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Molecular characterization of phosphorylcholine expression on the lipooligosaccharide of *Histophilus somni*

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ABSTRACT

Histophilus somni (*Haemophilus somnus*) is an important pathogen of cattle that is responsible for respiratory disease, septicemia, and systemic diseases such as thrombotic meningoencephalitis, myocarditis, and abortion. A variety of virulence factors have been identified in *H. somni*, including compositional and antigenic variation of the lipooligosaccharide (LOS). Phosphorylcholine (ChoP) has been identified as one of the components of *H. somni* LOS that undergoes antigenic variation. In this study, five genes (*lic1ABCD*$_{Hs}$ and *glpQ*) with homology to genes responsible for ChoP expression in *Haemophilus influenzae* LOS were identified in the *H. somni* genome. An *H. somni* open reading frame (ORF) with homology to *H. influenzae lic1A* (*lic1A*$_{Hi}$) contained a variable number of tandem repeats (VNTR). However, whereas the tetranucleotide repeat 5′-CAAT-3′ is present in *lic1A*$_{Hi}$, the VNTR in *H. somni lic1A* (*lic1A*$_{Hs}$) consisted of 5′-AACC-3′. Due to the propensity of VNTR to vary during replication and cause the ORF to shift in and out of frame with the upstream start codon, the VNTR were deleted from *lic1A*$_{Hs}$ to maintain the gene constitutively on. This construct was cloned into *Escherichia coli*, and functional enzyme assays confirmed that *lic1A*$_{Hs}$ encoded a choline kinase, and that the VNTR were not required for expression of a functional gene product. Variation in the number of VNTR in *lic1A*$_{Hs}$ correlated with antigenic variation of ChoP expression in *H. somni* strain 124P. However, antigenic variation of ChoP expression in strain 738 predominately occurred through variable extension/truncation of the LOS outer core. These results indicated that the *lic1*$_{Hs}$ genes controlled expression of ChoP on the LOS, but that in *H. somni* there are two potential mechanisms that account for antigenic variation of ChoP.
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1. Introduction

Histophilus somni (*Haemophilus somnus*) is a gram-negative coccobacillus that is an important cause of bovine respiratory disease and systemic infections in cattle, including septicemia, thrombotic meningoencephalitis, myocarditis, arthritis, abortion, and others [1]. *H. somni* possesses a variety of virulence factors, including immunoglobulin binding proteins that are similar to high molecular weight filamentous hemagglutinins [2,3], induction of endothelial cell apoptosis [4], survival in phagocytic cells [5], and production of lipooligosaccharide (LOS). *H. somni* LOS is an endotoxin, which can undergo phase variation in composition and structure *in vitro* or in response to a mounting immune response by the host [6,7]. The LOS can also be modified by the incorporation of sialic acid, which is associated with decreased binding by monoclonal antibodies (MAb) to LOS and enhanced resistance to serum killing [8].

The LOS of *H. somni* undergoes a high rate of random antigenic and compositional phase variation, similar to that of *Haemophilus influenzae* LOS [9]. However, serum-sensitive isolates from the urogenital tract do not undergo detectable antigenic variation or do so at a substantially lower rate [6]. Antigenic variation in *H. somni* LOS has been demonstrated in isolates obtained at different time intervals from calves challenged with *H. somni*. This variation correlates with an immune response to a previous LOS phenotype, indicating that emergence and predominance of new LOS variants

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are driven by the host's mounting immune response. However, LOS variation also occurs randomly *in vitro* at a relatively high rate of about 12% of the population [6].

Choline is a major component of eukaryotic cell membrane phospholipids and is present in the form of phosphatidylcholine. Choline has also been identified in the membranes of many bacterial species in the form of phosphorylcholine (ChoP). ChoP is incorporated into the teichoic acid and lipoteichoic acids of *Streptococcus pneumoniae* [10], on the LOS of *H. influenzae* [11], on the LOS and pili of *Neisseria* species [12,13], on the lipopolysaccharide of *Pasteurella multocida* [14], and on a 43-kDa protein in *Pseudomonas aeruginosa* [13]. Among bacterial isolates of different species from the human upper respiratory tract, 15% contained ChoP [15]. Expression of ChoP on *H. influenzae* LOS undergoes a high rate of reversible antigenic variation. In *H. influenzae*, ChoP is attached to the primary glycose on one of three heptoses present in the LOS inner core [16], and its expression is associated with bacterial colonization of the upper respiratory tract in an infant rat model [17]. The adherence and invasion of *H. influenzae* to host cells, including human respiratory cells, is the result of interaction of ChoP with platelet activating factor receptor (PAF-R) [18]. However, in the blood stream, ChoP binds to the acute phase reactant C-reactive protein (CRP), leading to the activation of complement through the classical pathway and killing of the bacteria. Therefore, systemic dissemination of *H. influenzae* is associated with loss of ChoP expression [17,19]. Thus, on and off expression of ChoP is important for *H. influenzae* host colonization and dissemination, respectively.

In *H. influenzae* the *lic1ABCD*$_{Hi}$ locus (*lic1ABCD*$_{Hi}$) is responsible for expression of ChoP. The gene *lic1A*$_{Hi}$ contains a variable number of tandem repeats (VNTR) of the tetranucleotide unit 5′-CAAT-3′ within its open reading frame (ORF) immediately downstream of potential start codons. Variation in the number of VNTR may occur through slipped strand mispairing (SSM), resulting in shifting of the downstream reading frame in or out of frame with the start codon. When the gene is out of frame translation of a truncated, non-functional protein occurs [20–23]. Therefore, the VNTR in *lic1A*$_{Hi}$ acts as a molecular translational switch responsible for the antigenic variation of ChoP on the LOS [11]. In addition to the *lic1*$_{Hi}$ locus, *H. influenzae glpQ* encodes for a glycerophosphoryl diester phosphodiesterase. In the host, and in the absence of free choline, GlpQ allows *H. influenzae* to obtain ChoP from glycerolphosphorylcholine, which is a degradation product of host cell phospholipids [24].

H. somni also expresses ChoP on its LOS [25]. In pathogenic strain 738, ChoP is expressed on the primary glucose attached to heptose I in the inner core [26]. Antigenic expression of ChoP on strain 738 is also subject to steric interference by expression of the β-galactose-(1-3)-β-GlcNAc (lacto-*N*-tetraose) outer core [25]. In this study we identified the genes required for expression of ChoP on *H. somni* LOS, and the molecular mechanisms involved in antigenic variation of ChoP. Our results indicated that a locus with homology to *lic1ABCD*$_{Hi}$ controls expression and antigenic variation of ChoP in *H. somni*, and that *H. somni lic1A* (*lic1A*$_{Hs}$) is a phase variable gene that encodes a choline kinase. We also determined that there are two possible mechanisms of ChoP antigenic variation of *H. somni* LOS that are strain variable.

2. Results

2.1. Identification of putative ChoP biosynthesis genes

Several attempts were made to amplify a homolog of *lic1A*$_{Hi}$ or *lic1D*$_{Hi}$ from *H. somni* genomic DNA by PCR using a variety of degenerate and non-degenerate primers under different reaction conditions. The reactions produced either no products or non-specific amplification products. Southern blotting experiments using a digoxigenin-labeled *lic1A*$_{Hi}$ probe with *H. somni* genomic DNA also did not hybridize to a specific DNA band (data not shown).

A BLAST analysis of the genome sequence of *H. somni* strain 2336 in comparison to the *lic1ABCD*$_{Hi}$ sequence revealed a locus that contained four ORFs with predicted amino acid homology. The first ORF shared 39% identity over 281 amino acids (AA) with *lic1A*$_{Hi}$, the second ORF had 35% identity over 301 AAs to *lic1B*$_{Hi}$, the third ORF shared 50% identity over 230 AAs with *lic1C*$_{Hi}$, and the forth ORF shared 66% AA identity with *lic1D*$_{Hi}$. Furthermore, an *H. somni* ORF that shared 79% identity over 343 AA with the *H. influenzae* glycerophosphoryl diester phosphodiesterase gene (*glpQ*) was also identified. *H. somni glpQ* also shared 80% identity over 363 AA with *P. multocida glpQ* and 60% identity over 360 AA with *Escherichia coli glpQ*.

Analysis of *lic1A*$_{Hs}$ predicted the gene to encode a protein containing the sequence HNDLVPENILM, which corresponds to the consensus sequence HXD*h*XXXN*hhh* (where *h* is F, L, I, M, V, W, or Y [a large hydrophobic AA] and X is any AA) [11]. This consensus sequence is reported to contain the catalytic domain for protein kinases and phosphotransferases [11,27], and is found in the sequence of *H. influenzae* Lic1A [11]. The sequence of *lic1A*$_{Hs}$ contained 25 repeats of the tetranucleotide unit 5′-AACC-3′ three base pairs downstream from the third of three potential start codons. These VNTR would be predicted to cause phase variable expression of ChoP. In contrast, *lic1A*$_{Hi}$ contains the VNTR 5′-CAAT-3′, which begins immediately downstream of a start codon [9]. The first and second potential start codons of *lic1A*$_{Hs}$ are in the same frame while the third start codon is in a different frame. This arrangement was similar to that of the start codons of *lic1A*$_{Hi}$. When 24 repeats were present in *lic1A*$_{Hs}$, the third start codon would be in frame with the stop codon at the end of the ORF, and a functional product would be expected to be expressed.

A *lic1ABCD*$_{Hs}$ locus was also identified in the genome sequence of *H. somni* preputial strain 129Pt, and contained 41 repeats of the VNTR. However, *lic1A*$_{Hs}$ in strain 129Pt was interrupted by an apparent IS1016 insertion sequence that began 61 bp downstream of the VNTR region. This IS1016 element has also been described in *bexA* of the *H. influenzae* type b *cap* locus, requiring a duplication of the locus in order for type b capsule to be expressed [28]. The sequence of the IS1016-like element in strain 129Pt contained 710 bp with 86–95% identity to that of the sequence in *H. influenzae*.

2.2. Constitutive expression of *lic1A*$_{Hs}$ in *E. coli*

The vector pSE1 was used for expression of *lic1A*$_{Hs}$ in *E. coli* BL21DE3pLysS cells (BL21DE3pLysS[pSE1]). Induction of BL21DE3-pLysS[pSE1] with IPTG resulted in expression of *lic1A*$_{Hs}$, as determined by SDS-PAGE analysis (Fig. 1, lanes 2–4). Maximum levels of expression were achieved 2 h post-induction and remained at the same level for 1 h. To express *lic1A*$_{Hs}$ that was not subject to potential phase variation, the 5′-AACC-3′ repeat region was removed from *lic1A*$_{Hs}$, as described in Materials and Methods, and was confirmed by PCR amplification (data not shown). Self-ligation of the PCR product resulted in the vector pSE3, which contained *lic1A*$_{Hs}$ lacking the VNTR in addition to three base pairs downstream of the repeat region [*lic1A*$_{Hs}$Δ(AACC)], thereby leaving the gene in -frame and translated from the start codon immediately upstream of the deleted repeats. The sequence of pSE3 was confirmed by sequencing, and expression of *lic1A*$_{Hs}$Δ(AACC) by *E. coli* containing pSE3 was comparable to that *E. coli* containing pSE1 (data not shown).

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