni* is CO_2 dependent, they can apparently adapt gradually to growth under fully aerobic conditions (Biberstein 1981). Variation in pigmentation has also been reported (Corboz 1981). Indole production is observed for most strains. However, strains earlier designated "*Haemophilus agni*" do not produce indole. Furthermore, considerable phenotypic variation between strains and laboratories has been reported (Garcia-Delgado et al. 1977; Biberstein 1981; Fussing and Wegener 1993; Kilian 2005), particularly in regard to carbohydrate fermentation. In contrast to Angen et al. (2003), production of acid has been reported from D-fructose, D-xylose, D-mannose, D-mannitol, D-sorbitol, L-arabinose, maltose and trehalose. The fastidious nature of *H. somni* likely contributes to their variable phenotypic appearance, which complicates bacterial isolation and identification. The phenotypic variability observed (such as in carbohydrate fermentation) may also be related to differences in the composition of fermentation media. Thiamine monophosphate (TMP) is also required for growth in the absence of fresh serum (Asmussen and Baugh 1981), and inadequate levels of TMP inhibit growth and metabolic processes.

4 Subtyping of *Histophilus somni*

Molecular methods have been used to investigate the epidemiology of *H. somni* infections. Corboz and Wild (1981) examined protein electrophoretic profiles by polyacrylamide gel electrophoresis (PAGE) to compare isolates from disease conditions and clinically healthy animals without detecting systematic differences. Subspecific divisions of *H. somni* have also been investigated by random amplified polymorphic DNA (RAPD) (Myers et al. 1993), plasmid profiling (Appuhamy et al. 1998; Fussing and Wegener 1993) and PCR-based methods (Appuhamy et al. 1997, 1998).

A collection of 105 *H. somni* strains from Denmark, Switzerland and the USA was investigated by ribotyping and restriction endonuclease analysis (REA) by Fussing and Wegener (1993). These authors demonstrated 33 different REA patterns and 16 different ribotypes in this collection. Most Danish isolates from pneumonia had the same REA type and ribotype, indicating the presence of a single clone. In contrast, strains isolated from the genital tract generally showed little homology to isolates from pneumonia using these typing methods.

PFGE has also been used by some researchers for subtyping of *H. somni*. D'Amours et al. (2011) investigated 606 isolates collected from four feedlots in Alberta, Canada, and found that most of them belonged to 7 closely related pulsotypes indicating a low genetic variability within this area.

Antigenic diversity within the species has also been demonstrated (Canto and Biberstein 1982). Serological differences were shown to reflect the geographical origin of the respective strains, but not their pathological or anatomical origin. Stephens et al. (1987) could demonstrate at least 11 different serotypes in addition

to a high number of untypable strains. No generally used serotyping scheme has until now been established for *H. somni*.

5 Phylogenetic Investigations

A phylogenetic analysis of the 16S rDNA sequences of 19 *H. somni* strains is shown in Fig. 1. All strains that were previously examined by DNA–DNA hybridization are marked by bold type. The sequence similarities between all investigated strains of *H. somni* were above 99.5 %. The 16S rDNA sequences of *H. somni* are very homogenous and show a very clear separation from all other described taxa.

The phylogenetic position based on 16S rDNA sequencing of the type strain of *H. somni* (8025^T) in the family *Pasteurellaceae* is shown in Fig. 2. By phylogenetic analysis of 16S rDNA sequences, thirteen monophyletic groups and ten single taxa not affiliated with any of these groups were identified. The results support the monophyletic groups published by Olsen et al. (2005), which verify the position of *H. somni* within cluster 20. *H. somni* did not show any close affiliation to any of the existing genera within the family. The highest 16S rDNA sequence similarity observed was to *Actinobacillus succinogenes* at 93.4 %. Deeper phylogenetic relationships were difficult to resolve based on the 16S rDNA sequences.

The relationship between the *rpo*B sequences of 8 selected strains of *H. somni* and the type species of the existing genera within the family *Pasteurellaceae* was reported by Angen et al. (2003) and is shown in Fig. 3. The strains of *H. somni* are

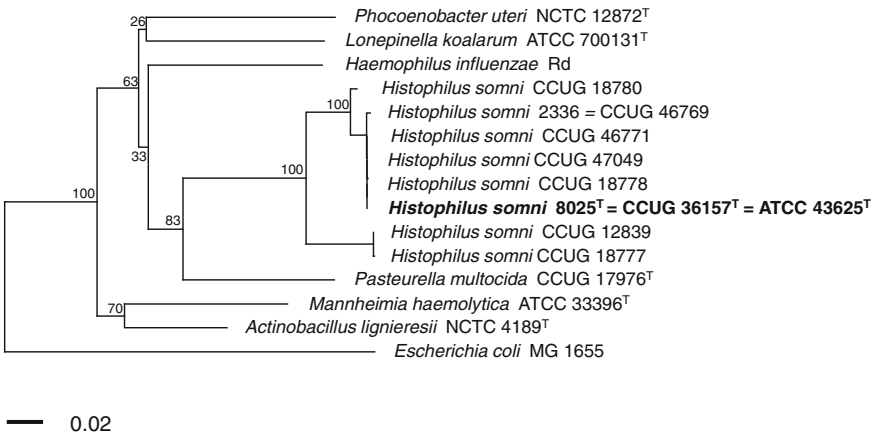

Fig. 3 Phylogenetic analysis based on partial *rpo*B sequences of *Histophilus somni* isolates and type species of the genera in the family *Pasteurellaceae*. The tree was built with the neighbour-joining method based on Jukes–Cantor corrected distances. Bootstrap values of 500 repetitions are indicated as numbers out of 100 at the major branching points. Copied with permission from Angen et al. (2003)

clearly separated from the other reference strains within the family. There is 15–20 % difference between the *rpo*B gene sequence of *H. somni* compared to that of the other type species of the *Pasteurellaceae*. Among the *H. somni* strains, two clusters were observed, which were separated by a sequence difference of about 6 %.

A more comprehensive investigation of the phylogeny of the family *Pasteurellaceae* that was based on the *rpoB* sequences was published by Korczak et al. (2004). In their analysis of the *rpoB* sequences from 72 type strains or reference strains within the family, *H. somni* was found to be most closely related to *Haemophilus haemoglobinophilus*, but unrelated to genus *Haemophilus* sensu stricto. However, analysis of the 16S rDNA sequences of the same collection of strains found *H. somni* to be most closely affiliated to *Actinobacillus succinogenes*. The authors concluded that the topology of the *rpoB* tree generally was in better agreement with the DNA–DNA hybridization results than the 16S rDNA tree.

Kuhnert and Korczak (2006) published a multisequence-based phylogeny of the family based on a combination of the genes *rrs*, *rpoB*, *infB*, *recN*, *rpoA* and *thdF*. In this analysis, only the type strain of *H. somni* 8025^{T} was included. In the consensus tree based on these six genes, *H. somni* was deeply branching and had no close affiliation to other genera of the family; *Avibacterium gallinarum* was the closest relative. Their results indicated that a MLST based on three of these genes (*recN*, *rpoA* and *thdF*) could approximate the DNA–DNA similarity among the species of the family. In this work, 16S rDNA was the gene showing the lowest correlation with other tree topologies.

From the different work cited so far, it can be seen that the phylogenetic position of *H. somni* is highly dependent on the gene or genes chosen. It is also evident that the genus *Histophilus* is not closely and consistently related to another genus of the family based on sequence analysis of a single gene or a small number of genes. Furthermore, DNA–DNA hybridizations are not suited to discern taxonomic relationships between genera due to low reproducibility at the lower hybridization levels and the difficulties in obtaining a complete matrix of hybridizations between members of the family.

6 Whole-Genome Sequencing

The increasing feasibility of whole-genome sequencing of bacterial genomes opens up new possibilities for elucidating the taxonomic relationships between species. Thus far, complete genome sequences are available for only two strains of *H. somni*. In 2007, the genome sequence of strain 129Pt, a non-pathogenic commensal preputial isolate, was published (Challacombe et al. 2007). The genome sequence of strain 2336, isolated from calf pneumonia, was published in 2011 (Siddaramappa et al. 2011). In a publication by Bonaventura et al. (2010), 12 genome sequences representing 10 species within the family *Pasteurellaceae* were investigated. In this analysis, *H. somni* was found to be most closely affiliated with *Pasteurella multocida* and more distantly related to *Haemophilus influenzae*, *Haemophilus*

aphrophilus and *Aggregatibacter actinomycetemcomitans*. Bonaventura et al. (2010) concluded that matrices using genome-level information are very robust to missing values and also that a large number of concatenated genes (>160) are needed to reliably obtain the same overall topology as the overall analysis. This also offers an explanation as to why phylogenies based on single or only a few genes tend to obscure the evolutionary history of bacterial species. On the other hand, one might expect that when including an increasing number of bacterial species and genomic diversity to the analysis, this will give an increasingly better estimate of the phylogeny of the genera and species of the family *Pasteurellaceae*.

Whole-genome sequencing will probably also contribute to a more accurate determination of the phylogenetic position of *H. somni*. Making comparisons between whole genomes is problematic due to the huge diversity between genomes of bacteria, both within species and to a much larger degree between species. A further obstacle to phylogeny-based bacterial characterization is that different loci within the same genome can have widely different evolutionary histories (Holmes et al. 1999). A gene-by-gene approach to population genomics has a number of advantages over methods of multiple genome comparisons that rely on whole-genome alignment and multiple pairwise comparisons (Jolley et al. 2012). Ribosomal multilocus sequence typing (rMLST) is an approach that indexes variations within 53 genes encoding the bacterial ribosome protein subunits (*rps* genes). The *rps* loci are ideal targets for a universal characterization scheme as they are present in all bacteria, distributed around the chromosome (Fujita et al. 1998), and encode proteins which are under stabilizing selection for functional conservation (Bashan and Yonath 2008). Jolley et al. (2012) showed that rMLST can be used to resolve bacteria into groups at all taxonomic levels, and also to a certain degree, for typing purposes. The use of rMLST, and certainly other analytical methods yet to be developed, will probably be able to determine the affiliation of *H. somni* to other species and indicate a "true" phylogenetic position of the species within the family *Pasteurellaceae.*

The analysis of the two available *H. somni* genome sequences has also provided valuable information regarding genetic events moulding the genomes within the species. The chromosome of strain 2336 contains 1980 protein-coding genes (2,263,857 bp), whereas the chromosome of strain 129Pt contains only 1792 protein-coding genes (2,007,700 bp). Siddaramappa et al. (2011) compared the genomes of strain 2336 and 129Pt and found that most of the genes and loci common to the two strains were located in different regions of the two chromosomes and occurred in opposite orientations indicating genome rearrangements since their divergence from a common ancestor. Their analysis also revealed that bacteriophage- and transposon-mediated horizontal gene transfer had occurred at several loci in the chromosomes of the two strains. Therefore, mobile elements seemed to have played a major role in creating genomic diversity and phenotypic variability among the two strains.

The two strains had 1586 protein-coding genes in common (Fig. 4). Strain 2336 was found to have a higher number of unique genes (315/2012, 16 %) than strain 129Pt (152/1844, 8 %) (Challacombe and Inzana 2008). It is also noteworthy that a number of genes present in strain 2336, but absent from strain 129Pt, encode

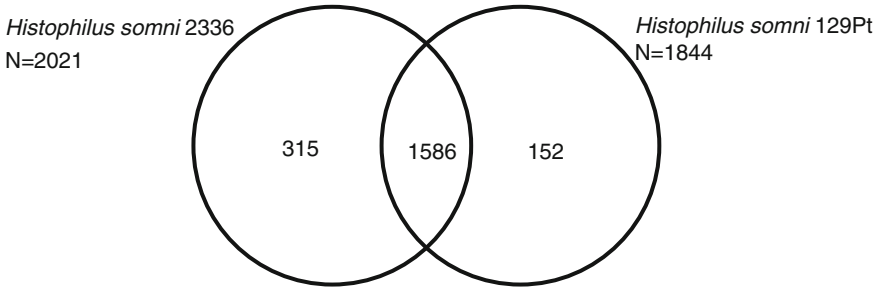

Fig. 4 Common and unique genes in the genomes of two *Histophilus somni* strains: 129P7 (commensal) and 2336 (pathogenic)

putative virulence factors such as auto-transporter adhesins, filamentous hemagglutinin (Fha) homologs, restriction–modification systems, prophage-like sequences and some phase-variable lipooligosaccharide (LOS) biosynthesis proteins (Sandal and Inzana 2009). However, broad generalizations regarding the genetics of *H. somni* cannot be applied to the entire species until the genomes of additional strains are sequenced. Another interesting observation is that several attributes are shared between *H. somni* and some important human pathogens (e.g. *Bordetella*, *Neisseria*, and *Haemophilus* spp.), including biofilm formation, LOS phase variation and sialylation, and the synthesis of homologous Fha and OMPs. *H. somni* infections in bovines might therefore prove to be useful models to study host-specific pathogens that cannot be studied in the human host (Sandal and Inzana 2009).

7 Conclusions

Histophilus somni seems to represent a relatively homogeneous species and is the only species within the genus *Histophilus*, showing no close affiliation to other species or genera within the family *Pasteurellaceae*. Whole-genome sequencing has revealed distinct genetic differences between a commensal and a pathogenic strain, for instance regarding putative virulence factors. However, broad generalizations regarding the genetics of *H. somni* cannot be applied to the entire species until the genomes of additional strains are sequenced.

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Histophilosis as a Natural Disease

D. O'Toole and K.S. Sondgeroth

Abstract *Histophilus somni* is responsible for sporadic disease worldwide in cattle and, to a lesser extent, in small ruminants, bighorn sheep (*Ovis canadensis*), and North American bison (*Bison bison*). The importance of *H. somni* diseases can be attributed to improved clinical and laboratory recognition, combined with the growth in intensive management practices for cattle. Although outbreaks of bovine histophilosis can occur year-round, in northern and southern hemispheres, it is most frequent in late fall and early winter. Weather, stress, dietary changes, and comingling of cattle are likely to be major triggers for outbreaks. The most frequent clinical expressions of histophilosis include undifferentiated fever, fibrinosuppurative pneumonia, encephalitis-leptomeningitis, necrotizing myocarditis, and diffuse pleuritis. Neurological disease occurs either as thrombotic meningoencephalitis (TME) or as suppurative meningitis with ventriculitis. Acute myocarditis is characteristically necrotizing and generally involves one or both papillary muscles in the left ventricular myocardium. Biofilm-like aggregates of bacteria occur in capillaries and veins in myocardium, in the central nervous system, and on endocardial surfaces. *H. somni* is a component of bovine respiratory disease (BRD) complex. In our experience, it is most commonly diagnosed in subacute-to-chronic polymicrobial pulmonary infections in combination with *Mannheimia haemolytica*, *Trueperella pyogenes, Pasteurella multocida*, or *Mycoplasma bovis.* Other, less common forms of *H. somni* disease present as polyarthritis/tenosynovitis, abortion with placentitis and fetal septicemia, epididymitis-orchitis, and ocular infections. It is likely that *H. somni* is under-recognized clinically and diagnostically. Most state and provincial laboratories in North America rely on bacterial isolation to confirm infection. The use of more sensitive detection methods on field cases of histophilosis will help resolve the pathogenesis of *H. somni* in natural outbreaks, and whether the disease is as common elsewhere as it is in Canada.

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Abbreviations

BRD	Bovine respiratory disease
CNS	Central nervous system
HSDC	*H somni* disease complex
IHC	Immunohistochemistry
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NET	Neutrophil extracellular trap
PCR	Polymerase chain reaction
TME	Thrombotic meningoencephalitis

Contents

1 Introduction

Research into the biology of *Histophilus somni* moved on apace in the last 20 years in the laboratories of Corbeil, Inzana, Czuprynski, Tegtmeier, and others (Yarnall et al. 1988; Inzana et al. 1992; Tegtmeier et al. 2000; Sylte et al. 2001; Sandal et al. 2007; Corbeil 2007; Sandal and Inzana 2010; Siddaramappa et al. 2011). In considerable contrast, new or corroborating information regarding the natural ecology of histophilosis in production settings is limited, particularly in the USA. What novel data we possess about clinical disease was developed by a small cadre of clinicians, epidemiologists, microbiologists, and pathologists in Canada and, to a lesser extent, Denmark (Humphrey et al. 1982; Harris and Janzen 1989; Gogolewski et al. 1989; Van Donkersgoed et al. 1990; Schuh and Harland 1991; Van Donkersgoed et al. 1995; Angen et al. 1998; Booker et al. 1999; Tegtmeier

et al. 1999b, 2000; Haines et al. 2001, 2004; Clark 2005). Diseases due to *H. somni* are a diagnostic challenge due to the agent's status as both a commensal and a pathogen, its tendency to occur in combination with other agents, and its fastidious growth characteristics (Quinn et al. 2007). It is a neglected truism that research hypotheses about pathogens should be anchored in field work on the natural disease. This is particularly true for *H. somni*.

Multiple knowledge gaps remain about bovine histophilosis. There is the practical aspect of veterinarians attempting to advise clients dealing with clinical histophilosis in their herds and flocks. One of the first questions asked about the disease was: Where in the host does vascular invasion by *H. somni* originate (Kennedy et al. 1960)? Half a century after Kennedy and colleagues posed that question, we still lack an answer (Maxie and Youssef 2007). Does it slip between pulmonary pneumocytes, digest underlying basement membrane, as suggested by Corbeil and colleagues (Zekarias et al. 2010; Agnes et al. 2013), and pass into the capillary beds of the lungs? What fraction of undifferentiated fever in fattening cattle is attributable to histophilosis? Why is it so difficult to experimentally reproduce the diffuse pleuritis that is one common clinical expression of histophilosis? How and why does intravascular infection localize with such extraordinary numbers of bacteria in papillary muscles of the left ventricular myocardium? Does formation of biofilms in brain, heart, and elsewhere limit the practical significance of in vitro antimicrobial sensitivity of isolates in specific outbreaks? How effective are commercial vaccines under feedlot conditions and which products, if any, does it make economic sense to use? If thrombotic meningoencephalitis (TME) indeed faded in importance since the 1980s as part of the *H. somni* disease complex (HSDC) (Orr 1992), what drove the process? A recent analysis of the literature sought to define basic aspects of responses by cattle to experimental challenge with infectious agents contributing to the bovine respiratory disease (BRD) complex (Grissett et al. 2015). The authors wished to characterize basic parameters such as time of exposure to onset of clinical signs, time to peak disease outbreak, time for signs to resolve, minimum time to shedding, time to maximum shedding, time for shedding to resolve, and time to seroconversion. Of eight major BRD agents examined, one stood apart. *H. somni* lacked the published studies of natural and experimental disease that met the authors' modest inclusion criteria.

This chapter is written by two diagnosticians based in a western US diagnostic laboratory. We attempt to summarize our understanding of histophilosis as a natural disease. It is informed in part by laboratory accessions from field outbreaks over the past 25 years.

Histophilosis was documented as a disease in feedlot cattle in the 1950s (Griner et al. 1956). Kennedy et al. (1960) identified the causative agent as *Haemophilus*-like. The name *Haemophilus somnus* was proposed by Bailie in 1969. The agent was renamed *H. somni* in 2003, and a new genus was established in the family *Pasteurellaceae* as a result (Angen et al. 2003). *H. somni* incorporates two species of previously uncertain placement that were isolated from sheep: '*Histophilus ovis'* and '*Haemophilus agni.' H. somni*-like agents were isolated from diseased sheep as early as the 1920s, where a septicemic disease was described in late fall and early

winter (Mitchell 1925). *H. somni* has since been associated with multiple clinicopathological syndromes in cattle, most of them presumed sequelae to septicemia. Major manifestations are fever, pneumonia, pleuritis, meningoencephalitis-myelitis, necrotizing myocarditis, and pericarditis. Two or more of these manifestations can occur in individual animals (Van Donkersgoed et al. 1990). Other diseases due to *H. somni* are abortion, conjunctivitis, arthritis-tenosynovitis, and reproductive diseases of the male and female reproductive tracts. Lesions may occur in other sites, typically as a facet of septicemia: nephritis, endophthalmitis, myositis, laryngitis, otitis media, and intestinal necrosis.

It is likely that histophilosis continues to be underdiagnosed in commercial herds and flocks. This is due to the ease with which small fatal lesions can be overlooked at necropsy, particularly in the heart, and due to difficulties with laboratory isolation of *H. somni*, especially from the lungs, when other pathogenic organisms are concurrently present (Moisan and Fitzgerald 1995; Haines et al. 2004). In addition, *H. somni* is susceptible to most antibiotics, so most treated animals would be culture-negative.

2 Clinical Diseases

2.1 Cattle

Although the incidence of thrombotic meningoencephalitis-myelitis (TME) appears to have declined in the past 20 years (Orr 1992; Haines et al. 2004), for many food animal veterinarians, the impression persists that TME remains the main manifestation of infection. If that were true, *H. somni* could be relegated to the status of an interesting, but relatively minor, pathogen. TME is sporadic in feedlots, backgrounding operations, and cow–calf herds. It occurs, relatively rarely, as a disease in dairy operations and among beef cattle on grass (Panciera et al. 1968; Saunders and Janzen 1980; Saunders et al. 1980; de Lahunta and Divers 2008). TME affected only 11 % of feedlots over one two-year investigation period (Little 1986). Average mortality due to TME was 0.1 % of the population at risk (Little 1986). A recent survey of diseases in US feedlot established that neurological disease of all kinds (including TME) affected 1.1 % of all feedlot cattle. To put this in perspective, 16.2 % of feedlot cattle were affected with respiratory disease (USDA 2011). It is more useful to consider *H. somni* as the cause of a disease complex in which many cattle are infected and only a few develop clinical symptoms. Some animals develop brief clinical signs and then carry *H. somni* in the lung for weeks, as demonstrated in one small study of experimental pneumonia (Gogolewski et al. 1989). The economic impact of subclinical histophilosis is largely unexplored, in spite of its likely importance (Kennedy et al. 1960). An experienced food animal veterinarian remarked recently, in the context of BRD complex, that subclinical disease is the monster we *don't* see (Griffin 2014). This is due to the practical

difficulties of attempting to follow specific cohorts of cattle through slaughter for sampling and testing, and matching those findings to those in individual animals, treated and untreated at the feed yard. Histophilosis is a component of BRD, which is the single most important cause of morbidity, mortality, and economic loss in stocker and feedlot cattle in North America (Loneragan et al. 2001; Gagea et al. 2006; Woolums 2015). Bacterial pneumonia of all causes (including *H. somni*) is second only to diarrhea as the leading cause of economic loss in dairy calves (Panciera and Confer 2010). *H. somni* was identified as the third most important bacterial pathogen found in BRD in one Midwestern study (1994–2002), with *Mannheimia haemolytica* ranked first (46.3 % of isolations), followed by *Pasteurella multocida* (34.7 %), and *H. somni* (19.0 %) (Welsh et al. 2004). BRD tends to peak around two weeks after animals arrive in feedlots, with 15–45 % of all incoming calves requiring treatment and death occurring in 1–5 % (Kelly and Janzen 1986).

In North America, clinical histophilosis is most commonly seen in stocker cattle (135–250 kg weaned calves), animals aged 6–12 months in feedlots, and young dairy calves (Griner et al. 1956; Panciera et al. 1968; Saunders et al. 1980; Bryson et al. 1990; Orr 1992; Moisan and Fitzgerald 1995; de Lahunta and Divers 2008; Francoz et al. 2015). In some parts of Europe, histophilosis is considered one of the most important causes of respiratory disease in young calves (Tegtmeier et al. 1995). Disease occurs year–round, but, in Northern Hemispheres, most clinical cases occur between October and January (Panciera et al. 1968; Orr 1992). In Wyoming, the bovine histophilosis season occurs in the fall when spring-born calves are comingled. This is associated with an abrupt drop in temperature in September–October and stress due to transportation, handling, vaccination, dietary change, and sorting. Clinical histophilosis is particularly associated with Western and Midwestern states and provinces of the USA and Canada. Similar weather-related stress in late fall and early winter in Southern Hemisphere countries coincides with the peak in histophilosis cases (pers. comm., Carlos Margineda).

2.1.1 *Histophilus somni* Disease Complex (HSDC) and Respiratory Disease

The HSDC encompasses septicemia and associated complications: undifferentiated fever, pneumonia, pleuritis, myocarditis, thrombotic meningoencephalitis-myelitis, tenosynovitis, and otitis media (Nation et al. 1983; Harris and Janzen 1989; Clark 2005). Signs of respiratory disease are typical of BRD: tachypnea, cough, nasal discharge, and depression. Some animals with pneumonia are found dead—this is the most common history associated with animals that have severe fibrinous pleuritis at necropsy (Clark 2005). Concurrent infections, particularly with viruses (bovine respiratory syncytial virus, bovine parainfluenza virus 3, bovine herpesvirus 1, bovine coronavirus, and bovine viral diarrhea virus), may predispose cattle to pulmonary histophilosis (Woolums 2015). Such agents are generally not detected in *H. somni*-infected cattle at the time of death. For reasons related to

specific virulence factors, *H. somni* can persist in lungs even when clinical signs are minimal (Gogolewski et al. 1989). Since *H. somni* alone causes pneumonia experimentally (Gogolewski et al. 1987a, b, 1988; Gogolewski et al. 1989; Geertsema et al. 2011), it is difficult to determine whether viral infection precedes histophilosis in cases where viruses are not detected at death.

The most comprehensive field studies of histophilosis have been performed in Canada. HSDC accounts for up to 40 % of all mortalities at some Canadian feedlots (Van Donkersgoed et al. 1994a, b), with most clinical cases recognized two-to-nine weeks after introduction (Van Donkersgoed et al. 1990). Serological studies suggest that animals are infected and seroconvert within 14–21 days of feedlot entry. A sequence of clinical manifestations at different time points has been reported (Van Donkersgoed et al. 1990). The clinical onset of fatal pneumonia of all causes, including histophilosis, has a median of 12 days after arrival (range 1–30 days). Other clinical syndromes occur somewhat later: overt septicemia (median time of onset 17 days; range 13–19), polyarthritis (18 days; range 5–41); pleuritis (22 days; range 11–37); myocarditis (22 days; range 3–36); and TME (17 days; range 19–29) (Van Donkersgoed et al. 1990). A subsequent feedlot study found that most cases of *H. somni* myocarditis occur around 60 days, with a peak in acute bronchopneumonia cases at 25 days (Gagea et al. 2006). A logical inference is that HSDC starts as a disease of the lower respiratory tract, and it is from here that septicemia originates (Clark 2005). Experimental studies established that *H. somni* can pass between alveolar epithelial cells and induce alveolar epithelial cells to secrete matrix metalloproteinases, digesting basement membrane collagen (Zekarias et al. 2010, Agnes et al. 2013).

2.1.2 Central Nervous System Disease

Thrombotic meningoencephalitis-myelitis (TME) due to *H. somni* is largely a disease of older calves and yearlings (Griner et al. 1956; Fecteau and George 2004). Originally called thromboembolic meningoencephalitis (Kennedy et al. 1960), it is now recognized that endothelial damage and thrombosis, rather than embolism, is central to the pathogenesis of the disease (Maxie and Youssef 2007). It is rare to see TME in animals less than 4 months of age, but cases are reported in neonatal calves, some as young as 2 days old (Headley et al. 2013; Saunders et al. 1980). The disease is particularly associated with feedlots, where losses may be high and continue over several weeks (Descarga et al. 2002). TME does not behave like a contagious disease, and during an outbreak, individual cases occur sporadically in separate pens throughout a feed yard. Some strains of *H. somni* that are isolated from the reproductive tract of healthy cattle are capable of inducing suppurative encephalitis following experimental intracisternal inoculation (Kwiecien and Little 1992). Clinical signs are typical of acute meningoencephalitis: depression, fever and blindness with or without seizures, coma, and sudden death (De Lahunta and Divers 2008). The closed-to-semi-closed eyes in many affected cattle gave rise to the common phrase 'sleeper syndrome' and the species name in *H. somni*. Fever is

generally present (Fecteau and George 2004). Otitis is a feature in some animals (McEwen and Hulland 1985). Animals may be so obtunded that it is not possible to tell whether they are blind, or instead are severely depressed (Harris and Janzen 1989). Lameness with a stiff gait, ataxia, and paresis is common. In extensive ranch operations, animals on grass are likely to be found dead. Untreated animals rarely live longer than 24–48 h (De Lahunta and Divers 2008). Only animals detected early in the clinical course (i.e., while still ambulatory) are likely to survive the following treatment. In Wyoming, it is generally impractical to subject animals with neurological signs to detailed neurological or ophthalmological examination, or to antemortem diagnostics such as collection and assessment of cerebrospinal fluid. Ancillary testing may be helpful, since hemorrhagic retinal infarcts have been reported in 20 % of cases, and meningeal exudate is generally abundant (Nayar et al. 1977; Little 1986; Orr 1992; Fecteau and George 2004). There is a consensus that the incidence of TME declined in the 1980s, following its recognition in the mid-1950s (Harris and Janzen 1989). In the 1960s, TME was the most common infectious neurological disease seen in US feedlots. It accounted for 60.5 % of all cases in Kansas in which a diagnosis of encephalic disease was made, compared to the second most common disease (polioencephalomalacia), which was responsible for 16.9 % (Bailie 1969). A proportion of animals with neurological histophilosis present with paresis due to the major lesion(s) occurring in the spinal cord.

2.1.3 Myocarditis and Sudden Death

Myocarditis-infarction is the most common form of *H. somni* infection that results in sudden death. TME, acute pneumonia, and diffuse pleuritis can also cause sudden death. The importance of myocarditis in histophilosis was recognized in the 1980s when a series of reports from Canada noted the association between this lesion and infection with *H. somni* (Janzen 1987; Guichon et al. 1988; Harris and Janzen 1989; Schuh and Harland 1991; Haines et al. 2004). Clients have reported seeing animals walk across a pen and drop dead without any premonitory signs—often one necrotic papillary muscle is found to be acutely ruptured. Chronic forms of myocardial histophilosis occur, leading to lethargy, ill thrift, and depression. This is less common in our experience.

Between October 2008 and May 2009, we monitored the occurrence of fatal histophilosis on one backgrounding operation and a large feedlot in southeast Wyoming. This was after the attending veterinarian (Dr. R. Hunter, Wheatland) alerted the Wyoming State Veterinary Laboratory to annually recurring necrotizing myocarditis. Our initial response was skepticism, since *H. somni* myocarditis was rarely diagnosed in mailed-in accessions. Like other diagnosticians, we assumed that papillary myocarditis was a peculiarity of feedlots in western Canada. Subsequent study confirmed the veterinarian's impression. Total morbidity and mortality rates (all causes) over 8 months (September–May) among 4612 cattle in the backgrounding operation were 15.9 % (734 animals) and 0.34 % (16 animals), respectively. The unusually low mortality was attributed to low-stress handling.

Morbidity and mortality rates in 4199 cattle in the feedlot during the same period were 34.1 % (1433 animals) and 4.5 % (187 animals), respectively. Necrotizing myocarditis accounted for 18.8 and 8.0 % of all deaths in the background operation and feedlot, respectively. No effort was made to assess what proportion of BRD cases contained *H somni*, with or without other agents. All cases of myocarditis examined at the laboratory were due to histophilosis, as established by culture and immunohistochemistry (IHC).

2.1.4 Other Manifestations of HSDC

H. somni is often found in the joints of animals with HSDC. Typically, arthritis-synovitis occurs following episodes of undifferentiated fever and TME. There is polyarthritis with firm swellings of joints, and animals display stiffness, lameness, and knuckling (Harris and Janzen 1989). Soft tissue around affected joints may be edematous. The atlanto-occipital joint is often affected (Maxie and Youssef 2007). *H. somni* can be isolated in pure culture from the joints of untreated animals. Otitis may coincide with the peak hemophilosis season in feedlot cattle (November–December). Morbidity is generally low, but in some herds, it reaches 10 % (Nation et al. 1983). Affected animals have a copious serous discharge from one or both external ear canals. Discharge drains from the pinna, moistening the hair coat and forming frozen balls of exudate. Affected cattle are febrile. In affected herds, there may be concurrent respiratory disease or TME (McEwen and Hulland 1985). *H. somni* can be obtained in pure culture from such cases. Routine short-term antibiotic treatment is effective. In some animals, infection tracks along the vestibulocochlear nerve and cause suppurative leptomeningitis. *H somni* is an occasional cause of conjunctivitis (Lamont and Hunt 1982).

2.1.5 Reproductive Tract Infection, Accessory Sex Glands, and Disease

Infection of the male and female reproductive tracts is an important aspect of infection with *H. somni*. Many clinically healthy bulls, and a smaller proportion of cows/heifers, harbor the bacterium in their reproductive tracts (61 and 15 %, respectively) for extended periods (Kwiecien and Little 1991; Corbeil et al. 1986). One early study reported that commensal infection was most common in young bulls with detection of *H. somni* in descending order in the following: preputial orifice > preputial cavity > urinary bladder > accessory sex glands > ampulla of ductus deferens (Humphrey et al. 1982). Infection in bulls has minimal clinical effects, apart from causing infrequent infertility and poor semen quality. Shedding of the organism in urine or discharges is one source of environmental contamination (Yaeger and Holler 2007).

In addition to commensal infection, endometritis accompanied by vaginitis-cervicitis (dirty cow syndrome) occurs due to *H. somni*, principally in Australia, Europe, Canada, and Africa. This is associated with early returns to service and infertility (Van Dreumel and Kierstead 1975; Saunders and Janzen 1980; Stephens et al. 1986; Corbeil et al. 1986). *H. somnus* is commonly isolated from the diseased female bovine reproductive tract in pure or mixed cultures (Kwiecien and Little 1991). Infection is characterized by purulent vaginal discharge and, in some herds, granular vaginitis-cervicitis following breeding (Chladek 1975; Saunders and Janzen 1980; Last et al. 2001; van der Burgt 2008; Headley et al. 2013; Bano et al. 2011). However, reproductive tract syndromes, other than sporadic abortion, have not been noted by some veterinary pathologists (Clark 2005). In a review of abortion cases investigated by the Washington Animal Disease Diagnostic Laboratory, 9 cases due to *H. somni* were detected over 9 months (Corbeil et al. 1986). *H. somni* was responsible for 0.23 % of all bovine abortions in one large survey in the American Midwest (Kirkbride 1993a). It ranked as the 11th most common cause of bacterial abortion and comprised 1.6 % (21/1299) of all cases in which a bacterial agent was detected. Laboratory-confirmed cases of spontaneous abortion typically occur in late gestation, reflecting in part the likelihood of an owner finding such larger fetuses. Experimental studies show that abortion may occur at any time during pregnancy and that blood-borne infection is an efficient route in causing placental infection (Corbeil et al. 1986). Use of vaginal–endometrial and preputial washes, which for our laboratory tend to be underused as a diagnostic tool to investigate infertility, can help establish the involvement of *H. somni* in some episodes (van der Burgt 2008). A review of the role of the bacterium in causing herd infertility considered the role of this pathogen tenuous (Yaeger and Holler 2007). Mastitis due to *H. somni* is seen on rare occasion and was reproduced experimentally (Hazlett et al. 1983).

2.1.6 Treatment and Control

There are three approaches to control anticipated outbreaks of histophilosis in cattle: vaccination, mass treatment with antimicrobial agents, and vaccination for other agents of the BRD complex that predispose to respiratory disease.

There are 26 USDA-approved *H. somni* vaccines manufactured by 6 companies for the American market. They are heavily used, with 69.7 % of all large feedlots in the US vaccinating cattle against *H. somni* (USDA 2011). Surprisingly, their ability to confer immunity in production settings is modest or unknown (Little 1986; Van Donkersgoed et al. 1994a, b; Booker et al. 1999; Bowland and Shewen 2000; Geertsema et al. 2011; Larson and Step 2012; Taylor and Confer 2014). Exceptions are bacterins that confer immunity against TEM following experimental intravenous or intracisternal challenge (Williams et al. 1978; Stephens et al. 1982, 1984). In experimental settings, vaccination reduces the severity of clinical respiratory disease following challenge. The proprietary data generated by companies

have been sufficient to persuade regulatory agencies to grant product licenses (Hall et al. 1977; Saunders and Janzen 1980; Amstutz et al. 1981; Morter et al. 1982; Morter and Amstutz 1983; Groom and Little 1988; Ribble et al. 1988; Silva and Little 1990; Stephens 1990).

The capacity of commercial products to reduce losses due to TEM is one possible explanation for the apparent shift in clinical expression of histophilosis from neurological (1960–late 1980s) to respiratory, cardiac, and septicemic disease (late 1980s–to date). Problems associated with *H. somni* products include loss of protective surface antigens during manufacture, induction of IgE-specific antibody responses, and anaphylactic/endotoxic reactions (Ellis and Yong 1997; Geertsema et al. 2011). Experiences with the latter are responsible for the reluctance of some clinicians to use *H. somni* bacterins, even in herds where histophilosis is likely each fall. Incorporation of protective components, such as recombinant IbpA subunits, might enhance the efficacy of commercial products (Zekarias et al. 2011; Lo et al. 2012).

An alternative to vaccination is metaphylactic treatment in anticipation of disease outbreaks, along with prompt individual treatment of sick cattle (Van Donkersgoed et al. 1994a). In a recent survey, it was found that 92.6 % of large feedlots (>8000 head) in the USA used mass treatment with antibiotics when a BRD outbreak was anticipated in lightweight calves (<320 kg)(USDA 2011). Many antibiotics have good in vitro activity against bacterial pathogens of BRD, including *H. somni* (Van Donkersgoed et al. 2009; Wollums 2015). Post-arrival metaphylaxis with long-acting oxytetracycline does not reduce the risk of hemophilosis mortality, but minimizes total losses due to BRD (Van Donkersgoed et al. 1994a, b; Van Donkersgoed et al. 2009). Tilmicosin (Micotil®), tulathromycin (Draxxin®), ceftiofur (Naxcel®, Excenel®, Excede®), enrofloxacin, trimethoprim–sulfadoxine, and florfenicol (with and without nonsteroidal anti-inflammatories such as flunixin meglumine) are used to suppress the occurrence of HSDC. However, there is concern about later problems that may result due to antibiotic resistance after metaphylaxis. *H. somni*-associated endometritis responds well to intrauterine treatment with antibiotics such as oxytetracycline, sexual rest, and switching from natural service to artificial insemination (Last et al. 2001; Yaeger and Holler 2007).

The third approach is to vaccinate against other agents of the BRD complex. Published information on the effectiveness of vaccines for histophilosis is limited. A recent meta-analysis suggested that the risk of BRD morbidity in vaccinated animals is not statistically different from that in controls. Such analyses are constrained by the small number of published vaccine studies that deal with respiratory disease and *H. somni* (Larson and Step 2012). It is likely that there are benefits to vaccinating feedlot cattle against infectious cofactors for *H. somni* infection (*Mannheimia haemolytica*; *Pasteurella multocida*; bovine respiratory syncytial virus; bovine herpesvirus 1). The magnitude of this effect is unknown (Larson and Step 2012; Wollums 2015).

2.2 Small Domestic Ruminants

H. somni (now incorporating *Haemophilus agni* and *Haemophilus ovis*) can be a pathogen or a commensal bacterium of the genital tract of sheep, with the highest prevalence in ram lambs aged 12–20 weeks (Walker and LeaMaster 1986). Histophilosis is a considerably less common disease in sheep than cattle, manifesting primarily as lameness, septicemia, and epididymitis-orchitis (Kennedy et al. 1958; Poonacha and Donahue 1984; Lundberg 1986). Other presentations are abortion, generalized pyemia, metritis, mastitis, and TME. Histophilosis tends to affect lambs, with polyarthritis in younger (1–4 weeks) and septicemia in older animals (4–7 months) (Philbey et al. 1991). Disease can occur as outbreaks. Morbidity is 1–10 %, though more commonly at the lower end of the scale (0.5–1.5 %). Mortality approaches 90 % in untreated symptomatic lambs. Sheep are either found dead or they expire within hours of developing clinical signs of fever, depression, recumbency, and cyanosis. Neurological disease occurs in some episodes, affecting both lambs and adults. As in cattle, CNS disease may occur as TME, or as suppurative leptomeningitis-ventriculitis-choroiditis (Philbey et al. 1991). The means of transmission is unknown. Septicemia can be induced experimentally by intranasal and intravenous inoculation (Rahaley 1978a, b). Suppurative leptomeningitis-ventriculitis without vasculitis can be induced by intracisternal inoculation of both septicemic and one of two preputial strains tested (Lees et al. 1994). As with cattle, stress and crowding are presumed to be precipitating factors.

Epididymitis-orchitis is a multifactorial bacterial disease in 6–15 month-old rams. *H. somni* is one of several causative agents that infect the epididymis and testis (Lees et al. 1990; Ridler and Sargison 2007). Disease has been reported in New Zealand, Australia, Canada, South America, and South Africa. The sheep at highest risk are vigorous rams in intensive systems and on high planes of nutrition. The disease is occasionally fatal with fever due to epididymitis-orchitis. Chronic epididymitis is more common and tends to affect older animals. In one experimental study using isolates from various sites and species, the most pathogenic was from a natural outbreak of ovine epididymitis-orchitis (Díaz-Aparicio et al. 2009). The main differential diagnosis in Wyoming is brucellosis due to *Brucella ovis*, as the clinical presentations are similar. At least two multiplex PCR assays have been developed to identify *H. somni*, as well as *B. ovis* and *Actinobacillus seminis*, in semen and urine (Saunders et al. 2007; Moustacas et al. 2013). Outbreaks of *H. somni*-induced vulvitis-vaginitis occur during the breeding seasons. Disease is transmissible venereally to rams, which develop ulcerations on the glans penis (Ball et al. 1991). *H. somni* has also been isolated from the vaginal fluid of apparently asymptomatic ewes (Higgins et al. 1981).

H. somni is a commensal in goats. It has been isolated from the vagina and prepuce, but not from nasal passages. On the basis of partial 16S rRNA sequences, three caprine isolates identical to an ovine septicemic isolate were assumed to have been derived from sheep (Jánosi et al. 2009). *H. somni* has not been associated with disease in goats to date.

2.3 Bighorn Sheep

H. somni has been isolated from bighorn lambs (*Ovis canadensis canadensis*) with respiratory disease and in free-ranging herds with poor lamb recruitment (Ward et al. 2006; Miller 2001). Bronchopneumonia is a population-limiting disease in bighorns, and its major infectious components are considered to be *Mannheimia haemolytica*, *Bibersteinia trehalosi,* and *Mycoplasma ovipneumoniae* (George et al. 2008; Besser et al. 2014). *H. somni* appears to be a minor component in natural outbreaks. As in domestic sheep, it is harbored in the reproductive tract, particularly the vagina (Ward et al. 2006). Isolates from bighorn sheep are similar to those from domestic sheep, although there are some differences in cultural characteristics (Ward et al. 2006).

2.4 Bison

Commercial bison (*Bison bison*) are susceptible to the same feedlot stressors as domestic cattle. Like cattle, they are predisposed to BRD due to pasteurellosis and mycoplasmosis, and to pulmonary histophilosis following weather and comingling stress in feedlots (Dyer and Ward 1998; Dyer 2001). Strains of *H. somni* that are almost identical to those from cattle have been isolated from tonsils of bison (Ward et al. 1999a, b). Sudden death is the most common indication of histophilosis in bison (Berezowski, undated). *H. somni* is most often a pathogen in recently weaned bison calves. Animals are found recumbent, depressed, blind, and—shortly before death—convulsing. Affected bison calves generally die within 24–48 h of clinical onset.

3 Pathology

3.1 Cattle

3.1.1 Respiratory Disease in Cattle

Acute pulmonary lesions of histophilosis take several forms. One is acute fibrinosuppurative bronchopneumonia (Fig. 1a). Grossly, these changes resemble those of acute pasteurellosis due to *P. multocida*. In our experience and that of others (Griffin 2012), it is not possible to draw a definitive conclusion about the likely involvement of *H. somni* based on the gross appearance of lungs. These lobular lesions are bilateral and affect cranial–ventral portions of the lungs. Affected parenchyma is consolidated and gray to red-gray, with intraluminal exudate in small airways. Areas of consolidation contain multiple small abscesses, arising in and

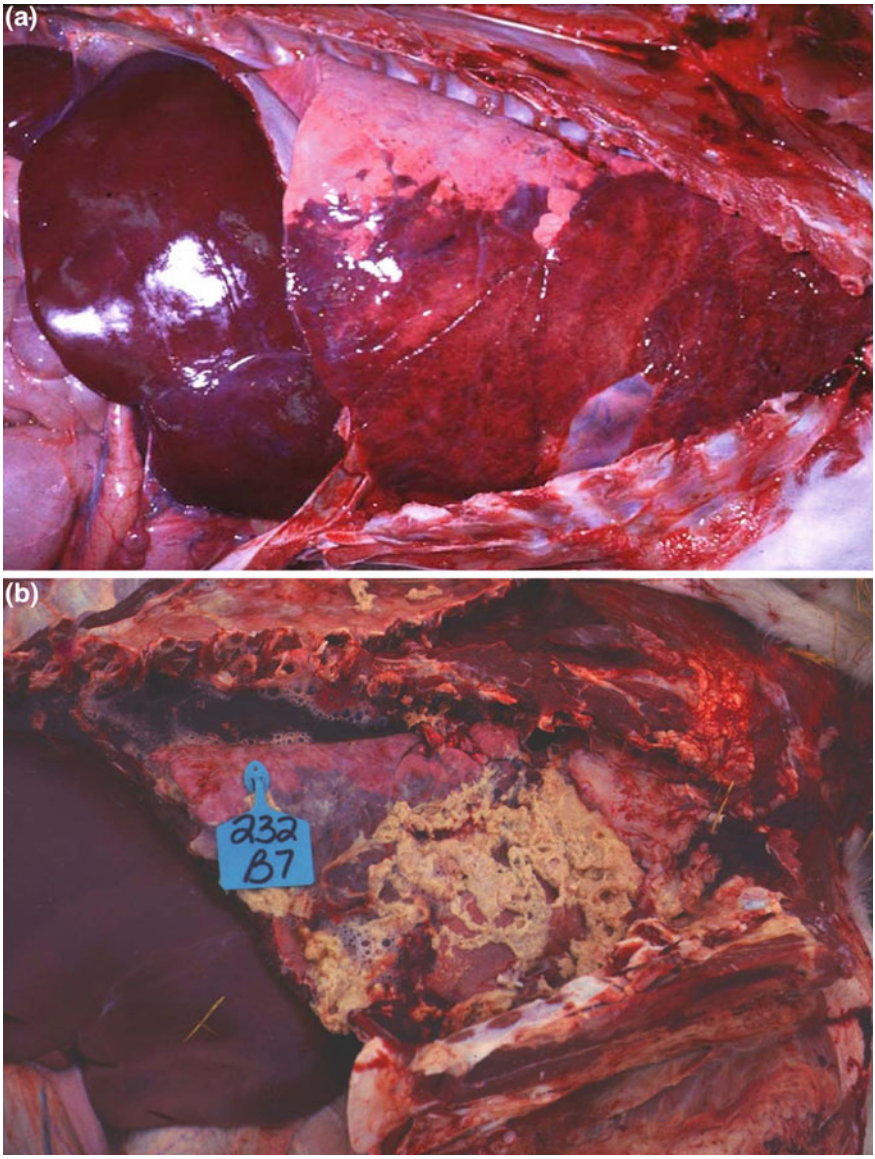

Fig. 1 **a**, **b** Two major forms of acute pneumonia in *H. somni*. **a** Classical anteroventral fibrinosuppurative bronchopneumonia; grossly such lungs resemble those infected with *Pasteurella multocida*. *Image credit* Dr. Bruce Anderson, Noah's Arkive. **b** Fibrinosuppurative bronchopneumonia with severe associated pleuritis. *Image credit* Dr. Ted Clark, Calgary, AB

effacing bronchioles (Andrews et al. 1985; Tegtmeier et al. 1995). Mediastinal and tracheobronchial lymph nodes are mildly edematous. In a small proportion of cases of acute histophilosis, gross lesions resemble those of acute mannheimiosis, with

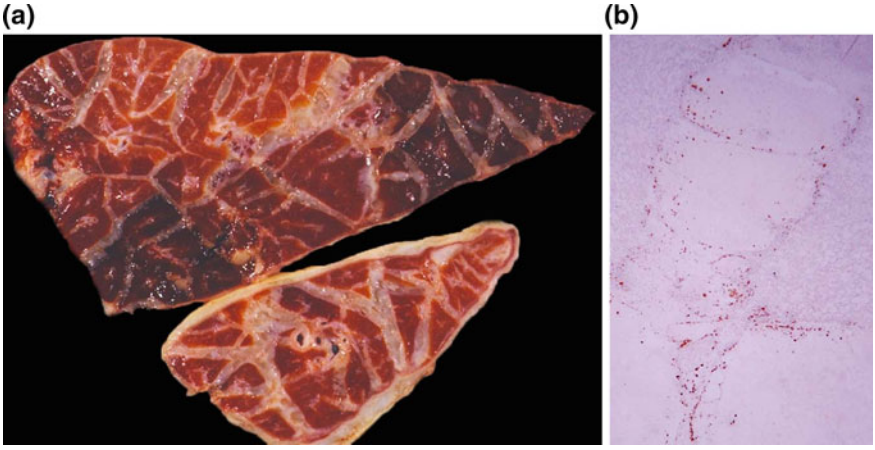

Fig. 2 **a**, **b** Fibrinous pleuritis with septal inflammation. **a** Gross appearance of affected lung. **b** Histopathological appearance of a. Inflammation extends into septal areas, associated with septal edema and lymphangitis. Red = aggregates of *H. somni* antigen. Pulmonary parenchyma is free of inflammation. Immunohistochemistry preparation (IHC). *Photo credit* **a**, **b** Dr. Ted Clark, Calgary, AB

similarly florid bronchopneumonia and pleuritis. Pleuritis, when present in acute pulmonary disease due to *H. somni*, can occur as a solitary lesion, with varying volumes of exudate in the pleural sacs (Andrews et al. 1985) (Fig. 1b). Severe acute fibrinous pleuritis occurring 30–90 days after arrival in feedlots is the most common manifestation of histophilosis in feedlots in western Canada (Clark 2005). Many animals die acutely with few premonitory signs. It is noteworthy that most acute cases lack lesions of concurrent pneumonia (Fig. 2a, b).

Microscopically, as first noted by Gogolewski et al. (1987), acute pulmonary lesions are characterized by a distinctive pattern of degeneration in leukocytes, which lack distinct cytoplasmic margins and form ballooned, homogenized nuclei (Fig. 3a–d). These may be early neutrophil extracellular traps (NETs), which have been shown to occur in leukocytes following in vivo exposure to *H. somni* (Brinkmann and Zychlinsky 2007; Hellenbrand et al. 2013) (Fig. 3c). These changes may be distinct from nuclear streaming ('oat cells'), which are a pronounced feature of mannheimiosis (Fig. 3d). Foci of coagulative necrosis, and intralesional colonies of Gram-negative coccobacilli, are present (Fig. 4a, b) (Jackson et al. 1987; Potgieter et al. 1988; Gagea et al. 2006). Vasculitis and thrombosis, a prominent feature of TME, develop in a proportion of acutely affected lungs (Fig. 4a) (Andrews et al. 1985). This is characteristic of experimental pneumonia at 24 h after inoculation (Gogolewski et al. 1987). In calves dying with pleuritis alone, there is severe septal edema and interlobular thrombo-lymphangitis associated with the presence of *H. somni* antigen (Clark 2005). Histologically, the picture is of a purulent bronchiolitis and bronchopneumonia. Fibrin-rich exudate floods alveolar spaces. Necrosis affects bronchiolar epithelium and muscle, leading

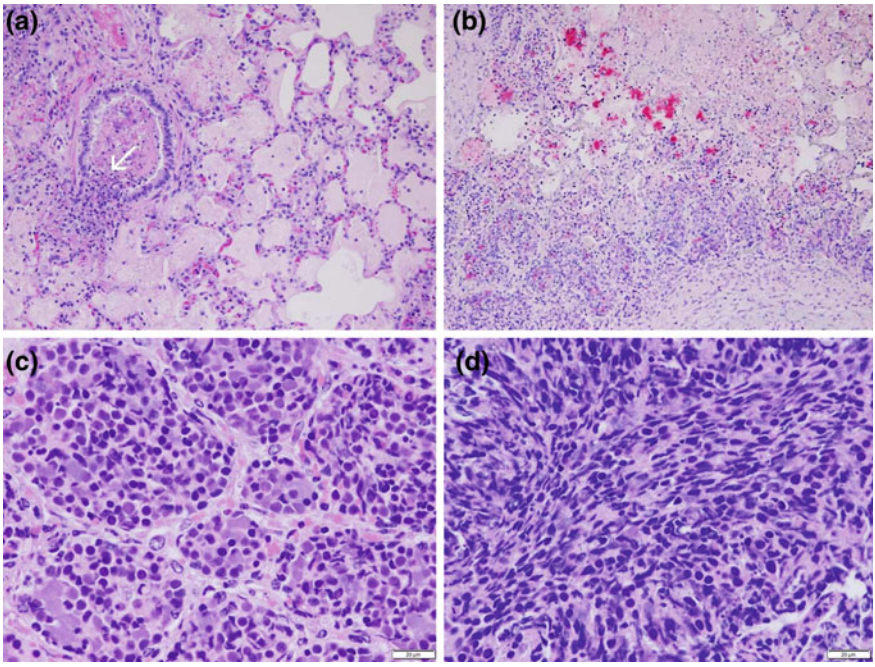

Fig. 3 **a–d** Microscopic appearance of acute pulmonary histophilosis. **a** Acute bronchopneumonia with focal necrosis of bronchiolar wall (*arrow*). Alveoli are flooded with fibrin-rich fluid: natural infection. Hematoxylin and eosin (HE stain). **b** Acute necrosis in pulmonary lobule. Large aggregates of *H. somni* antigen are in the necrotic tissue, invested by a zone of degenerating leukocytes in which smaller amounts of presumably intracellular *H somni* antigen are present: natural infection (IHC). **c** Necrosis of leukocytes, with the formation of swollen, homogenous nuclei suggestive of neutrophil extracellular traps (NETs) in alveoli; 2 days post-challenge: experimental infection. **d** Necrosis of leukocytes, with the formation of 'oat cells' and streaming of chromatin: natural infection

to *bronchiolitis obliterans* in survivors. Epithelial ulceration is evident as early as 3.5–7.0 h post-infection (Tegtmeier et al. 1999a, b). Necrotic foci, originating in distal airways, are scattered throughout affected (anteroventral) portions of the lung. *H. somni* is found in areas of acute inflammation and in foci of necrosis (Gogolewski et al. 1987; Bryson et al. 1990; Tegtmeier et al. 1995). Bacterial antigen is present as extracellular aggregates in bronchi/bronchioles and in necrotic foci (Fig. 4b). Bacterial antigen is also present in apparently intracellular locations in viable margins around such foci, but it is not possible to identify which inflammatory cells are involved. Bacteria can be seen in the cytoplasm of neutrophils and pulmonary alveolar macrophages as early 3.5 and 7.0 h after intrabronchial challenge, but were not seen in pneumocytes or in pulmonary intravascular macrophages (Tegtmeier et al. 1999a, b). Occlusive thrombosis affects septal lymphatics. Florid and widespread necrotizing phlebitis, such as found in the

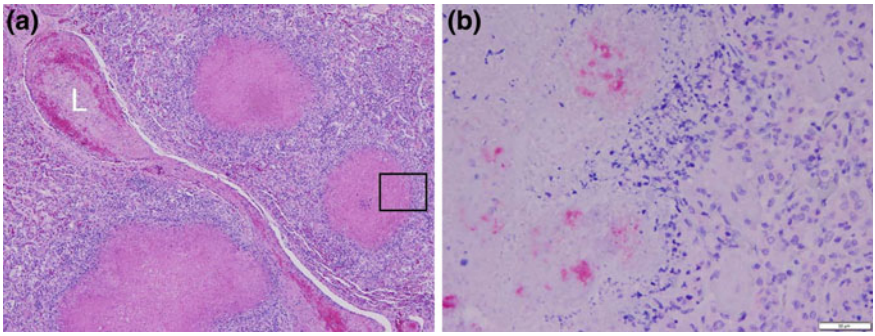

Fig. 4 **a**, **b** Microscopic appearance of chronic pulmonary histophilosis: natural infection. **a** Two common features are necrosis, in many instances arising in destroyed distal airways, and lymphangitis with fibrin thrombi undergoing fibrous organization (L). Portion of a focal area of necrosis illustrated in **b**. HE stain. **b** Necrotic focus with intralesional *H. somni* antigen (IHC). In the absence of the immunohistochemistry, such foci can be assumed to be due to *Mycoplasma bovis*

meninges in TME, is in our experience uncommon in acutely affected lungs in natural histophilosis.

Polymicrobial bronchopneumonia, in which *H. somni* is one component, is common, particularly after infection has been established for several weeks, and antibiotic treatments have been used (Schiefer et al. 1978; Andrews et al. 1985; Corbeil et al. 1986; Gagea et al. 2006; Booker et al. 2008). Up to 60 % of cattle with pneumonia in which *H. somni* is detected at necropsy have one or more other respiratory pathogens (Booker et al. 2008). Such lungs have suppurative bronchopneumonia with bronchiectasis and bronchiolitis obliterans. Common coinfecting agents are *Pasteurella multocida, Mannheimia haemolytica*, *Mycoplasma bovis*, *Trueperella pyogenes,* and/or *Bibersteinia trehalosi* (Schiefer et al. 1978; Panciera and Confer 2010). A common microscopic finding is the presence of multifocal necrosis, which can be confused with Pyogranulomatous caseonecrotic foci that typify chronic pneumonia due to *Mycoplasma bovis* (Caswell et al. 2010). It is noteworthy that *M. bovis* and *H. somni* are often found together in calves with chronic pneumonia (Booker et al. 2008). The presence of bacteria in necrotic foci may partly explain the ability of *H. somni* to persist chronically in lungs. Pneumonia due to *M. bovis* and *H. somni* has many common features, such as feedlot diseases that occur early in the feeding period, concurrent arthritis, tenosynovitis and otitis, tendency to chronicity following unsuccessful antibiotic treatment, and an anteroventral distribution. It is useful to establish by IHC whether necrotic foci contain one or both agents (Fig. 4). A third and relatively common pulmonary lesion of histophilosis is interstitial pneumonia due to septicemia/endotoxemia. Such pneumonia is characterized by intra-alveolar edema and hemorrhage (Caswell and Williams 2007; O'Toole et al. 2009) and is often seen when myocardial lesions are present (Janzen 1987), and some contribution

may be made by vascular changes secondary to left-sided heart failure. Affected lungs are heavy due to edema and red as a result of hemorrhage and congestion. The etiological agent may be misinterpreted as evidence of primary viral pneumonia and should prompt prosectors to examine the heart closely for focal myocarditis.

3.1.2 CNS Disease in Cattle

The classical lesion of TME is moderate-to-severe acute multifocal hemorrhage with necrosis throughout the brain (Stephens et al. 1981). It is a useful necropsy practice to sample cerebrospinal fluid by syringe at the cisterna magna when dealing with neurological cases (Nietfeld 2012). In TME, such fluid is cloudy and blood-tinged, and can be used for cytospin cytology and bacterial culture to expedite diagnosis. Individual red-brown infarcts are 1–30 mm in diameter and vary from 1 to 3 to >50. Few or no grossly evident lesions may be present in the brain and spinal cord when animals are promptly treated with antibiotics (Clark 2005). The extent of lesions is best revealed by slicing brain at 1-cm intervals after it has fixed in formalin for 48 h. We occasionally see cases where the primary or sole lesions are in the spinal cord. Chronic TME, with the formation of mature abscesses, is rare in our experience. Presumably, most cattle with neurological histophilosis either die acutely, or recover following prompt treatment. Nevertheless, particularly in herds where histophilosis is active, any 'brainer' animal with long-standing neurological signs and intracerebral abscesses should be considered a histophilosis suspect until proven otherwise. Suppurative leptomeningitis, ventriculitis, and choroiditis that lack the textbook lesions of necrohemorrhage and vasculitis in brain can be a facet of histophilosis (McEwen and Hulland 1985). Examination of the globes after fixation is helpful diagnostically, since the high proportion of TME-affected cattle have keratitis, retinal edema ('cotton spots'), and hemorrhage (Dukes 1971).

Histologically, septic thrombosis affects capillaries, post-capillary venules, and veins in TME. *H. somni* forms intravascular aggregates resembling biofilms. In very early cases, as noted by Kennedy et al. (1960), dense aggregates of bacteria plug capillaries. Bacterial adherence to endothelium allows *H. somni* to breach the blood–brain barrier, resulting in characteristic angiocentric microabscesses. There is usually some degree of vasculitis, primarily phlebitis, affecting vessels that contain intraluminal *H. somni*. It is noteworthy that the seminal report of TME in 1956 noted this association, although it was interpreted at the time as bacterial phagocytosis by microvascular endothelium (Griner et al. 1956). On the basis of in vitro studies, an intracellular location is unlikely (Czuprynski et al. 2004; Behling-Kelly et al. 2006). Although ultrastructural studies of TME are limited, it is likely that the cardinal intravascular event is microvascular plugging by bacteria that results in apoptosis of endothelium (Sylte et al. 2001).

3.1.3 Myocardial and Vascular Disease in Cattle

Cardiac lesions associated with histophilosis were noted briefly in the first account of TME. Of 21 animals with TME, 10 had cardiovascular lesions, including myocardial infarction, myocarditis, and pericarditis (Griner et al. 1956). It was not until the mid-1980s that the importance of cardiac lesions was recognized as one important cause of sudden 'pen deaths' (Janzen 1987; Harris and Janzen 1989; Schuh and Harland 1991). Such lesions generally affect one or both papillary muscles of the left ventricular myocardium (Fig. 5). These may be the only major changes found postmortem. Where concurrent lesions are present, they may involve the brain, spinal cord, and meninges. In our experience, florid pneumonia is uncommon when animals die of necrotizing myocarditis due to *H. somni*, suggesting that myocarditis is a sequel to silent septicemia rather than concurrent pneumonia. The endocardium overlying affected papillary muscles is often discolored (Fig. 6a–d). It is a good practice to incise papillary muscles in 'sudden death' calves in search of 1–3 cm areas of purple hemorrhage (acute) to tan (subacute) discoloration that typify early cases. Rarely, disseminated small lesions are evident throughout the myocardium (Fig. 6b). In a proportion of cases, such disseminated myocarditis may only be evident microscopically. Subacute and chronic lesions are fibrotic with multiple small pockets of exudate. Chronic lesions have a well-developed fibrous capsule and may incorporate a necrotic sequestrum (Fig. 6d). Inconsistent findings are ruptured chordae tendineae, endocardial rupture, adherent intraventricular thrombosis, valvular endocarditis, fibrinous endocarditis, and pericarditis. Bacteria on endocardial surfaces, and to a lesser extent in necrotic myocardium, form large, biofilm-like aggregates. Similar aggregates are seen following experimental infection (Sandal et al. 2009; Elswaifi et al. 2012). Other opportunistic or pathogenic bacteria (*E. coli*, *Pseudomonas* spp., *Streptococcus* spp., and *Mycoplasma bovis*) can cause suppurative or necrotizing myocarditis, but these are associated with randomly distributed lesions. These bacteria do not tend to target papillary muscles, unlike *H. somni*.

As the term TME suggests, particularly when disease is acute, bacteria are found in blood vessels, primarily capillaries, venules, and veins (Fig. 7a–e). Biofilm-like aggregates form in vessels in cardiac papillary muscles and elsewhere in the heart.

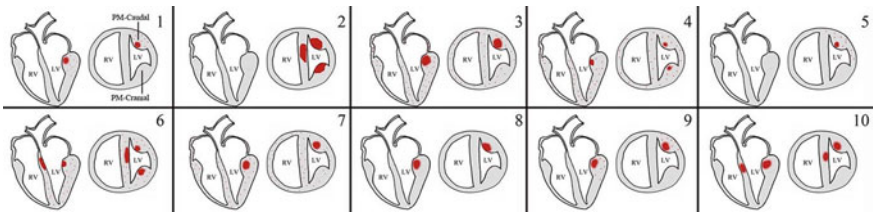

Fig. 5 Cardiac histophilosis. Cartoon of 10 cases illustrating tendency to involve anterolateral and posteromedial papillary muscles of left ventricular myocardium. Numbers refer to individual animals. Each panel shows heart in dorsoventral and horizontal section. Gross lesions are *red*

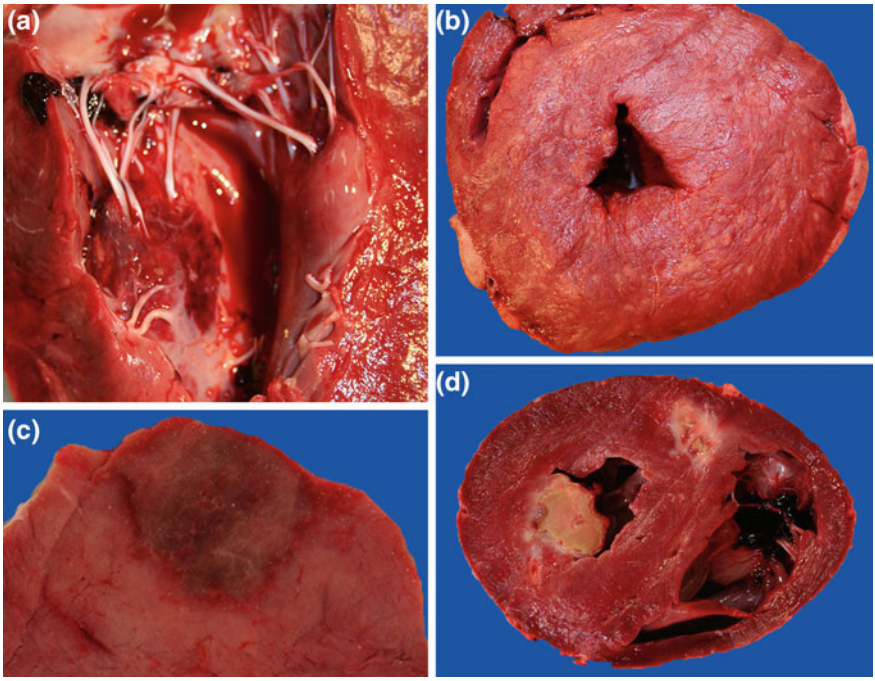

Fig. 6 **a**–**d** Gross images of cardiac histophilosis. **a** Endocardial hemorrhage overlying necrosis in papillary muscle of left ventricle, typical of acute necrotizing myocarditis. **b** Acute disseminated myocardial necrosis throughout all portions of the heart. **c** Typical acute lesion in papillary muscle of left ventricular myocardium. Such lesions are typically 1–3 cm. **d** Chronic abscess in one of two papillary muscles, with a smaller lesion in interventricular septum. Rupture of such sequestra at the endocardial surface causes acute death

Large colonies up to 400 μm thick form on endocardial surfaces, including valves (Fig. 7d). In older, more subacute lesions, there is necrosis or apoptosis of endothelium and necrosis of perivascular cardiocytes, with varying degrees of myocarditis. The resultant lesion—angiocentric myocarditis with intralesional bacteria—characterizes histophilosis microscopically. It should be corroborated by IHC. Ultrastructurally, bacterial communities are closely associated with degenerating endothelial cells. Endothelial cells contract, so that bacteria rest on denuded vascular basement membranes. Why *H. somni* tends to lodge in vessels in one or both papillary muscles of the left ventricle is unclear. These muscles are susceptible to hypoxia due to a dependence on subendocardial perfusion since most coronary blood flow occurs during diastole, unlike more superficial parts of the ventricular myocardium. Variation in the anatomy of individual animals' hearts (presence of a 'papillary muscle artery'; few or no anastomotic connections with the extrapapillary subendocardial plexus; balance between class A and class B arterial network) may predispose them to bacterial infection and infarction (Teixeira Filho et al. 2001; Ranganathan and Burch 1969).

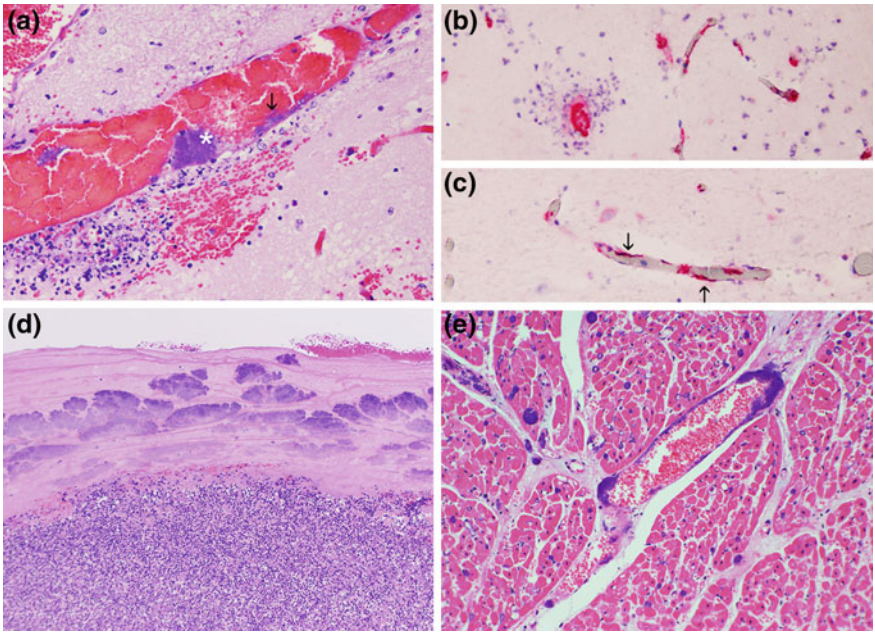

Fig. 7 **a**–**e** Biofilm-like formation in vivo in field cases of bovine histophilosis. **a** Thrombotic encephalitis (TME) with large (*asterisk*) and small colonies (*arrow*) of *H. somni* in close apposition to endothelium. There is early perivascular inflammation and hemorrhage. Brain. HE stain. **b** TME with *H. somni* stained by IHC (*red*) in capillaries, some of which appear occluded. Brain. **c** Adherence of *H. somni* to endothelium in beaded patterns (*arrows*). Brain. IHC. **d** Ulcerated endocardium with thick layer of *H. somni* in fibrin layer adherent to inflamed myocardium. Heart. HE stain. **e** Layer of *H. somni* adherent to venous endothelium, forming an almost continuous film of bacteria. Many capillaries are plugged with *H. somni*. Heart. HE stain

It is sometimes implied or stated in reviews of *H. somni* that vasculitis is a systemic feature of histophilosis. Our impression is that vasculitis is a prominent feature of TME, and microvascular and venous lesions are common in acute and peracute cardiac histophilosis. However, vasculitis in other tissues, such as in the lung, is relatively modest and probably cannot be used as a marker for likely *H. somni* infection.

3.1.4 Other Tissue Lesions in Cattle

Abortion due to *H. somni* is associated with necrotizing placentitis involving cotyledons and intercotyledonary placenta, combined with medial arteritis (van Dreumel and Kierstead 1975; Miller et al. 1983a, b; Widders et al. 1986). Embryonic loss has also been attributed to histophilosis (Ruegg et al. 1988; Kaneene et al. 1986a, b). Unlike the situation with pneumonia, abortion is generally associated with the isolation of *H. somni* alone (Corbeil et al. 1986). Placental

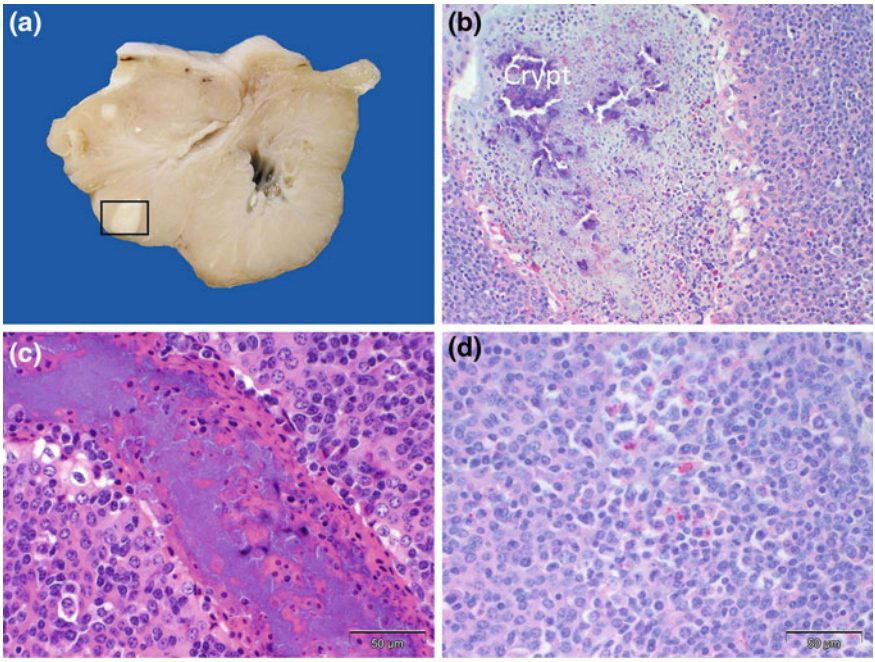

Fig. 8 **a–d** Palatine tonsil. **a** Gross image of inspissated white exudate in crypts of palatine tonsil of calf. Box = area comparable to that illustrated microscopically in **b**. **b** Lumen of tonsillar crypt filled with partly mineralized exudate. Most *H. somni*-positive antigen is present in the lumen of the crypt. There are smaller amounts of antigen in cells in crypt wall. IHC. **c** Abundant extracellular bacterial antigen (purple material) in crypt lumen; bacteria stained positively for *H. somni* in adjacent section. HE stain. **d** Intracytoplasmic *H. somni*-positive antigen in cells, presumably macrophages, in crypt wall (*arrow*). IHC

lesions may be sufficiently severe to be evident grossly. Systemic lesions vary in the fetus but, when present, include angiocentric meningoencephalitis with vasculitis and generalized vascular lesions consistent with bacterial septicemia and disseminated intravascular coagulation (Van Dreumel and Kierstead 1975; Miller et al. 1983b; Widders et al. 1986; Headley et al. 2013, 2015). There have been few recent morphological studies of the lesions associated with *H. somni* in the female reproductive tract and none using IHC. Infected tracts may be free of lesions, or have mucopurulent vaginitis with adenitis of the major vestibular gland (Miller et al. 1983a).

A neglected aspect of histophilosis is the potential importance of paired tonsillar tissues comprising Waldeyer's ring. The crypts of bovine palatine tonsils, like those of bison (Ward et al. 1999a, b), contain *H. somni* embedded in exudate, often in an inspissated, partly mineralized form (Fig. 8a–d). Some bacteria are in the cytoplasm of mononuclear cells in tonsillar lymphoid aggregates. This suggests one extrapulmonary mechanism whereby boluses of bacteria might enter the circulation, initiating septicemia. Laryngitis is an intermittent feature in histophilosis. Some

pathologists wonder whether this too might be a portal of entry, since bilateral ulcerative or erosive laryngitis with thrombophlebitis is not uncommon in cattle dying of TME (T. Clark, personal communication). We have not found consistent lesions in the larynges of cattle dying of acute histophilosis, including TME.

3.2 Small Ruminants

The principal findings at necropsy of fattening lambs with generalized histophilosis are those typical of septicemia—disseminated petechial hemorrhages, particularly in liver and skeletal muscle and, in longer-surviving lambs, nascent microabscesses. Multifocal hepatic necrosis is a helpful if not invariable finding. Lesions in other organs such as the kidney and myocardium consist of pale 0.5–2.0-mm-diameter foci (Rahaley 1978a, b; Orr et al. 1992). Lambs surviving for >1 day typically develop CNS disease and polyarthritis with periarticular inflammation. The histological lesions are those of disseminated septicemia with necrosis, vasculitis, and thrombosis, most evident in liver and skeletal muscle (Kennedy et al. 1958; Rahaley 1978a). *H. somni* occasionally causes outbreaks of perinatal death in lambs (Rahaley and White 1977). Pregnant ewes at 65–80 days' gestation that are challenged intravenously abort due to acute placentitis (Rahaley 1978b). It is likely that septicemia with preferential localization in the uterus and placenta is the basis for late-term and perinatal losses in lambs. Spontaneous abortion due to *H. somni* appears to be rare in sheep (Kirkbride 1993b), but has been reported as a result of polymicrobial infection that includes *H. somni* (Hajtós 1987). CNS disease in sheep, as in cattle, occurs in two forms: a TME-like syndrome, essentially indistinguishable from the bovine disease, and suppurative leptomeningitis-ventriculitis-choroiditis, particularly in lambs that survive an initial episode of septicemia (Kennedy et al. 1958; Philbey et al. 1991; Romero et al. 2013). The distinctive relationship that occurs in cattle between microvascular endothelium and flat adherent colonies of *H. somni* can also be seen microscopically in the brains of sheep (Cassidy et al. 1997). Lesions consistent with a prior episode of septicemia in non-CNS tissues are often found (Philbey et al. 1991). Stress due to transportation and close confinement or crowding appears to contribute to the disease process. There are also similarities to the pneumonia found in cattle. *H. somni* is recovered from pneumonic lungs in mixed growth with pathogens such as *T. pyogenes* and *M. haemolytica* (Philbey et al. 1991).

Uncomplicated epididymitis-orchitis is rarely a cause of death. It is unusual for samples from this infection to be submitted for histology unless a regulated disease such as ovine brucellosis is suspected. Lesions are of bacterial orchitis and epididymitis, with marked testicular swelling (acute disease) or induration and atrophy (chronic disease), along with spermatic granulomas. No vaccine is currently available in the USA. Control is by including antibiotics in the feed (typically chlortetracycline) (Kimberling 1988).

3.3 Bighorn Sheep

As noted above, bacterial pneumonia in bighorn sheep is an important polymicrobial epizootic disease that limits the expansion of the species in its natural habitat in western North America (Post 1962). In the only investigation published to date on a possible role for *H. somni*, four of 12 sheep had immunohistochemical evidence of the bacterium in pneumonic lungs (Ward et al. 2006). It is assumed that *H. somni* is a minor component in epizootics.

3.4 Bison

H. somni was found by IHC in a retrospective study of 7 of 21 bison with terminal pneumonia. A subsequent investigation found that some of the virulence factors for histophilosis in cattle, such as IbpA, are present in *H. somni* isolated from the palatine tonsils of healthy, commercially managed bison (Ward et al. 1999a, b; Zekarias et al. 2011). Pneumonia due to a complex of microorganisms is common and important in ranched bison, particularly when they are finished at feed yards. It is unclear at present what proportion of bison with pneumonia have detectable *H. somni* in lesions. Leptomeningitis and hemorrhagic infarcts in the brain and spinal cord, presumably TME-like, have also been found in bison (Berezowski, undated).

4 Laboratory Diagnosis and Antimicrobial Resistance Testing

The most appropriate animals to select for necropsy are those with early clinical signs typical of the syndrome confronting the practitioner. Ideally, animals should be untreated by antibiotics. When treated, details of any antimicrobials used, including duration of administration, should be provided to laboratory personnel. There is a natural reluctance for owners to kill animals early in the clinical course and for them to prefer to submit chronic 'lunger' animals that were treated repeatedly. Unfortunately, laboratory results from long-standing cases are difficult to interpret due to the likelihood of finding multiple pathogens. Several excellent articles provide general approaches to field necropsies of cattle and on diagnostic sample submission (Caswell et al. 2012; Cooper and Brodersen 2010; Griffin 2012). Most necropsy tissues examined at our laboratory are generated by field necropsies done by veterinarians. The best laboratory accessions come with a detailed clinical history, uncontaminated fresh samples, and digital images of gross lesions (Griffin 2012).

Due to the varied clinical presentations of histophilosis, veterinarians should submit multiple fresh and fixed tissue samples. If *H. somni* is one of the pathogens

that is suspected, optimal samples come from lung, heart, brain, and synovial fluid, as well as any other tissues with gross lesions. Appropriate samples from the cranial vault are one or more pieces of grossly discolored cerebral tissue; swabs of exudate in leptomeninges or ventricles; and CSF collected postmortem by needle and syringe before the head is disarticulated. If a veterinarian suspects TME and is unable collect tissues aseptically under field conditions, one or more heads should be submitted intact to the laboratory. This allows skulls to be opened and samples taken for culture using sterile instruments in a controlled environment. Lung samples should be taken at the junction of affected and unaffected parenchyma. Pulmonary tissue with abnormal structural changes (lesioned) can be problematic because of bacterial overgrowth by organisms other than *H. somni*, particularly in subacute-to-chronic pneumonias (Corbeil et al. 1986; Tegtmeier et al. 2000). *Pasteurella multocida* and *Mannheimia haemolytica* propagate quickly and overgrow *H. somni*. Like others, we find that IHC of formalin-fixed tissue is useful in that it gives a sense of how much *H. somni* antigen is present and its relationship to lesions and therefore its likely importance (Haines et al. 2004; Clark 2005). Lesions in the heart, typically in anterior or posterior papillary muscle of left ventricle, are good candidates for culture. It is rare that confounding bacteria, such as *Mycoplasma bovis*, are detected at this site (Haines et al. 2004).

For culture, if only one fresh sample is taken per tissue, it is better to submit pieces of tissue rather than a swab. Lesioned tissue generates the largest volume of bacteria and increases the odds of a successful culture. An appropriate sample size is 5.0 × 5.0 × 2.5 cm (2 × 2 × 1 in.). If the sample is fluid, 1 ml should be collected and transferred to a red-top tube before shipment to the laboratory. Where arthritis is the main presentation, it is useful to ask the practitioner to saw long bones proximal and distal to the joint, and submit the affected joint with capsule intact for laboratory sampling. The value of using nasopharyngeal swabs from live cattle is limited and so is rarely used; there is some evidence that *H. somni* preferentially colonizes the lower respiratory tract rather than nasal mucosa in calves with pulmonary disease (Corbeil 2007). Some animals are nasopharyngeal carriers of nonpathogenic *H. somni*. Therefore, isolation of *H somni* from such swabs may not indicate that it is the primary pathogen found in the lower respiratory tract. Trans-tracheal washes circumvent this problem, but these are almost never used by our clients. Isolation of *H. somni* from the vagina in cases of herd infertility needs to be interpreted with caution. A role is plausible when there is heavy bacterial growth, combined with post-breeding endometritis, vaginitis, and a purulent vaginal discharge (Yaeger and Holler 2007).

Isolation of *H. somni* by culture is less sensitive than PCR for *H. somni* (Tegtmeier et al. 2000; Bell et al. 2014). Multiplex PCR assays have been developed for *H. somni* in lung and in sheep semen (Tegtmeier et al. 2000; Saunders et al. 2007; Moustacas et al. 2013). Quantitative PCR, whether multiplex or in an individual assay, is a sensitive way to detect *H. somni.* Several North American laboratories using an *H. somni* PCR bundle it with probes for other respiratory pathogens. Detecting *H. somni* in samples by PCR is valuable, but no antimicrobial information is generated unless bacterial isolation is successful. Culture has the

advantage of providing the laboratory isolates for use in antimicrobial susceptibility assays, MALDI-TOF mass spectroscopic characterization, other comparative analyses, and epidemiological studies (Tegtmeier et al. 2000; Portis et al. 2012; Kuhnert et al. 2012; Frey and Kuhnert 2015). Antimicrobial susceptibility assays for *H. somni* include disk diffusion (Kirby-Bauer, Edisk) and broth microdilution (D'Amours et al. 2011; Goldspink et al. 2015). *H. somni* is susceptible to multiple antimicrobial classes in vitro. Susceptibility to individual antimicrobials includes ceftiofur, penicillin, enrofloxacin, and florfenicol. Resistance to tetracyclines is emerging in the USA and Australia (D'Amours et al. 2011; Portis et al. 2012).

H. somni can be isolated from the upper respiratory tract, semen, vagina, and preputial mucosa. Bovine and ovine *H. somni* isolates can be differentiated using PCR amplification and restriction enzyme digests of the *rpoB* gene (Tanaka et al. 2005). At present, it is difficult to readily distinguish pathogenic from non-pathogenic isolates of *H. somni* in a conventional diagnostic laboratory. This requires inoculating cattle and the involvement of a *H. somni* specialist. When pneumonia is chronic and multiple pathogens are isolated, it would be helpful to clients to determine which agents are primary and which are secondary. This is not usually possible. IHC increases the overall rate of positive diagnosis of pulmonary and cardiac histophilosis, particularly in tissues from antibiotic-treated animals (Tegtmeier et al. 1995; Bryson et al. 1990).

Given the pitfalls of attempting to grow a fastidious bacterium like *H. somni* from tissue or swabs from tissues with varying degrees of autolysis, sometimes in the presence of other pathogens, and following antibiotic treatment, one approach to diagnosis is a combination of IHC and PCR when *H. somni* is suspected. Surprisingly, only one (California) of 33 state/provincial laboratories in the USA and Canada is listed on the AAVLD PCR Web site as offering a PCR test for *H. somni* (Graham 2015). The most commonly used protocol is based on one developed in Denmark, which detects the 16S rRNA gene of *H. somni* (Angen et al., 1998). In addition to the California laboratory, which now uses a culture MALDI-TOF MS approach, three state laboratories in the USA (Kansas, Iowa, and Nebraska) have deployed or are implementing a PCR test for *H. somni*.

A tentative identification of *H. somni* is based on the growth of small tan/yellow-white colonies on blood agar plates incubated in 10 % CO_2, with no or very little growth in air. A final identification is based on Gram staining (pleomorphic Gram-negative bacilli or coccobacilli, 1 μm × 1–3 μm in size), oxidase-positive, catalase-negative, and variable indole production, fermentation of glucose, and nitrate reduction (Markey et al. 2013). *H. somni* is often isolated from lungs along with other bacterial pathogens such as *P. multocida* or *M. haemolytica*. Identification of *H. somni* based on culture alone is likely to underestimate the incidence of disease. In one Danish study that compared culture with IHC, strong immunostaining was seen in 15 of 17 culture-positive cases, as well as in 20 pneumonic lungs from which *H. somni* was not isolated, although *Histophilus*-compatible lesions were present (Tegtmeier et al. 1995). Similar findings were obtained in a retrospective study of *H. somni* in bison, comparing isolation with IHC (Dyer 2001). These results suggest that laboratories relying on culture alone for an

etiological diagnosis will underestimate the presence of *H. somni* in pneumonic lungs.

Microagglutination assays have been used to monitor populations of cattle for infection, particularly in the early feedlot period. Unfortunately, many clinically healthy cattle have high serological titers (Widders et al. 1986). Currently, the use of serological assays for diagnostic purposes, based on antibodies to whole *H. somni*, is of limited value; moreover, such assays are not widely available. Their value is small due to *H. somni* inhabiting mucosal sites in asymptomatic cattle, because of the possibility of subclinical infections, cross-reactive antibodies with other bacteria, or the confounding effects of vaccination (Kania et al. 1990; Corbeil 2007; Pan et al. 2014). The recent development of an enzyme-linked immunosorbent assay (ELISA) using antibodies to *H. somni* exopolysaccharide (EPS) (described in a later chapter), a major component of the *H. somni* biofilm matrix, holds promise of having an application in diagnostic settings (Pan et al. 2014). Exopolysaccharide is generated only when *H. somni* forms biofilm or is under stress conditions, so serological responses are less likely in healthy and recently vaccinated cattle. A disadvantage of the EPS ELISA is that EPS is a relatively weak immunogen, and serological responses can be short-lived (6–10 weeks post-infection). An ELISA has also been developed to the virulence factor IbpA, particularly its A5 subunit, which is more immunogenic. The serological response of infected calves, as detected by the A5 IbpA ELISA, tends to be more prolonged (up to 10 weeks post-infection) (Lo et al. 2012). The diagnostic value of the EPS and IbpA ELISAs in confirming natural outbreaks of *H. somni* remains to be established.

Once a laboratory diagnosis of HSDC is made, it is helpful to alert veterinarians that continuing sporadic illness may continue for weeks or months, particularly when the initial case is seen in fall–early winter.

5 Summary

We mentioned in the introduction that a disparity exists between the perceived significance of histophilosis in Canada, where it is high, versus elsewhere in the world, not least in the USA, where it is considerably lower. It is possible that its documented importance in Canada is related to regional factors, such as strain differences, feedlot practices, or harsh climate. What strikes us is the excellence of Canadian investigational work since the mid-1980s, when Dr. Peter Little's group was first active, which helped clarify the importance of histophilosis in the feedlot industry. Comparable field studies have not been performed in the USA. Such studies are unfashionable and expensive, albeit critical for a large and concentrated animal industry (Kelly and Janzen 1986). There may be a tendency to group all bacterial agents of BRD complex under one rubric and assume that they behave similarly in response to stress, clinical recognition, treatment, and vaccination (Griffin et al. 2010). Yet the overall yearly bovine mortality rates have not changed appreciably, and the risk of death attributable to respiratory tract disorders has

increased compared to the risk of death in 1994 (Loneragan et al. 2001). Additional field studies seem essential to assess the importance of the disease to the cattle industry in the USA.

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Histophilus somni Genomics and Genetics

Shivakumara Siddaramappa

Abstract *Histophilus somni* is a commensal and an opportunistic bacterial pathogen associated with multisystemic diseases in cattle and sheep. Some strains of *H. somni* isolated from the genital tract of cattle are biochemically and serologically similar to the pathogenic strains, but are relatively innocuous. Several virulence factors/mechanisms have been identified in *H. somni*, of which the phase-variable lipooligosaccharide, induction of apoptosis of host cells, intraphagocytic survival, and immunoglobulin Fc-binding proteins have been well characterized. The genomes of *H. somni* pneumonia strain 2336 and preputial strain 129Pt have also been sequenced, and comparative analyses of these genomes have provided novel insights into the role of horizontal gene transfer in the evolution of the respective strains. Continued analyses of the genomes of *H. somni* strains and comparing them to the newly sequenced genomes of other bacteria facilitated the identification of a putative integrative and conjugative element (designated ICE*Hso*2336) encoding tetracycline resistance. Comparative genomics also showed that the uptake signal sequence (5′-AAGTGCGGT) of *Haemophilus influenzae* is abundant in *H. somni* and provided a genetic basis for the recalcitrance of some strains of this species to natural transformation. The post-genomic era for *H. somni* offered an opportunity for the functional characterization of genes identified by computational methods. This opportunity has been realized to a great extent by transcriptomic studies that have identified several small noncoding RNAs and new genes. These new discoveries and developments are expected to stimulate further in-depth investigations of *H. somni*, especially from the systems biology viewpoint.

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Contents

1 Introduction to Genomic Analyses

The terms "genomics" and "genomic methods" describe "the molecular and bioinformatics techniques that employ all or part of the genome to answer a question about an organism or a group of organisms" (Carruthers et al. 2012). Genomics has immense applications in the quest to understand nature, and comparative microbial genomics is an indispensable tool for molecular pathogenic bacteriology. The first complete genome sequence from a free-living organism was that of *Haemophilus influenzae* strain Rd KW20 (Fleischmann et al. 1995), a close relative of *H. somni*. This pioneering work at the erstwhile Institute for Genomic Research (TIGR) popularized the concept of whole-genome random sequencing by the "shotgun" approach. Since the completion of the first bacterial genome sequence, thousands of bacterial and archaeal genomes have been sequenced and annotated using novel tools and techniques. The genomes of several species of *Pasteurellaceae* have also been sequenced, and whole-genome comparisons have provided new insights into the physiology and evolution of members within this very important bacterial family (Challacombe and Inzana 2008).

Numerous in vitro and in vivo studies during the pre-genomic era have shed light on the differences in virulence properties and genetic traits between *H. somni* pathogenic isolates from sick animals and commensal isolates from the genital tract (Corbeil et al. 1995). *H. somni* pneumonia strain 2336 (NCBI taxonomy ID 228400) and preputial strain 129Pt (NCBI taxonomy ID 205914) have been phenotypically well characterized in the laboratory and utilized in several comparative studies (Corbeil et al. 1997; Inzana et al. 1992, 2002). The genomes of these two strains have been completely sequenced and compared (Challacombe et al. 2007; Siddaramappa et al. 2011). This chapter will provide an overview of the pre-genomic investigations, comparative genomic analyses, and post-genomic studies of *H. somni* strains.

2 Comparative Genomics

Several temperate bacteriophages that infect strains of *H. influenzae* have been purified and described (Williams et al. 2002). However, temperate bacteriophages that infect strains of *H. somni* remain to be isolated and characterized. Nevertheless, prophages and their associated sequences appear to be rife in the genome of *H. somni* strain 2336, but less abundant in the genome of strain 129Pt (Siddaramappa et al. 2011), indicating that the natural repertoire of bacteriophages that potentially infect some strains of *H. somni* strains could be large. Furthermore, a large portion of strain-specific sequences occurring in strains 2336 and 129Pt appear to be due to prophages and their associated sequences (Siddaramappa et al. 2011). Although the Mu-like prophage (FluMu) found in *H. influenzae* strain Rd KW20 is absent in the genomes of *H. somni* strains, the genome of *H. somni* strain 2336 contains a prophage that appears to be partially related to the *Mannheimia haemolytica* serotype A1 lysogenic bacteriophage ϕMhaA1-PHL101.

In addition to the prophages and their associated sequences, the genomes of *H. somni* strains contain several genomic islands that appear to be unrelated to each other (Siddaramappa et al. 2011). A genomic island that is homologous to ICE*Hin1056*, which is a 59,393-bp integrative and conjugative element (~39 % G+C) containing genes encoding ampicillin, chloramphenicol, and tetracycline resistance in *H. influenzae* type b strain 1056, has also been identified in *H. somni* strain 2336 (Mohd-Zain et al. 2004). The genomic island of *H. somni* strain 2336 was more precisely delineated upon comparison with ICE*Pmu1*, which is an integrative and conjugative element (~42 % G+C) containing genes encoding resistance to multiple antibiotics in *Pasteurella multocida* strain 36950 (Michael et al. 2012). This genomic island of *H. somni* strain 2336 appears to be a putative integrative and conjugative element and is referred to as ICE*Hso*2336 (~40.5 % G+C). An integrative and conjugative element (ICE*Mh1*, ~40 % G+C) containing genes encoding resistance to multiple antibiotics and closely related to ICE*Pmu1* is also present in *M. haemolytica* strain 42548 (Eidam et al. 2015). Whereas the nucleotide identity between ICE*Mh1* and ICE*Hin1056* is only ~70 %, the nucleotide identity between ICE*Mh1*, ICE*Pmu1*, and ICE*Hso*2336 is ~99 %.

Furthermore, ICE*Pmu1* and ICE*Mh1* are integrated site-specifically into $tRNA^{Leu}$ in the chromosomes of *P. multocida* strain 36950 and *M. haemolytica* strain 42548, respectively (Eidam et al. 2015; Michael et al. 2012). A comparison of these loci as well as ICE*Hso*2336 indicated that each element contains 11-bp (5′-GATTTTGAATC) terminal direct repeats and an 86-bp $tRNA^{Leu}$ at the right terminus (Fig. 1a). Although ICE*Pmu1* is smaller in size than ICE*Mh1* by ~10,000 bp, it contains more antimicrobial resistance genes than the latter (Eidam et al. 2015; Michael et al. 2012). As reported previously, ICE*Hso*2336 contains the tetracycline repressor gene *tetR* and the tetracycline resistance gene *tetH* (Michael et al. 2012; Mohd-Zain et al. 2004; Siddaramappa et al. 2011), and *H. somni* strain 2336 is resistant to tetracycline (MIC 8 μg/ml) (Ueno et al. 2014). A schematic map of ICE*Hso*2336 is shown in Fig. 1b, and a comparison of the ORFs that occur

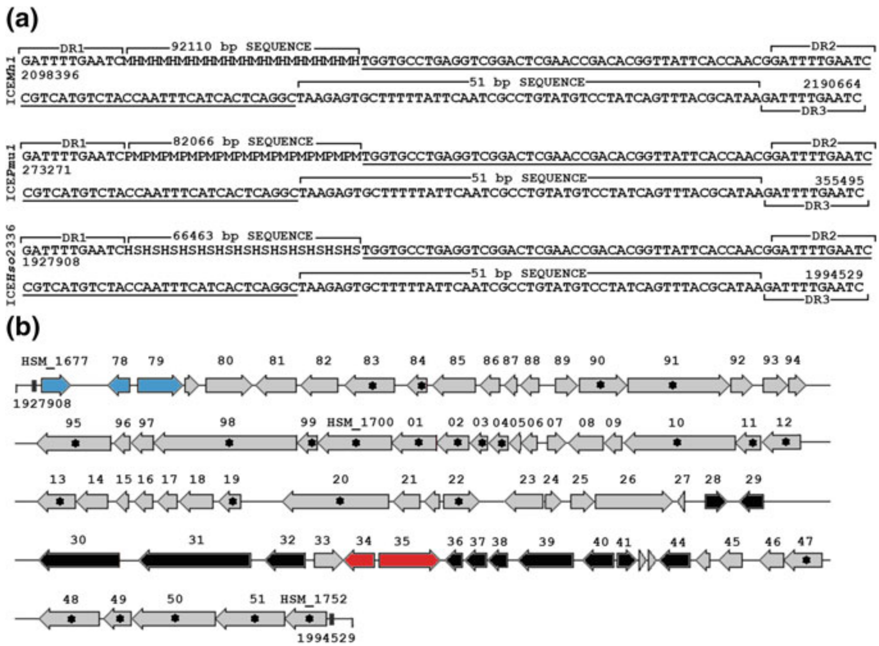

Fig. 1 **a** Comparison of ICE*Mh1*, ICE*Pmu1*, and ICE*Hso*2336. Each ICE contains 11-bp terminal direct repeats (DR1 and DR3) and an additional direct repeat (DR2) within the 86-bp tRNA^{Leu} gene (underlined). A 51-bp sequence occurs between the tRNA^{Leu} gene and DR3. These features are identical among the three ICEs. The sequence between DR1 and the tRNA^{Leu} gene (92110 bp in ICE*Mh1*, 82,066 bp in ICE*Pmu1*, and 66463 bp in ICE*Hso*2336) contains genes that distinguish the three ICEs. Numbers above/below the maps (e.g., 2098396 and 2190664) indicate nucleotide positions within the respective chromosomes. **b** Schematic map of ICE*Hso*2336. Terminal direct repeats (DR1 and DR3) shown in Fig. 1a are indicated by vertical black bars. *Arrows* represent ORFs found within ICE*Hso*2336 and compared in Table 1. *Blue arrows* represent ORFs that have orthologs in ICE*Pmu1*, but not in ICE*Mh1*. *Gray arrows* represent ORFs that have orthologs in ICE*Pmu1* and ICE*Mh1*. *Black arrows* represent ORFs that have no orthologs in ICE*Pmu1* and ICE*Mh1*. *Red arrows* represent *tetR* and *tetH* (have orthologs in ICE*Pmu1* and ICE*Mh1*). Gray arrows containing asterisks represent ORFs that have full-length or partial homologs in *H. somni* strain 129Pt

among ICE*Pmu1*, ICE*Mh1*, and ICE*Hso*2336 is shown in Table 1. Although these elements are closely related, they are not identical and it is evident from Table 1 that they display a mosaic structure with alternating conserved and variable regions. In particular, ICE*Hso*2336 lacked 22 ORFs found in ICE*Pmu1*, and 13 of these 22 ORFs are also absent in ICE*Mh1*. In contrast, ICE*Pmu1* and ICE*Mh1* lack 12 ORFs found in ICE*Hso*2336. Interestingly, *H. somni* strain 129Pt lacks an analogous ICE, but contains short stretches of homologous sequences. Not surprisingly, most of the ORFs identified in ICE*Pmu1* and/or ICE*Hso*2336 have distant homologs outside of the *Pasteurellaceae*.

Table 1 Comparison of the ORFs that occur among ICE*Pmu1*, ICE*Mh1*, and ICE*Hso*2336

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_02680, 192 aa	Hypothetical protein	HSM_1677, 192 aa, 100 %	*Mannheimia varigena*, AHG79487, 192 aa, 99 %	*Streptococcus mutans*, EMC55849, 193 aa, 42 %
Pmu_02690, 145 aa	Putative phage transposase	HSM_1678, 145 aa, 100 %	*Mannheimia varigena*, AHG79486, 94 aa, 100 %	None
Pmu_02700, 299 aa	Tyrosine recombinase-1 family protein (phage integrase)	HSM_1679, 299 aa, 100 %	*Mannheimia varigena*, AHG79485, 303 aa, 100 %	*Salmonella enterica*, ESG49884, 308 aa, 40 %
Pmu_02710, 307 aa	ISApl1 transposase	HSM_1680, 324 aa, 99 %	ICE*Mh1*, AGK02702, 201 aa, 99 %	*Streptococcus pyogenes*, EIK41786, 331 aa, 57 %
Pmu_02740, 271 aa	Aminoglycoside 3'-phosphotransferase protein	None	ICE*Mh1*, AGK02696, 271 aa, 100 %	*Acinetobacter baumannii*, KHW21245, 271 aa, 99 %
Pmu_02760, 278 aa	Aminoglycoside 6'-phosphotransferase protein	None	ICE*Mh1*, AGK02697, 276 aa, 100 %	*Klebsiella pneumoniae*, AHG50664, 279 aa, 100 %
Pmu_02770, 252 aa	Aminoglycoside 3'-phosphotransferase protein	None	ICE*Mh1*, AGK02698, 286 aa, 99 %	*Vibrio cholerae*, EHI02781, 252 aa, 99 %
Pmu_02780, 234 aa	Dihydropteroate synthase	None	*Bibersteinia trehalosi*, AGH37389, 342 aa, 100 %	*Escherichia coli*, EMD02302, 282 aa, 100 %
Pmu_02790, 430 aa	ISCR21 transposase	None	*Bibersteinia trehalosi*, AGH37390, 430 aa, 100 %	*Geobacter* sp. *GSS01*, KIE42276, 430 aa, 97 %
Pmu_02820, 404 aa	Florfenicol/chloramphenicol efflux protein	None	*Bibersteinia trehalosi*, AGH37387, 404 aa, 99 %	*Escherichia coli*, AAS16362, 404 aa, 99 %
Pmu_02830, 101 aa	LysR transcriptional regulator	None	*Actinobacillus pleuropneumoniae*, KIE87619, 101 aa, 100 %	*Vibrio cholerae*, AAV84894, 101 aa, 99 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_02840, 497 aa	ISCR2 transposase	None	*Bibersteinia trehalosi*, AGH37386, 357 aa, 99 %	*Shigella flexneri*, NP_838055, 497 aa, 100 %
Pmu_02850, 301 aa	rRNA (adenine-N6-)-methyltransferase	None	*Bibersteinia trehalosi*, AHG85539, 234 aa, 100 %	*Morganella morganii*, CDK68643, 303 aa, 99 %
Pmu_02880, 254 aa	Tyrosine recombinase-2 family protein (phage integrase)	None	ICE*Mh1*, AGK02704, 254 aa, 100 %	*Salmonella enterica*, YP_002149427, 308 aa, 46 %
Pmu_02890, 659 aa	Integrating conjugative element relaxase	None	ICE*Mh1*, AGK02705, 659 aa, 100 %	*Pseudomonas xanthomarina*, CEG51019, 625 aa, 37 %
Pmu_02900, 265 aa	Putative type I restriction–modification system methyltransferase subunit	HSM_1681, 265 aa, 99 %	ICE*Mh1*, AGK02706, 265 aa, 100 %	*Providencia stuartii*, AIN62209, 269 aa, 41 %
Pmu_02910, 248 aa	Hypothetical protein	HSM_1682, 248 aa, 100 %	ICE*Mh1*, AGK02707, 248 aa, 100 %	*Neisseria gonorrhoeae*, KDM99755, 259 aa, 39 %
Pmu_02920, 324 aa	Hypothetical protein	HSM_1683[a], 324 aa, 100 %	ICE*Mh1*, AGK02708, 324 aa, 100 %	*Yersinia ruckeri*, KGX82889, 332 aa, 46 %
Pmu_02930, 135 aa	Hypothetical protein	HSM_1684[a], 135 aa, 100 %	ICE*Mh1*, AGK02709, 135 aa, 100 %	None
Pmu_02940, 280 aa	Hypothetical protein	HSM_1685, 280 aa, 100 %	ICE*Mh1*, AGK02710, 280 aa, 100 %	*Alcanivorax* sp. 19-m-6, KGD61388, 280 aa, 35 %
Pmu_02950, 131 aa	Hypothetical protein	HSM_1686, 131 aa, 100 %	ICE*Mh1*, AGK02711, 131 aa, 100 %	None

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_02960, 81 aa	Hypothetical protein	HSM_1687, 81 aa, 100 %	ICE*Mh1*, AGK02712, 81 aa, 100 %	None
Pmu_02970, 117 aa	Hypothetical protein	HSM_1688, 117 aa, 100 %	ICE*Mh1*, AGK02713, 117 aa, 100 %	None
Pmu_02980, 147 aa	Integrating conjugative element protein	HSM_1689, 147 aa, 100 %	ICE*Mh1*, AGK02714, 147 aa, 100 %	*Serratia* sp. ATCC 39006, ESN64104, 134 aa, 38 %
Pmu_02990, 313 aa	Integrating conjugative element protein	HSM_1690[a], 313 aa, 100 %	ICE*Mh1*, AGK02715, 313 aa, 100 %	*Klebsiella oxytoca*, KFC43925, 310 aa, 55 %
Pmu_03000, 670 aa	Integrating conjugative element protein	HSM_1691[a], 670 aa, 100 %	ICE*Mh1*, AGK02716, 670 aa, 100 %	*Salmonella enterica*, ESG40339, 461 aa, 44 %
Pmu_03010, 142 aa	Hypothetical protein	HSM_1692, 142 aa, 100 %	ICE*Mh1*, AGK02717, 142 aa, 100 %	None
Pmu_03020, 156 aa	Hypothetical protein	HSM_1693, 156 aa, 100 %	ICE*Mh1*, AGK02718, 156 aa, 100 %	*Leptotrichia* sp. ERK50264, 146 aa, 36 %
Pmu_03030, 113 aa	Hypothetical protein	HSM_1694, 113 aa, 100 %	ICE*Mh1*, AGK02719, 113 aa, 100 %	None
Pmu_03040, 490 aa	TraG-like domain-containing protein	HSM_1695[a], 490 aa, 100 %	ICE*Mh1*, AGK02720, 490 aa, 100 %	*Salmonella enterica*, ESG40341, 498 aa, 46 %
Pmu_03050, 105 aa	Hypothetical protein	HSM_1696, 105 aa, 100 %	ICE*Mh1*, AGK02721, 105 aa, 100 %	None
Pmu_03060, 142 aa	Hypothetical protein	HSM_1697, 142 aa, 100 %	ICE*Mh1*, AGK02722, 142 aa, 100 %	None
Pmu_03070, 945 aa	Conjugative transfer ATPase TraC-like protein	HSM_1698[a], 945 aa, 100 %	ICE*Mh1*, AGK02723, 945 aa, 100 %	*Salmonella enterica*, ESG40327, 902 aa, 56 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_03080, 132 aa	Conjugative transfer region lipoprotein	HSM_1699[a], 132 aa, 100 %	ICE*Mh1*, AGK02724, 132 aa, 100 %	*Pseudomonas brassicacearum,* AHL34884, 138 aa, 53 %
Pmu_03090, 484 aa	Integrating conjugative element protein	HSM_1700[a], 484 aa, 100 %	ICE*Mh1*, AGK02725, 484 aa, 100 %	*Salmonella enterica*, ESG40325, 468 aa, 47 %
Pmu_03100, 294 aa	Integrating conjugative element protein	HSM_1701[a], 294 aa, 100 %	ICE*Mh1*, AGK02726, 294 aa, 100 %	*Xenorhabdus bovienii*, CDH06738, 277 aa, 53 %
Pmu_03110, 214 aa	Integrating conjugative element protein	HSM_1702[a], 214 aa, 100 %	ICE*Mh1*, AGK02727, 214 aa, 100 %	*Klebsiella pneumoniae*, ESL45356, 208 aa, 48 %
Pmu_03120, 121 aa	Conjugative transfer region protein	HSM_1703[a], 121 aa, 100 %	ICE*Mh1*, AGK02728, 121 aa, 100 %	*Salmonella enterica*, ABX22958, 117 aa, 43 %
Pmu_03130, 129 aa	Integrating conjugative element protein	HSM_1704[a], 129 aa, 100 %	ICE*Mh1*, AGK02729, 129 aa, 100 %	None
Pmu_03140, 77 aa	Hypothetical protein	HSM_1705, 78 aa, 100 %	ICE*Mh1*, AGK02730, 77 aa, 100 %	*Salmonella enterica*, EHL38285, 78 aa, 36 %
Pmu_03150, 108 aa	Integrating conjugative element protein	HSM_1706[a], 108 aa, 100 %	ICE*Mh1*, AGK02731, 108 aa, 100 %	*Salmonella enterica*, ESH30146, 98 aa, 45 %
Pmu_03160, 127 aa	Hypothetical protein	HSM_1707, 127 aa, 100 %	ICE*Mh1*, AGK02732, 127 aa, 100 %	None
Pmu_03170, 231 aa	Integrating conjugative element membrane protein	HSM_1708, 231 aa, 100 %	ICE*Mh1*, AGK02733, 231 aa, 100 %	*Halomonas* sp., ERL52507, 234 aa, 45 %
Pmu_03180, 117 aa	Hypothetical protein	HSM_1709, 117 aa, 100 %	ICE*Mh1*, AGK02734, 117 aa, 100 %	None

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_03190, 733 aa	Conjugative coupling factor TraD	HSM_1710[a], 733 aa, 99 %	ICE*Mh1*, AGK02735, 733 aa, 100 %	*Salmonella enterica*, ESG40317, 703 aa, 61 %
Pmu_03200, 168 aa	Integrating conjugative element protein	HSM_1711[a], 168 aa, 100 %	ICE*Mh1*, AGK02736, 168 aa, 100 %	*Pseudomonas xanthomarina*, CEG54628, 180 aa, 42 %
Pmu_03210, 256 aa	Putative lysozyme-like protein	HSM_1712[a], 256 aa, 100 %	ICE*Mh1*, AGK02737, 256 aa, 100 %	*Acinetobacter calcoaceticus*, EOQ63901, 723 aa, 48 %
Pmu_03220, 257 aa	Integrating conjugative element protein	HSM_1713[a], 257 aa, 100 %	ICE*Mh1*, AGK02738, 257 aa, 100 %	*Escherichia coli*, EFM51825, 235 aa, 44 %
Pmu_03230, 209 aa	Integrating conjugative element protein	HSM_1714, 209 aa, 100 %	ICE*Mh1*, AGK02739, 209 aa, 100 %	*Pseudomonas* sp. 12M76, CEA04059, 183 aa, 37 %
Pmu_03240, 86 aa	Hypothetical protein	HSM_1715, 86 aa, 100 %	ICE*Mh1*, AGK02740, 86 aa, 100 %	*Phycisphaera mikurensis*, BAM05377, 90 aa, 49 %
Pmu_03250, 120 aa	Hypothetical protein	HSM_1716, 120 aa, 100 %	ICE*Mh1*, AGK02741, 120 aa, 100 %	*Neisseria gonorrhoeae*, YP_003600430, 113 aa, 32 %
Pmu_03260, 135 aa	Hypothetical protein	HSM_1717, 135 aa, 99 %	ICE*Mh1*, AGK02742, 135 aa, 100 %	*Acinetobacter* sp. NIPH 899, ENV00920, 119 aa, 42 %
Pmu_03270, 226 aa	Hypothetical protein	HSM_1718, 226 aa, 99 %	ICE*Mh1*, AGK02743, 226 aa, 100 %	None
Pmu_03280, 143 aa	Hypothetical protein	HSM_1719[a], 143 aa, 100 %	ICE*Mh1*, AGK02744, 143 aa, 100 %	*Pseudomonas savastanoi*, EFW82260, 184 aa, 59 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_03290, 683 aa	DNA topoisomerase III	HSM_1720[a], 683 aa, 100 %	ICE*Mh1*, AGK02745, 683 aa, 100 %	*Bordetella hinzii*, KCB25912, 671 aa, 51 %
Pmu_03300, 172 aa	Hypothetical protein	HSM_1721, 172 aa, 100 %	ICE*Mh1*, AGK02746, 172 aa, 100 %	None
Pmu_03310, 94 aa	Hypothetical protein	None	ICE*Mh1*, AGK02747, 94 aa, 100 %	None
Pmu_03320, 236 aa	Hypothetical protein	HSM_1722[a], 236 aa, 100 %	ICE*Mh1*, AGK02748, 236 aa, 100 %	*Proteus mirabilis*, KGA90870, 241 aa, 40 %
Pmu_03330, 255 aa	Aldo/keto reductase	HSM_1723, 255 aa, 100 %	ICE*Mh1*, AGK02749, 255 aa, 100 %	*Flavobacterium subsaxonicum*, KGO90979, 381 aa, 55 %
Pmu_03340, 106 aa	Hypothetical protein	HSM_1724, 106 aa, 100 %	ICE*Mh1*, AGK02750, 106 aa, 100 %	None
Pmu_03350, 151 aa	Hypothetical protein	HSM_1725, 51 aa, 100 %	ICE*Mh1*, AGK02751, 151 aa, 100 %	*Pseudomonas aeruginosa*, ERV17255, 161 aa, 55 %
Pmu_03360, 515 aa	Multicopper oxidase protein	HSM_1726, 515 aa, 100 %	ICE*Mh1*, AGK02752, 515 aa, 100 %	*Kingella denitrificans*, EGC16775, 99 %
None	Putative transcriptional regulator, MerR family	HSM_1728, 134 aa	*Mannheimia varigena*, AHG73107, 149 aa, 100 %	*Kingella kingae*, EGK11561, 129 aa, 62 %
None	Protein of unknown function	HSM_1729, 105 aa	*Mannheimia varigena*, AHG79433, 145 aa, 100 %	*Neisseria wadsworthii*, EGZ49932, 129 aa, 61 %
None	Multicopper oxidase type 3	HSM_1730, 534 aa	*Mannheimia varigena*, AHG73105, 534 aa, 99 %	*Kingella denitrificans*, EGC16775, 515 aa, 60 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
None	Heavy metal translocating P-type ATPase	HSM_1731, 730 aa	*Mannheimia varigena*, AHG73102, 717 aa, 99 %	*Bordetella bronchiseptica*, KAK67234, 831 aa, 86 %
None	Protein of unknown function	HSM_1732, 248 aa	*Mannheimia varigena*, AHG73100, 248 aa, 100 %	*Neisseria wadsworthii*, EGZ46421, 270 aa, 94 %
Pmu_03390, 172 aa	Hypothetical protein	HSM_1733, 172 aa, 100 %	ICE*Mh1*, AGK02755, 172 aa, 100 %	*Acinetobacter baumannii*, EXS20166, 314 aa, 100 %
Pmu_03400, 207 aa	Tetracycline repressor protein (TetR)	HSM_1734, 207 aa, 100 %	ICE*Mh1*, AGK02756, 207 aa, 100 %	*Proteus hauseri*, EST57920, 207 aa, 96 %
Pmu_03410, 209 aa	Aminoglycoside 2”-O-adenyltransferase protein	None	None	*Escherichia coli*, YP_009082244, 195 aa, 99 %
Pmu_03420, 263 aa	Aminoglycoside 3”-O-adenyltransferase protein	None	*Pasteurella aerogenes*, YP_006961169, 258 aa, 91 %	*Salmonella enterica*, ABL95942, 263 aa, 98 %
Pmu_03440, 275 aa	Beta-lactamase OXA-2 protein	None	None	*Salmonella enterica*, NP_511223, 275 aa, 100 %
Pmu_03450, 234 aa	IS26 transposase	None	*Bibersteinia trehalosi*, AGH37398, 238 aa, 99 %	*Escherichia coli*, YP_006953879, 239 aa, 100 %
Pmu_03460, 491 aa	Macrolide efflux protein	None	*Bibersteinia trehalosi*, AGH37396, 491 aa, 100 %	*Citrobacter freundii*, NP_775053, 491 aa, 100 %
Pmu_03470, 294 aa	Macrolide 2’-phosphotransferase protein	None	*Bibersteinia trehalosi*, AGH37397, 294 aa, 99 %	*Acinetobacter baumannii*, YP_001736317, 294 aa, 99 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
Pmu_03480, 234 aa	IS26 transposase	None	*Bibersteinia trehalosi*, AGH37398, 238 aa, 100 %	*Salmonella enterica*, YP_209349, 240 aa, 100 %
Pmu_03490, 207 aa	Tetracycline repressor protein (TetR)	HSM_1734, 207 aa, 100 %	ICE*Mh1*, AGK02756, 207 aa, 100 %	*Proteus hauseri*, EST57920, 207 aa, 96 %
Pmu_03500, 400 aa	Tetracycline efflux protein, class H (TetH)	HSM_1735, 400 aa, 99 %	ICE*Mh1*, AGK02757, 400 aa, 99 %	*Gilliamella apicola*, KES17253, 400 aa, 99 %
Pmu_03510, 436 aa	Transposase	None	ICE*Mh1*, AGK02758, 436 aa, 100 %	*Escherichia coli*, ELJ62457, 436 aa, 92 %
Pmu_03520, 100 aa	Hypothetical protein	None	ICE*Mh1*, AGK02760, 100 aa, 100 %	None
Pmu_03530, 62 aa	Hypothetical protein	None	ICE*Mh1*, AGK02761, 62 aa, 100 %	*Vibrio fluvialis*, EPP21553, 71 aa, 73 %
None	Small multidrug resistance protein	HSM_1736, 110 aa	*Mannheimia varigena*, AHG73095, 110 aa, 100 %	*Cardiobacterium valvarum*, EHM53491, 99 aa, 63 %
None	Transcriptional regulator, MarR	HSM_1737, 149 aa	*Mannheimia varigena*, AHG73094, 149 aa, 100 %	*Clostridium] clostridioforme*, ENZ04504, 152 aa, 39 %
None	Protein of unknown function	HSM_1738, 123 aa	*Mannheimia varigena*, AHG73093, 123 aa, 100 %	*Neisseria flavescens*, EER56534, 123 aa, 64 %
None	Conserved hypothetical protein	HSM_1739, 366 aa	*Mannheimia varigena*, AHG73092, 366 aa, 100 %	*Tannerella forsythia*, AEW20145, 367 aa, 49 %
None	Cation efflux protein	HSM_1740, 204 aa	*Mannheimia varigena*, AHG73091, 172 aa, 100 %	*Lautropia mirabilis*, EFV94656, 212 aa, 55 %

(continued)

Table 1 (continued)

ICE*Pmu1* locus tag, protein	Annotation	*H. somni* strain 2336 (ICE*Hso*2336), ortholog locus tag, protein, identity	Closest homolog within *Pasteurellaceae*; (other than *H. somni*), species/ICE, locus tag, protein, identity	Closest homolog outside *Pasteurellaceae*, species, locus tag, protein, identity
None	Putative transcriptional regulator, MerR	HSM_1741, 132 aa	*Mannheimia varigena*, AHG73090, 132 aa, 100 %	*Enhydrobacter aerosaccus*, EEV23030, 150 aa, 43 %
None	Alcohol dehydrogenase zinc-binding domain protein	HSM_1744, 202 aa	*Mannheimia varigena*, AHG73087, 201 aa, 100 %	*Pseudomonas* sp., KEY89048, 330 aa, 64 %
Pmu_03540, 150 aa	Single-stranded DNA-binding protein	HSM_1745, 150 aa, 100 %	ICE*Mh1*, AGK02762, 150 aa, 100 %	*Cycloclasticus* sp., EPD13323, 154, 60 %
Pmu_03550, 161 aa	Hypothetical protein	HSM_1746, 161 aa, 100 %	ICE*Mh1*, AGK02763, 161 aa, 100 %	*Salmonella enterica*, CCF89832, 164 aa, 31 %
Pmu_03560, 252 aa	Integrating conjugative element protein	HSM_1747[a], 252 aa, 100 %	ICE*Mh1*, AGK02764, 252 aa, 100 %	*Citrobacter youngae*, EFE07328, 232 aa, 40 %
Pmu_03570, 396 aa	Hypothetical protein	HSM_1748[a], 396 aa, 99 %	ICE*Mh1*, AGK02765, 396 aa, 100 %	*Salmonella enterica*, ABX22929, 377 aa, 37 %
Pmu_03580, 185 aa	Hypothetical protein	HSM_1749[a], 185 aa, 100 %	ICE*Mh1*, AGK02766, 185 aa, 100 %	*Salmonella enterica*, ESJ18378, 179 aa, 38 %
Pmu_03590, 1548 aa	Integrating conjugative element ParB family protein	HSM_1750[a], 548 aa, 100 %	ICE*Mh1*, AGK02767, 548 aa, 100 %	None
Pmu_03600, 428 aa	Replicative DNA helicase	HSM_1751[a], 453 aa, 99 %	ICE*Mh1*, AGK02768, 453 aa, 99 %	*Klebsiella pneumoniae*, EMI36970, 432 aa, 49 %
Pmu_03610, 274 aa	Chromosome partitioning ATPase	HSM_1752[a], 274 aa, 100 %	ICE*Mh1*, AGK02769, 274 aa, 100 %	*Salmonella enterica*, ESJ18374, 294 aa, 46 %

[a]Full-length or partial homolog is present in *H. somni* strain 129Pt

It is interesting to note that *M. haemolytica* strain 42548, *P. multocida* strain 36950, and *H. somni* strain 2336 were isolated from cases of naturally occurring bovine respiratory tract infections in different parts of the USA (Pennsylvania, Nebraska, and Washington, respectively) in different years (2007, 2005, and 1980s, respectively), but harbor closely related genomic elements containing antibiotic resistance determinants. Multidrug-resistant isolates of these respiratory pathogens appear to be more common among animals in bovine feedlots (Klima et al. 2014). Furthermore, horizontal transfer of ICEs that mediate antibiotic resistance from *M. haemolytica* and *H. somni* to *P. multocida*, and from *P. multocida* to *Escherichia coli*, has been demonstrated (Klima et al. 2014). It is possible that these ICEs have a common evolutionary origin, and indiscriminate use of antibiotics favors their preservation and dispersal in the field.

3 Comparative Transcriptomics

Computational gene prediction at best provides a "first pass" structural annotation of genomes and has many limitations, which could be overcome using experimental approaches that involve the analyses of the transcriptome. Attempts have been made to obtain a high-resolution transcriptome map of *H. somni* strain 2336 using the Illumina RNA-Seq technology (Kumar et al. 2012). Comparison of the transcriptome map of strain 2336 with the computationally annotated genome facilitated the identification of 94 small noncoding RNA (sRNA) of various sizes (70–695 bp, average G+C content 39.3 %). A vast majority of these sRNA (82 of 94) were reported to be novel (unidentified in previous bacterial transcriptome studies) and proposed to play roles in housekeeping and virulence, in addition to gene regulation. Sequence analyses of the 94 sRNA indicated that 31 were specific to strain 2336, 41 were specific to strains 2336 and 129Pt, 11 had homologs only in the genomes of *P. multocida*, *H. influenzae*, and *H. parainfluenzae*, and 11 had homologs in the genomes of other distantly related bacteria (Kumar et al. 2012). Furthermore, the start sites of five predicted genes (HSM_0031, 0525, 0789, 1019, and 1729) were corrected using the RNA-Seq data and comparison with other phylogenetically related homologs.

Genome annotation had predicted that putative proteins encoded by HSM_0603, 0748, 1385, 1666, and 1744 (hypothetical protein, α-L-fucosidase, 3-hydroxydecanoyl-ACP dehydratase, DNA damage-inducible protein, and alcohol dehydrogenase, respectively) were shorter than their homologs in other species. RNA-Seq data of strain 2336 showed the presence of full-length mRNA for these genes and confirmed that the putative proteins were truncated at the *N*-terminus due to either frameshift mutations (for HSM_1385 and 1744) or non-functional start codons (for the other three genes) (Kumar et al. 2012). Analyses of the RNA-Seq data indicated that 1636 of the 1980 predicted protein-coding genes were transcribed and there were 278 operons consisting of 730 genes in *H. somni* strain 2336 (Kumar et al. 2012).

4 Plasmids and Shuttle Vectors

Plasmid-borne resistance to multiple antibiotics is a relatively common feature among some members of the *Pasteurellaceae*. Isolates of *H. somni* resistant to tetracycline and harboring *tetH*, albeit lacking plasmids, have been cultured from nasal swabs of feedlot calves from Alberta, Canada (D'Amours et al. 2011). Furthermore, plasmid profiling as a means of identification and characterization of field isolates of *H. somni* has been reported (Appuhamy et al. 1998; Fussing and Wegener 1993). Efforts have also been made toward deciphering and describing the complete nucleotide sequences of plasmids from *H. somni* strains (Izadpanah et al. 2001; Siddaramappa et al. 2006). All four *H. somni* circular plasmids whose sequences have been deciphered/described are referred to as cryptic plasmids since they lack the genes that encode functions other than those necessary for their own replication (Izadpanah et al. 2001; Siddaramappa et al. 2006). Interestingly, the largest *H. somni* circular plasmid that has been completely sequenced (pHS129, 5178 bp) appears to be a dimer (Siddaramappa et al. 2006), and the natural occurrence of such plasmid dimers among bacteria is relatively rare.

The possibility of using native or non-native plasmids, after suitable modifications, as shuttle vectors that function in *E. coli* and *H. somni* has been explored. *H. somni* strain HS91 was transformed with plasmid pD70Kan^R, which is derived from *M. haemolytica* plasmid pD70 (Briggs and Tatum 2005). Interestingly, in vitro modification of pD70Kan^R using a commercially available *HhaI* methylase significantly improves the transformation efficiency (Briggs and Tatum 2005). Furthermore, *H. somni* strain 129Pt, which contains plasmid pHS129, can be transformed with pLS88, which is a broad-host-range plasmid purified from *Haemophilus ducreyi* (Sanders et al. 1997). In vivo modification of pLS88 using the recombination-deficient *H. influenzae* strain DB117 improves the transformation efficiency (Sanders et al. 1997).

H. somni strain 129Pt has also been transformed with a modified version of *H. somni* circular plasmid pHS649 (Siddaramappa et al. 2006). Derivatives of pLS88 that transform *H. somni* with a higher efficiency (e.g., pNS3K) have also been developed using kanamycin resistance as the selectable marker (Sandal et al. 2008). Therefore, it appears that pHS129 and pLS88 do not belong to the same incompatibility group, as are pHS129 and pHS649. The possibility of improving these vectors or other forms of pLS88 (such as pLSSK and pLSKS) (Wood et al. 1999) for efficient transformation of *H. somni* strains remains to be explored.

5 Mutagenesis

Although chemical mutagenesis is a popular technique in bacterial genetics and ethyl methanesulfonate has been used to obtain non-capsulated mutants of *Actinobacillus pleuropneumoniae* (Inzana et al. 1993), it has not been widely used

in other members of the *Pasteurellaceae*. Transformation and mutagenesis of strains of *H. somni* using exogenous DNA molecules is difficult, at least in part due to an omnipresent restriction–modification system.

Molecular genetic analyses of *H. somni* were invigorated following the demonstration that in vitro or in vivo modification improves the transformation efficiency of shuttle plasmids for *H. somni* (Briggs and Tatum 2005; Sanders et al. 1997). Successful transformation of *H. somni* strain 129Pt with a putative virulence-associated gene of *H. somni* strain 2336, and the stable expression of the gene in the transformed strain, demonstrated the utility of *H. somni* preputial isolates for genetic analyses (Sanders et al. 1997). Furthermore, transformation of strain 129Pt was also used to demonstrate that *lob1* is involved in lipooligosaccharide (LOS) biosynthesis in *H. somni* and that the 5′-$(CAAT)_n$ repeats within *lob1* are involved in LOS phase variation (McQuiston et al. 2000) (see chapter on "The Many Facets of Lipooligosaccharide as a Virulence Factor for *Histophilus somni*"). These studies used a commercial electroporator to introduce heterologous DNA into *H. somni* rendered electrocompetent by growth in brain–heart infusion broth or Columbia broth and washing the bacterial pellets with 272 mM sucrose solution (Briggs and Tatum 2005; McQuiston et al. 2000; Sanders et al. 1997).

A non-replicative suicide plasmid methylated in vitro by *HhaI* methylase was used for mutagenesis of a *H. somni* strain 738 DNA locus involved in LOS biosynthesis by homologous recombination-mediated allelic exchange (Wu et al. 2000). The mutant strains had an altered LOS profile in comparison with the wild-type strain, indicating that *lob2A* could be involved in LOS biosynthesis (Wu et al. 2000). However, the prototype mutant strain (*H. somni* 738-*lob2A1::Km*) could not be complemented using shuttle vector pLSlob2A, reportedly due to inefficient electrotransformation (Wu et al. 2000) (see chapter on "The Many Facets of Lipooligosaccharide as a Virulence Factor for *Histophilus somni*").

A combination of methylation in vivo using the *H. influenzae* cloning strain DB117 and in vitro using *HhaI* methylase has been shown to improve the transformation efficiency of plasmids for *H. somni* strain 8025 (Sanders et al. 2003). A fivefold increase in transformation efficiency is observed after plasmids derived from *H. somni* strain 8025 are reintroduced into the same strain by electroporation (Sanders et al. 2003), indicating that the restriction–modification systems among *H. influenzae* and *H. somni* strains could be different. Furthermore, homologous recombination-mediated allelic exchange was used for partial deletion of a locus encoding high molecular weight immunoglobulin-binding proteins (HMW IgBPs) in *H. somni* strain 8025 (Sanders et al. 2003). A significant difference ($p < 0.001$) in the adherence of the mutant or wild-type strain to bovine pulmonary artery endothelial cells was also reported (Sanders et al. 2003). Of interest is that both *lob1* and the gene encoding for HMW IgBPs contain the *H. influenzae* uptake signal sequence (see Sect. 6).

A temperature-sensitive plasmid was developed to obtain in-frame, unmarked *aroA* deletion mutants of *H. somni* (Tatum and Briggs 2005). *M. haemolytica* native plasmid pD70 was modified by inserting the Tn903 kanamycin resistance cassette and the modified plasmid ($pD70Kan^R$) mutagenized using hydroxylamine. A single

base-pair mutation from G to A at position 301 within the origin of replication renders this plasmid temperature sensitive. The *aroA* gene from *H. somni* was amplified by PCR and cloned into the temperature-sensitive plasmid pGA301*ori*C to create pTs*Hsaro*C. An in-frame deletion was engineered within pTs*Hsaro*C to create the replacement plasmid pTs*Hs*Δ*aroA*C (Tatum and Briggs 2005). This replacement plasmid is methylated in vitro using *HhaI* methylase, electroporated into *H. somni* strain 2336, and recovered at the permissive temperature of 30 °C for 2 h on medium containing 50 μg/ml kanamycin. The plates are then incubated at the non-permissive temperature (41 °C) for 16 h to select for single-crossover mutants containing the temperature-sensitive replacement plasmid integrated into the chromosome by homologous recombination (Tatum and Briggs 2005). Single-crossover mutants are cultured in broth without kanamycin at the permissive temperature for 16 h to facilitate a second crossover event and plasmid excision. This process is repeated twice, and bacteria from the third-pass culture are streaked onto plates without kanamycin. The plates are incubated at 37 °C for 16 h, and colonies are further replica-plated with or without kanamycin selection. After incubation at 37 °C, kanamycin-sensitive colonies are selected and the absence of the kanamycin gene is tested by PCR. Deletion of *aroA* is also confirmed by PCR (Tatum and Briggs 2005).

A non-replicative suicide plasmid methylated in vitro using *HhaI* methylase can also be used for complete deletion of the *ibpA* open reading frame encoding HMW IgBPs in *H. somni* strain 2336 by homologous recombination-mediated allelic exchange (Hoshinoo et al. 2009). The isogenic mutant strain was less cytotoxic than wild-type strain 2336 for bovine FBM-17 macrophage-like cells, murine J774.1 macrophage-like cells, and bovine primary monocyte cells (Hoshinoo et al. 2009). Although wild-type strain 2336 significantly compromised the ability of murine J774.1 macrophage-like cells and bovine primary monocyte cells to phagocytize microspheres, the isogenic mutant strain had no such effect, indicating that IbpA (specifically the Fic region; see chapter on "*Histophilus somni* Surface Proteins") of *H. somni* may play a role in pathogenesis (Hoshinoo et al. 2009).

Homologous recombination-mediated exchange of genes encoding the major outer membrane protein (MOMP) between *H. somni* strains 129Pt and 2336 has been described (Ueno et al. 2014). Since plasmid-based cloning of the *H. somni* gene encoding MOMP proved difficult, a vector-free strategy that utilizes the direct electroporation of PCR-amplified, *HhaI*-methylated linear DNA into *H. somni* was developed (Ueno et al. 2014). Following allelic exchange, strain 129Pt stably expresses the gene encoding MOMP from strain 2336 (HSM_1447, *ompH*/OmpH, 1443 bp/380 aa) and strain 2336 stably expresses the gene encoding MOMP from strain 129Pt (HS_0971, *ompH*/OmpH, 951 bp/316 aa), and the proteins can be detected by Western and dot blots using strain-specific anti-MOMP monoclonal antibodies. Furthermore, strains 129Pt and 2336 stably express a chimeric gene encoding MOMP (due to combining parts of genes encoding MOMPs from the two strains) after allelic exchange, and the fusion proteins can be detected using strain-specific anti-MOMP monoclonal antibodies in Western and dot blots (Ueno et al. 2014). The serum susceptibilities of strain 129Pt expressing HSM_1447 and

strain 129Pt expressing the fusion protein (containing portions of HSM_1447 at the *C*-terminus) are significantly greater than those of the wild type (Ueno et al. 2014). This is not surprising since the genomes of strains 129Pt and 2336 differ from each other, and the genes encoding the OmpH homologs have only 56 % identity.

To overcome the inherent low efficiency of transformation and recombination of non-replicative suicide plasmids used for allelic exchange in *H. somni*, improved methods of mutagenesis need to be developed. Mutagenesis of *H. somni* using a commercially available transposon (Sandal et al. 2009) represents a significant step in this direction. Electroporation of *H. somni* strain 2336 yields up to 100 kanamycin-resistant colonies per 20 ng of the EZ-Tn5™ <KAN-2> Tnp Transposome™ (Epicentre, Madison, WI). Of 500 transposon mutants of *H. somni* strain 2336 screened for biofilm formation using the crystal violet assay, 55 formed either more or less biofilm than the wild-type strain. Of the several transposon mutants confirmed to produce less biofilm than the wild-type strain by scanning electron microscopy, six contained a transposon insertion in a region of the *ibpA* gene that encodes a putative filamentous hemagglutinin. This indicates that filamentous hemagglutinins, which are important attachment factors in other pathogenic bacteria [such as *Bordetella* (Villarino Romero et al. 2014)], likely contribute to *H. somni* biofilm formation and possibly pathogenesis (Sandal et al. 2009). Mutagenesis of *H. somni* strain 2336 genes putatively encoding S-ribosylhomocysteinase (*luxS*), universal stress protein E (*uspE*), major facilitator transport protein, and a protein of unknown function has also been achieved using the EZ-Tn5™ <KAN-2> Tnp Transposome™ (Sandal et al. 2009; Shah et al. 2014). Interestingly, both *luxS* and *uspE* mutants are attenuated in an acute septicemia mouse model, whereas only the *uspE* mutant is deficient in biofilm formation (Shah et al. 2014).

6 Natural Transformation

The ability of bacteria to internalize chromosomal fragments and/or plasmids under natural conditions is referred to as *competence*. Competence is proposed to be regulated by biochemical as well as environmental cues, and the purposes for internalizing DNA within the host cell could be non-genetic (e.g., nutrition) or genetic (e.g., transformation) (Mell and Redfield 2014). Although most naturally competent bacteria are indiscriminate in DNA internalization, members of the *Pasteurellaceae* and the *Neisseriaceae* are known to prefer conspecific DNA. The preferential internalization of conspecific DNA by members of these two families appears to be facilitated by short uptake signal sequences (Mell and Redfield 2014). In *H. influenzae*, the uptake signal sequence is a nonamer (5′-AAGTGCGGT, or its reverse complement), and comparative genomic analyses have demonstrated the abundance of this sequence in *Actinobacillus actinomycetemcomitans*, *P. multocida*, and *H. somni* (Bakkali et al. 2004; Redfield et al. 2006).

Although several members of the *Pasteurellaceae* are believed to be competent, only *H. influenzae*, *A. actinomycetemcomitans*, and *A. pleuropneumoniae* have been shown to undergo natural transformation under laboratory conditions (Redfield et al. 2006). In other species, the lack of competence or transformation in the laboratory is believed to be due to the failure to mimic native conditions and/or dysfunctional genetic systems (Redfield et al. 2006). Notably, *H. somni* strains 2336 and 129Pt lack a *comD* homolog and appear to encode a shortened ComE homolog. Since it has been demonstrated that functionality of each gene within the *com* operon is essential for transformation of *H. influenzae* (Carruthers et al. 2012), it could be presumed that *H. somni* strains 2336 and 129Pt are non-transformable. This appears to be valid in the case of strain 129Pt since it fails to be transformed when plasmid pNS3K (Sandal et al. 2008), genomic DNA from a *lob2A* mutant of strain 738 (Wu et al. 2000), or genomic DNA from transposon mutants of *luxS* or *uspE* of strain 2336 is used (Shah et al. 2014). However, strain 2336 can be transformed with a low efficiency when plasmid pNS3K or genomic DNA from a *lob2A* mutant of strain 738 is used. Nevertheless, strain 2336 fails to transform when genomic DNA from transposon mutants of *luxS* or *uspE* is used. Moreover, *H. somni* strains 649, 8025, and M14-622 also fail to transform when genomic DNA from a *lob2A* mutant of strain 738 is used (Shah et al. 2014). Therefore, it is likely that *H. somni* strains differ in their competency/transformability, and this could be due to the lack of specific *com* genes and/or the variety of restriction–modification systems that occur in this species (Briggs and Tatum 2005; Siddaramappa et al. 2011). Differences in competence and transformation have also been observed among strains of *H. influenzae* lacking specific *com* genes (Maughan and Redfield 2009). Furthermore, transformation of *H. influenzae* with restriction endonuclease-digested conspecific DNA is dependent on fragment size (Beattie et al. 1982), and it has been hypothesized that restriction endonucleases released by lysed cells may cut donor DNA fragments destined for uptake and reduce recombination efficiency (Mell and Redfield 2014).

7 Conclusions

Biochemical and genetic studies in the pre-genomic era firmly establish *H. somni* as a potent opportunistic pathogen. Complete genome sequencing reveals the pathogenic repertoire of this species, and comparative genomic analyses facilitate the identification of chromosomal regions that resemble the pathogenicity islands of other virulent bacteria. One such pathogenicity island has now been identified as ICE*Hso*2336 and appears to represent a classical horizontally transferred element. Transcriptome analyses indicate that ~80 % of the predicted genes of *H. somni* strain 2336 are readily transcribed, and ~44 % of these genes are operonic. Furthermore, electrotransformation of *H. somni* appears to be more efficient than natural transformation. In addition, genetic manipulation of *H. somni* is achievable through either suicide plasmid-based homologous recombination (targeted mutagenesis) or transposomes

(random mutagenesis), and several plasmids are now available that can serve as shuttle vectors. Future investigations of *H. somni* are expected to be guided by the principles, technologies, and developments discussed in this chapter.

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Interactions of *Histophilus somni* with Host Cells

Erica Behling-Kelly, Jose Rivera-Rivas and Charles J. Czuprynski

Abstract *Histophilus somni* resides as part of the normal microflora in the upper respiratory tract of healthy cattle. From this site, the organism can make its way into the lower respiratory tract, where it is one of the important bacterial agents of the respiratory disease complex. If *H. somni* cells disseminate to the bloodstream, they frequently result in thrombus formation. A series of in vitro investigations have examined potential mechanisms that might contribute to such thrombus formation. Earlier work showed that *H. somni* can stimulate some bovine endothelial cells to undergo apoptosis. More recent studies indicate that *H. somni* stimulates endothelial cell tissue factor activity and disrupts intercellular junctions. The net effect is to enhance procoagulant activity on the endothelium surface and to make the endothelial monolayer more permeable to molecules, leukocytes, and perhaps *H. somni* cells. *H. somni* also activates bovine platelets, which also can enhance tissue factor activity on the endothelium surface. When exposed to *H. somni*, bovine neutrophils and mononuclear phagocytes form extracellular traps in vitro. Ongoing research is investigating how the interplay among endothelial cells, platelets, and leukocytes might contribute to the thrombus formation seen in infected cattle.

Contents

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1 Introduction

The ability of pathogenic bacteria to colonize, infect, and cause clinical manifestations of disease depends on how host cells respond to the bacterial cells. The wide array of clinical syndromes caused by *Histophilus somni* speaks to the capacity of this particular opportunistic pathogen to interact with a variety of tissue cells and evade both local and systemic immune responses. In this chapter, we will focus on the bacteria–host cell interactions that lead to the most severe clinical manifestations of *H. somni* infection, with an emphasis on events that lead to systemic dissemination and thrombotic disease (Angen et al. 1998).

Because respiratory disease typically precedes more severe presentations of *H. somni* infection, we will begin by considering the interactions of *H. somni* with cells of the respiratory tract (Ames 1987; Andrews et al. 1985; Angen et al. 1998, 2003; Appuhamy et al. 1998). Cattle can harbor *H. somni* asymptomatically in the upper respiratory tract (Allen et al. 1991; Ward et al. 1984). The ability of *H. somni* to cause upper respiratory disease first requires a tropism for, and adherence to, epithelial cells of the nasal mucosa. Binding of *H. somni* cells to bovine nasal turbinate epithelial cells was shown to vary among isolates propagated in chicken embryos (Ward et al. 1984). The bacterial factors responsible for the differences in adherence were not further characterized in this early study.

Adherence of the related human pathogen, *Haemophilus influenza*e, to human respiratory epithelial cells is dependent on encapsulation of the organism. Electron microscopy studies have shown that nonencapsulated strains of *H. influenzae* cause significant epithelial cell damage and are adherent to the epithelial cell's surface (Wilson et al. 1992). Conversely, encapsulated strains are seen in association with a thick matrix that forms above the epithelial cell surface and are rarely cell-associated. Fimbriae also confer adherence to buccal, but not respiratory, epithelium (Read et al. 1992). Nontypeable *H. influenzae* can cause focal damage to respiratory mucosa in human organ cultures and only adhere to damaged epithelial cells, as indicated by transmission electron microscopy (Read et al. 1992). A similar increase in adherence secondary to cellular damage has been demonstrated in vivo, as robust adherence of *H. influenzae* is evident in children with chronic sinusitis (Harada et al. 1990). These findings suggest a role for increased bacterial adherence in the context of cellular damage. Although there is no direct evidence for damage to the upper respiratory tract epithelium by adherence of *H. somni* cells, *H. somni* is often part of a polymicrobial complex. Perhaps endothelium damage by other pathogens could facilitate adherence of *H. somni* cells.

H. somni adherence to epithelial cells of the lower airway has been inferred from histopathological examination of pneumonia cases, although the cellular interactions responsible for adherence have not been defined. Molecular studies identified various motifs that may be involved in *H. somni* adherence, including heparin-binding and RGD domains (Tagawa et al. 2005).

Uncomplicated cases of *H. somni* respiratory illness involve interactions with nasal and respiratory epithelial cells, as well as local immune cells in the alveolar

space. Clinical progression from pneumonia to septicemia indicates that *H. somni* can breach the air–blood barrier. However, little is known as to how *H. somni* does so. Perhaps direct transmigration of *H. somni* cells across the respiratory tract may occur. Alternatively, the bacterial cells could gain access to the bloodstream via infected leukocytes, or by other mechanisms that require compromised function of the bronchoalveolar epithelium. Currently, there is no evidence that *H. somni* cells transmigrate across intact epithelial cells. In animal inoculation studies, intrabronchial administration of *H. somni* resulted in the development of pulmonary lesions. Bacterial antigens were confined to the luminal spaces of the airways, with the exception of one area of bronchio-associated lymphoid tissue (Tegtmeier et al. 1999).

2 Interactions with the Innate Immune System

H. somni is adept at evading destruction by cells of the innate immune system. One of the first cells *H. somni* must either evade, or defeat, is the neutrophil. Several lines of evidence indicate the bactericidal activity of the neutrophil is compromised by its interactions with *H. somni* cells. Neutrophils isolated from *H. somni*-infected cattle display a generalized decrease in antibacterial activity (Pfeifer et al. 1992). In vitro studies elucidated some of the mechanisms behind the lackluster performance of neutrophils in combating *H. somni*. Neutrophil ingestion of opsonized *H. somni* cells triggers a modest respiratory burst compared with uptake of opsonized *E. coli* (Czuprynski and Hamilton 1985). *H. somni* release of nucleic acids into the culture medium impairs neutrophil function in vitro (Chiang et al. 1986). Viable *H. somni* cells can eliminate reactive oxygen intermediates released from bovine neutrophils and are capable of removing H_2O_2 from solution. The removal of H_2O_2 requires metabolically active *H. somni* cells and does not occur when the bacteria are heat-killed, formalin-fixed, or sonicated (Sample and Czuprynski 1991). Perhaps the ability of *H. somni* to impair neutrophil function contributes to the polymicrobial nature of *H. somni* infections in the bovine respiratory disease complex. Ingestion of *H. somni* cells triggers morphological changes in neutrophils consistent with apoptosis (Yang et al. 1998). More recently, it was shown that incubation with *H. somni* cells or outer membrane vesicles also triggers the formation of neutrophil extracellular traps (NETs). This response was not recapitulated with *H. somni* lipooligosaccharide (LOS) (Hellenbrand et al. 2013).

The neutrophil is not alone in its plight to kill this evasive pathogen. Mononuclear phagocytes also have limited ability to destroy *H. somni* cells, which are capable of surviving and, to a limited extent, replicating within bovine monocyte-derived macrophages and alveolar macrophages in vitro (Gomis et al. 1998; Lederer et al. 1987). The ability of the *H. somni* cells to inhibit mononuclear

phagocyte production of reactive oxygen and nitrogen intermediates corresponds with their ability to survive within mononuclear phagocytes (Gomis et al. 1997a; Howard et al. 2004). Macrophage release of superoxide anion required exposure to viable *H. somni* cells; it did not occur with killed cells or LOS (Howard et al. 2004). *H. somni*-mediated inhibition of nitric oxide production does not occur when the macrophages encounter bacterial cells opsonized with hyperimmune serum, or killed *H. somni* cells (Gomis et al. 1997b). Perhaps the ability of *H. somni* to survive within phagocytic cells contributes in part to the duration of *H. somni* infections. Furthermore, perhaps *H. somni*-laden leukocytes act as a "Trojan horse" and carry the bacterial cells through the vasculature to distant tissue sites where the bacterial cells can establish new foci of infection (Chladek 1975; Cole et al. 1993; Hajtos 1987).

3 Interactions with the Vasculature

Both circulating free *H. somni* cells and *H. somni*-infected leukocytes in the blood can interact with endothelial cells lining the blood vessel wall. *H. somni* cells have been demonstrated to adhere to, but not invade, bovine aortic endothelial cells (BAEC), and bovine brain microvascular endothelial cells (BBMEC) in vitro (Behling-Kelly et al. 2006; Sylte et al. 2001). Adherence of *H. somni* cells to endothelial cells is altered by *H. somni*-driven activation of adjacent endothelial cells or nearby leukocytes. Activation of endothelial cells by the inflammatory cytokine TNF-α increased adherence of *H. somni* to aortic explants, BAEC, and bovine brain endothelial cells (Behling-Kelly et al. 2006; Kwiecien et al. 1994; Sylte et al. 2006; Thompson et al. 1987). Although there was some variability in the above experimental results, no difference among pathogenic and nonpathogenic (i.e., preputial) strains of *H. somni* were noted in these studies. *H. somni* cells themselves can induce TNF-α secretion from cultured endothelial cells, which might augment the above responses (Behling-Kelly et al. 2006).

4 Thrombus Formation

The interplay among the inflammatory, coagulation, and fibrinolysis cascades is an active area of investigation in both human and veterinary medicine. The propensity of *H. somni* to elicit thrombosis is rather unique among bovine bacterial pathogens and is similar to that of human pathogens (e.g., *Neisseria meningitidis*) that are associated with a high risk of coagulopathy. Manifestations of thrombosis in *H. somni* infection range from septicemia and disseminated intravascular coagulation (DIC) to the acute neurological syndrome thrombotic meningoencephalitis

(TME) (Momotani et al. 1985; Roberts et al. 1979; Thompson et al. 1987). It was previously thought that emboli were a key feature of the latter syndrome, but data suggest that local alterations of the endothelium contribute to thrombi formation in the brain (Behling-Kelly et al. 2007a, b).

An array of mechanisms contribute to the bacterial cells' ability to trigger a thrombus. A need to understand the mechanisms responsible for *H. somni*-induced thrombotic complications has been the underlying rationale for studies investigating the roles of platelets and endothelial cells in both the coagulation and fibrinolytic cascades. A detailed discussion of this work is beyond the scope of this review. However, a brief synopsis of coagulation will facilitate understanding of these mechanisms in the context of *H. somni* infection.

Fluidity of the blood is maintained by tight regulation of: (1) thrombogenicity of the endothelium; (2) the proteins that activate and inhibit coagulation; and (3) the fibrinolytic enzymes that degrade clots. Investigations of the underlying mechanisms that contribute to clot formation in *H. somni* infections focused primarily on interactions between intact *H. somni* cells, or the outer membrane vesicles they secrete, with host cellular constituents intimately involved in coagulation including platelets, leukocytes, and endothelial cells. *H. somni* interacts with and alters the function of all three cell types in a procoagulant manner. In contrast, investigations of the interactions of *H. somni* cells with soluble clotting factors are lacking.

Activation of bovine platelets by *H. somni* has the potential to activate coagulation, modulate the immune response, and contribute to dissemination of the infection. *H. somni* and its LOS can directly activate bovine platelets. Phase-variable phosphorylcholine on *H. somni* LOS binds to and cross-links the platelet-activating factor receptor on bovine platelets (Kuckleburg et al. 2007). This activation is characterized by increased expression of P-selectin, a protein released from platelet alpha granules that would be expected to promote adherence of *H. somni* cells to the endothelium (Kuckleburg et al. 2008). Interestingly, coincubation of *H. somni*-activated platelets with endothelial cell monolayers resulted in endothelial cell uptake of platelets in vitro (Kuckleburg et al. 2008). Perhaps this is a mechanism used by *H. somni* to breach vascular barriers. Contact between *H. somni*-activated platelets and endothelial cells also induced endothelial cell apoptosis. The apoptotic process required activation of caspases 8 and 9 and was reduced by inhibition of reactive oxygen species (Kuckleburg et al. 2005). Apoptosis of endothelial cells in vivo would be expected to promote additional adherence of activated platelets to the underlying subendothelial collagen and von Willebrand factor that could further activate platelets (Corbeil et al. 2006). *H. somni*-activated platelets also express increased levels of CD40L and FasL (Kuckleburg et al. 2008). Once activated by *H. somni* or LOS, platelets transmit inflammatory signals to pulmonary artery endothelial cells that result in increased production of proinflammatory cytokines and chemokines including IL-1α, MCP-1,

and MIP-1α (Kuckleburg et al. 2008). Production of IL-1α and other inflammatory cytokines by the endothelium can increase vascular permeability, promote adherence of additional bacterial cells, and increase thrombogenicity of the endothelial cell surface.

Maintaining the antithrombotic state of the quiescent endothelial cell is central to regulation of hemostatic balance. The endothelium normally has an anticoagulant profibrinolytic surface that repels platelets and coagulation factors and secretes mediators that inhibit platelet activation and promote dissolution of blood clots. One of the key inhibitors on the luminal surface of endothelial cells is a coating of heparan sulfate proteoglycans. These molecules form a densely charged layer that acts as a cofactor for activation of antithrombin, the main anticoagulant protein in vivo (Opal et al. 2002). Interestingly, adherence of *H. somni* to endothelial cells can be inhibited by exogenous heparin (Behling-Kelly et al. 2006), and the outer membrane vesicles of *H. somni* cells are rich in proteins with heparin-binding domains (Tagawa et al. 2005). The direct impact of bacterial attachment to sulfated proteoglycans on the endothelial cell is not known at this time. However, one might hypothesize that the normal ability of heparin to activate the anticoagulant protein antithrombin might be compromised by adherent *H. somni* cells.

Coagulation results when a series of proteolytic enzymes are activated in the presence of an appropriate negatively charged surface (Fig. 1). The main trigger for coagulation in vivo is tissue factor. This protein is present in an inactive form on the surface of resting, healthy endothelial cells or monocytes, but is highly expressed by subendothelial fibroblasts. Vascular injury, or (as will be discussed later in the context of TME) retraction of endothelial cells, can expose tissue factor to small amounts of factor VIa in the bloodstream. This in turn initiates the coagulation

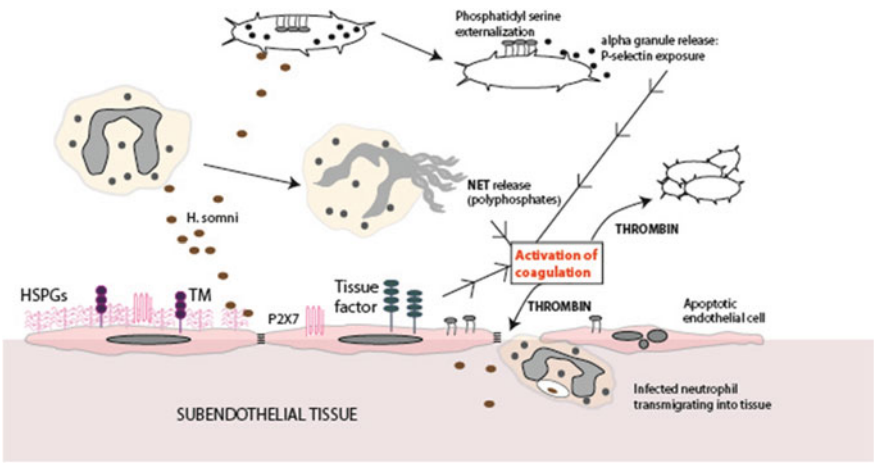

Fig. 1 Overview of the interactions among *H. somni cells*, endothelial cells, platelets, and leukocytes that could result in thrombus formation

cascade. Many investigations of bacterial activation of the coagulation cascade have focused on *N. meningitidis*, whose outer membrane vesicles and LOS induce tissue factor expression on monocytes and endothelial cells (Heyderman et al. 1997; Mirlashari et al. 2001; Ovstebo et al. 2011). Exposure of BBMEC to *H. somni* cells increased tissue factor expression and activity (Behling-Kelly et al. 2007a, b). Unlike *N. meningitidis*, purified *H. somni* LOS did not increase BBMEC procoagulant activity (Behling-Kelly et al. 2007a, b). An *H. somni*-induced increase in BBMEC procoagulant activity is detectable within 2 h, suggesting that it may result from de-encryption of tissue factor at the cell surface rather than de novo protein expression. However, tissue factor encryption in the endothelial cell membrane is controversial and beyond the scope of this review. Regardless of the source of tissue factor, *H. somni* elicits changes in endothelial cells that make their surface able to support fibrin deposition.

H. somni and its LOS cause apoptosis of pulmonary artery endothelial cells in vitro (Sylte et al. 2001). This response was dependent on production of reactive nitrogen and oxygen intermediates and caspase activation (Sample and Czuprynski 1991). Not surprisingly, *H. somni*-induced endothelial cell apoptosis is enhanced by addition of exogenous TNF-α (Sylte et al. 2004, 2006). *H. somni*-induced apoptosis is modulated by purinergic receptors; pharmacological antagonists of P2X receptors decrease LOS-induced apoptosis, whereas P2X agonists have an opposing effect (Sylte et al. 2005). Because platelets release ADP-containing alpha granules upon exposure to *H. somni*, ADP activation of P2X receptors may be one mechanism by which platelets modulate endothelial cell apoptosis (Kuckleburg et al. 2008). Links between endothelial cell apoptosis and activation of the coagulation cascade are well documented. Activation results in inversion of the endothelial cell membrane and exposure of phosphatidylserine on the outer leaflet of the cell membrane. This in turn increases tissue factor activity and provides a dense negatively charged surface that facilitates assembly of active coagulation complexes. Activation of the coagulation cascade culminates in production of thrombin, which then cleaves fibrinogen into fibrin.

Once in the bloodstream, the bacterial cells encounter an arsenal of host-defense mechanisms (complement, phagocytic cells). Virulence factors utilized by *H. somni* to evade destruction by the complement system are addressed elsewhere in this volume (Host Immune Response to Histophilus somni, L. Corbeil, Curr. Topics Microbiol. Immunol., 2015). Here, we focus on the interactions of *H. somni* with cells of the vasculature that are thought to facilitate thrombosis and dissemination of the organism to various tissues including the heart, placenta, joints, and brain (Chiang et al. 1990; Cole et al. 1992, 1993; Colles 2007; Corbeil 1990, 2007).

Thrombosis and DIC can occur secondary to a number of disease states, including septicemia. In cattle, DIC has been associated with certain forms of mastitis, metritis, abomasal displacement, and *H. somni* infections (Braun et al. 1990; Irmak et al. 2006; Irmak and Turgut 2005; Momotani et al. 1985). Human patients with meningococcemia suffer from a higher rate of DIC than patients

suffering from other forms of septicemia (Nieuwland et al. 2000). Platelet and granulocyte-derived microparticles are found in greater numbers in meningococcemia patients compared to controls (Nieuwland et al. 2000). These microparticles bear tissue factor and support the generation of thrombin, indicating they trigger the coagulation cascade.

In addition to promoting tissue factor expression, interaction with *H. somni* cells reduces endothelial cell levels of thrombomodulin, a key inhibitor of coagulation (Behling-Kelly et al. 2007a, b). Thrombomodulin is a protein expressed on healthy endothelial cells that is essential for the activation of protein C. Excess thrombin binds thrombomodulin on the surface of endothelial cells. The thrombin:thrombomodulin complex, in association with the endothelial protein C receptor and protein S (a circulating cofactor for protein C), changes the conformation of activated protein C, a potent anticoagulant molecule. Activated protein C then inhibits factors V and VIII, which are key cofactors in the coagulation cascade. Exposure of BBMEC to live *H. somni* cells causes a substantial decline in endothelial cell activation of protein C and expression of thrombomodulin. These alterations occur concurrently with increased production of IL-6 and TNF-α (Behling-Kelly et al. 2007a, b; Rochfort and Cummins 2015). However, the role of inflammatory cytokines in the endothelial cell response to *H. somni* in vivo remains to be shown. These prothrombotic changes are not unique to infection with *H. somni*. A number of pathogenic bacteria (e.g., *N. meningitidis*) and viruses elicit similar changes in the endothelial cell surface. For example, children with meningococcemia with the evidence of vascular compromise (e.g., purpura or petechial lesions) exhibit decreased expression of thrombomodulin and the endothelial protein C receptor in skin lesion biopsies. Plasma levels of activated protein C were also low in infected children (Faust et al. 2001). A decline in protein C activation is a well-recognized sequela to septicemia and is predictive of outcome (Shorr et al. 2006). Pharmacological replacement of activated protein C is not efficacious in mitigating sepsis and is associated with an increased risk of bleeding (Marti-Carvajal et al. 2012). More investigation is needed to discern the roles of host inflammatory and bacterial factors in promoting thrombotic changes in the vasculature in *H. somni* infection (Claxton and Everett 1966).

Many of the pathologic sequelae of Gram-negative septicemia are thought to be a consequence of the detrimental impacts of lipopolysaccharide, or as in the case of *H. somni* and *Neisseria* spp., LOS. For example, in human meningococcemia, plasma LOS levels correlate with more rapid and robust generation of thrombin in calibrated automated thrombin generation assays (Hellum et al. 2014). In that case, LOS is not released as a singular molecule, but as a part of vesicle trafficking in the form of an outer membrane vesicle (Kulp and Kuehn 2010). These findings led to investigations of microparticles, both pathogen and host-derived, as potent triggers of coagulation (Hellum et al. 2014). The possible role of microparticles in the thrombotic changes elicited by *H. somni* deserves investigation.

5 Role of Neutrophil Extracellular Traps (NETs) in Thrombus Formation

NETs form via a process by which activated neutrophils release their nuclear DNA peppered with antimicrobial peptides such as histones, elastase, cathepsin G, and myeloperoxidase (Brinkmann et al. 2004). The resulting mesh-like structures are capable of ensnaring and inactivating a variety of bacteria, fungi, viruses, and protozoa (Brinkmann et al. 2004; Guimarães-Costa et al. 2009; Rocha et al. 2015; Saitoh et al. 2012). Upon stimulation, the neutrophil's nuclear envelope fragments and the chromatin undergo decondensation. Antimicrobial proteins from neutrophil azurophilic granules subsequently mix with and adhere to the decondensed chromatin. Finally, the chromatin protein mixture is released into the nearby microenvironment. The end result is an active form of death that differs from apoptosis or necrosis.

Recently, there has been considerable interest in the role of NETs in host defense. *H. somni* cells cause extracellular trap formation by bovine neutrophils and macrophages in a time- and concentration-dependent manner (Hellenbrand et al. 2013). The mechanism by which *H. somni* cells trigger this response is not clear, but it may be dependent in part on binding of outer membrane proteins and fibrils. Incubation of bovine neutrophils with *H. somni* outer membrane vesicles (which contain LOS, outer membrane proteins, and fibrils, such as IbpA) triggers NET formation in a concentration-dependent manner (Hellenbrand et al. 2013). In contrast, purified *H. somni* LOS does not induce NET formation. In contrast, there are reports that bacterial endotoxin can stimulate NET formation by human or murine neutrophils. For example, *N. meningitidis* and its outer membrane vesicles induce NET formation. Interestingly, *N. meningitidis* evades killing by the NETs via modification of its LOS and uptake of Zn. Release of outer membrane vesicles serves as a potential decoy, eliciting NET formation that entraps the vesicles, but not the bacterial cells, which evade killing (Lappann et al. 2013). Some investigators report that costimulation with LOS and cytokines, or some other signal, is required to induce NET formation. However, LOS from a related bacterial species, nontypable *H. influenza*e, induces extracellular trap formation by human neutrophils (Juneau et al 2011).

It appears that at least some *H. somni* cells are inactivated when interacting with bovine NETs in vitro (Hellenbrand et al. 2013). Bovine neutrophils preincubated with cytochalasin D to inhibit phagocytosis are able to trap and kill *H. somni* cells. Addition of DNase I to degrade extracellular DNA decreases killing of *H. somni* cells, providing additional evidence for NETs in bacterial killing. These observations are particularly interesting because *H. somni* is resistant to phagocytosis and intracellular killing. Perhaps NET-mediated bacterial killing represents one mechanism by which bovine neutrophils can limit *H. somni* infection in vivo.

NET formation can be a double-edged sword. Although NETs can benefit the host by trapping, killing, and limiting pathogen spread, they also have been implicated in hemostatic disorders such as vasculitis, deep venous thrombosis, and pulmonary thromboembolism (Brill et al. 2012; Fuchs et al. 2010; Savchenko et al. 2014). Histones, chromatin, and serine proteases, all of which are present in NETs, can increase thrombin generation, leading to venous thrombosis (Fuchs et al. 2010; Higuchi et al. 1992; Massberg et al. 2010; Perrin et al. 2010). Intravenous histone administration exacerbated thrombus formation in a mouse model of deep venous thrombosis (Martinod et al. 2013). In contrast, intravenous administration of DNase I, which degrades the DNA scaffold of NETs, protects mice from venous thrombosis (Martinod et al. 2013). These findings implicate histone-containing extracellular DNA fibrils in hemostasis. There also is evidence that serine proteases within NETs, such as cathepsin G, elastase, and myeloperoxidase, can activate coagulation factors directly or indirectly via blocking the effects of anticoagulant plasma proteins, such as tissue factor pathway inhibitor (Goel 2003; Massberg et al. 2010; Nogami et al. 2011).

Although the importance of NETs in *H. somni*-infected cattle is not clear at this time, evidence suggests trap formation is a dynamic process and relevant to sepsis and other disorders in humans. DNase present in serum, or DNase II expressed in macrophage lysosomal compartments, degrades extracellular traps (Farrera and Fadeel 2013; Hakkim et al. 2010). Moreover, human macrophages clear extracellular traps via an endocytic pathway facilitated through C1q opsonization. Thus, during *H. somni* infection, one would predict a dynamic and ongoing process of trap formation and remodeling via DNase activity or endocytosis. If the balance of these activities tips in favor of trap formation rather than degradation, the predicted outcome would be thrombus formation that is deleterious to vascular function.

6 Vascular Permeability and Vessel Transmigration

The ability of a bacterial pathogen to breach the endothelial barrier is essential to its dissemination to distant sites. This is particularly evident for those pathogens that cause septicemia and have a propensity to infect the meninges and central nervous system. In adult humans, *Streptococcus pneumoniae*, *N. meningitidis* (meningococcus), and *H. influenzae*-type B (HiB) are the most common culprits, while *Listeria monocytogenes*, *Escherichia coli* K1, and Group B *Streptococcus* cause the majority of meningitis cases in the neonate (van Sorge and Doran 2012). This process can occur via a number of distinct mechanisms, including direct bacterial invasion of endothelial cells, paracellular migration of bacterial cells, induction of necrosis or apoptosis in endothelial cells, or transport of bacterial cells within an infected leukocyte. In some cases, the level of bacteremia is associated with the incidence of neurological disease. The difficulty in consistently reproducing *H. somni* infection in animal models has precluded answering this question. One possibility is that activation of the coagulation cascade culminates in production of

thrombin, which increases vascular permeability that could facilitate paracellular migration of *H. somni* cells. Another possibility is that production of histamine by *H. somni* could disturb endothelium integrity and promote dissemination of the *H. somni* cells (Ruby et al. 2002). Perhaps production of inflammatory cytokines in response to *H. somni* also contributes to vascular leakage. Systemic levels of TNF-α have been linked to increased permeability of the blood–brain barrier in human patients and experimental mouse models (Barichello et al. 2011; Kim et al. 1997; Sharief et al. 1992). Interestingly, *H. somni* LOS did not increase endothelial cell monolayer permeability in vitro (Behling-Kelly et al. 2007a, b). These results contrast with in vivo studies of HiB infection, where the intracisternal injection of outer membrane vesicles induced permeability of the blood–brain barrier (which was abrogated by polymyxin B) in normal, but not leukopenic, rats (Wispelwey et al. 1989).

One mechanism by which a pathogen can breach the endothelial barrier is by triggering mechanical contraction of the endothelial cell's cytoskeleton. Phosphorylation and activation of myosin light chain kinase by enteropathogenic *E. coli* lead to a diminution of barrier function by intestinal epithelial cells (Simonovic et al. 2000). Previous in vitro investigations showed that adherence of *H. somni* cells to BBMEC activated myosin light chain kinase, leading to retraction of the endothelial cell and increased paracellular permeability. Endothelial cell contraction coincided with altered localization of tight junction proteins that confer barrier properties to the endothelial cell monolayer. This response required viable *H. somni* cells, as it was not recapitulated with purified LOS, nor heat-killed or formalin-fixed *H. somni* cells (Behling-Kelly et al. 2007a, b). Perhaps the initial adherence of *H. somni* cells to endothelial cells promotes localized shedding of bacterial outer membrane vesicles that elicit the endothelial cell response. Contraction of endothelial cells in vivo could promote dissemination of the bacterial cells and expose subendothelial collagen and fibroblast-associated tissue factor, thus providing yet another means for amplifying localized activation of coagulation.

There is considerable interest in how *H. somni* cells cross the blood–brain barrier. Disruption of the proteins that form junctional complexes between endothelial cells is one mechanism by which bacterial cells can gain access to the brain. Tight junctions in brain microvascular endothelial cells are composed of four integral membrane proteins (occludin, claudins, junctional adhesion molecules, and cell-selective adhesion molecules) that are linked to the actin cytoskeleton through cytoplasmic anchoring proteins (ZO-1, -2, and -3 and cingulin) (Furuse et al. 1994; Martin-Padura et al. 1998; Wolburg and Lippoldt 2002). *N. meningitidis* disrupts the localization of occludin, ZO-1, and Claudin-1 in cellular junctions by production of matrix metalloprotease 8 (Schubert-Unkmeir et al. 2010). Likewise, loss of occludin from the cell membrane also occurs when BBMEC are incubated with *H. somni* (Behling-Kelly et al. 2007a, b). The mechanisms that underlie the redistribution of tight junction proteins in *H. somni* infection are yet to be elucidated.

H. somni-mediated contraction of the endothelial cell monolayer might facilitate transmigration of leukocytes across the blood–brain barrier where they would contribute to the focal necrosis that characterizes TME. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is localized at the intercellular interface between endothelial cells. This molecule plays a crucial role in the final steps of leukocyte transmigration across vascular barriers (Muller 1995). Incubation of BBMEC monolayers with *H. somni* cells increased neutrophil transmigration that was abrogated by addition of anti-PECAM antibodies (Tiwari et al. 2009).

7 Summary

In closing, *H. somni* is a versatile ruminant pathogen that can quietly reside in the microflora of the upper respiratory tract, be a significant player in the respiratory disease complex, and in some cases go on to cause septicemia and infections at multiple systemic sites, including fulminant infection of the central nervous system (TME). What causes *H. somni* cells to undergo these transitions, and how the bacterial cells trigger changes in the vasculature that results in the more profound manifestations (e.g., thrombus formation) of *H. somni* infection, is still poorly understood. Future investigations will unlock the multifactorial events that disrupt the normal hemostatic balance and cause thrombus formation and tissue damage.

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Histophilus somni Surface Proteins

Lynette B. Corbeil

Abstract The pathogen surface is usually the first site of interaction with the host. *Histophilus somni* was earlier thought to only have an outer membrane on its surface. Now it is known that the surface is composed of many virulence factors, including outer membrane proteins, lipooligosaccharide or endotoxin, a fibrillar network, and an exopolysaccharide. Outer membrane blebs, endotoxin, the fibrillar network, and the exopolysaccharide are also shed from the surface. This review will focus on the surface proteins of this pathogen that may colonize the mucosal surface of ruminants as a commensal or may cause pneumonia, septicemia, myocarditis, thrombotic meningoencephalitis, arthritis, and/or abortion. The major outer membrane protein has been well studied. Since its size and epitopes vary from strain to strain, it may be useful for typing strains. Iron-regulated OMPs have also received much attention because of their role in iron uptake for in vivo growth of *H. somni.* Other OMPs may be protective, based on passive immunization with monospecific antibodies and active immunization experiments. The surface and shed fibrillar network has been shown to be an immunoglobulin-binding protein in that it binds bovine IgG2 by the Fc portion. Two repeat domains (DR1 and DR2) have cytotoxic Fic motifs. Vaccine studies with recombinant DR2 are promising. Studies of the bacterial genome as well as comparison of surface proteins of different strains from the various *H. somni* syndromes and carrier states will be discussed and have provided much insight into pathogenesis and protection.

Abbreviations

AMP	Adenosine monophosphate
BAT2	Bovine alveolar type 2
BT	Bovine turbinate
CCS	Concentrated culture supernatant
EPS	Extracellular polysaccharide
FHA	Filamentous hemagglutinin

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IbpA	Immunoglobulin-binding protein A
IgBP	Immunoglobulin-binding protein
Hbp	Hemoglobin-binding protein
IROMPs	Iron-regulated outer membrane proteins
LOS	Lipooligosaccharide (or endotoxin)
MOMP	Major outer membrane protein
OMP	Outer membrane protein
Tbp	Transferrin-binding protein
TME	Thrombotic meningoencephalitis

Contents

1 Introduction

Histophilus somni is a capnophilic pleomorphic gram-negative rod that was once called *Haemophilus somnus, Haemophilus agni,* or *Histophilus* ovis, depending on the country of isolation and whether it infected cattle or sheep. Some time ago, there was consensus that all three organisms should be considered as members of a single taxon, so both bovine and ovine organisms were called *Haemophilus somnus* (Lees et al. 1994). These three bacterial species are now classified as *Histophilus somni* (Angen et al. 2003), so quoted references may include any of the above names. The organism causes a great variety of syndromes in several different ruminants. It colonizes mucous surfaces of cattle, sheep, goats, bison, bighorn sheep, and perhaps others that have not been detected yet (Corbeil 2007; Janosi et al. 2009). Not only does this pathogen infect several ruminant hosts, but it can either exist in a carrier state on the upper respiratory and genital surfaces or cause economically important diseases such as pneumonia, epididymitis (especially in sheep), septicemia, myocarditis, arthritis, thrombotic meningoencephalitis (TME), or other sequelae of septicemia (Corbeil 2007). The various manifestations of disease may occur alone or in combination. The pathogenesis of disease is complex, but is important background for addressing the role of virulence factors in the etiology of these various overlapping syndromes.

Early steps in pathogenesis involve cell attachment and evasion of host defenses. *H. somni* attaches to several cell types, including bovine pulmonary artery endothelial cells, bovine turbinate (BT) cells, and bovine alveolar type 2 (BAT2) epithelial cells in vitro (Sanders et al. 2003; Tagawa et al. 2005; Zekarias et al. 2010). Evasion of host defenses may be the next step in pathogenesis. Disease isolates are resistant to killing by bovine complement (serum resistant) or by polymorphonuclear and mononuclear phagocytes (Cole et al. 1992; Corbeil et al. 1985; Czuprynski and Hamilton 1985; Gomis et al. 1998; Lederer et al. 1987; Yang et al. 1998; Zekarias et al. 2011). Most asymptomatic carrier isolates are also serum resistant, although a few preputial carrier isolates are serum sensitive (Cole et al. 1992, 1993; Corbeil et al. 1985) (Table 1). In serum killing assays, different clones from the same clinical isolate sometimes demonstrate differences in serum resistance (Corbeil et al. 1985). So it is understandable if results vary from laboratory to laboratory or when done in the same laboratory with the same methods, but years apart.

Resistance to killing by phagocytes and inhibition of various phagocyte functions has been reported. For example, it has been shown that *H. somni* inhibits macrophage and PMN superoxide anion production (Gomis et al. 1997; Howard et al. 2004). However, these studies did not discuss the duration of viability of the phagocytes after exposure to *H. somni.* More recently, *H. somni* was reported to inhibit phagocytosis by primary bovine monocytes and murine J774.1 macrophages (Hoshinoo et al. 2009). The virulent *H. somni* strain used in that study was also cytotoxic for FBM-17 bovine macrophage-like cells, primary bovine monocytes and J774.1 macrophages (Hoshinoo et al. 2009). This implies that *H. somni* survives phagocytosis not only because it inhibits phagocyte functions, but also because it killed the phagocytes. Thus, the pathogen may not survive a long time inside healthy phagocytic cells (like a facultative intracellular parasite), but rather survives by inhibiting phagocytosis and production of bactericidal factors, as well as by killing the phagocytes. Therefore, *H. somni* probably does not meet the definition of a facultative intracellular pathogen, which was a suggested classification some time ago (Lederer et al. 1987). Bacterial factors associated with this inhibition and cytotoxicity will be discussed below.

Not only does this pathogen need to resist serum and cellular killing to survive in the host, but acquisition of nutrients is also crucial. Mammalian hosts tend to sequester iron as one mechanism of limiting pathogen multiplication. Although

Table 1 Serum resistance of *H. somni* isolates from clinical disease or asymptomatic carriers

Source	*H. somni* Isolates			
	n	S	R	I
Pneumonia	5	0	4	1
TME	5	0	5	0
Abortion	5	0	5	0
Vaginal carrier	11	0	9	2
Preputial carrier	19	5	13	1

N—number of isolates, S = serum sensitive, R = serum resistant, I = intermediate

many bacterial pathogens overcome this host defense by secreting siderophores, *H. somni* accomplishes iron acquisition by binding host iron-binding proteins, such as transferrin and other proteins. The bacterial proteins involved will be discussed below.

Interaction with respiratory epithelial cells is also important in pathogenesis. We found that *H. somni* causes upper respiratory cells (bovine turbinate or BT cells) to retract, but to a lesser extent than bovine alveolar type 2 (BAT2) cells (Zekarias et al. 2010). Our studies suggest that invasion to cause septicemia and its sequelae occurs across the pulmonary alveolar membrane (Agnes et al. 2013; Zekarias et al. 2010). The bacteria do not invade the epithelial cells, but pass between the retracted cells. Crossing the alveolar barrier between alveolar epithelial cells would not only involve retraction of those cells, but also digestion of the basement membrane. This can be attributed to the ability of *H. somni* to stimulate BAT2 cells to increase expression of matrix metalloproteinases that digest basement membrane collagen (Agnes et al. 2013). To invade the blood stream, *H. somni* then causes retraction (Corbeil and Zekarias, unpublished data) and apoptosis (Czuprynski et al. 2004) of endothelial cells. Once across the endothelial barrier, *H. somni* circulates in the blood stream and then colonizes the endothelium at least in the heart and probably in other sites (O'Toole et al. 2009). Inzana's group showed that *H. somni* produces biofilms in the heart, but did not determine the cellular localization (Sandal et al. 2009). Thus, it is likely that the masses of *H. somni* found on the cardiac endothelium of clinical cases of bovine myocarditis constitute biofilms (O'Toole et al. 2009). A pathogen that causes such an array of clinical pictures is likely to express many virulence factors. Inzana's group has been instrumental in defining lipooligosaccharide (LOS) and extracellular polysaccharide (EPS) virulence factors (Sandal and Inzana 2010; Sandal et al. 2011; Siddaramppa and Inzana 2004), as will be described in separate chapters. Several protein virulence factors have also been identified, including several outer membrane proteins (OMPs) and a surface fibrillar network. Some of these factors are also shed from the surface. Surface and shed proteins will be the subject of this review.

2 Outer Membrane Proteins (OMPs)

Early studies of the *H. somni* surface revealed no capsule, pili, flagella, or surface structures other than the outer membrane (Stephens and Little 1981). No toxins, other than endotoxin, were known. Therefore, it was assumed that bacterial host interactions primarily involved the outer membrane proteins and endotoxin. This report deals with surface proteins. Endotoxin has been extensively covered elsewhere (Inzana et al. 1992, 1997, 1988; Sandal and Inzana 2010; Siddaramppa and Inzana 2004). Later, an exopolysaccharide of *H. somni* was identified and characterized (Sandal et al. 2011). Both the LOS and the EPS will be reviewed separately in this volume. The integral OMPs and LOS form a bilayer on the outer side of the murein (or peptidoglycan) layer of the cell wall and are characteristic of

gram-negative bacteria. They can be differentiated from peripheral membrane proteins (on the surface of the outer membrane), since integral OMPs are sarcosyl insoluble and peripheral membrane proteins are sarcosyl soluble.

2.1 Major Outer Membrane Protein (MOMP)

The MOMP is the most abundant OMP in the outer membrane of gram-negative bacteria. The *H. somni* MOMP was initially defined as a 40 K OMP, which was immunogenic when purified and inoculated with Freund's adjuvants in rabbits and calves (Tagawa et al. 1993b). Reactivity with monoclonal antibodies revealed antigenic heterogeneity. Further analysis of 53 strains showed that the molecular mass and monoclonal antibody reactivity could be used to place strains in groups (Tagawa et al. 2000). Most strains had a molecular mass of about 40 K but a few asymptomatic carrier strains had a truncated MOMP of about 33 K. This study also showed that the bovine immune response to the MOMP during *H. somni* infections is relatively weak, as we had reported earlier (Yarnall and Corbeil 1989). Investigation of the immunogenic OMPs of *H. somni* in our laboratory revealed that there are 3 different antigens that are approximately 40 K in mass, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Corbeil et al. 1991). This includes the MOMP at about 41 K, the immunodominant OMP at about 40 K, and a 39 K OMP that reacts with monoclonal antibody 3G9. There is some strain heterogeneity in molecular mass in these OMPs, so we have arbitrarily categorized them as p41, p40, and p39, respectively (Corbeil et al. 1991). Since the MOMP had been characterized as a 40 K OMP, but runs just above the immunodominant 40 K protective antigen by SDS-PAGE and Western blotting, there has been some confusion in the literature. The protective p40 OMP that will be discussed below is not the MOMP, even though the MOMP of some strains of *H. somni* does have a relative molecular mass of 40 K by SDS-PAGE. We defined these differences some years ago in an attempt to make the situation more clear (Corbeil et al. 1991). Molecular characterization of the MOMP showed genetic similarity among type 1 strains, but considerable differences among those strains and 3 other strains in type 3a and type 3c (Khan et al. 2005). That manuscript also reported significant homology between the *H. somni* MOMP and the porin proteins of other *Pasteurellaceae*. The same research group later made mutations in the MOMP using an allelic exchange method (Ueno et al. 2014). By exchanging MOMP domains between asymptomatic strain 129Pt (which has a truncated MOMP of 33 K) and virulent strain 2336, they indicated that serum susceptibility may be due to MOMP characteristics, especially in strain 129Pt. However, the serum susceptibility of the wild types of these two strains relative to one another was the opposite of the originally reported relative serum sensitivities (Corbeil et al. 1985). Perhaps the differences could be attributable to changes introduced in these strains in the 30 years between the two studies or differences in the fresh normal bovine serum used in the two studies. Their conclusions were that serum resistance

of virulent strain 2336 is due to several factors, including IbpA and LOS with modifications by sialic acid, as reported by others (Cole et al. 1992; Inzana et al. 2012). Asymptomatic carrier strain 129Pt lacks these factors, so the MOMP is likely to be most important in its serum sensitivity (Ueno et al. 2014). Overall, the weak immunological reactivity of the MOMP with serum from infected convalescent cattle and its antigenic variability suggests that the MOMP may not be a good antigen for diagnostic assays or as a subunit vaccine candidate. On the other hand, the MOMP may be useful in devising a grouping system for *H. somni.*

2.2 OMPs Involved in Iron Acquisition

Bacterial pathogens need to compete with their hosts for iron in order to survive in vivo. Many bacteria accomplish this by secreting siderophores, but screening of 9 clinical isolates of *H. somni* did not reveal production of siderophores (Ogunnariwo et al. 1990). Rather, these authors reported that iron-deficient cells bound bovine transferrin, but not human or porcine transferrin. Low iron conditions induced three iron-regulated outer membrane proteins (IROMPs) with transferrin receptor activity. Schryvers group also showed that 8 bovine strains of *H. somni* bound bovine and ovine transferrin, but not goat, horse, pig, or human transferrin (Yu et al. 1992). An iron-deficient strain of *H. somni* grew in iron-deficient medium with added $FeCl_3$ or bovine transferrin, but not with ovine, goat, or human transferrin. Niven's group then investigated production of transferrin receptors by strains of *H. somni* isolated from sheep, then called *Histophilus ovis* (Ekins and Niven 2001). They found that all strains could use ovine, bovine, and goat transferrins as iron sources, but not porcine or human transferrins. Two of the five strains expressed transferrin-binding proteins (Tbp) A and B under iron-restricted conditions, but the other 3 strains required both iron restriction and the presence of transferrin to induce expression. Another study showed that bovine strain 649 could acquire iron from bovine, ovine, and goat transferrin (Ekins et al. 2004). This strain has two iron-binding systems, one specific for bovine transferrin and the other binding bovine, ovine, or goat transferrin. These studies also showed phase variable expression of transferrin-binding receptors. Later, the same group compared two bovine strains (649 and 2336) with two ovine strains (9L and 3384Y) in iron acquisition from hemoglobin (Tremblay et al. 2006). The two bovine isolates could utilize bovine hemoglobin as an iron source, but not ovine, porcine, or human hemoglobins. We have reproduced bovine pneumonia and abortion with strain 2336 and 649, respectively, so it is clear they are pathogenic strains for cattle (Gogolewski et al. 1987b; Widders et al. 1986). The ovine isolates did not utilize any of these hemoglobins (Tremblay et al. 2006). Expression of hemoglobin-binding activity is associated with production of a 120 K outer membrane hemoglobin-binding protein, HgbA. Clearly, iron acquisition by *H. somni* is complex and not yet fully understood.

In vivo studies of the role of bovine plasma proteins in *H. somni* virulence provide some insight into the role of iron acquisition in production of murine septicemia (Geertsema et al. 2007). We found that preincubation of *H. somni* strain 2336 in 5 % fetal calf serum enhanced virulence in a mouse model of septicemia. Incubation of the bacteria with several different purified bovine serum or plasma proteins revealed that transferrin increased virulence. Growth in iron-restricted conditions increased transferrin binding, and bovine transferrin supported growth of *H. somni* on iron-depleted agar. Transferrin bound to both whole cells and culture supernatant of *H. somni. H. somni* does release outer membrane blebs from the surface (Lin C and Corbeil LB, unpublished data). Thus, it is likely that the binding of transferrin by culture supernatant, especially under iron-depleted conditions, is due to IROMPs in the OM blebs. Bovine lactoferrin also bound to *H. somni,* but such binding appeared to be due to electrostatic interactions rather than to interactions with IROMP receptors. We concluded that transferrin bound to IROMPs provides iron to the pathogen. Decreased virulence in mice was concluded to be due to the lack of binding of mouse transferrin to *H. somni* IROMPs and low uptake of iron for bacterial growth in the mouse model.

2.3 Other OMPs

Several other *H. somni* OMPs have been investigated. We found that two antigens in sarcosyl-extracted OMP preparations react most strongly with protective convalescent serum from calves with experimental *H. somni* pneumonia (Gogolewski et al. 1987a). These would be integral membrane proteins, since they are in the sarcosyl-insoluble fraction and electron microscopy of the insoluble fraction demonstrate vesicles and ribbons of bilaminar membranes. The immunodominant 40 K OMP is detected just below the 41 K MOMP in Western blots over-stained with amido black (to show OMPs that do not react with convalescent phase serum at 1:1000). As indicated above, the MOMP, the immunodominant 40 K OMP (p40), and a 39 K OMP (p39) run very closely together by SDS-PAGE. Both p39 and p40 are conserved in all bovine *H. somni* isolates tested (Corbeil et al. 1991). Antibodies to p40 react with antigens of other *Pasteurellaceae*, but the cross-reactive antigens range in relative molecular mass from 55 to 28 K. Monoclonal antibodies to p39 (3G9) only react with *H. somni* from a bank of *Pasteurellaceae* and other gram-negative bacteria (Corbeil et al. 1991). An equally immunodominant OMP is detected at 78 K. Absorption of the convalescent phase serum with live *H. somni* followed by subsequent washing and elution of adsorbed antibodies with glycine HCl shows that antibodies react with surface-exposed epitopes of the immunodominant 40 and 78 K OMPs (Gogolewski et al. 1987a). This information was critical for subsequent immunization studies because it showed immunogenic epitopes are not hidden. These two immunodominant antigens were then purified, calves immunized, and the monospecific serum antibodies used for passive protection. Interestingly, antibodies to the 40 K OMP protected,

but those with specificity for the 78 K OMP did not (Gogolewski et al. 1988). In fact, incubation of *H. somni* with subagglutinating amounts of antiserum to the 78 K OMP before intrabronchial inoculation resulted in about twice as much pneumonia as in calves inoculated with *H. somni* in preimmune serum. Possible reasons for the protective ability of antibodies to one OMP, but not the other, are discussed in the chapter "Host Immune Response to *Histophilus somni*." Other investigators then cloned, sequenced, and characterized two 40 K lipoproteins of *H. somni*: LppA and LppB (Theisen et al. 1992, 1993). Which of these lipoprotein OMPs corresponded to the 40 K protective OMP was not reported in those manuscripts (Theisen et al. 1992, 1993). However, a more recent study expressed and purified two *H. somni* OMPs: LppB and p31 (to be discussed below) (Guzman-Brambila et al. 2012). These investigators concluded that LppB was distinct from the 40 K OMP previously shown to elicit passively protective antibodies (Gogolewski et al. 1988). This conclusion was based on a table in a review by Sandal and Inzana, which did not indicate that the two 40 K OMPs differed (Sandal and Inzana 2010). So it is not clear from the literature whether these two reported 40 K antigens are the same or different proteins. The recent study of immunogenicity of a vaccine composed of both p40 (LppB) and p31 recombinant antigens revealed immunogenicity in rabbits and sheep as well as protection of mice against septicemia when both antigens were given together with a multivalent *Clostridium* spp. vaccine and $Al(OH)_3$ adjuvant (Guzman-Brambila et al. 2012). Whether both OMPs were necessary for protection was not clear.

The p31 antigen was selected on the basis that a 31 K OMP gene had previously been cloned and sequenced (Won and Griffith 1993). Expression of p31 in *Escherichia coli* resulted in transformants that were hemolytic on bovine blood agar. As indicated by Won and Griffith, only some isolates of *H. somni* are hemolytic on bovine blood agar. The gene for the 31 K protein was derived from a genomic library strain 8025, which did not produce complete hemolysis on bovine blood agar in their studies. Since they also could not purify sufficient active protein to obtain hemolytic activity, it was not clear whether the 31 K OMP was actually a hemolysin in *H. somni* or whether its expression in *E. coli* resulted in production of an *E. coli* hemolysin. Screening of isolates in our laboratory from various *H. somni* syndromes in cattle showed that some strains were hemolytic on bovine blood agar and others were not (Table 2). Most of our studies of experimental pneumonia in cattle have been done with strain 2336 (Berghaus et al. 2006; Gershwin et al. 2005; Gogolewski et al. 1987a, b, 1988, 1989). That strain is not hemolytic on bovine blood agar, so hemolysis is not a necessary factor for virulence.

Some other *H. somni* OMPs have been studied by the Tagawa Laboratory. A heat-modifiable OMP was identified at 28 K when sarcosyl preparations were solubilized at 60 °C, but was 37 K if solubilized at 100 °C (Tagawa et al. 1993a). Monoclonal antibody 27.1 to this 37 K OMP reacts with all strains tested, as well as with some other gram-negative bacteria. Convalescent phase serum from calves with experimental *H. somni* pneumonia also reacts strongly with surface-exposed epitopes of this heat-modifiable OMP. Immunological cross-reactivity and

Table 2 Hemolytic activity of *H. somni* isolates from cattle with various clinical syndromes or carrier states

Source	*H. somni* isolates	
	n	# hemolytic
Pneumonia	5	1
TME	4	1
Abortion	5	3
Myocarditis	6	2
Vaginal carrier	29	29
Preputial carrier	23	9

Hemolytic activity was determined by streaking for isolation on bovine blood agar plates, incubating for 48 h at 37 °C under 10 % CO_2 and for another 48 h at room temperature in air

N-terminal sequence homology with *E. coli* OMP A has also been demonstrated. This homology could lead to insights into the role of the *H. somni* heat-modifiable protein in pathogenesis and protection.

3 Immunoglobulin-Binding Proteins (IgBPs or IbpA)

Early studies from our laboratory revealed that there is an additional protein virulence factor on the surface of *H. somni* that bound the Fc region of bovine IgG2. This was discovered serendipitously when it was noted that *H. somni* grown on bovine blood agar reacted with the anti-bovine IgG conjugate in an enzyme-linked immunosorbent assay (ELISA), even when no primary bovine serum had been added (i.e., in the conjugate controls). The binding was shown to be independent of the antigen combining site of the immunoglobulin by binding with anti-dinitrophenol (DNP), which did not cross-react antigenically with *H. somni* (Widders et al. 1988). The "immunoglobulin receptors" were then isolated and characterized (Yarnall et al. 1988a, b). The major receptor consisted of a series of bands at 120, 270, and 350 K in Western blots probed with convalescent phase serum from cattle infected with *H. somni* or with purified IgG2 anti-DNP (Yarnall et al. 1988b). These high molecular weight (HMW) Fc receptors were isolated from culture supernatant (Yarnall et al. 1988b). The terminology of bacterial Fc receptors was changed around 1990 to immunoglobulin-binding proteins (IgBPs) in order to differentiate mammalian cell FcRs from bacterial surface proteins that bind immunoglobulin Fcs. We originally thought that the *H. somni* high molecular weight IgBPs were outer membrane proteins that were also shed as outer membrane blebs. This conclusion was drawn because these proteins were found both on the bacterial surface and in the culture supernatant. Earlier ultrastructural studies detected no structures beyond the outer membrane (Stephens and Little 1981). Therefore, it seemed that the IgBPs must be OMPs. However, we later discovered that the HMW series of IgBPs included a 76 K protein and that this protein was found mainly in the membrane fraction, but as a peripheral membrane protein rather than an integral membrane protein since it was detected in

the sarcosyl-soluble fraction of the bacterial pellet (Corbeil et al. 1997). The higher molecular weight IgBPs (120–350 K) were found predominately in the sarcosyl-soluble fraction of the bacterial supernatant (Corbeil et al. 1997). In the same study, we reported that there is a network of very fine flexible fibrils (1–2 nm in diameter) on the surface of *H. somni* and that this fibrillar network binds bovine IgG2 (Corbeil et al. 1997). These fibrils were only detected if the bacteria were heavily over-stained for transmission electron microscopy (Fig. 1). Strains that bound IgG2

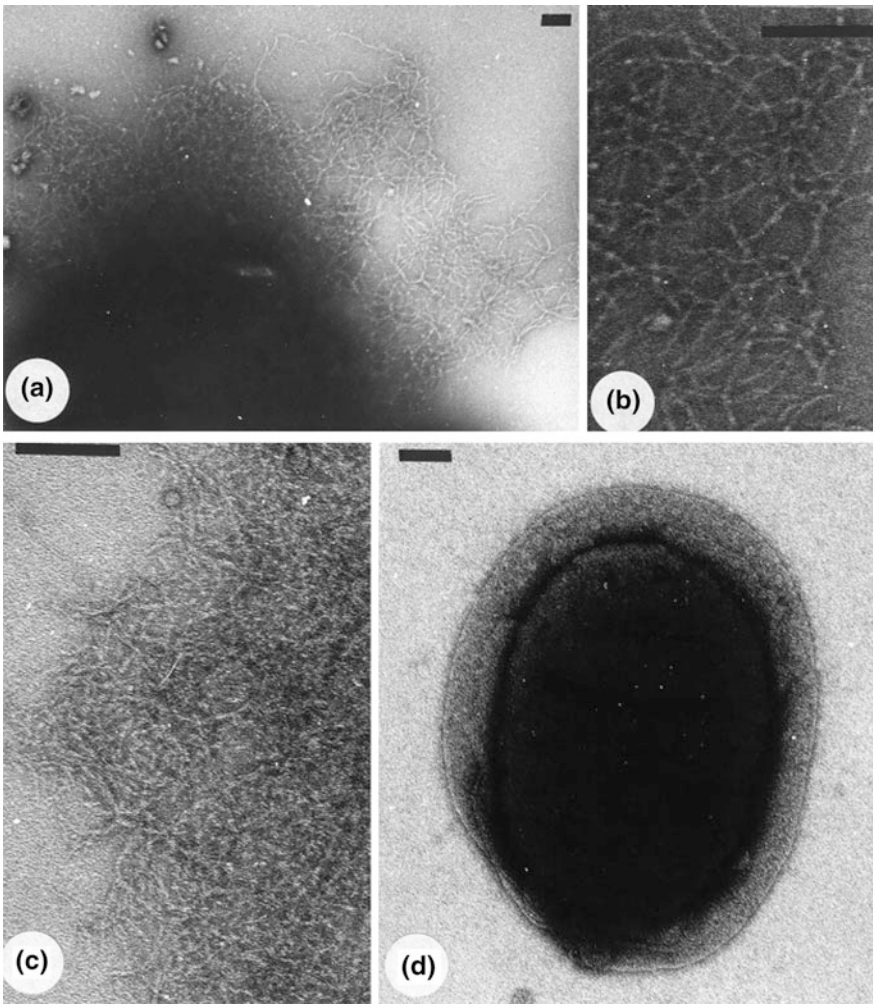

Fig. 1 Transmission electron microscopy of *H. somni* surface fibrils negatively stained heavily with ammonium molybdate. Panels **a** and **b** show the surface of pathogenic strain 2336, and panel **c** is of pathogenic strain 649 surface. Panel **d** is IgBP-negative strain 129Pt from an asymptomatic carrier with no surface fibrils. Bars in panels a and d are 100 nm, bars in panels **b** and **c** are 20 nm. Reproduced from previously published data (Corbeil et al. 1997) with permission

had surface fibrils and strains that did not bind IgG2 did not have surface fibrils. Thus, the IgBPs consist of fibrils on the surface that are also shed into the supernatant. These IgBPs are conserved in all strains tested, with the exception of a minority of isolates from asymptomatic preputial carriers (Cole et al. 1992; Yarnall et al. 1988b; Zekarias et al. 2011).

The nomenclature of the high molecular weight IgBPs was changed to IbpA when the gene was sequenced (Tagawa et al. 2005). We originally thought that there were two genes involved, for two outer membrane proteins, p76 and p120 (Cole et al. 1992). Both had immunoglobulin-binding activity and both were missing in strain 129Pt and three other serum-sensitive isolates from asymptomatic carriers. Later, the "gene" for p76 was sequenced and found to contain two 1.2-kb direct repeats (DR1 and DR2) with the characteristics of insertion sequence elements (Cole et al. 1993). This study also showed that there were multiple ATG start sites functional in *E. coli* and possibly in *H. somni*. Now, it is clear that IbpA is one very large protein, including both p76 and the upstream p120, encoded by the *ibpA* gene (Tagawa et al. 2005) and that it forms fibrils on the surface rather than being an OMP, as discussed above (Corbeil et al. 1997). An upstream 1.76-kb ORF was called *ibpB*. These two genes meet the definition of a two-partner secretion system. IbpB has homology to the transporter proteins of large fibrillar exoproteins of gram-negative bacteria, such as the filamentous hemagglutinin (FHA) of *Bordetella* spp. and HMW 1 and 2 of *Haemophilus influenzae* (Yeo et al. 2007). The 12.3-kb ORF for the complete *ibpA* gene has 18 potential ATG start sites, which may account for the many protein bands in Western blots with convalescent phase serum or anti-DNP IgG2, as described above. The protein encoded by this very large (12.2 kb) gene has many functional domains and motifs (Fig. 2).

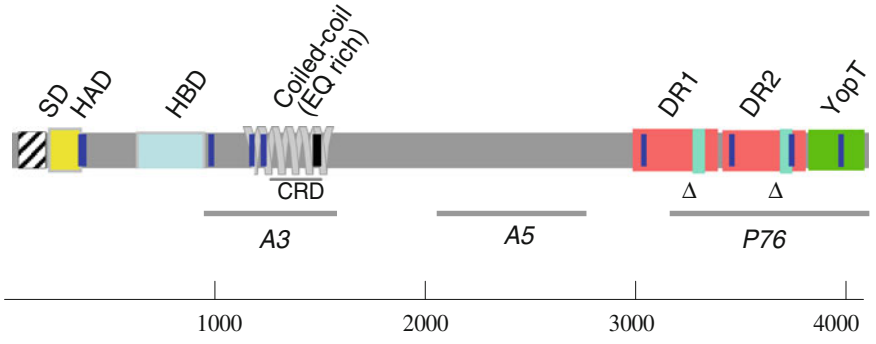

Fig. 2 Diagram of IbpA, including predicted functional domains: *SD* Secretion domain, *HAD* Hemagglutinin domain, *HBD* Heparin-binding domain, *CRD* Carbohydrate-binding domain, *DR1/DR2* Direct repeats with Fic motifs (*open triangle*), *YopT* Cysteine proteinase, *dark blue lines* —cell binding sites (RGD, KEK or TK—D). A3, A5, and DR2 were purified as GST fusion proteins and used for vaccination of calves (see below). P76 was the first IgBP characterized (Cole et al. 1992). Amino acid numbers are at the *bottom*. (Diagram courtesy of Bereket Zekarias)

The N-terminal portion of IbpA has homology with FHA of *Bordetella spp*, PfhB1 and PfhB2 of *Pasteurella multocida,* and LspA1 of *Haemophilus ducreyi.* Both FHA and IbpA have a heparin-binding domain (HBD) and a carbohydrate-binding domain (CBD), the later with an integrin-binding motif (RGD), which may be involved in adherence. Competitive inhibition studies showed that RGD did not inhibit adherence of *H. somni* to bovine pulmonary artery endothelial cells, but heparin (or dextran sulfate, a comparable sulfated polysaccharide) did inhibit adherence in a dose-dependent fashion, suggesting that the heparin-binding domain of IbpA may be involved in adherence (Tagawa et al. 2005). Subsequently, *H. somni* strain 2336 was shown to have 4 loci containing 12 genes encoding proteins homologous to FHA (Siddaramappa et al. 2011). One of the genes in Locus III was homologous to *ibpA.* None of the other FHA homologs in the genome of strain 2336 included the C-terminal portion of *ibpA*, which encodes DR1, DR2, and YopT (a cysteine proteinase virulence factor of *Yersinia* spp.). The only other large exoproteins with homology to IbpA DR1 and DR2 were PfhB1 and PfhB2 of *P. multocida* strain Pm70. We detected the DR2 domain in avian strains Pm70 and 5A by PCR methods described previously (Zekarias et al. 2011), but not in 7 bovine strains of *P. multocida,* including clinical strains and the standard challenge strain 1062 (Zekarias B, Lehmann J and Corbeil LB, unpublished data). However, IbpA was detected by Western blotting of culture supernatant of bovine *H. somni* strains from pneumonia, abortion, TME, myocarditis, vaginal carriers, and 2 of 4 preputial carriers (20P and 127P, but not 129Pt or 1P) (Zekarias et al. 2011). The sequences of IbpA DR2 in strains positive for IbpA were then compared and shown to be conserved in all strains examined (Zekarias et al. 2011).

Functional studies of the C terminus of IbpA began with the hypothesis that the YopT homolog would be cytotoxic for epithelial cells as in *Yersinia* spp. (Worby et al. 2009). In *Yersinia* spp., YopT is introduced to target cells by a type III secretion system, whereas the YopT homolog in *H. somni* is a domain of the very large IbpA exoprotein with a two-partner secretion system, as noted above. Analysis of IbpA cytotoxicity revealed cell rounding when HeLa cells were transfected with the p76 sequence containing a portion of DR1, as well as the DR2 domain and the YopT sequence with the critical catalytic domain (C/H/D). However, deletion analysis revealed that the YopT homolog of IbpA was not cytotoxic, but the DR1 and DR2 domains were (Worby et al. 2009). Cell retraction was due to the DR2 and DR2 Fic motif, HPFxxGNGR, which transferred AMP to the tyrosine in the switch region of Rho GTPases (RhoA, Rac, and Cdc42), resulting in collapse of the actin cytoskeleton (Worby et al. 2009). If the critical **H**istidine in the conserved Fic motif was mutated to **A**lanine (DR2HA), cell rounding did not occur (Worby et al. 2009). Transfection of HeLa cells with IbpA DR1 or IbpA DR2 sequences for cellular expression was useful for defining molecular mechanisms involved in cell retraction. However, transfection of human cells does not mimic in vivo pathogenesis in cattle because HeLa cells are immortalized human cervical carcinoma cells and transfection is probably not the way IbpA gets into bovine respiratory cells. To evaluate pathogenesis in relevant

bovine cells, we treated bovine turbinate (BT) or bovine alveolar type 2 (BAT2) epithelial cells with either *H. somni* or its concentrated culture supernatant (CCS), which is enriched for shed IbpA fibrils (Zekarias et al. 2010). BT cells were used as a surrogate for upper respiratory colonization and BAT2 cells for lung infection since *H. somni* is detected mostly in the alveolus at 24 h after infection of calves (Gogolewski et al. 1987b). Both live *H. somni* and its CCS were shown to cause BT or BAT2 cell retraction, with BAT2 cells being more sensitive than BT cells (Zekarias et al. 2010). HeLa cells were the least sensitive. Purified recombinant IbpA DR2, but not IbpA DR2HA, also caused retraction. Confocal microscopy showed that although IbpA DR2 was present on the surface of non-permeabilized BAT2 cells infected with virulent *H. somni* strain 2336, much more of this antigen could be detected in BAT2 cells permeabilized after treatment with live *H. somni* strain 2336, CCS, or rDR2 Fic. This indicates the IbpA DR2 antigen (and probably the whole IbpA molecule) was taken up by BAT2 cells, even though *H. somni* did not invade the cells (Fig. 3) (Zekarias et al. 2010). Since *H. somni* does not invade cells and IbpA is not "injected" into cells by a type III or IV secretion system, but is secreted by a two-partner system, it is likely that IbpA is taken up by pinocytosis in BAT2 cells.

Rabbit and bovine antibodies to IbpA DR2 neutralized cytotoxicity for BAT2 cells (Zekarias et al. 2010). The role of IbpA DR2 Fic in cytotoxicity was confirmed by the fact that antisera to IbpA DR2, as well as mutation of the critical histidine in the Fic active site, prevented BAT2 cell retraction. Antibodies to IbpA DR2 also protected mice against *H. somni* septicemia (Zekarias et al. 2011). Tagawa's group further defined the role of IbpA in pathogenesis by deleting the IbpA gene from virulent strain 2336 (Hoshinoo et al. 2009). The wild-type strain, but not the deletion mutant (2336.A1), caused disruption of actin filaments and cytotoxicity for bovine macrophage-like FBM-17 cells, as well as in murine J774.1 macrophages and primary bovine monocytes. Phagocytosis of microspheres by J774.1 cells and primary bovine monocytes was also inhibited by strain 2336, but not by the mutant 2336.A1. They concluded that *H. somni* IbpA inhibits macrophage and monocyte phagocytosis probably by disruption of actin filaments.

The many domains of IbpA, its surface location, its role in virulence, and neutralization of cytotoxicity by antibodies in convalescent serum or anti-IbpA DR2 suggest that IbpA may be protective. Therefore, we chose 3 domains for preparation of recombinant proteins: IbpA3, IbpA5, and IbpA DR2 (Fig. 1). IbpA3 is an N-terminal coiled coil region, IbpA5 is a central domain with several 200-base-pair repeats, and IbpADR2 is a cytotoxic domain described above. The IbpA DR2 recombinant vaccine protected mice and calves against *H. somni* septicemia and pneumonia, respectively (Geertsema et al. 2008, 2011). Strong antibody responses were reported in both vaccine studies. Therefore, we tested the hypothesis that cattle infected with *H. somni* may have antibody responses to these three recombinant domains of IbpA that could be used diagnostically. Diagnostic serology with whole *H. somni* as antigen has very high background due to cross-reactive antigens. We had already shown that the 270 K FcR (now called IbpA) had little cross-reactivity with other gram-negative pathogens of cattle and

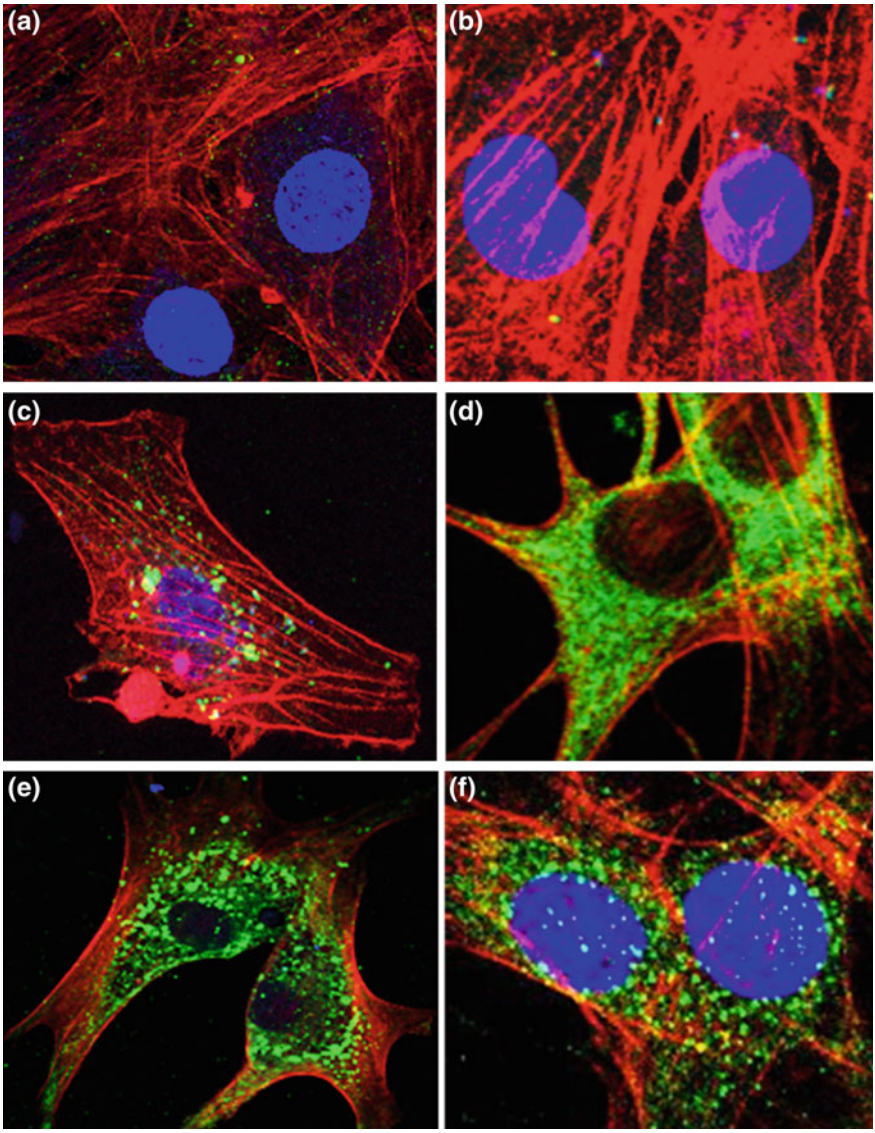

Fig. 3 Confocal microscopy of BAT2 cells treated with *H. somni,* concentrated culture supernatant (CCS), or recombinant IbpA DR2. Nuclei stained blue with TOPRO 3, F actin stained with rhodamine phallodin and antibody to rIbpA DR2 labeled with Alexa Fluor 448 (*green*). **a** Control BAT2 cells. **b** Cells infected with strain 129 Pt (with no IbpA), permeabilized with Triton X-100, and reacted with antibody to rDR2—lack of green fluorescence shows lack of IbpA DR2. **c** Cells infected with *H. somni* strain 2336, but not permeabilized. *Green* fluorescence shows IbpA DR2 on the surface due to adherent *H. somni* or IbpA fibrils. **d**, **e**, and **f** Cells treated with *H. somni* strain 2336, CCS, or rIbpA DR2, respectively, and permeabilized after fixation. *Green* fluorescence shows internalized DR2 antigen. Reproduced with permission from previously published work (Zekarias et al. 2010)

could distinguish asymptomatic carriers and normal healthy cattle from clinical *H. somni* cases (Yarnall and Corbeil 1989). Then, we designed ELISA assays using IbpA3, IbpA5, IbpA DR2, whole *H. somni*, or culture supernatant on the solid phase. Sera were diluted 1:2000 to detect robust antibody responses. Calves experimentally infected with *H. somni* had the lowest background ELISA values as well as the earliest and highest responses of greatest duration to IbpA5 (Lo et al. 2012). Calves vaccinated with a commercially available *H. somni* formalin-inactivated vaccine did not react with IbpA5 antigen until at least 2 weeks after infection, suggesting that this antigen may also differentiate between vaccinated and infected cattle. An additional attribute of IbpA5 as an ELISA diagnostic antigen is the fact that it represents a series of repeats, so has several copies of reactive epitopes. The repeats are not present in the large exoproteins of other gram-negative bacteria that have homology with the N-terminal or C-terminal portions of IbpA (Tagawa et al. 2005). Therefore, IbpA5 is not likely to cross-react with those proteins. It would be worthwhile to further investigate IbpA5 as a diagnostic antigen for *H. somni* infection.

4 Conclusions

Several surface proteins of *H. somni* have now been well studied. The MOMP, or most abundant membrane protein, appears to be an approximately 41 K porin. As a porin, it would likely be essential for bacterial viability. It only stimulates a weak antibody response in infected cattle, but gives a strong response if animals are vaccinated with purified protein. This suggests that the antigenic epitopes may not be very accessible on the bacterial surface. Since the MOMP has been shown to undergo antigenic variation it may not be a good vaccine candidate. The antigenic variation and low immune response in infected cattle also suggest that the MOMP may not be a good diagnostic antigen. However, it may be useful in developing a typing system for *H. somni.* IROMPs have also been well studied. Transferrin-binding proteins TpbA and TbpB have been shown to bind transferrin and to result in iron uptake and utilization by *H. somni.* A hemoglobin-binding protein, HgbA, is also involved in iron acquisition. The immunodominant 40 K antigen is probably equivalent to LppA or LppB. Antiserum to this OMP passively protects against experimental bovine pneumonia, whereas antiserum to the 78 K immunodominant OMP did not, even though epitopes recognized by both antisera were surface exposed. Very fine surface fibrils of *H. somni* are encoded by the *ibpA* gene. This very large exoprotein is multifunctional due to its many different domains. A heparin-binding domain appears to be involved in binding to host cells. C-terminal DR1 and DR2 domains with Fic motifs cause respiratory cell retraction and transmigration of *H. somni* across the alveolar barrier. This cytotoxicity is due to adenylylation of Rho GTPases, which disrupts the actin cytoskeleton. Replacement of the critical histidine in the Fic motif or treatment with antibody to IbpA DR2 Fic prevents cytotoxicity. Since IbpA and the DR1/DR2 Fic cytotoxic

domains are conserved in all disease isolates tested, the IbpA surface fibrils constitute important virulence factors in the pathogenesis of disease caused by *H. somni*. The neutralization of cytotoxicity by antibodies to IbpA DR2 suggests that this IbpA domain may be a subunit vaccine candidate.

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Host Immune Response to *Histophilus somni*

Lynette B. Corbeil

Abstract *Histophilus somni* is known to cause several overlapping syndromes or to be found in genital or upper respiratory carrier states in ruminants. Vaccines have been used for decades, yet efficacy is controversial and mechanisms of protective immunity are not well understood. Since *H. somni* survives phagocytosis, it has sometimes been considered to be a facultative intercellular parasite, implying that cell-mediated immunity would be critical in protection. However, *H. somni* not only inhibits phagocyte function, but also is cytotoxic for macrophages. Therefore, it does not live for long periods in healthy phagocytes. Protection of calves against *H. somni* pneumonia by passive immunization is also evidence that *H. somni* is more like an extracellular pathogen than an intracellular pathogen. Several studies showed that bovine IgG2 antibodies are more protective than IgG1 antibodies. Even the IgG2 allotypes tend to vary in protection. Of course, antigenic specificity also determines protection. So far, there is most evidence for protection by a 40 K outer membrane protein and by Immunoglobulin binding protein A fibrils. Serology and immunohistochemistry have both been used for immunodiagnosis. Many evasive mechanisms by *H. somni* have been defined, including decreased phagocyte function, antibodies bound by shed antigens, decreased immune stimulation, and antigenic variation. Interaction of *H. somni* with other bovine respiratory disease organisms is another layer of pathogenesis. Studies of bovine respiratory syncytial virus (BRSV) and *H. somni* in calfhood pneumonia revealed an increase in IgE antibodies to *H. somni*, which were associated with more severe disease of longer duration than with either agent alone. Innate immune mechanisms at the epithelial cell level are also affected by dual infection by BRSV and *H. somni* as compared to either pathogen alone. Although much more work needs to be done, the complex mechanisms of *H. somni* immunity are becoming clearer.

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Abbreviations

BAT2	Bovine alveolar type 2
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BT	Bovine turbinate
EPS	Extracellular polysaccharide
Ig	Immunoglobulin
IbpA	Immunoglobulin-binding protein A
IHC	Immunohistochemistry
IgBP	Immunoglobulin-binding protein
LOS	Lipooligosaccharide (or endotoxin)
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
OMP	Outer membrane protein
TME	Thrombotic meningoencephalitis

Contents

1 Introduction

Attempts have been made for decades to control *H. somni* infection by vaccination (Humphrey and Stephens 1983). Efficacy in prevention of the various *H. somni* syndromes (pneumonia, septicemia, abortion, myocarditis, thrombotic meningoencephalitis or TME, and arthritis) has been variable. Commercially available bacterins (killed whole cell vaccines) have been somewhat effective, but better

immune prophylaxis has been a goal for some time (Harris and Janzen 1989). Since the dogma is that Th2 immune responses are most important in protection against extracellular bacterial pathogens and Th1 immune responses are critical in defense against facultative intracellular bacterial pathogens, it is necessary to understand the pathogen type before delving into protective host immunity. *Haemophilus somnus*, now called *Histophilus somni*, was once thought to be a facultative intracellular pathogen of bovine mononuclear phagocytes (Lederer et al. 1987). This was based on data showing that opsonized *H. somni* was ingested, but not killed, by bovine alveolar macrophages or blood monocytes. The bacteria did multiply over an 18-hour period, but electron micrographs showed that the infected monocytes were "undergoing dissolution" and releasing *H. somni* by 18 h of incubation. So the mononuclear phagocyte was not a site of long-term residence for the bacteria, rather the bacteria killed the phagocytes. Extracellular bacteria have several mechanisms of avoiding killing by phagocytic cells. Capsules and other surface structures may prevent phagocytosis, released enzymes may digest immunoglobulins to prevent opsonization, or the pathogens may interfere with phagocyte function. Evasion of host responses by *H. somni* will be discussed further below. The topic is raised here to suggest that activation of cellular defenses via Th1 immunity may not be the predominant immune defense, as was indicated in the past (Lederer et al. 1987), but humoral immunity may be critical. The fact that calves can be passively protected by antibody lends support to this idea (Gogolewski et al. 1987a, 1988). Although cell-mediated immunity probably also plays a role, not much information is available on this arm of the immune response. Therefore, this review will primarily address the humoral response.

2 Antibody Responses to *H. somni*

Antibody responses during *H. somni* infection or after vaccination have been reported utilizing various serological assays (Humphrey and Stephens 1983). For example, vaccinated cows and their calves have significantly higher ELISA antibody titers to outer membrane protein extracts of *H. somni* after vaccination with a commercial vaccine composed of *H. somni* extracts (Van Donkersgoed et al. 1995). We found that experimental *H. somni* pneumonia or abortion increases bovine serum immune responses dramatically and for extended periods (Gogolewski et al. 1989; Widders et al. 1986). This was also true of antibody levels in bronchial lavage fluids for a 10-week period after intrabronchial inoculation of *H. somni* (Gogolewski et al. 1989). Therefore, it is clear that antibody responses occur. Further definition of these responses is necessary to more fully understand their role in protection or even adverse reactions.

2.1 *Protection by Passive Antibody Treatment*

One of the oldest tests for Th1 immunity versus Th2 immunity is passive transfer of antibody or T lymphocytes. Of course, cell-mediated Th1 immunity cannot be tested by lymphocyte transfer in outbred animals such as cattle and sheep. Passive antibody transfer is feasible, so we used convalescent phase serum from calves with experimental *H. somni* pneumonia to passively protect a subsequent group of calves (Gogolewski et al. 1987a). The passively protective serum recognized two immunodominant antigens in the outer membrane preparations: 40 K and a 78 K outer membrane proteins (OMPs). These antigens were purified, calves immunized, and the immunoglobulin fraction from the immune sera used at non-agglutinating doses in a second set of passive protection experiments. Interestingly, the antibodies to the 40 K antigen were protective and those to the 78 K antigen were not (Gogolewski et al. 1988). In fact, antibodies to the 78 K OMP resulted in twice as much pneumonia as in the preimmune controls. Adsorption studies with live *H. somni* showed that antibodies to both OMPs recognized surface-exposed epitopes. One difference in the two antibody preparations, in addition to specificity, was the fact that the antibodies to the 78 K OMP were entirely of the IgG1 subclass, whereas the antibodies to the 40 K OMP consisted of both IgG1 and IgG2 subclasses. These experiments lead us to conclude that passively administered antibody of appropriate specificity does transfer protection, so humoral immunity can protect against *H. somni* pneumonia. The intriguing difference in IgG classes of the antibodies in this experiment induced us to further study the role of different Ig classes and subclasses in protection against this infection.

2.2 *Role of Immunoglobulin Isotypes and Allotypes in* H. somni *Immunity*

IgM antibodies are often broadly cross-reactive, especially against gram-negative bacteria. This was found to be the case with experimental *H. somni* abortion or pneumonia in that IgM levels were relatively high in preinfection sera, did not increase much post-infection, and declined soon afterward (Gogolewski et al. 1989; Widders et al. 1986). Background IgG1 levels to whole *H. somni* also were relatively high in those studies, peaked quickly, and endured. IgG2 antibodies peaked a bit later and endured. However, the increase over the preimmune levels of antibody to *H. somni* was greatest at and after the peak for IgG2 antibodies. Passive protection studies were then done with purified antibody of IgG1 or IgG2 subclasses with specificity for the 40 K OMP. The IgG subclass preparations were purified from the same antiserum to the 40 K OMP in order to limit differences in antibody specificity as the reason for protection. IgG1 antibodies to this OMP protected less effectively than IgG2 antibodies to the 40 K OMP. Although the differences were not statistically significant, the trend suggests that IgG2 may be more protective.

When this is combined with the data showing that IgG2 levels increase the most after infection (Gogolewski et al. 1989; Widders et al. 1986), and that calves convalescent from experimental pneumonia resisted 10 times the initial dose at 10 weeks after the original infection (Gogolewski et al. 1989), the importance of IgG2 in protection is apparent. A later study in vaccinated calves showed that IgG2 antibody levels to *H. somni* were inversely related to disease severity in challenged calves (Berghaus et al. 2006). Lastly, we investigated differences in IgG2 allotypes because the two allotypes (IgG2A1 and A2, later called $IgG2^a$ and $IgG2^b$) may have slightly different functions. Differences were detected in complement activation, binding of IgBPs (now called IbpA), and resistance to microbial proteinases (Bastida-Corcuera et al. 1999a, b, 2000). Bovine IgG1 and IgG2 both initiate the complement cascade, and complement has been shown to be important in defense against pyogenic infections such as *H. somni* infection (Bastida-Corcuera et al. 1999a). Since IgG2 allotypes may differ in function, we examined their ability to initiate the complement cascade. $IgG2^b$ consistently had twice as much activity in complement-mediated lysis as $IgG2^a$ (Bastida-Corcuera et al. 1999a). Therefore, animals with the $IgG2^a$ allotype may be more susceptible to *H. somni* infection than cattle with the $IgG2^b$ allotype. Also, we found that $IgG2^b$ bound to *H. somni* IbpA by the Fc component, but $IgG2^a$ did not (Bastida-Corcuera et al. 1999b). Interestingly, we found that there were differences in age-related development of IgG2 allotype expression in calves (Corbeil et al. 1997). IgG2A1 (or $IgG2^a$) expression was detected early in the life of calves, whereas IgG2A2 (or $IgG2^b$) was not usually detected until 3–4 months of age (Corbeil et al. 1997). Since calfhood pneumonia most often occurs after waning of colostral passive immunity from the dam and before effective active immunity is in place, the delayed IgG2A2 response could be an important factor in susceptibility of young calves to *H. somni* pneumonia. These collective data lead us to conclude that IgG2 antibodies of appropriate allotype and specificity are important for immune defense against *H. somni* infection. However, it should be remembered that bovine IgG2 is a Th1 response, so it also could be correlated with cell-mediated immune defense, which has not been well investigated.

IgA responses are often thought to be most important on mucosal surfaces. Our studies of antibody responses after experimental pneumonia showed that IgA antibodies to *H. somni* in the bronchial lavage fluid did increase after infection (Gogolewski et al. 1989). The role of these IgA antibodies in protection is problematic. The infection persisted throughout the 10-week experiment in the face of this immune response. When IgG2 antibody activity increased and IgA antibody activity decreased at 4–6 weeks of infection, the number of *H. somni* also decreased. So we hypothesized that the IgA antibodies may be blocking IgG2. IgG2 is most opsonic, so it may have been that IgA was blocking epitopes for IgG2 opsonization (Gogolewski et al. 1989). Also, there was a positive correlation between IgA, IgG1, and IgM antibody levels and *H. somni* numbers throughout infection, but not between IgG2 and *H. somni* counts, another confirmation that IgG2 may be critical for protection, and that IgA (and perhaps other Ig classes/subclasses) may have blocking antibody activity (Gogolewski et al. 1989).

Commercially available *H. somni* whole cell vaccines have been reported to induce IgE antibody responses to *H. somni* (Ruby et al. 2000). The respiratory signs relating to IgE-related immediate hypersensitivity reactions may be compounded by histamine released by *H. somni*, under certain conditions (Ruby et al. 2002). We hypothesized that infection with *H. somni* may also induce antigen-specific IgE responses and that this response may be associated with more severe disease (Gershwin et al. 2005). To test this hypothesis, we also included an examination of viral–bacterial synergy because BRSV also induces IgE responses. In experimental bovine pneumonia with *H. somni*, we found increased IgE responses in calves inoculated intrabronchially with *H. somni* or with BRSV plus *H. somni* as compared to controls. The dual infection increased IgE antibodies to *H. somni* the most and was correlated with more severe pneumonia. These studies of IgE and bacterial–viral interactions will be further discussed below.

2.3 *Specificity of Antibody Responses to* H. somni *and Protection*

The importance of antibody specificity was introduced briefly in the section of passive immunity. The immunoglobulin fractions of antisera to 40 K and 78 K OMP at similar titers, but at subagglutinating levels, were incubated for 15 min at room temperature with *H. somni* before intrabronchial inoculation of calves. Although both antibody preparations reacted with surface epitopes of *H. somni*, the antibodies to the 40 K OMP protected and those to the 78 K OMP did not. Therefore, surface reactivity alone was not adequate for protection. This illustrated that specificity was one critical aspect of protection. Not many studies have shown specific *H. somni* antigens to induce protective immune responses in cattle or sheep. One group recently reported a vaccine composed of recombinant LppB (one of two *H. somni* 40 K OMPs), and p31 mixed with a commercial killed-cell vaccine containing 6 *Clostridium* spp and $Al(OH)_3$. This combination produced antibody responses in rabbits and sheep, and protected mice against *H. somni* septicemia (Guzman-Brambila et al. 2012). No protection studies were reported for cattle or sheep. Although LppB was equated with the 40 K OMP recognized by protective bovine antibodies as described above (Gogolewski et al. 1988), it is not clear from published data whether these protective antibodies recognize LppA or LppB (Theisen et al. 1992, 1993).

Specificity for the surface and shed fibrils of *H. somni* has been detected in cattle with several *H. somni* syndromes. When IbpA was still called an immunoglobulin Fc receptor, we showed that immune responses could be detected to this purified native antigen in sera from cattle with experimental *H. somni* abortion, pneumonia, or commercial vaccination, but not asymptomatic carriers or cattle with disease due to *Pasteurella multocida* or *Mannheimia haemolytica* (Yarnall and Corbeil 1989). Active and passive immunization with the DR2 recombinant subunit of IbpA protects both mice and calves (Geertsema et al. 2008, 2011; Zekarias et al. 2011).

The immunoglobulin fraction of convalescent phase serum recognized IbpA and protected calves against pneumonia (Gogolewski et al. 1987a; Yarnall and Corbeil 1989). Rabbit anti-IbpA DR2 protected mice against septicemia (Zekarias et al. 2011). Based on these studies, we actively immunized mice and then calves with three recombinant subunits of IbpA and found that the IbpA DR2 subunit protected best against experimental *H. somni* bovine pneumonia and murine septicemia (Geertsema et al. 2008, 2011). IbpA DR2 has a cytotoxic Fic motif, which causes bovine turbinate (BT) and bovine alveolar type 2 (BAT2) cells to retract (Worby et al. 2009; Zekarias et al. 2010). In vitro studies of antibody neutralization showed that either convalescent phase serum from calves that recovered from experimental *H. somni* pneumonia or antibodies to recombinant IbpA DR2 neutralized the cytotoxicity of live *H. somni* or culture supernatant concentrated 20X (Fig. 1).

The data show that the specificity of the antibody response to IbpA DR2 is functional in protecting respiratory epithelial cells against IbpA cytotoxicity. Antibodies to IbpA DR2 or bovine convalescent phase serum also prevented migration of *H. somni* across a BAT2 monolayer, suggesting that antibody neutralization of IbpA cytotoxicity for alveolar epithelial cells would also prevent

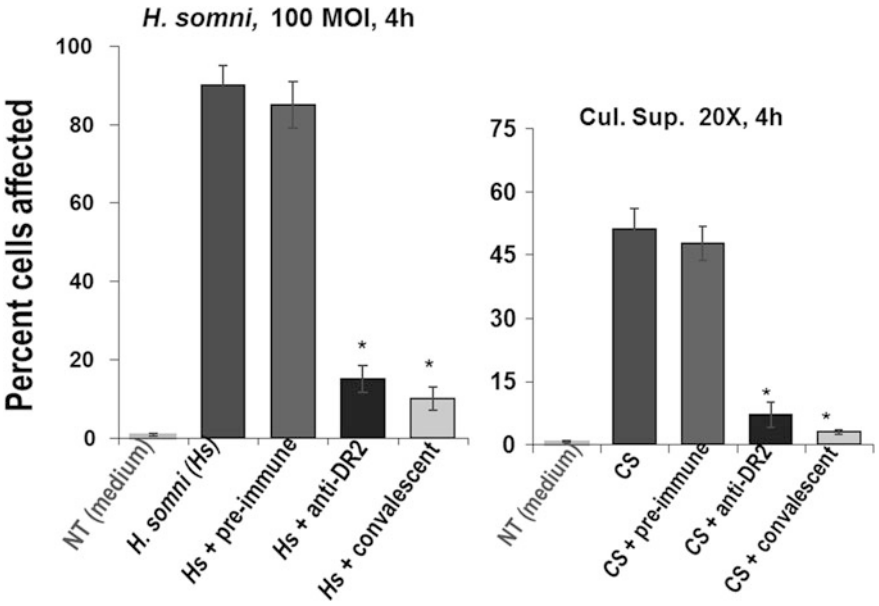

Fig. 1 Convalescent phase serum or antibody to IbpA DR2 neutralizes cytotoxicity for BAT2 epithelial cells whether due to live *H. somni* at a multiplicity of infection (MOI) of 100, or culture supernatant (CS) concentrated 20X. Treatment with medium alone (no treatment—NT) resulted in essentially no retracted cells. *H. somni* or CS pretreated with antiserum at 1:100 reduced the percent of cells affected (retracted or rounded up) dramatically after 4 h incubation (Zekarias et al. 2010). *Significantly different from preimmune serum ($P < 0.05$). Data previously published in a slightly different form and used by permission (Zekarias et al. 2010)

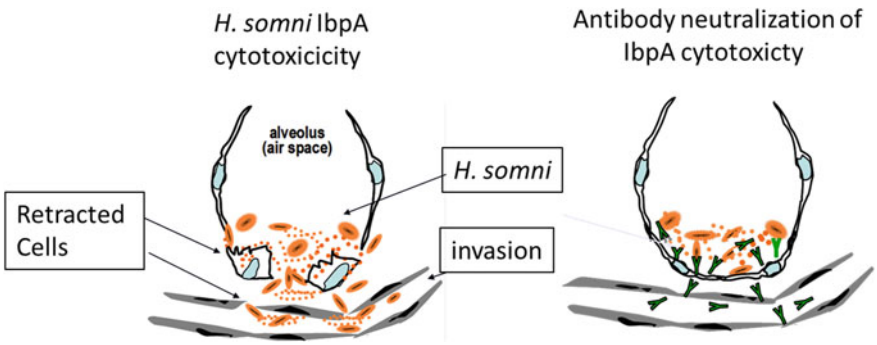

Fig. 2 Bovine alveolar epithelial cells retract when exposed to IbpA (*orange*) on the surface of *H. somni* or released from the bacterial surface (*orange dots*). Transmigration of *H. somni* between retracted alveolar cells has been shown in vitro (Zekarias et al. 2010). Endothelial cells then retract (Corbeil and Zekarias, unpublished data) to allow invasion of the blood stream. Antibodies to IbpA DR2 (*green*) neutralize the cytotoxic Fic motifs in DR1 and DR2 domains preventing cell retraction and transmigration. Figure on the *left* previously published in slightly different form and reused by permission (Zekarias et al. 2010)

invasion across the alveolar barrier to cause pneumonia and septicemia (Zekarias et al. 2010). The model for protection against invasion of the lung parenchyma and blood stream is diagramed in Fig. 2.

2.4 Immunodiagnosis

Several immunodiagnostic tests for *H. somni* have been used or proposed, but different assays lead to different conclusions (Stephens et al. 1981). One commonly used assay has been the micro-agglutination test (Widders et al. 1986). We found high background levels of antibody by the micro-agglutination test before inoculation of *H. somni* in an experimental abortion study and not much increase after inoculation (Widders et al. 1986). Isotypic ELISA assays with whole cell *H. somni* antigen showed that IgM antibodies were also high before inoculation of *H. somni* and increased little after inoculation. Since IgM is the most efficient Ig class in agglutination and is also quite cross-reactive, it may be that the high background levels in the former assay are due to IgM. On the other hand, IgG2 *H. somni*-specific antibodies were lowest before challenge, increased the most, and persisted the longest. It was concluded that IgG2 antibody responses may be most useful diagnostically as well as likely being most protective. A recent study of antibodies to *H. somni* exopolysaccharide (EPS) in sera from the same calves with experimental pneumonia, as described below, as well as some clinical cases and normal calves revealed immune responses to EPS in sera from infected calves, but significantly lower responses in uninfected calves (Pan et al. 2014). The analysis was elegant, but the responses were a little week in that the sera were only diluted 1:100

and the absorbance values were quite low. The conjugate used was an anti-IgG (H&L chain) conjugate, which would have detected IgG1 and IgG2 (and perhaps the L chain antibodies may have detected some IgM). The response of asymptomatic carriers or animals with other bacterial pneumonias was not determined. More extensive older studies with a high molecular weight *H. somni* surface antigen (later called IbpA) investigated antibody responses of normal cattle with no history of *H. somni* disease, preputial and vaginal carriers, clinical cases of culture-positive *H. somni* pneumonia, and animals with experimental pneumonia or abortion as compared to cattle having disease due to *P. multocida* or *M. haemolytica* (Yarnall and Corbeil 1989). The immunodot assay used in that study would not be as practical as an ELISA assay, but the results were revealing. Both IgG1 and IgG2 titers to IbpA increased, but there was no significant increase in IgM titers of animals with experimental *H. somni* infection. Since the net increase in IgG2 titers was a little higher than IgG1 and other studies had shown IgG2 to increase most, peroxidase-conjugated protein A was used to detect IgG2 reactivity in a second set of assays. These assays showed that cattle with *H. somni* abortion or pneumonia could be differentiated from cattle with *P. multocida* or *M. haemolytica* pneumonia and from asymptomatic vaginal or preputial carriers or normal culture-negative cattle (Yarnall and Corbeil 1989). A more recent small study on recombinant subunits of IbpA was done with preinfection sera as controls, but without comparison to sera from animals with other bacterial BRD or asymptomatic carriers. Three GST fusion protein subunits of IbpA (A3, A5 and DR2) were chosen as ELISA antigens and serum was diluted 1:2000 (Lo et al. 2012). IgG2 antibody responses of calves with experimental *H. somni* pneumonia gave low background readings before infection. Of the three subunits, responses to IbpA5 (a central IbpA domain with several approximately 200 bp repeats) increased the most and lasted throughout the 10-week experimental period. This response was detected in animals with experimental *H. somni* pneumonia, but not after commercial vaccination. Such a diagnostic antigen is desirable because IbpA has been shown not to cross-react with most other gram-negative bacteria tested (Yarnall and Corbeil 1989). Other *H. somni* antigens, such as the 40 K and 78 K OMPS, are very cross-reactive with antigens of other *Pasteurellaceae* (Corbeil et al. 1991; Kania et al. 1990). Now that IbpA and its recombinant subunits are available as antigens for diagnostic ELISAs, it would be worthwhile to further investigate isotypic antibody responses to IbpA or IbpA5 for immunodiagnostic assays.

Immunohistochemistry (IHC) is useful for diagnosis at necropsy. One study showed that most feedlot cattle dying with myocarditis were positive for *H. somni* by IHC of cardiac lesions (Haines et al. 2004). Later, IHC studies of clinical cases of bovine myocarditis found disseminated intravascular *H. somni* in cardiac capillaries. Rabbit antibodies to *H. somni* were also used to define the location of the bacteria 24 h after infection in experimental pneumonia (Gogolewski et al. 1987b). The latter study revealed that most bacteria were in the alveolus or associated with degenerate macrophages. So IHC has been used diagnostically or as a research tool to precisely locate the organism in the tissue (see Chap. “Histophilosis as a Natural Disease”). Since most *H. somni* isolates are very sensitive to antibiotics, it may not

be possible to culture the organism in animals treated with antibiotics before death. In that case, IHC may still detect the *H. somni* in the lesions.

3 Evasion of Immune Responses

Immunity and evasion of immune defense constitute a dynamic interaction between the host and the pathogen. As noted above, cattle have strong immune responses against *H. somni*. Yet, the infection persists for weeks in the lungs of calves with experimental *H. somni* pneumonia in the face of a strong antibody response (Gogolewski et al. 1989). In that study, three calves were rechallenged with 10 times the original dose of *H. somni* 10 weeks after the original infection and were able to clear *H. somni* in 2–3 days with minimal pneumonia. Thus, the immune response was capable of clearing a new infection, but not the original persisting pulmonary infection. The question is what mechanisms of immune evasion permitted *H. somni* to persist in the face of an effective host immune response? Several mechanisms have been reported.

3.1 Interference with Phagocyte Function

It has been known for some time that *H. somni* interferes with host phagocyte morphology and function. Early studies showed that *H. somni* was resistant to killing by neutrophils and that subcellular fractions of the bacteria suppressed *Staphylococcus aureus* ingestion and iodination by bovine neutrophils (Hubbard et al. 1986). The same group then reported that the inhibitory components of fractionated samples consisted of guanine, adenine, and GMP (Chiang et al. 1986). This fascinating work seems not to have been carried further by the same group or others. Most of the reports after those from Roth's group focused on the inhibitory function of the phagocytes, not the virulence factors of *H. somni* that interfere with that function. Since *H. somni* is a very autolytic organism, it would not be surprising to find released ribonucleotides, ribosides, adenine, and guanine in the vicinity of infection. Czuprynski's group reported about the same time that bovine neutrophils ingest, but do not kill, *H. somni* (Czuprynski and Hamilton 1985). Later, Gomis et al. found that live log-phase *H. somni* inhibited phagocytosis by bovine macrophages (Gomis et al. 1997). Inzana's group showed that live *H. somni*, but not killed *H. somni*, lipooligosaccharide (LOS), or cell free supernatant inhibits production of superoxide anion by both neutrophils and macrophages of cattle (Howard et al. 2004). More recently, Tagawa's group investigated the role of IbpA in macrophage inhibition (Hoshinoo et al. 2009). Nearly all of the *IbpA* gene was knocked out of virulent *H. somni* strain 2336. The virulent strain, but not the mutant strain 2336.A1, was cytotoxic for bovine primary monocytes and murine J774.1 macrophages. Similarly, phagocytosis by both cell types was inhibited by the virulent strain, but

not the IbpA knockout strain. They demonstrated disruption of actin stress fibers, which is consistent with the function of IbpA Fic motifs (Worby et al. 2009). IbpA is released into the culture supernatant, so it is surprising that others did not find that *H. somni* culture supernatant interfered with phagocyte function (Howard et al. 2004). In that paper, supernatant was collected after only 2 h of incubation of *H. somni* in fresh media, so perhaps the concentration of IbpA was too low for inhibition of phagocyte function. The decreased functions of mononuclear phagocyte function and the IbpA cytotoxicity may be related to the degenerated alveolar macrophages seen in histopathologic studies of pneumonic lungs of calves 24 h after intrabronchial inoculation of *H. somni* strain 2336 (Gogolewski et al. 1987b).

3.2 Decoys or Antigen–Antibody Interactions Away from the Bacterial Surface

Convalescent phase bovine serum antibodies from calves recovering from experimental *H. somni* pneumonia recognize shed IbpA and OMP in culture supernatant (Geertsema et al. 2008; Zekarias et al. 2010) (Fig. 3). When these shed antigens react with antibody, initiation of the complement cascade may be too far away from the bacterial surface to deposit macromolecular complexes on the bacterium. Both Fc-mediated and complement-mediated opsonization may not result because antigen–antibody complexes are away from the bacterial surface. Perhaps this is also one of the reasons that *H. somni* strains lacking IbpA, such as 129Pt and 1P, are killed by complement (serum sensitive) while strains producing and shedding IbpA fibrils are not killed. *H. somni* LOS is also shed from the surface in soluble and particulate (probably in outer membrane blebs) fractions (E Ziegler, A Wunderlich and LB Corbeil, unpublished data). Activation of complement away from the bacterial surface by shed LOS may be one reason why most strains are serum-resistant. Serum-sensitive strains also cannot sialylate their LOS (see Chap. "The Many Facets of Lipooligosaccharide as a Virulence Factor for *H. somni*"), which may also contribute to serum sensitivity (Sandal and Inzana 2010).

3.3 Lack of Immune Stimulation and Reactivity

The major outer membrane protein (MOMP) of *H. somni* has an interesting relationship with the bovine immune system. When the protein was purified and inoculated into mice, calves, or rabbits to elicit an immune response, both monoclonal and polyclonal antibodies were produced (Tagawa et al. 1993). Yet reactivity with convalescent phase serum was very weak compared with the reactivity of the same convalescent phase serum with other surface or shed *H. somni* antigens (Tagawa et al. 2000). This suggests that the MOMP antigenic epitopes may not be exposed on the bacterial surface to stimulate an immune response, but when the

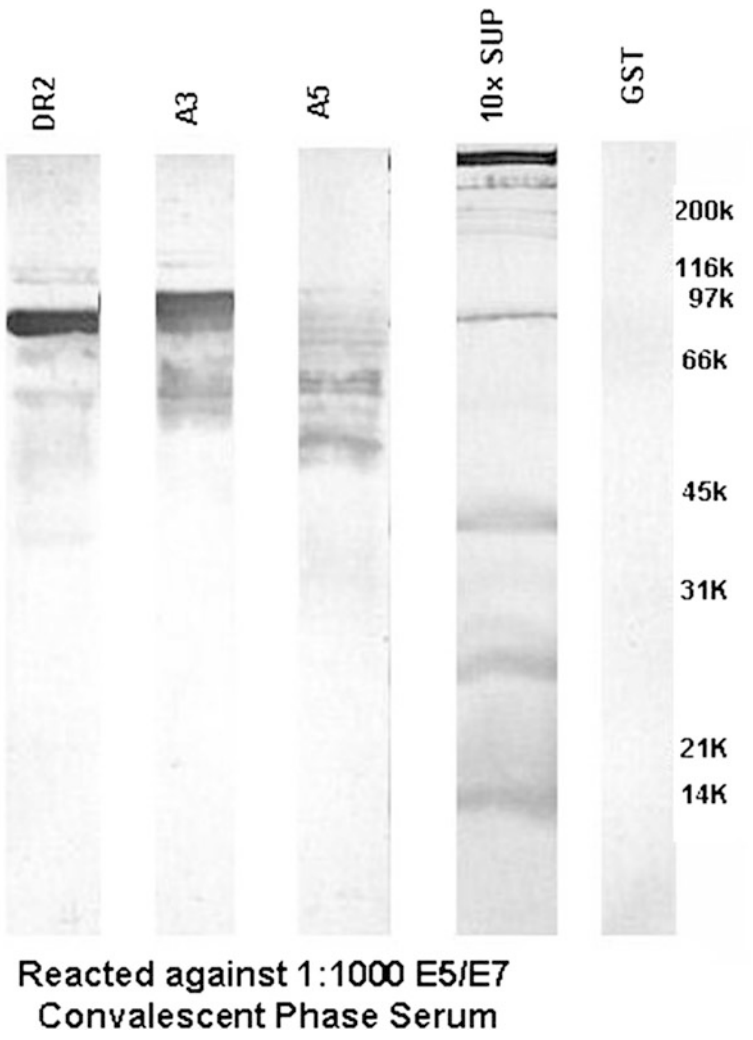

Fig. 3 Convalescent serum from calves E5 and E7 recovering from experimental *H. somni* pneumonia detects IbpA in culture supernatant (10X SUP) as well as three recombinant IbpA subunits—GST fusion proteins DR2, A3, and A5, but not the GST control. The immunodominant 78 K and 40 K OMPs are also present in the culture supernatant. Molecular weight markers are indicated on the *right;* proteins inoculated into lanes are indicated at the *top*. Lanes were cut and trimmed from the Western blot for neatness

protein is purified and mixed with adjuvant it is immunogenic in calves as well as in laboratory animals. Perhaps other surface structures such as the IbpA fibrils cover the MOMP to protect it from stimulating the immune system.

Biofilms are another means of hiding from host immune defenses (see Chap. "Exopolysaccharide Production and Biofilm Formation by *H. somni*").

Virulent *H. somni* strain 2336 forms thick mature biofilms in vitro, whereas asymptomate carrier strain 129Pt only forms smaller biofilms under the conditions of the experiments (Sandal et al. 2007). Later, the same group reported *H. somni* biofilm formation in the heart during experimental myocarditis (Sandal et al. 2009). Large numbers of bacteria surrounded by extracellular substance were detected in the cardiac tissue by transmission electron microscopy. The presence of *H. somni* in these cardiac biofilms was confirmed by FISH. These elegant studies can be compared with clinical studies, published about the same time, showing that the biofilms in *H. somni* myocarditis are found on or in the endothelial surface of capillaries in the myocardial lesions (O'Toole et al. 2009). Subsequent investigation of clinical cases of *H. somni* myocarditis confirmed that masses of *H. somni* bacteria colonize the endothelial surface of myocardial small vessels during clinical myocarditis (O'Toole and Corbeil, unpublished data). The formation of such masses should result in the protection of the bacteria from both antibiotics and the host immune responses.

Antigenic mimicry also fits in the classification of immune evasion by the lack of immune stimulation or reactivity. The *H. somni* LOS outer core mimics host glycosphinoglipids and also many strains sialylate the terminal galactose of LOS (Sandal and Inzana 2010). Both of these mechanisms of antigenic mimicry camouflage the organism and result in resistance to phagocytosis and/or complement-mediated killing. This concept was confirmed in that serum-sensitive aysmptomatic carrier strains such as 1P and 129Pt could not sialylate their LOS (Sandal and Inzana 2010) (see Chap. "The Many Facets of Lipooligosaccharide as a Virulence Factor for *H. somni*").

3.4 Antigenic Conservation and Variation

Another means of evasion of host defense is antigenic variation in the face of an immune response. A critical feature of antigens with promise as vaccine candidates is conservation in a wide variety of disease isolates. Both the 40 K and the 78 K OMP antigens were shown to be conserved by Western blotting, as was a 39 K OMP antigen (Corbeil et al. 1991; Kania et al. 1990). Conservation was not determined by sequencing. The MOMP is conserved in disease isolates, but undergoes antigenic variation (Tagawa et al. 1993, 2000) as well as nucleotide and amino-acid sequence variation, especially in predicted surface-exposed loops (Khan et al. 2005). This degree of variability indicates that the MOMP may have limitations in stimulating immune responses that would protect against most disease isolates. Thus, MOMP evasion of host responses involves both antigenic variation and lack of stimulating responses, probably by most exposed loops being covered by more surface-exposed structures, such as the IbpA fibrils. Some of the iron-regulated OMPs also undergo phase variation (Ekins and Niven 2003; Tremblay et al. 2006), decreasing their usefulness in stimulating widely protective responses. Lastly, antigenic variation in the face of an immune response has been

well defined for *H. somni* LOS (see Chap. "The Many Facets of Lipooligosaccharide as a Virulence Factor for *H. somni*"). This was first noticed serendipitously when calves were infected intrabronchially in the right caudal lobe with a subculture of strain 2336 that had been calf-passed and cloned three times by subculturing from a single colony each time (Inzana et al. 1992). The three inoculated calves were sampled weekly by fiberoptic bronchoscopy and lavage of the inoculated site for 10 weeks. The persistent infection in the face of a robust immune response in the calves was reported separately (Gogolewski et al. 1989). Why the infection of the lung persisted was mysterious. Possible blocking IgA antibody and toxicity for alveolar macrophages was proposed and is described above (Gogolewski et al. 1989). However, LOS profiles from weekly lung and nasal isolates showed phenotypic variation of the isolates from each of the 3 calves following somewhat different patterns (Inzana et al. 1992). Western blotting and ELISA analysis with serum from the infected calves revealed antigenic variation in addition to phenotypic LOS electrophoretic variation in silver-stained polyacrylamide gels. Since the calves were all infected with the same clone, it could be concluded that this was true antigenic variation, not just selection among a variety of antigenic types in the inoculum. Inzana's group has thoroughly investigated mechanisms of this variation. One aspect of the variation was probed by monoclonal antibodies to various LOS epitopes (Howard et al. 2000). This revealed conserved epitopes in the inner core of LOS but variation in the outer core phosphorylcholine. The mechanisms of phase variation of *H. somni* LOS are reviewed fully elsewhere (Sandal and Inzana 2010; Siddaramppa and Inzana 2004) and in the Chap. "The Many Facets of Lipooligosaccharide as a Virulence Factor for *H. somni*" in this volume.

4 Role of Microbial Interactions in Immunity to *H. somni* Infection

Bovine respiratory disease (BRD) is a multifactorial disease often associated with stress, crowding, as well as viral and bacterial pathogens. Stress has been mimicked by treatment with dexamethasone. In this model, dexamethasone-treated calves challenged with *H. somni* developed significantly increased volumes of pneumonic lung than untreated challenged calves (Chiang et al. 1990). Crowding results in both stress and higher doses of a variety of pathogens. It is well known that several viruses predispose calves to bacterial pneumonia. The most common viruses include Bovine Herpes Virus 1 (BHV1), Parainfluenza Virus 3 (PI3), Bovine Respiratory Syncytial Virus (BRSV), and Bovine Virus Diarrhea Virus (BVDV). Common bacterial pathogens in BRD include *P. multocida*, *M. haemolytica*, *H. somni*, and *Mycoplasma bovis*. We chose to investigate mechanisms of viral–bacterial interactions using the BRSV-*H. somni* model in calves because we had experience with this model and much was known about the roles of different immunoglobulin classes and specificities in pathogenesis and protection.

4.1 Shifting Immunoglobulin Class and Subclass Responses

We hypothesized that challenging calves with BRSV, followed by *H. somni,* would result in higher IgE responses, since both pathogens were known to stimulate IgE antibodies (Ruby et al. 2000; Stewart and Gershwin 1989a, b). To test this hypothesis, calves were infected first with BRSV by aerosol and 6 days later with *H. somni* strain 2336 by intrabronchial inoculation (Gershwin et al. 2005). Other groups of calves were given BRSV or *H. somni* alone. The clinical scores were much higher and persisted much longer in the dually infected calves as compared to groups treated with BRSV or *H. somni* alone. Serum IgE antibody responses to *H. somni* were highest in the dually infected group and correlated with high clinical scores on day 21, just after the peak clinical score. Although we had expected a shift from a Th1 response to a Th2 response, with higher IgG1 antibody levels and lower IgG2 levels, both IgG subclasses of antibodies were increased in dually infected calves. Necropsy was at 28 days after BRSV infection. Lung and bronchial cultures were positive for *H. somni* at necropsy in dually infected calves, but not in any of the singly infected calves. So, increased IgE antibody responses were correlated with greater severity and duration of pneumonia as well as persistent pulmonary infections with *H. somni*. Next, we wondered whether the *H. somni* antigenic specificity of IgE antibodies in serum from these calves would be different from that of the IgG antibodies. IgE antibodies in sera from 6 calves infected with *H. somni* (with or without BRSV) for 21 days (at the peak response) reacted predominantly with the 41 K MOMP, whereas IgG antibodies from the same calves at 21 days reacted primarily with the 40 K OMP (Corbeil et al. 2006). The lack of strong reactivity of IgG antibodies with the MOMP is consistent with our earlier studies as outlined above. It is not clear why IgE anti-MOMP antibodies are high in these calves, but it is another reason why the MOMP would probably not be a good vaccine antigen (in addition to its antigenic variation). Based on these studies, we hypothesized that dual vaccination may increase IgE responses and result in more severe disease. Concurrent vaccination with both BRSV and *H. somni* vaccines resulted in higher levels of IgG2 antibodies to *H. somni* that correlated to protection (Berghaus et al. 2006). There were IgE antibodies to BRSV, especially in the calves with the most severe pneumonia, and serum IgE antibody levels to BRSV correlated with severity of disease in this vaccine study.

4.2 Innate Immunity

After studying the specific antibody response and Ig isotypes, we decided to look at the innate immune responses at the level of the alveolar epithelial cell. The effect of BRSV and *H. somni* on BAT2 cells was studied in vitro because the alveolus is the

site where most *H. somni* is detected by IHC 24 h after inoculation of calves. Also, BRSV infects this cell type. We showed that pretreatment with BRSV increased the retraction of BAT2 cells after treatment with *H. somni* IbpA, as well as increasing the paracellular transmigration of *H. somni* across a monolayer of BAT2 cells (Agnes et al. 2013). In order to examine the innate immune mediator expression, gene expression profiles of BAT2 cells were determined by microarray analysis after infecting cells with BRSV for 60 h and then treating with *H. somni* concentrated culture supernatant (CCS) for 4 h (Agnes et al. 2013). Other BAT2 cells with only medium, only BRSV, or only *H. somni* were also analyzed. Results showed that there was a synergistic up-regulation of IL-6, Il-8, prostaglandin synthase, and some chemokines when cells were treated with both BRSV and *H. somni* (Shao M. X.; Corbeil, L.B.; Gershwin, L.J. unpublished data). This up-regulation of IL-6, IL-8, and prostaglandin synthase was consistent with the increased inflammation in dually infected calves over that in singly infected calves as previously reported (Gershwin et al. 2005). Furthermore, treatment of BAT2 cells with both BRSV and *H. somni* synergistically up-regulated the expressions of *mmp1* and *mmp3* over that of singly treated cells (Agnes et al. 2013). MMP1 and MMP3 proteins were also synergistically up-regulated when BAT2 cells were treated with BRSV and *H. somni* CCS. Culture supernatant from dually treated BAT2 cells digested more collagens 1 and 4 than supernatant from BAT2 cells treated with either pathogen alone. Collagens 1 and 4 are the targets of MMP1 and MMP3, respectively. Collagen 1 comprises a major part of lung extracellular matrix and collagen 4 is a major component of the basement membrane. Increased MMP3 would degrade the basement membrane, increasing passage of *H. somni* between the alveolar epithelial cells and the endothelial cells of the underlying capillary (Fig. 4). MMP1 would break down the interstitial matrix allowing dissemination of *H. somni* into the lung. Since BRSV preinfection of BAT2 cells also resulted in increased retraction of the BAT2 cells, the end result would be greater invasion of *H. somni*. The overall effect of BRSV plus *H. somni* on epithelial cell mediators of innate immunity implies greater invasion of the bacteria and increased inflammation, as was detected from bovine infection experiments (Gershwin et al. 2005).

Most studies of viral–bacterial interactions involve investigation of the effects of viral infection on subsequent bacterial infection (Bosch et al. 2013; Murphy et al. 2009). The effect of prior bacterial infection on subsequent viral infection is not well studied. However, most bacterial pathogens in BRD can colonize the upper respiratory tract in an asymptomatic carrier state. This means that BRD bacterial pathogens could very well be present before the host is exposed to the viral pathogens. Further examination of microarray results of BAT2 cells treated with BRSV, *H. somni* CCS, or BRSV followed by *H. somni* revealed that treatment with *H. somni* CCS alone results in up-regulation of four antiviral proteins (Lin, C.; Agnes, J.; Shao, M.X.; Behrens, N.; Gershwin, L.J.; Corbeil, L.B., In Preparation). Dual treatment or BRSV treatment alone resulted in very little up-regulation of these proteins. In vitro functional studies showed that pretreatment of BAT2 cells with *H. somni* CCS before infection with BRSV did inhibit production of BRSV virus. Infection of calves with *H. somni* before BRSV has not been done, but the

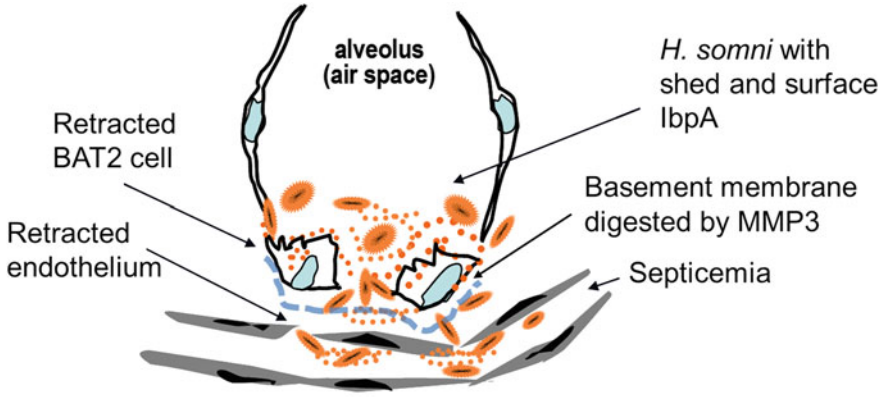

Fig. 4 Diagram of the effect of infection of alveolar epithelial cells with BRSV before *H. somni* infection. BRSV infection before *H. somni* results in greater retraction of alveolar epithelial cells and transmigration of *H. somni* between the epithelial cells. Increased secretion of MMP1 and MMP3 by the epithelial cells digests interstitial collagen and basement membrane collagen to increase invasion of the bacteria into the lung and into the blood stream. Modified from a previous publication (Zekarias et al. 2010) by permission, courtesy Bereket Zekarias and Joe Agnes

in vitro experiments predict that *H. somni* infection before BRSV may result in less pneumonia than either alone (the opposite of synergy).

These few studies of viral–bacterial interactions in *H. somni* immunity reveal that the interactions are complex. If the viral infection is first, IgE responses to *H. somni* are increased in vivo and are associated with more severe pneumonia. If the virus infects BAT2 cells before *H. somni* in vitro, innate immune mediators from BAT2 cells are increased, which increases *H. somni* invasion in an in vitro model. However, when BAT2 cells are treated with *H. somni* CCS first, antiviral proteins are up-regulated. This suggests that *H. somni* colonization of the upper respiratory tract may inhibit viral infection. It is expected that interactions of other viruses and bacterial pathogens causing BRD may be equally complex.

Another aspect of innate immunity involves the interactions among bacteria in the microbiome. Many years ago, we approached this topic by determining the ability of aerobic bacterial isolates present in the normal upper respiratory tract to inhibit or enhance the growth of *H. somni* in vitro (Corbeil et al. 1985). Bacterial isolates that enhanced the growth of *H. somni* outnumbered inhibitors 4 to 1. Similar results were obtained for microbiome isolates that enhanced or inhibited *P. multocida and M. haemolytica* growth in vitro (Corbeil et al. 1985). If these interactions occur in vivo, the composition of the microbiome of individual calves could be one factor influencing their susceptibility or resistance to infection with *H. somni* as well as for the other BRD bacterial pathogens. It needs to be determined whether nonpathogenic bacterial isolates from the microbiome that inhibit growth of pathogens could be used as probiotics to colonize the upper respiratory tract to aid in preventing BRD.

5 Conclusions

The ability of *H. somni* to exist in an asymptomatic carrier state, to cause severe local disease, or to invade to cause septicemia, myocarditis, TME, or abortion suggests that many factors are likely involved in tipping the dynamic interaction between host and parasite toward health or disease. Host innate and acquired immune mechanisms shift the outcome toward health, and pathogen immune evasive mechanisms may shift the outcome to disease or death. Protection against experimental bovine pneumonia caused by *H. somni* is best achieved with IgG2 antibodies and specificity for a 40 K OMP and/or the IbpA fibrillar protein, especially the DR1 and DR2 domains with the cytotoxic Fic motifs. Undoubtedly, other antigens are important as well. Cell-mediated immunity has not been well studied, but since bovine IgG2 is a Th1-mediated response such as CMI, it is likely that the latter is also involved. Increases in IgE responses to *H. somni* after dual infection with BRSV seem to be associated with increased clinical signs and pulmonary pathology. On the other side of the dynamic interaction between pathogen and host, *H. somni* has several mechanisms of immune evasion. It has been shown to interfere with bovine neutrophil and macrophage function, and to resist complement-mediated killing probably due to activation of complement away from the surface by shed OMPs, fibrils and LOS, as well as by other mechanisms. The low antibody response to the MOMP after infection is an example of immune evasion by not stimulating an antibody response, perhaps because antigenic epitopes are not surface exposed. Several *H. somni* antigens undergo antigenic variation including the MOMP, LOS, and some IROMPS, providing another way of evading immune responses. Lastly, the whole area of microbial interactions in pathogenesis and immunity is barely investigated. BRSV and *H. somni* interactions produce higher IgE antibody levels to *H. somni* and increase several innate responses at the respiratory epithelial cell level. Most investigations of viral–bacterial interactions involve exposure to the virus first. If *H. somni* is inoculated first, one study found increases in antiviral responses of respiratory epithelial cells. This implies bacterial interference with viral pathogenesis rather than the synergy that is seen when viruses infect first. Obviously, the host defense mechanisms are complex.

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The Many Facets of Lipooligosaccharide as a Virulence Factor for *Histophilus somni*

Thomas J. Inzana

Abstract The lipooligosaccharide (LOS) of *Histophilus somni* is a multifaceted molecule that provides critical protection to the bacterium against host defenses, may act as an adhesin, and like similar molecules of gram-negative bacteria, is an endotoxin that signals through toll-like receptor 4 and NF-κB to cause inflammation. The lipid A component is responsible for the endotoxic and apoptotic activity of the LOS. The *H. somni* LOS lacks O-side chains typically characteristic of gram-negative bacteria that have lipopolysaccharide, but has a complex, microheterogeneous outer core. The LOS of disease isolates is capable of undergoing structural and antigenic phase variation of its outer core due to slip-strand mispairing of glycosyltransferase genes that contain repetitive sequences of DNA base pairs. Such variation enables the bacteria to evade bactericidal antibodies made to oligosaccharide antigens. In addition, the LOS can be decorated with phase-variable phosphorylcholine (ChoP), which binds to platelet-activating factor receptor on host cells, thereby aiding in colonization of the upper respiratory tract. However, ChoP is likely not expressed when the bacteria are in systemic sites because ChoP also binds to C-reactive protein, resulting in activation of host complement and promoting bactericidal activity. The structure of some LOS outer core chains is identical to oligosaccharides on host glycosphingolipids of red blood cells, other cells, and merconium (lacto-*N*-neotetraose, lacto-*N*-biose, *N*-acetyllactosamine, etc.). Furthermore, terminal galactose residues on LOS and elsewhere are decorated with sialic acid, which blocks antibody binding, activation of complement, phagocytosis, and intracellular killing. Therefore, antigenic mimicry of host antigens is an important defense mechanism provided by the oligosaccharide component of the LOS to avoid innate and adaptive host defense mechanisms. However, some strains of *H. somni* isolated from the bovine genital tract, particularly the normal bovine prepuce, are incapable of LOS phase variation, sialylation of the LOS, and expression of ChoP. At least 1 such strain has been shown to be avirulent, underscoring the importance of the LOS as a virulence factor, although this strain is deficient in other factors as well. The structure and arrangement of the inner core glycoses (heptose and

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3-deoxy-D-*manno*-2-octulosnic acid) is remarkably similar to the inner core oligosaccharide on some strains of *Neisseria* spp., and mutants that contain a truncated LOS oligosaccharide are considerably more serum-sensitive than the parent strain. Therefore, the LOS is a critical component that enables *H. somni* to resist host defenses and cause disease.

Contents

1 Introduction

Lipopolysaccharide (LPS) is an integral component of the outer membrane of gram-negative bacteria. The LPS of *Escherichia coli* and other enteric bacteria has been studied in great detail. The LPS consists of a highly conserved diglucosamine backbone that is linked through hydroxyl and amino groups with acyl chains of various lengths, and the diglucosamine residues are linked by phosphate bridges at hydroxyl groups 1 and 4′ (Rietschel et al. 1984). LPS is also known as endotoxin, and the toxic properties of the LPS are due to the lipid A acylation pattern, and to the presence of two phosphate residues or modification of these phosphate residues, as determined through chemical alteration of the lipid A and analysis of lipid A from bacterial LPSs that are not toxic (Alexander and Rietschel 2001; Phillips et al. 2004). Enteric bacteria typically have C12, C14, C16, and 3-hydroxy-C14 fatty acids in their lipid A. However, a wide variety of fatty acid chain lengths are present in bacteria of different genera (Alexander and Rietschel 2001; Rietschel et al. 1984). Endotoxic activity is the result of an inflammatory response attributed to lipid A,

which effects inflammation through interaction with toll-like receptor (TLR) 4. TLR4 is one of the multiple pathogen recognition receptors present on macrophages, dendritic cells, neutrophils, and other cells (Barton et al. 2004). In order for inflammation to occur, LPS must be processed through LPS-binding protein, which binds to lipid A, and soluble glycoprotein CD14. The LPS is then transferred to membrane-bound CD14 and then to TLR4-MD-2, which are primarily responsible for the host response to LPS. TLR4 is a transmembrane protein, and many other proteins (such as MyD88, IRAK, and others) work downstream of TLR4 to activate MAP kinase pathways and nuclear factor-κB to induce interferon α/β resulting in subsequent release of pro-inflammatory cytokines. For additional details on the inflammatory responses resulting from LPS interactions with responsive cells, see the excellent reviews that have been written on this subject (Alexander and Rietschel 2001; Barton et al. 2004; Raetz and Whitfield 2002).

While lipid A is the predominant component of the outer leaflet of the outer membrane of gram-negative bacteria, interaction of the bacteria with the environment is through carbohydrate components of various lengths. Most gram-negative bacteria have an LPS that contains KDO (3-deoxy-D-*manno*-2-octulosonic acid), which in enteric bacteria is linked to the diglucosamine backbone through the 6′ hydroxyl group (Rietschel et al. 1984). In enteric LPS, a variable number of KDO sugars is attached to one or more heptose molecules (L-*glycero*-D-*manno*-heptose), making up what is referred to as the inner core oligosaccharide. The lipid A, heptose, and KDO can be further modified by attachment of phosphate, phosphatidylethanolamine, or phosphoethanolamine groups (Raetz 1990). Attached to the heptose are common sugars that make up the outer core oligosaccharide, notably glucose, galactose, and *N*-acetylglucosamine (Raetz and Whitfield 2002). Although the arrangement of these sugars can vary, they are highly conserved in core LPSs. In some bacteria, heptose is replaced with mannose or other sugars. A detailed description of LPS core oligosaccharides is described by Holst (1999). Attached to the terminal core, oligosaccharide sugar is a repeating oligosaccharide referred to as O-antigen. The O-antigen, as implied, is the component to which the host immune response is primarily directed. Some sugars in the O-antigen are unusual and foreign to the host, making them highly immunogenic. Furthermore, there is considerable structural and compositional variation of the O-antigen, even within a species, resulting in a wide variety of potential serotypes. In addition, the O-antigen is a polymer, with 40 or more repeating units, which can be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into a ladder-like organization (Fig. 1, lanes 5–8). In addition to antigenic specificity, the O-antigen serves to protect the bacterium from host defenses, such as complement-mediated bactericidal activity, and exposure to stress and severe environmental conditions (Whitfield and Valvano 1993).

Many gram-negative bacteria that inhabit mucosal sites, such as *Haemophilus, Pasteurella, Neisseria*, and *Campylobacter* spp., lack expression of a polymeric O-antigen, but rather express a more complex core oligosaccharide only. These molecules have been referred to as lipooligosaccharide (LOS) (Hitchcock et al. 1986; Inzana et al. 1985). Since many of these bacteria have adapted to being

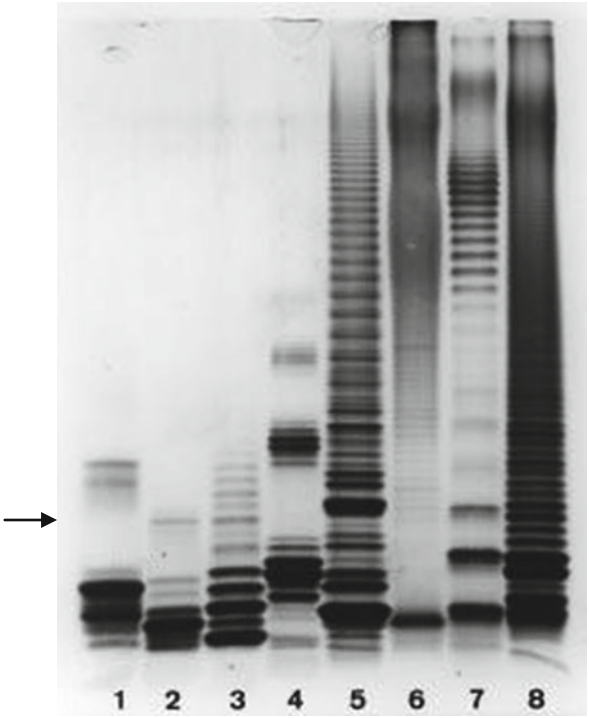


Fig. 1 Electrophoretic profiles of LOSs (lanes 1–4) and LPSs (lanes 5–8) from various bacterial genera following SDS-PAGE and staining with ammoniacal silver. Lanes, bacteria from which LOS/LPS samples were isolated: (*1*) *Haemophilus influenzae* type b; (*2*) *Histophilus somni*; (*3*) *Neisseria gonorrhoeae*; (*4*) *Pasteurella multocida*; (*5*) *Actinobacillus pleuropneumoniae* serotype type 7; (*6*) *Brucella abortus*; (*7*) *Escherichia coli*; (*8*) *Salmonella typhimurium*. Repetitive, higher molecular weight bands (above *arrow*) in lanes 1–4 only are aggregates of lower molecular size profiles and are ignored. Reprinted from Inzana and Apicella (1999) with permission

obligate inhabitants of mucosal sites, they may no longer need the long, polymeric O-antigen many gram-negative bacteria need to persist in environmental conditions that rapidly change and where they are exposed to extremes in temperature and pH. In contrast, gram-negative opportunist pathogenic or commensal bacteria that primarily or solely reside on mucosal surfaces express an outer core oligosaccharide that may be decorated with sialic acid and/or phosphorylcholine (ChoP), and the sugars that make up one or more oligosaccharide chains may mimic oligosaccharides expressed on host glycosphingolipid molecules, such as lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and others (Moran et al. 1996). Furthermore, more than one oligosaccharide chain can be attached through the multiple heptose residues on the inner core, resulting in microheterogeneity of the LOS (Fig. 1, lanes 1–4). The presence, structure, and even linkages of these oligosaccharide chains can undergo high-frequency phase variation through multiple mechanisms, but a common mechanism well characterized in *H. influenzae* and *Neisseria* spp. is

slip-strand mispairing of glycosyltransferase genes, which add sugars to the growing oligosaccharide chain (Berrington et al. 2002; Power et al. 2009). Slip-strand mispairing occurs when microsatellites of variable number tandem nucleotide repeats (VNTR) (commonly 1, 2 or 4 VNTR) occur just downstream of the start codon, or anywhere within the open reading frame (ORF). During replication, one or more repeats may be added or lost, resulting in a frame shift that may cause premature transcriptional termination (Levinson and Gutman 1987). In addition to phase variation of sugar moieties, genes required for decoration of the LOS with sialic acid and ChoP have also been shown to phase vary (Clark and Weiser 2013; Fox et al. 2006).

2 *Histophilus somni* Lipooligosaccharide Purification, Structure, and Biological Activity

While water-soluble, native LPS (also known as smooth) is most efficiently isolated by hot aqueous phenol extraction (Johnson 1993), rough (lacking O-antigen) enteric LPS has greater hydrophobicity than smooth LPS and is more efficiently purified by a mixture of aqueous phenol, chloroform, and petroleum ether (PCP) (Galanos et al. 1969). However, although lacking O-side chains, *H. somni* LOS is more efficiently isolated by modified enzyme digestion, aqueous phenol extraction, and ultracentrifugation (Inzana et al. 1988), rather than by PCP extraction or enzyme and chemical extraction without aqueous phenol and differential centrifugation (Darveau and Hancock 1983). As for *H. influenzae* LOS, the LOS of *H. somni* is microheterogeneous (2–6 bands on SDS-PAGE, ranging in size from about 3200–5000 kDa). However, the LOS of disease isolates is more complex than that of the few commensal preputial isolates that have been examined. Antiserum to the LOS is bactericidal, even for strains resistant to killing by normal serum (Corbeil et al. 1985), and LOS antiserum contains antibodies to both the oligosaccharide and the lipid A (Inzana et al. 1988).

2.1 Lipid A

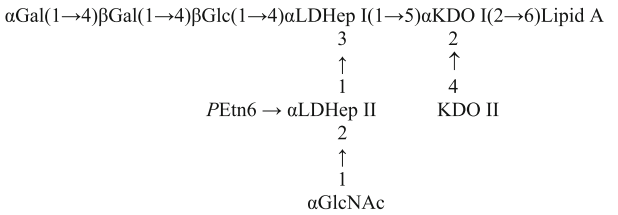
Relatively, little work has been done on the lipid A component of *H. somni* LOS. Full structural analysis of *H. somni* lipid A has not been done. Compositional analysis indicates that, like *E. coli* lipid A, *H. somni* lipid A consists of dodecanoic acid ($C_{12:0}$), tetradecanoic acid ($C_{14:0}$), and 3-hydroxytetradecanoic acid (3-OH-$C_{14:0}$). However, the ratio of the lipid A fatty acids differs between *E. coli* and *H. somni* (Inzana et al. 1988). Based on agglutination of *Limulus* amoebocyte lysate, a positive dermal Schwartzman reaction, and mouse lethality assays, *H. somni* lipid A clearly has endotoxic activity that is similar to that of *E. coli* lipid A (Inzana et al. 1988). Therefore, *H. somni* LOS likely contributes substantially to the vasculitis and other inflammatory lesions characteristic of *H. somni* systemic

infections. The lipid A component can be released from the oligosaccharide by mild acid hydrolysis of the acid-labile ketosidic linkage of KDO (Inzana and Todd 1992). Following detoxification of solubilized lipid A with mild alkali treatment, and conjugation to chicken ovalbumin or keyhole limpet hemocyanin, cattle immunized with the lipid A–protein conjugate make a substantial antibody response to lipid A after 2 immunizations (Inzana and Todd 1992). Most pregnant mice challenged with an abortion isolate of *H. somni* aborted, but only 1 of 8 C3H/HeJ mice (which lack TLR4 and are recalcitrant to the effects of endotoxin) aborted, indicating abortion was related to the effects of endotoxin. Although fewer mice immunized with the lipid A–protein conjugate aborted than controls, the difference was not significant. However, a strong immune response to lipid A was made in the immunized mice, but it is likely that the location of lipid A within the outer membrane made it inaccessible to antibodies on viable whole cells.

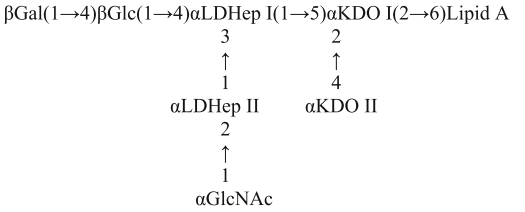
In addition to endotoxic activity, *H. somni* LOS can induce apoptosis of bovine endothelial cells, which may contribute to the vasculitis commonly seen in systemic infections due to *H. somni* (Gogolewski et al. 1987). Apoptosis can be inhibited by addition of Polymyxin B, suggesting that lipid A is responsible for this activity (Sylte et al. 2001). Additional factors interact with LOS to contribute to apoptosis and are reviewed in detail in the Chapter "Interactions of *Histophilus somni* with Host Cells."

2.2 Core Oligosaccharide Structures and Antigenic Mimicry

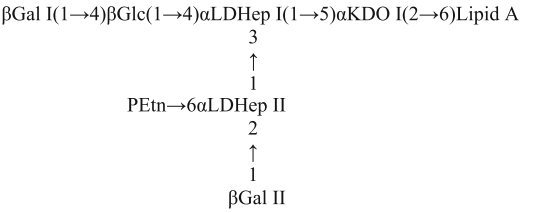
The structures of the oligosaccharide component of the LOS of 3 disease isolates and two commensal, non-pathogenic preputial isolates have been determined and are shown in Fig. 2 (Cox et al. 1998, 2003; St Michael et al. 2004, 2005, 2006). As shown by SDS-PAGE (Inzana et al. 1988), the structures of the outer core oligosaccharides of disease isolates (strains 2336, 738, and 8025) are often more complex than those of commensal isolates (strains 129Pt and 1P). Of interest is that the oligosaccharide structures of *H. somni* LOS are very similar to those of *Neisseria meningitidis* and *H. influenzae*. The inner core region of *N. meningitidis* LOS (two KDO and two heptose residues) is most similar to that of *H. somni*, except for strain 8025, which is reported to have only one KDO residue (St Michael et al. 2006). In contrast, the inner core of *H. influenzae* LOS typically contains one KDO and three heptose residues. However, the glycosyltransferases that attach the respective sugars to the outer core oligosaccharide chain have amino acid similarity to glycosyltransferases from *H. influenzae* and contain similar microsatellite VNTR in their genes (see below). However, *H. influenzae, N. meningitidis*, *N. gonorrhoeae, H. somni*, and other related species all share expression of common sugars (glucose, galactose, and *N*-acetylglucosamine) and are arranged in sequences that mimic oligosaccharides on glycosphingolipids of host cells (lacto-*N*-neotetraose [βGal (1 → 4)βGlcNAc(1 → 3)βGal(1 → 4)Glc], *N*-acetyllactosamine [βGal(1 → 4) GlcNAc], and others) (Cox et al. 2002; Mandrell et al. 1988; St Michael et al. 2005). Furthermore, these bacteria further decorate their LOS with *N*-acetyl-5-neuraminic



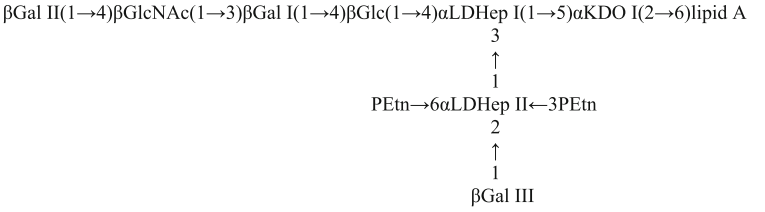
A. Oligosaccharide structure of LOS from preputial isolate *H. somni* strain 129Pt (St Michael et al. 2004).



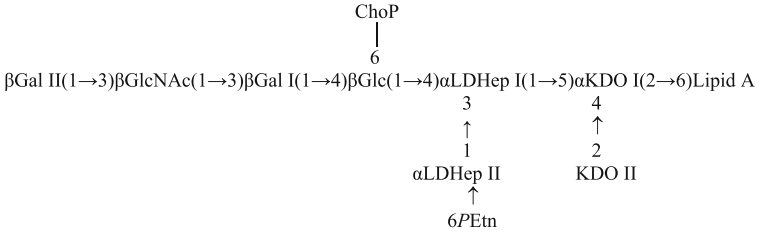
B. Oligosaccharide structure of LOS from preputial isolate *H. somni* strain 1P (Cox et al. 2003).



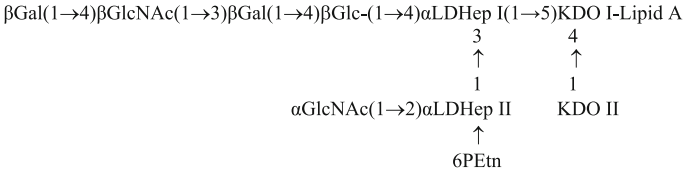
C. Oligosaccharide structure of LOS from thrombotic meningoencephalitis isolate *H. somni* strain 8025 (St Michael et al. 2006).



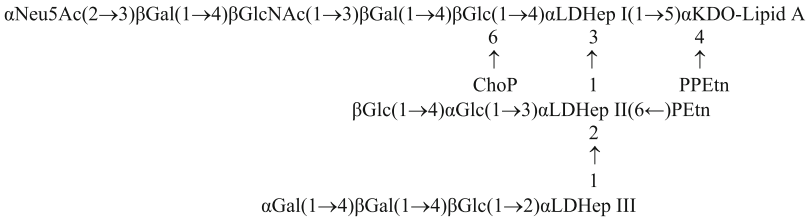
D. Oligosaccharide structure of LOS from pneumonia isolate *H. somni* strain 2336 (St Michael et al. 2005).



E. Oligosaccharide structure of LOS from *H. somni* phase variant strain 738, an LOS phase variant derived from pneumonia isolate strain 2336 (Cox et al. 1998). An additional form of this LOS contained a β-Glc II adjacent to β-Glc I.



F. Oligosaccharide structure of LOS from *N. meningitidis* strain M986 (Tsai et al. 2009). Oligosaccharide structures of other strains may be different.



G. Oligosaccharide structure of sialylated LOS from *H. influenzae* strain RM118 (Cox et al. 2002). Oligosaccharide structures of other strains may be different.

Fig. 2 Oligosaccharide structures of *H. somni* disease and commensal isolates, *N. meningitidis* strain M986, and *H. influenzae* strain RM118. KDO, 3-deoxy-D-*manno*-2-octulosonic acid; LDHep, L-*glycero*-D-*manno*-heptose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetyl-5-neuraminic acid; PEtn, phosphoethanolamine; PPEtn, pyrophosphoethanolamine; ChoP, phosphorylcholine. **a** Oligosaccharide structure of LOS from preputial isolate *H. somni* strain 129Pt (St Michael et al. 2004). **b** Oligosaccharide structure of LOS from preputial isolate *H. somni* strain 1P (Cox et al. 2003). **c** Oligosaccharide structure of LOS from thrombotic meningoencephalitis isolate *H. somni* strain 8025 (St Michael et al. 2006). **d** Oligosaccharide structure of LOS from pneumonia isolate *H. somni* strain 2336 (St Michael et al. 2005). **e** Oligosaccharide structure of LOS from *H. somni* phase variant strain 738, an LOS phase variant derived from pneumonia isolate strain 2336 (Cox et al. 1998). An additional form of this LOS contained a β-Glc II adjacent to β-Glc I. **f** Oligosaccharide structure of LOS from *N. meningitidis* strain M986 (Tsai et al. 2009). Oligosaccharide structures of other strains may be different. **g** Oligosaccharide structure of sialylated LOS from *H. influenzae* strain RM118 (Cox et al. 2002). Oligosaccharide structures of other strains may be different

acid (Neu5Ac; sialic acid), which is also commonly expressed on mammalian cell surfaces (see below).

3 *Histophilus somni* Lipooligosaccharide Phase Variation and Phase Variable Components

3.1 Evasion of Host Defenses by LOS Antigenic Phase Variation

Some mucosal pathogens, including *Haemophilus* spp., *Neisseria* spp., *Pasteurella* spp., and others, such as H. *somni*, lack O-antigen, but have a very complex core oligosaccharide. Like *H. influenzae*, some of the genes that encode for *H. somni* LOS glycosyltransferases contain VNTR immediately downstream of the gene's start codons or within the ORF (Inzana et al. 1997; McQuiston et al. 2000; Wu et al. 2000). Regardless of their location, these VNTR can be responsible for slip-strand mispairing (Levinson and Gutman 1987), potentially placing the gene out of frame and resulting in translation of a truncated, nonfunctional protein. Of particular interest is that the phase-variable glycosyltransferase genes consist of both 4-bp and 2-bp VNTR, and the encoded proteins have amino acid similarity to glycosyltransferases of *H. influenzae.* In one gene (*lob1*), the VNTR are identical to those in three glycosyltransferase genes of *H. influenzae* (5′-CAAT-3′) (Inzana et al. 1997), but in other genes the VNTR are different (Elswaifi et al. 2009; Wu et al. 2000). Therefore, either convergent or divergent evolution has occurred from a common ancestor of *H. influenzae* and *H. somni.* Such phase variation is random, but occurs in about 12 % of the population (Inzana et al. 1997). However, some commensal preputial isolates, such as strain 129Pt, lack VNTR in LOS glycosyltransferases and do not undergo LOS phase variation (Inzana et al. 1997).

Of greatest significance is that this structural variation is also associated with antigenic variation. Probing colonies of a single *H. somni* strain with monoclonal antibodies to the LOS of *H. influenzae, H. aegyptius,* or *N. gonorrhoeae* will identify reactive *H. somni* colonies, and the electrophoretic profile of those reactive colonies is qualitatively distinct from the LOS profile of the parent (Inzana et al. 1997). For example, *H. somni* strain 738 is derived from a single colony of strain 2336 that was used for respiratory challenge of a calf, and recovered from that calf (Gogolewski et al. 1987). Strain 738 has been confirmed to be the same strain as strain 2336 by pulsed field gel electrophoresis (unpublished), but the LOS structure changed in several ways (described above). Antigenic phase variation likely contributes to the persistence of the bacterium in response to the host immune response. Following experimental challenge of three calves with strain 738 (Gogolewski et al. 1987), isolates recovered weekly from bronchial washes of each calf occasionally underwent LOS phase variation, as determined by a qualitative alteration in their electrophoretic profile by SDS-PAGE (Inzana et al. 1992). Furthermore, when the

LOSs of these isolates were tested for reactivity with serum obtained at weekly intervals from these calves by ELISA and Western blotting, the antibodies in the sera only reacted with LOSs from isolates recovered two or more weeks earlier. In calves that cleared their infection by the end of the experiment (10 weeks), antibodies in the serum at that time period reacted with LOSs of all isolates. In contrast, antibodies from serum obtained during the last few weeks from calves that had not cleared their infection did not react with LOSs from the last few isolates, but did react with earlier isolates (Inzana et al. 1992). Therefore, it appeared that once an antibody response was made to a particular LOS antigen, bacteria expressing that antigen were cleared, whereas phase variants that were antigenically distinct and to which antibodies were not yet made survived and gave rise to a new population. Once antibodies were made against all potential phase variants, the host could then begin to clear *H. somni* from the lungs (Inzana et al. 1992). However, it is not clear whether only an antibody response is needed to clear—*H. somni* from the lungs and other tissues—or if a cellular immune response also contributes to clearance of the bacterium from some host sites. Furthermore, passage of a single colony of *H. somni* daily for 2 weeks consistently results in a population that has undergone LOS phase variation (Inzana et al. 1992). Therefore, it is important not to pass *H. somni* in vitro, but when passage is necessary a large volume of colonies should be used. A preferred phenotype appears to be relatively stable, and we have not had unintentional phase variation of a strain following large volume transfer of colonies or during growth in broth.

3.2 Molecular Basis of LOS Phase Variation

The *H. influenzae* genes *lic1, lic2, and lic3* encode for enzymes responsible for attaching glycoses or ChoP to the outer core of the LOS and are phase variable due to tandem VNTR of $(5'\text{-CAAT-}3')_n$ (see Introduction and (Maskell et al. 1991)). Southern blotting of the genome of *H. somni* strain 2336 using a probe of $(5'\text{-CAAT-}3')_n$ identified a region with an identical repeat. Sequencing of this gene showed that Lob1 has the greatest amino acid homology (59 %) to the *H. influenzae* glycosyltransferase Lex2B (McQuiston et al. 2000), although later genome sequencing indicated that Lob1 also has 52 % amino acid homology with a glycosyl transferase from *Actinobacillus succinogenes* (Siddaramappa et al. 2011). As for *H. influenzae,* the VNTR in *lob1* are located immediately downstream of putative start codons. Sequencing of regions containing these VNTR from distinct colonies showed that the number of VNTR can vary between 31 and 35, but that 94 % of colonies contain 33 VNTR, which places the gene in frame with the second (S2) of two potential upstream start codons. When 32 or 35 VNTR are present, the ORF stops shortly after the last 5′-CAAT-3′ repeat if the S2 start codon is used, which was supported by an altered LOS electrophoretic profile in such colonies. Repeated attempts to mutate *lob1* were unsuccessful, but *lob1* was cloned into shuttle vector pLS88 and transformed into strain 129Pt, which has not been demonstrated to phase

vary. Following daily passage of single colonies of two clones of strain 129Pt expressing Lob1 [129Pt(pLSlob1-33)], both clones underwent phase variation within one week of passage, but the parent strain containing only the shuttle vector did not. Following gel electrophoresis of 129Pt(pLSlob1-33) LOS there was an increase in the amount of one of three bands compared to the parent, but phase variants appeared that were deficient in one or two bands. Chemical analysis, particularly gas chromatography–mass spectrometry, nano-electrospray–mass spectrometry (nES-MS), and nuclear magnetic resonance (NMR), identified the presence of additional galactose in the LOS of the recombinant strain (McQuiston et al. 2000). Therefore, Lob1 is a putative phase-variable galactosyl transferase.

Following identification of *lob1* additional sequencing identified *lob2ABCD* adjacent to *lob1*, but on the opposite DNA strand. The *lob2ABCD* genes also encode for putative glycosyl transferases with the greatest homology to glycosyl transferases in the *Pasteurellaceae* and *Neisseria* spp. The genes *lob2ABCD* reside in a locus that is transcribed together. Unlike the location of the VNTR in *lob1*, *lob2A* contains 18-20 5′-GA-3′ VNTR 141 bp upstream of the termination codon within the ORF (Wu et al. 2000). Twenty VNTR of 5′-GA-3′ were found to be most common in individual colonies and correlated with the typical LOS electrophoretic profile. However, when 19 VNTR are present, translation of the ORF is predicted to stop 1 bp after the last repeat. *lob2A* was able to be deleted by allelic exchange, which was the first *H. somni* gene mutated (Wu et al. 2000). Strain 738Δ*lob2A* has an altered electrophoretic profile, predominately a deficiency in production of the two highest molecular size bands, which is similar between several clones of *lob2A* mutants and a clone with 19 5′-GA'3′ VNTR. Cloning of *lob2A* in pLS88 and expression in strain 129Pt *in trans* [129Pt(pLSlob2A)] resulted in a recombinant strain with an increase in molecular size of the largest mass unit. Chemical analysis (nES-MS and NMR) demonstrated that 738Δlob2A lacked *N*-acetylglucosamine (see structure of strain 738 LOS above), and that 129Pt(pLSlob2A) contained additional *N*-acetylglucosamine (Wu et al. 2000). Therefore, Lob2A was confirmed to be an *N*-acetylglucosamine glycosyltransferase. The gene *lob2B* is almost identical in sequence to *lob2A* and also has the same $(5'\text{-GA-}3')_n$ VNTR in the same location as *lob2A*. Therefore, *lob2B* is likely a backup gene to *lob2A*, suggesting that the presence of *N*-acetylglucosamine is important to the function of *H. somni* LOS. Glycosyl transferases with the greatest homology to Lob2AB are found in *Aggregatibacter actinomycetemcomitans* (Siddaramappa et al. 2011). Little is known of *lob2C* except that it is the only gene in this locus that does not contain any VNTR, and Lob2C is most similar (61 %) to a protein from *P. multocida* (Siddaramappa et al. 2011). It is tempting to propose that Lob2C may attach the first glucose to the inner core heptose, because this sugar is required for extension of the core and for the attachment of phosphorylcholine (see below), and therefore, this primary glucose should not phase vary. *lob2D* has VNTR of $(5'\text{-CAGT-}3')_n$ within the body of its ORF and is the first phase-variable gene shown to contain a 4-bp repeat within its ORF. *lob2D* is truncated in strain 2336 (157 amino acids) and has only 18 VNTR. Based on structural differences, *lob2D* may attach galactose to the end of the primary chain of the oligosaccharide or GlcNAc to the secondary

heptose (see LOS structures of strains 2336 and 129Pt above). The more truncated oligosaccharide in stain 129Pt LOS can be explained by the presence of only the 5′ ends of *lob2A* and *lob2C* and the complete absence of *lob2B* (Siddaramappa et al. 2011).

3.3 Role of Phosphorylcholine on LOS

Phosphorylcholine (ChoP) is a phase-variable component present on the LOS of *H. influenzae*, *H. somni*, and some other bacteria. In *H. influenzae,* four genes are responsible for the synthesis and placement of ChoP: *lic1ABCD* (Lysenko et al. 2000; Weiser et al. 1997). Lic1A is a choline kinase and its gene contains the VNTR $(5'\text{-CAAT-}3')_n$ immediately downstream of putative start codons. Lic1B is most similar to choline permease; Lic1C is a pyrophosphorylase; Lic1D is proposed to be a diphosphonucleoside choline transferase and is responsible for transferring ChoP to a specific glycose on the LOS outer core. Polymorphisms in *licD* between strains affect which heptose extension ChoP is added to (Lysenko et al. 2000). ChoP has been proposed to contribute to adherence of *H. influenzae* to epithelial cells of the upper respiratory tract by binding to the receptor for platelet-activating factor present on cell surfaces (Swords et al. 2000; Weiser et al. 1998). However, in the presence of calcium, C-reactive protein (CRP) can bind to the ChoP epitope and activate complement through the classical pathway, and is bactericidal for *H. influenzae* (Weiser et al. 1998). Therefore, it appears that the bacteria express ChoP to aid in colonization of the host upper respiratory tract, but if they enter the bloodstream phase variants that do not express ChoP will have a survival advantage. These experiments were done in an infant rat model and with human bronchial epithelial cells.

H. somni strain 738, but not strain 2336 (Fig. 2e, d, respectively), also expresses phase-variable ChoP, but on the primary glucose of the outer core and is only accessible to specific antibody if most of the outer core is not synthesized (Howard et al. 2000). Homologs of *lic1ABCD* are present in *H. somni*, but the $lic1A_{Hs}$ homolog (beginning at genome coordinate 619615) in strain 2336 contains 25 VNTR of 5′-AACC-3′ at the same location as the 5′-CAAT-3′ VNTR in *H. influenzae lic1A* (Elswaifi et al. 2009). Twenty-five VNTR would place $lic1A_{Hs}$ out of frame in strain 2336, whereas in strain 738 there are 24 VNTR of 5′-AACC-3′ in $lic1A_{Hs}$, the gene is in frame, and ChoP is present on the LOS (Fig. 2e). Therefore, in order for ChoP to be accessible to host sites $lic1A_{Hs}$ must be in frame and ChoP expressed on the LOS, and the oligosaccharide chain extension beyond the primary glucose must be truncated. The gene $lic1A_{Hs}$ was amplified from strain 738, cloned into an expression vector, the VNTR removed, and the plasmid amplified by PCR to contain $lic1A_{Hs}$ lacking the VNTR region and the gene being constitutively "on." When cloned into *E. coli,* functional enzyme assays confirmed $lic1A_{Hs}$ encodes for a choline kinase and is functional without the 5′-AACC-3′ VNTR (Elswaifi et al. 2009). Therefore, as for the 5′-CAAT-3′ VNTR in *H. influenzae lic2B* (High et al.

1996), the VNTR in the genes responsible for synthesis of *H. somni* LOS act only as a molecular switch, even when present within the ORF.

In the natural bovine host, Elswaifi et al. (2012) has shown that an *H. somni* clone predominately expressing ChoP colonizes the upper respiratory tract of challenged calves efficiently, but most isolates recovered from systemic sites, particularly the lungs and heart, no longer express ChoP. Likewise, after challenge of calves with a clone predominately not expressing ChoP most isolates recovered from the upper respiratory tract now express ChoP, whereas those from systemic sites remain ChoP negative (Elswaifi et al. 2012). Furthermore, more cells of the clone expressing ChoP adhered to bovine turbinate cells than cells from the clone not expressing ChoP. In addition, *H. somni* cells expressing ChoP on their LOS, but not ChoP-negative cells, efficiently aggregated bovine platelets, apparently through ChoP binding to bovine platelet-activating factor receptor (Kuckleburg et al. 2007). Therefore, ChoP on the LOS, along with likely pili and other factors, contributes to *H. somni* colonization of the bovine upper respiratory tract, but the ChoP moiety is phase variable to avoid complement-mediated bactericidal activity in the bloodstream and systemic sites. In strain 129Pt, there are 41 VNTR of 5′-AACC-3′ in $lic1A_{Hs}$, but the gene is interrupted by an IS1016 insertion sequence 61 bp downstream of the VNTR (Elswaifi et al. 2009).

4 *Histophilus somni* Lipooligosaccharide Sialylation

4.1 Sialic Acid Metabolism and Decoration of the H. somni Surface with Sialic Acid

Both *H. influenzae* and *H. somni* scavenge Neu5Ac from the host or growth medium, catabolize it, and add it to cytidine-5′-monophosphate (CMP) through CMP-Neu5Ac synthetase. Neu5Ac can then be transferred to terminal galactose residues on the cell surface by sialyltransferases (Inzana et al. 2002; Vimr et al. 2000). In contrast, *N. gonorrhoeae* utilizes CMP-Neu5Ac directly from its environment to transfer Neu5Ac to terminal galactose residues on the LOS because it is unable to synthesize CMP-Neu5Ac (Vimr and Lichtensteiger 2002). In the presence of Neu5Ac or CMP-Neu5Ac, all terminal galactose residues on most *H. somni* disease isolates can be sialylated. In contrast, commensal preputial isolates that have been tested thus far are unable to sialylate their cell surface. Sialylation of the LOS can easily be detected by an increase in molecular size of the largest size bands by SDS-PAGE (Inzana et al. 2002). All the genes necessary for LOS sialylation are present in *H. somni*, including two sialyltransferases, a CMP-*N*-Neu5Ac synthetase, and an *N*-Neu5Ac lyase. However, the sialyltransferase and synthetase genes in commensal strain 129Pt are either absent or truncated. All the genes necessary for sialic acid metabolism are also present, including in strain 129Pt (Howard et al. 2011). Terminal galactose residues on both the LOS (St Michael et al. 2005) and the biofilm exopolysaccharide (EPS) (Sandal et al. 2011) can be sialylated. When

the EPS is sialylated, the biofilm is much thicker, smoother, and more robust (Sandal et al. 2011). Therefore, it appears that any terminal galactose can be sialylated. A *lob2A* mutant that is unable to attach the terminal βGal II (1 → 4)βGlcNAc disaccharide on the LOS can still have its primary galactose sialylated (see structure above) when the bacteria are grown in the presence of Neu5Ac (Inzana et al. 2002). However, if *H. somni* is grown in vitro in the absence of Neu5Ac, little or no sialic acid is present on the cell surface. Therefore, investigators studying pathogenesis or vaccine development need to be aware that if *H. somni* is grown in the absence of sialic acid, then the bacteria will not have the same phenotype they have in the host, where the bacterial cell surface is well sialylated.

4.2 Adaptation of Sialyltransferases to Phase Variation

Unlike *H. influenzae*, in which two sialyltransferase genes (*lic3A* and *lic3B*) contain VNTR of the tetra nucleotide 5′-CAAT-3′ and is subject to phase variation (Apicella 2012), VNTR have not been found in any *H. somni* genes involved in sialylation of the cell surface (Siddaramappa et al. 2011). Therefore, sialylation of *H. somni* is not subject to phase variation. However, two LOS phase variants (strains 2336 and 738) differ in the linkage of their terminal galactose residue. Strain 738 expresses a lacto-*N*-tetraose with a terminal lacto-*N*-biose (βGal (1 → 3)βGlcNAc) disaccharide, whereas strain 2336 expresses lacto-*N*-neo-tetraose with a terminal *N*-acetyllactosamine (βGal(1 → 4)βGlcNAc) disaccharide. Two sialyltransferase genes have been identified in the *H. somni* strain 2336 genome (Howard et al. 2011). Using capillary electrophoresis of aminopyrene trisulfonic acid (APTS)-labeled oligosaccharides, one sialyltransferase preferentially sialylates the galactose residue with the βGal(1 → 3) linkage present in strain 738, and the other sialyltransferase preferentially sialylates the galactose residue with the βGal(1 → 4) linkage present in strain 2336. Therefore, although the sialyltransferases are not subject to phase variation, the bacterium makes a sialyltransferase that can sialylate the terminal galactose regardless of its linkage, which does appear to be subject to phase variation.

4.3 Effect of Lipooligosaccharide Sialylation on Resistance to Host Defenses

Disease isolates of *H. somni* have been shown to be more serum resistant, and hence potentially more virulent, than some commensal isolates from preputial sites (Corbeil et al. 1985). One reason for this enhanced serum resistance in disease isolates is the capability to sialylate their LOS (Inzana et al. 2002). When Neu5Ac is added to the growth medium and the LOS is sialylated less monoclonal antibody

5F5.9 (specific for the ChoP epitope on LOS) and less monoclonal antibody 3F11 (specific to the Galβ-(1-4)-GlcNAc epitope) can bind to the bacteria (Inzana et al. 2002), as well as IgG in normal bovine serum (Inzana et al. 2012). Furthermore, although antiserum to LOS is bactericidal for *H. somni*, bacteria that have been grown in the presence of sialic acid are significantly more resistant to killing by normal bovine serum, whereas strains that cannot sialylate their LOS are susceptible to killing by complement only and are not more resistant when grown with Neu5Ac (Inzana et al. 2002). Subsequent studies also showed that when the LOS is sialylated *H. somni* is significantly less susceptible to the bactericidal activity of pre-colostral calf serum (antibody-free complement source) by the alternative complement pathway. Sialylated *H. somni* LOS activates and consumes less complement. This diminished consumption of complement is likely due to the fact that sialylated *H. somni* binds more factor H and iC_3b, and less C_3, than non-sialylated bacteria (Inzana et al. 2012). In addition to binding less complement, sialylated *H. somni* cells bind less specific antibody in normal bovine serum, are less efficiently phagocytosed by bovine polymorphonuclear leukocytes, and those that are phagocytosed are less efficiently killed by these cells (Inzana et al. 2012). Following incubation of sialylated LOS with bone marrow-derived macrophages from C57BL/6 mice, the chemokine response (MIP-2) is significantly lower than from macrophages incubated with desialylated LOS, and as for other endotoxins, *H. somni* LOS signals through TLR4. Furthermore, sialylated LOS is significantly less active in stimulating an NF-κB response from bone marrow-derived macrophages than desialylated LOS (Howard et al. 2011). Therefore, sialylation of the *H. somni* LOS provides the bacteria with a substantial advantage in resistance to host innate immune defenses and is likely a major contributor in the ability of strains that can sialylate their cell surface to cause lower respiratory tract and systemic diseases.

5 Summary

The LOS of *H. somni* is remarkably similar to the LOSs of *H. influenzae* and *N. gonorrhoeae*. The LOS oligosaccharide outer core of disease isolates interacts with host cell surfaces and has multiple mechanisms to resist host defenses in systemic sites. The outer core oligosaccharide structure mimics oligosaccharide structures on glycosphingolipids of mammalian cells to minimize host response. However, the oligosaccharide can also undergo random structural and antigenic phase variation to avoid the antibody response made. In addition, *H. somni* can attach phase-variable ChoP and sialic acid to the LOS. Expression of ChoP on *H. somni* enhances colonization of the bacteria to the upper respiratory tract, whereas when ChoP phases off the bacteria may be more resistant to complement-mediated bactericidal activity in systemic sites. Sialylation of terminal galactose residues on the LOS further contributes to evasion of host defense systems, including reduced antibody binding, resistance to complement-mediated killing, and reduced phagocytosis and intracellular killing. In contrast, the biological activity of the LOS resides in the

lipid A component. Although not as biologically active as the endotoxin of *E. coli*, it is nonetheless proinflammatory, signals through TLR4, induces apoptosis in epithelial cells, and likely contributes to, if not a major player in, the vasculitis and inflammatory processes typically characteristic of *H. somni* diseases.

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Exopolysaccharide Production and Biofilm Formation by *Histophilus somni*

Briana Petruzzi and Thomas J. Inzana

Abstract The biofilm matrix of *Histophilus somni* is a complex architecture that differs substantially in structure between a pathogenic and commensal isolate examined. Overall, most pathogenic isolates produce more biofilm than commensal isolates. A major component of the biofilm is exopolysaccharide (EPS), which is also produced in greater quantity in the pathogenic isolate than in the commensal isolate studied. The EPS is composed of a D-mannan polymer, with occasional galactose residues present on side chains, similar in composition to that of yeast mannan. When grown in the presence of sialic acid, the biofilm EPS becomes sialylated and the amino sugars *N*-acetylglucosamine and *N*-acetylgalactosamine can be detected. In vitro biofilm formation follows a typical 4-stage growth curve, characterized by attachment, growth, maturation, and detachment. Following experimental challenge, formation of an *H. somni* biofilm has been demonstrated in cardiopulmonary tissue, often with *Pasteurella multocida* cohabitating the biofilm. A recently developed diagnostic test can detect antibodies to the EPS only in animals with systemic disease due to *H. somni* and is therefore capable of distinguishing between healthy animals colonized with *H. somni* and animals with systemic disease.

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1 *Histophilus somni* Biofilm Formation and Its Relevance During Infection

1.1 An Introduction to Biofilm Formation

Biofilms formed following an infection within the host are a complex matrix of bacterial cells, host cells, exopolysaccharide (EPS), nucleic acid, trapped nutrients and water, enzymes, and protein. Due to the advances in microbiological research, it is now clear that most bacterial species have the capacity to form a biofilm. Planktonically grown bacteria in rich media are poor models for studying disease, since this does not represent the way bacteria most commonly interact within the host. During infection, bacteria are exposed to the host environment, which harbors antibodies and phagocytic cells. Bacterial populations living within a biofilm are tightly adhered to a surface, where they are provided with a constant source of nutrients and are protected by the matrix. Once planktonic bacteria are shed from the biofilm, they can travel through the host to find a new surface to colonize (Kuramitsu et al. 2007). Biofilm formation is an essential survival mechanism utilized by bacteria associated with chronic or otherwise persistent infections (Bjarnsholt 2013; James et al. 2008). Therefore, it is not surprising that *H. somni* is capable of forming a biofilm and can do so in its only habitat: mucosal surfaces and systemic sites of cattle and sheep. In vitro, *H. somni* always grows as a biofilm in stationary or in slowly rotating broth cultures with little or no headspace (Sandal et al. 2011). Biofilm formation by other bacterial pathogens of bovine respiratory disease (BRD), such as *Mannheimia haemolytica* (Boukahil and Czuprynski 2015) and *Pasteurella multocida* (Olson et al. 2002), has also been described. However, *H. somni* biofilm formation is more prominent in myocardial tissue than in pulmonary tissue, suggesting that biofilm formation can be associated with any systemic infection as well as with BRD (Sandal et al. 2009).

1.2 Polymicrobial Relationships Within Biofilm

Biofilms in nature are often polymicrobial, and there may be advantages to bacteria in polymicrobial relationships (Ehrlich et al. 2005). Within a polymicrobial biofilm, the genetic diversity of the population is increased. In the same way that planktonic growth does not often represent bacteria in their natural environment, single species biofilms may also not be representative. Genetically diverse populations are more likely to be resistant to a broader range of antibiotics, they may be more metabolically diverse and be better protected from host defenses or the external environment (Ehrlich et al. 2005; Harriott and Noverr 2009; Molin and Tolker-Nielsen 2003; Weigel et al. 2007). BRD is often described as being a polymicrobial infection (Gagea et al. 2006), which further suggests the potential importance of biofilm formation to disease progression (Corbeil 2007). Isolation of more than one causative bacterium or virus from a BRD infection is common, which may be the evidence of a polymicrobial etiology. For example, *P. multocida* and other BRD pathogens have been isolated from *H. somni* pneumonia (Elswaifi 2012; Gogolewski et al. 1987) in calves that tested negative for respiratory pathogens prior to experimental challenge. Other reports have indicated relationships between *H. somni* and Bovine Respiratory Syncytial virus (BRSV) during BRD, and that this relationship correlates with disease severity (Agnes et al. 2013; Gershwin 2007; Gershwin et al. 2005).

1.3 Biofilm Formation During Respiratory Infection and Septicemia

Infections involving biofilms can occur throughout host tissues and have been identified in dental caries (Marsh 2006; Nyvad et al. 2013), osteomyelitis (Johansen et al. 2012), endocarditis (Hussain et al. 2015), and otitis media (Bakaletz 2012; Nguyen et al. 2012; Puig et al. 2014), to name a few. Subclinical symptoms allow infections to go undetected, which is common in bovine chronic pneumonia. Calves with subclinical infections can be difficult to identify in herds and can contribute to disease transmission (Francoz et al. 2015; Griffin 2014). Experimental infection of calves with *H. somni* via the respiratory tract can result in the biofilm formation within the myocardium and the pulmonary tissues. The biofilm found in the myocardium is more prominent than the biofilm observed within the lungs, which would correlate with the more prominent formation of biofilm during growth in tissues with reduced levels of oxygen (Sandal et al. 2009). Of significance is that *P. multocida* is often isolated with *H. somni* from BRD infections, in which biofilm is present (Gogolewski et al. 1987; Sandal et al. 2009).

2 Differences in Biofilm Structure Between Pathogenic and Commensal Isolates

2.1 Methods of Studying Biofilm Formation Reviewed

H. somni appears to prefer the biofilm lifestyle forms a biofilm in vitro when grown under environmental conditions with low oxygen availability (non-shaken cultures or in flasks filled with medium) or NaCl concentrations above that of saline (Sandal et al. 2011). Thus, stressful, poor growth conditions under which relatively few cells are present result in the greatest amount of biofilm. For example, low oxygen conditions result in increased biofilm growth, while aerobic conditions result in little to no biofilm formation. Although *H. somni* is a facultative anaerobe, growth is poor anaerobically, resulting in exopolysaccharide (EPS) formation and a biofilm. Low oxygen conditions can be simulated experimentally using a flask sealed and filled with broth medium, with minimal shaking (50 or less rotations per minute) in order to reduce aeration and distribution of nutrients. *H. somni* biofilm growth is most pronounced during the late stationary phase of grow, which represents a period of decreased availability of nutrients.

Continuous flow cell systems provide an almost natural environment for biofilm formation to occur. In such a flow system, there is a continuous supply of nutrients across the developing biofilm, allowing biofilms to be sustained for longer periods of time. The resulting biofilms are most commonly analyzed by microscopy: confocal scanning laser microscopy (CLSM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). These microscopy techniques can be used to analyze the thickness, biomass, substratum coverage, surface-area-to-volume ratio, architecture, viability, over-all morphology, and other characteristics of the biofilm that may be essential for their function. These techniques were used to show that the biofilm of pathogenic strain 2336 was far more robust than the biofilm of commensal strain 129Pt under in vitro growth conditions.

2.2 The H. somni Biofilm Life Cycle

Four distinct stages of *H. somni* biofilm growth occur in vitro over seven days when grown under continuous flow conditions. Stage one involves attachment of the cells to a surface. During stage two, growth/multiplication occurs after approximately three days. The third stage corresponds to maturation, which occurs in five-day-old biofilms. Stage four is the detachment of some planktonic cells, and occurs by the time biofilms are seven days old (Sandal et al. 2007). Details of the stages of biofilm development in *H. somni* are described below:

Stage 1. Attachment: Sparse cell aggregates adhering to the abiotic or biotic surface are typical during the attachment stage. Adhesion proteins (such as Fha and type IV pili) are essential at this stage in the biofilm life cycle. The cell aggregates are composed predominantly of live cells, as determined by CSLM live/dead staining and are not yet surrounded by a substantial extracellular matrix (ECM).

Stage 2. Growth: Cell clusters predominantly of live cells increase in size during this stage. Biofilms are still relatively sparse. Thickness, biomass, and substratum coverage are still increasing in size. The surface-area-to-volume ratio is high, as is to be expected in early biofilms.

Stage 3. Maturation: During maturation, the biofilms reach their maximum thickness and most of the cells within the biofilm are still living. The surface-area-to-volume ratio is low and the architecture of the biofilm is most complex. In *H. somni*, maturation of biofilms for all strains tested is approximately five days in vitro.

Stage 4. Detachment: At this time, large microcolonies start to disperse individual planktonic cells in order to colonize a new location. The biofilm is composed primarily of dead cells and there is an overall decrease in substratum coverage, mean thickness, and biomass.

2.3 Comparison of Biofilm Formation Between Strains 2336 and 129Pt

During attachment, microcolonies of strain 2336 are present in greater quantity than strain 129Pt. The early architecture of strain 2336 biofilm forms a structure of large microcolonies interconnected by an ECM, which is not present in the biofilm of strain 129Pt. Strain 129Pt microcolonies are smaller and more elongated, with little connective ECM visible. The fully mature biofilms of strains 2336 and 129Pt display the most distinct differences. Strain 2336 has a mean thickness one hundred times greater than the mean thickness of strain 129Pt. The mature biofilm of strain 2336 is a complex structure of microcolonies with visible water channels seen in the ECM. The substratum coverage is decreased while the surface-area-to-volume ratio, mean thickness, and mean biomass are increased. Mature biofilms formed by strain 129Pt display almost the opposite features: increased substratum coverage and a decrease in surface-area-to-volume ratio. The biofilm architecture of 129Pt is composed of tower-shaped microcolonies intertwined with strands of EPS. The roughness coefficient 'r' is significantly different between strains 2336 ($r = 0.1$) and 129Pt ($r = 2$). However, during the detachment stage, both strains contain primarily dead cells in their matrices.

EPS production is significantly greater in pathogenic strain 2336 than commensal strain 129Pt, particularly if sialic acid is added to the medium. This is expected, since the average biofilm thickness of strain 2336 is approximately 100 times greater than that of strain 129Pt. Assays using crystal violet staining to measure the amount of biofilm formed by various strains indicated that most isolates from systemic sites formed more biofilm than isolates from genital sites, suggesting that biofilm formation is important in resistance to systemic host defenses and correlated inversely with the roughness coefficient. Biofilms that have a roughness coefficient closer to 0 are considered to be smoother. Smooth biofilms have fewer towers and gaps between microcolonies than biofilms with a roughness coefficient closer to 2. Biofilms that are rougher have higher towers and spaces that are devoid of biofilm matrix. The biofilm of strain 2336 is significantly smoother than the biofilm of strain 129Pt, which may be related to the amount of EPS produced, as EPS functions to coat and connect the components of the biofilm matrix. The bacterial populations within biofilms of both strains grow at an equal pace, indicating they are both able to survive and thrive in this lifestyle (Sandal et al. 2007).

2.4 Gene Products Essential for Biofilm Formation

Random mutagenesis of strain 2336 with the EZ::Tn5^{TM}(KAN-2)Tnp Transposome^{TM} (Epicentre) has identified genes whose products are important for biofilm formation, in addition to those already described for EPS production and export (Sandal et al. 2009). Mutations in IbpA, which has homology to the very large molecular size surface proteins of *Bordetella pertussis* (Fha) (Villarino Romero et al. 2014), *Haemophilus ducreyi* (LspA) (Ward et al. 1998), and others results in mutants that are biofilm deficient, indicating that Ibpa plays a role in biofilm formation. Since IbpA is an important adhesin in those *H. somni* strains in which it has been studied, it likely contributes to attachment during the initial stage of biofilm formation, resulting in less biofilm or one that takes much longer to form. Furthermore, expression of ibpA *H. somni* is increased to fourfold during biofilm formation in comparison to bacteria grown plank tonically (Sandal et al. 2009). However, further research is needed to confirm the role of IbpA in *H. somni* biofilm formation virulence. A transposon mutation in the Major Facilitator Superfamily 1 (MFS_1) protein increased biofilm formation by an unknown mechanism that warrants further study (Sandal et al. 2009).

3 *Histophilus somni* Exopolysaccharide Identification and Production

3.1 *H. somni* Exopolysaccharide Production

Unlike many of the Pasteurellaceae, *H. somni* does not produce a capsular polysaccharide (CP). However, *H. somni* does produce an EPS, which is a major component of the biofilm matrix. A CP remains closely associated with the individual bacterium and remains adherent to the cell surface through an outer membrane-associated lipid anchor (Gotschlich et al. 1981). In contrast, EPS is released from the bacterial cell, does not maintain close contact with the cell surface, and therefore can envelop a large community of bacteria that share in the EPS, as well as proteins and extracellular DNA, which together make up the architecture of the biofilm matrix material.

The EPS produced by *H. somni* has been characterized as a highly branched α-(1 → 6)-*p*D-mannan polymer. The comb-like branches are composed of mannopyranose units attached to the backbone at C-2. C-3 branches extend from the C-2-substituted mannose residues, creating trisaccharide lateral chains. Occasional galactose residues are also found at the non-reducing ends of the mannose side chains creating tetrasaccharide branches (Fig. 1). As described for the lipooligosaccharide (LOS) (see Chapter "The Many Facets of Lipooligosaccharide as a Virulence Factor for Histophilus somni"), the terminal galactose residues can be sialylated in the presence of *N*-acetyl-5-neuraminic acid (Neu5Ac), making the biofilm thicker and more prominent (Sandal et al. 2011). Although the structure of the EPS is similar to yeast mannan, the *H. somni* EPS can be isolated from growth

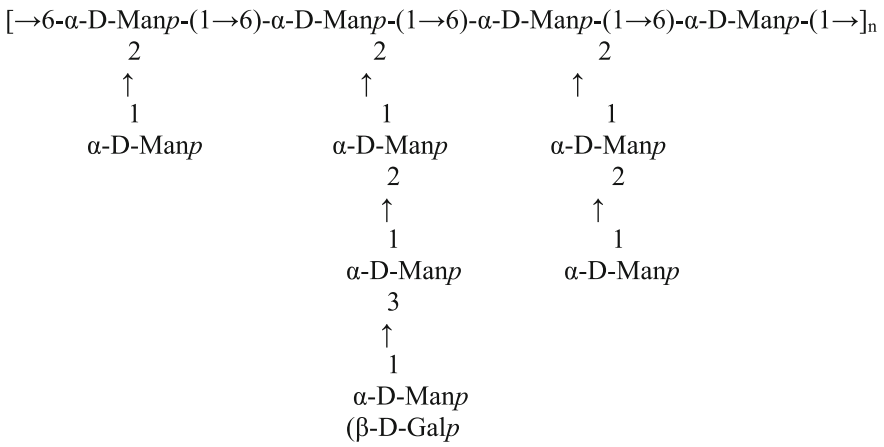

Fig. 1 Structure of the EPS of *H. somni* strain 2336 (Sandal et al. 2011). The *H. somni* EPS backbone is composed of *p*D-mannan residues, with branching *p*D-mannose and *p*D-galactose residues

medium lacking any yeast extract and the biofilm matrix specifically reacts with fluorescein-conjugated *Moringa* M lectin (specific for mannose). Therefore, the EPS is not derived from the bacterial growth medium (Sandal et al. 2011).

Proteins are also present in the *H. somni* biofilm matrix and may comprise approximately 50 % of its dry weight (unpublished data). One protein that has been identified in the biofilm matrix is Hsp60, and antibodies to this protein are effective in inhibiting biofilm formation in vitro (Zarankiewicz et al. 2012). The activity of Hsp60 in the biofilm may have little to do with protein folding, but may be important for ligand binding (Henderson et al. 2013).

3.2 Genes Responsible for Exopolysaccharide Formation

The expression levels of putative EPS-associated genes during *H. somni* biofilm formation and planktonic growth have been determined using quantitative real-time PCR (qRT-PCR). Genes with significant increases in expression have been identified that encode for periplasmic substrate binding proteins and transmembrane constituents of the ribose ABC transporter. These gene products represent domain II of the carbohydrate uptake proteins responsible for transport of monosaccharides and forming the transport system responsible for high-affinity ribose transport. Expression of another putative ABC transporter, *rbs2a*, is eight times greater during biofilm growth than during planktonic growth (Sandal et al. 2011).

Other genes that may be responsible for synthesis of the *D*-mannan polymer have been annotated as *manB*, *galU*, and *csrA*. The *galU* gene is upregulated sevenfold during biofilm growth in comparison with planktonic growth. GalU is a glucose-1-phosphate uridylyltransferase responsible for catalyzing the reversible production of UDP-glucose. GalU is important for the synthesis of many carbohydrate components necessary for glycolipids and proteoglycans and has been associated with biofilm formation in *Haemophilus parasuis* (Zou et al. 2013). *CsrA* encodes a putative carbon storage regulator (LiGuo et al. 2014), while *manB* encodes for a phosphomannomutase homolog responsible for the conversion of mannose-6-phosphate to mannose-1-phosphate (Sandal et al. 2011).

Another gene cluster of approximately 19 kb is predicted to encode for transport proteins, glycosyltransferases, and proteins involved in polysaccharide biosynthesis that are also predicted to be important for EPS and biofilm formation. This cluster includes *pldB*, *ybhA*, *araD*, *sgbU*, *rmpA*, *xylB*, *rbs1C*, *rbs1A*, *rbs1B*, *glsS*, *rbs2A*, *dctP*, *dctM,* and *dctQ*. Between the two loci mentioned, 14 genes are expressed at higher levels during biofilm formation in pathogenic strain 2336. The genes encoding proteins important for the production of EPS are also present in the genome of commensal strain 129Pt. However, only 5 genes are significantly expressed at higher levels during biofilm formation in strain 129Pt (Sandal et al. 2011). The biofilms formed by pathogenic strain 2336 are thicker and display a different architecture than those formed by commensal strain 129Pt. The differences in gene expression between strains 2336 and 129Pt during biofilm formation may

account for their differing biofilm phenotypes (described later in this chapter), but may be well suited for their respective ecological niches (strain 129Pt is restricted to the genital tract, whereas strain 2336 can disseminate and persist in systemic sites).

3.3 Sialylation of the Exopolysaccharide

Terminal galactose residues of *H. somni* LOS, such as the LOS of *H. influenzae* (Mandrell et al. 1992), are sialylated when the bacteria are grown in the presence of Neu5Ac (Howard et al. 2011; Inzana et al. 2012) (see chapter "The Many Facets of Lipooligosaccharide as a Virulence Factor for Histophilus somni"). A small amount of galactose is also present in the EPS, which can also be sialylated when sialic acid is present in the growth medium. When *H. somni* is grown as a biofilm in the presence of Neu5Ac, the density of the EPS increases, sialic acid can be extracted from the EPS, and two amino sugars (*N*-acetylglucosamine and *N*-acetylgalactosamine) are detected in the biofilm of strain 2336 (Sandal et al. 2011). These amino sugars are absent when sialic acid is not present. Strain 129Pt lacks functional sialyltransferases and Neu5Ac-synthetase, and therefore even when grown in the presence of Neu5Ac cannot sialylate its LOS (Inzana et al. 2002) (see following chapter) or EPS, and the amount of biofilm formed is not altered. In addition, expression of the *siaB* gene in strain 2336, which encodes for an α-2, 3-sialyltransferase, is increased 15-fold during biofilm growth in comparison with growth under planktonic conditions, further supporting a role for sialylation in development of a mature biofilm (Sandal et al. 2011). Sialylation also contributes to biofilm formation in other bacteria. Sialic acid, apparently on the LOS, contributes to the integrity of biofilms of non-typable *Haemophilus influenzae* (NTHI), as NTHI is unable to produce a typical EPS (Langereis and Hermans 2013; Swords et al. 2004).

3.4 Diagnostic Application of the Exopolysaccharide

The galactomannan polymer of *H. somni* EPS may not be an obvious antigenic marker as it is composed entirely of simple sugar monomers. However, detection of anti-EPS antibodies by enzyme-linked immunosorbent assay (ELISA) has been shown to be a useful diagnostic assay to differentiate infected from colonized animals (Pan et al. 2014). A hindrance of current serological assays is the inability to distinguish between colonization with commensal isolates, previously infected, or currently infected cattle. Current diagnostic procedures utilize isolation of *H. somni* from a normally sterile site as indication of infection. The EPS diagnostic test is based on the principle that pathogenic isolates form biofilms *in vivo*, but not when growing planktonically, which is how they are normally grown in the laboratory. Furthermore, antibodies are more likely to be produced to foreign antigens in systemic sites than to antigens in sites commonly colonized by many commensal

bacteria, such as mucosal sites. Since EPS is a major component of the biofilm, detection of antibodies to the EPS should indicate the presence of infection rather than colonization. In addition, there is no cross-reactivity between polysaccharides of other common bovine respiratory pathogens and the *H. somni* EPS. In a preliminary study, the EPS diagnostic test had a specificity of 92.5 % and a sensitivity of 90.5 % (Pan et al. 2014). In cases where isolation of the bacteria is difficult or is too time-consuming, the EPS ELISA may help to diagnose and quickly initiate corrective measures and prevent future herd infections by *H. somni*.

4 Concluding Summary

The biofilm matrix of *H. somni* is a complex architecture that differs significantly between a pathogenic and a commensal isolate, with the pathogenic isolate producing more EPS than the commensal isolate. Furthermore, there is a correlation between the amount of biofilm formed and the virulence of the strain. EPS produced during biofilm formation is essential in bridging microcolonies within the biofilm and is responsible for the smooth biofilm formation observed in pathogenic isolates. The EPS is composed of a D-mannan polymer, with occasional galactose residues present on side chains, which is similar to the composition of yeast mannan. The EPS is also the target of a recently developed diagnostic test that is capable of distinguishing between healthy and currently infected bovines with promising results. This test is based on the production of antibodies to EPS in animals during infection, but not during colonization. Two putative genetic loci that are likely responsible for EPS production have been identified. However, whether both loci are necessary for production of the EPS has yet to be determined. The expression of most of the genes in these loci is upregulated during biofilm growth in comparison with planktonic growth. Differences in gene expression are evident during biofilm formation in strains 2336 and 129Pt, which explains the differences in EPS production and biofilm architecture observed in these two strains. When grown in the presence of sialic acid, the EPS of strain 2336, but not the EPS of strain 129Pt, becomes sialylated and the amino sugars *N*-acetylglucosamine and *N*-acetylgalactosamine can be detected within the EPS. In vitro biofilm formation follows a typical 4-stage life cycle (attachment, growth, maturation and detachment) for both strains studied.

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