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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.

Keywords

Histophilus somni; Phosphorylcholine; Lipooligosaccharide; Adherence; Antigenic and phase variation

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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 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).

2.3. Choline kinase assay for Lic1A

The ability of *H. somni* Lic1A to phosphorylate choline in the presence of ATP and produce ChoP was determined by comparing the activity of recombinant Lic1A$_{Hs}$ to that of yeast choline kinase (CKI) [29]. Choline kinase from yeast strain KS106, a double mutant that does not express ethanolamine kinase and choline kinase, but overexpresses wild type choline kinase, was used [30]. The amount of ChoP produced by Lic1A$_{Hs}$ that was expressed in *E. coli* was 8.39 nmol/min/mg protein, while the control yeast choline kinase produced 11.86 nmol/min/mg protein (Fig. 2), confirming that Lic1A$_{Hs}$ was a functional choline kinase.

2.4. LOS composition and phase variation of lic1A$_{Hs}$

ChoP$^+$ and ChoP$^-$ clonal derivatives of strains 738 and 124P were selected using MAbs to ChoP and identified as such, as described in Materials and Methods (Table 1). To assess the mechanism of ChoP phase variation in each strain, the number of VNTR in *lic1A*$_{Hs}$ was determined and the LOS composition was analyzed from clonal derivatives of both strains (Table 2). The LOS of the ChoP$^+$ isolate of strain 124P (124P$^+$) contained one glycoform that contained ChoP with 2 hexoses in the outer core. The ChoP$^-$ isolate of the same strain (124P$^-$) contained three glycoforms, none of which contained ChoP, but contained 3 hexoses, hexNAc, and sialic acid in the largest glycoform. Thus, there was correlation between a truncated outer core and the presence of ChoP on the LOS. The presence of sialic acid was of particular interest since sialic acid has not previously been found in the LOS of other serum-sensitive preputial isolates of *H. somni* [8,31,32]. The number of VNTR in *lic1A*$_{Hs}$ of 124P$^+$ was 27. When compared to the sequence of *lic1A*$_{Hs}$ from the genome sequence of strain 2336, this number of VNTR was consistent with the gene being in frame with a stop codon, and expressing a full length and functional product. The number of VNTR in *lic1A*$_{Hs}$ of strain 124P$^-$ was 29, which was consistent with the gene translating a truncated, non-functional protein.

The LOS of both ChoP$^+$ and ChoP$^-$ clonal isolates of strain 738, which were selected based on reactivity with an anti-ChoP MAb, contained ChoP as determined by electrospray mass spectrometry (ES-MS). The ChoP$^+$ isolate (738$^+$) contained three glycoforms, of which two contained ChoP. The ChoP$^-$ isolate (738$^-$) contained seven glycoforms, five of which contained ChoP. The LOS of 738$^+$ contained a higher proportion of glycoforms that contained fewer hexose and hexosamine units and was consistent with the LOS being more

truncated than that of 738$^-$ LOS (Table 3). The number of VNTR in *lic1A*$_{Hs}$ of both 738$^+$ and 738$^-$ was 24, indicating that *lic1A* in both isolates would be in frame with the start codon and express a functional gene product (Table 2). The number of VNTR in *lic1A*$_{Hs}$ of both ChoP$^+$ and ChoP$^-$ isolates of strain 738 did not vary, whereas the VNTR number did vary between the ChoP$^+$ and ChoP$^-$ isolates of strains 7735, 93, and 124P. However, the number of VNTR in strain 7735 was 42 or 43, almost twice the number in most of the other strains. Furthermore, unlike all other strains examined a polymorphism in the sequence of the coding region downstream of the VNTR in strain 7735 resulted in the presence of an additional nucleotide and a frame-shift in the ORF. That frame-shift resulted in *lic1A*$_{Hs}$ being in frame when 43 VNTR were present, and out of frame in the presence of 42 repeats. In contrast, 43 repeats in the sequence of *lic1A* from the other strains examined would have resulted in expression of a non-functional product. There were also 41 repeats in *lic1A*$_{Hs}$ of strain 129Pt, but ChoP could not be expressed due to the IS1016 insertion downstream of the VNTR. The number of VNTR in strain 2336, which lacks ChoP, was 25, predicting the gene would be out of frame.

The number of VNTR in *H. somni lob2A*, which encodes for an *N*-acetylglucosamine (GlcNAc) transferase, was examined to determine if there was any correlation between expression of the terminal LOS disaccharide and reactivity with anti-ChoP MAb. The full outer LOS core blocks antigenic reactivity of ChoP with MAb, and *lob2A* mutants fail to express the terminal lacto-*N*-tetraose unit [33]. The number of VNTR in *lob2A* of the strains examined was either 20 or 21. However, the number of repeats in *lob2A* was independent of, and did not correlate with, ChoP expression (Table 2).

2.5. SDS-PAGE analysis of LOS

The electrophoretic profile of LOS from ChoP$^+$ and ChoP$^-$ isolates of strains 738, 7735, and 124P are shown in Fig. 3. LOS from strains 2336 and 129Pt, neither of which express ChoP [32,34], were included as controls (lanes 10 and 11). The LOS profile from isolate 738$^-$ contained high molecular size bands that were similar to those present in parent strain 738 (lanes 4 and 1, respectively). Overall, the LOS of 738$^+$ contained lower molecular size bands compared to the LOS of isolate 738$^-$ (lanes 3 and 4). However, the LOS of 738$^+$ expressed bands of higher molecular size compared to LOS from a ChoP$^+$ isolate of strain 738 obtained in a previous study (738P) (lanes 3 and 2, respectively) [25]. The highest molecular size LOS bands of isolate 7735$^+$ were similar to those of the LOS from isolate 7735$^-$ (lanes 6 and 7, respectively). However, 7735$^+$ LOS contained a unique lower molecular size band of high intensity. The LOS of isolate 124P$^+$ also contained a single band of a lower molecular size than that of the predominant band present in the LOS of isolate 124P$^-$ (lanes 9 and 10, respectively), which was consistent with the results of ES-MS analysis (Table 3).

The sequence of *lic1A*$_{Hs}$ from this study is available on GenBank (http://www.nbci.nlm.nih.gov) under the accession number BK001334 and *glpQ* sequence is available under the accession number BK001335.

3. Discussion

H. influenzae is capable of variable expression of ChoP on its LOS, which plays an important role in the organism's ability to colonize and invade host tissues [17]. Variable expression of ChoP also occurs at a high rate on *H. somni* LOS and is reversible [7,25]. In *H. influenzae* the pathway for incorporation of ChoP into *H. influenzae* LOS by the *lic1*$_{Hi}$ locus has been proposed by Weiser et al. [11]. The gene *lic1A*$_{Hi}$ encodes a putative choline kinase, which phosphorylates choline to form ChoP, while *lic1B*$_{Hi}$ encodes a high affinity choline transporter that may be involved in uptake of choline from the environment [35]. The gene *lic1C*$_{Hi}$ encodes a predicted pyrophosphorylase [11,35] that may be involved in

activation of ChoP to form nucleoside diphosphocholine. The gene *lic1D*$_{Hi}$ encodes a putative diphosphonucleoside choline transferase that plays a role in transfer of ChoP onto a specific LOS glycose [36]. The amino acid similarity and identical arrangement of the *lic1* genes between *H. somni* and *H. influenzae* suggest that the choline uptake and utilization pathways in *H. somni* are similar to those of *H. influenzae*. However, the nucleotide sequences of these genes did not show high similarities, thereby explaining why PCR and hybridization were not successful in identifying these genes in *H. somni*. The low nucleotide similarity and differences in the sequence of the VNTR in *lic1A*$_{Hs}$ indicate possible divergent or convergent evolution between the two organisms. However, the identical organization of genes in *lic1*$_{Hs}$, and the presence and arrangement of three potential start codons and VNTR in *lic1A*$_{Hs}$ with that of *H. influenzae* indicates the two species may share a common ancestry. As more genomes of the family Pasteurellaceae become available the evolution of these and other genes may become clearer.

We also identified a gene in *H. somni* with homology to *H. influenzae glpQ*, which in *H. influenzae* encodes an enzyme with glycerophosphoryl diester phosphodiesterase activity. GlpQ enables *H. influenzae* to obtain choline from glycerolphosphorylcholine, which is a degradation product of mammalian cell phospholipids, allowing the bacteria to obtain choline directly from epithelial cells in the absence of free choline [24]. The presence of a homolog of *H. influenzae glpQ* in *H. somni* suggests that the latter pathogen may use a similar mechanism of acquiring choline from the bovine host.

The insertion sequence IS1016 that interrupts *lic1A*$_{Hs}$ in strain 129Pt is the only insertion sequence in *lic1*$_{Hs}$ and no similar sequences appear to flank the locus. This insertion sequence is also present in the *bex* capsule gene cluster in *H. influenzae* and is responsible for duplication of the *cap* locus in order for the type b capsule to be expressed [28]. The IS1016 in the genome of strain 129Pt may have contributed to the evolution of that strain, but the significance of its presence in *lic1A*$_{Hs}$ is not clear.

The role of *lic1A*$_{Hi}$ in expression of ChoP was determined through sequence homology to eukaryotic choline kinases [11], and generation of a gene deletion mutant that lacked expression of ChoP [36]. Further confirmation of *lic1A*$_{Hi}$ function was achieved through complementing the mutant strain with a copy of the gene that was missing the VNTR, resulting in constitutively restoring ChoP expression. However, the choline kinase activity of *lic1A*$_{Hi}$ has not been biochemically confirmed. In this study a *lic1A*$_{Hs}$ mutant was not generated due to the lack of genetic tools to manipulate *H. somni* [37]. However, the homology between the *H. influenzae* and *H. somni* Lic1A proteins, including amino acid repeats, indicated that *lic1A*$_{Hs}$ likely controls expression of ChoP on *H. somni* LOS. The absence of ChoP on the LOS of *H. somni* strain 129Pt [32], which has an interruption in *lic1A*$_{Hs}$, further supports the role of *lic1A*$_{Hs}$ in expression of ChoP. Finally, choline kinase activity was confirmed for recombinant *H. somni* Lic1A using a strain with the VNTR removed so that *lic1A*$_{Hs}$ was constitutively on. Furthermore, the translation of an active product from a gene missing the 5′-AACC-3′ VNTR indicated that the repeat region was not required for expression of a functional protein, confirming similar results by High et al., who showed that the 5′-CAAT-3′ VNTR are not necessary for expression of *lic2A* by *H. influenzae* [38].

The primary mechanism of antigenic variation of ChoP expression in *H. influenzae* is *lic1A*$_{Hi}$ phase variation. SSM during DNA replication varies the number of VNTR in *lic1A*$_{Hi}$ resulting in shifting the reading frame downstream of the repeats in or out of frame with the start codon. Therefore, *lic1A*$_{Hi}$ phase varies ON or OFF according to the number of VNTR present [11,23], with concomitant phase variable expression of Lic1A. *H. somni lob1* and *lob2A* contain VNTR in their ORFs and SSM in the repeats of both genes contribute to

phase and antigenic variation of *H. somni* LOS [33,39]. However, phase variation of *lic1A*$_{Hs}$ due to SSM was not entirely responsible for antigenic variation of ChoP expression in strain 738. ChoP^{+} and ChoP^{-} isolates of strain 738 contained the same number of 5′-AACC-3′ repeats in *lic1A*$_{Hs}$, consistent with the gene being in the ON phase, which was confirmed by the chemical identification of ChoP on the LOS of both variants. Although the LOS of both variants contained ChoP, the LOS of the ChoP^{+} variant, as well as another ChoP^{+} variant from a previous study [25], contained more truncated LOS glycoforms. Furthermore, Western blot analysis of LOS from several *H. somni* strains containing ChoP showed that only the lowest molecular size glycoforms reacted with MAb to ChoP [25]. Thus, variation in the composition and extension of the oligosaccharide outer core is responsible, at least in part, for the antigenic variation of ChoP expression on strain 738. ChoP is linked to the primary glucose of the LOS outer core in strain 738 [26], and therefore, the addition of glycoses beyond the primary glucose and the change in their linkages may lead to steric interference of ChoP binding to anti-ChoP MAb [25]. Therefore, variation of the LOS outer core may be a mechanism of antigenic variation of ChoP expression in many *H. somni* strains that are capable of incorporating ChoP into their LOS. When *H. influenzae* strain RM118 is grown in the presence of sialic acid, the oligosaccharide (Neu5Ac-Gal-GlcNAc-Gal) is added to the primary glucose, which is modified by ChoP [40]. However, whether ChoP is accessible to antibody binding when in this configuration has not been determined (Derek Hood, personal communication).

The selection of isolates with an equal number of VNTR may be due to the random nature of selecting clonal isolates or may reflect a selective preference or stability of the VNTR in *lic1A*$_{Hs}$ of strain 738. Effective DNA repair mechanisms play a role in stability of base pair repeat regions through effective mismatch repair after SSM [41–43]. Therefore, a high fidelity DNA repair mechanism may be responsible for reduced variation of the repeats in strain 738. However, in other strains examined (7735, 93, 124P, and 2336) there was direct correlation between the number of VNTR and expression of ChoP. Nonetheless, all ChoP+ isolates had lower molecular size LOS glycoforms, indicating a shorter oligosaccharide chain. One possibility is that the linkage site for ChoP attachment is blocked by specific glycoses or extension of the outer core. However, this is not the case in strain 738, which is a phase variant of strain 2336 that lacks ChoP [34]. In strain 2336 the glycose extension from the primary glucose is similar to that in 738, but the number of VNTR in strain 2336 *lic1A*$_{Hs}$ was 25, thereby placing the gene out of frame. Therefore, in strain 2336 lack of expression of ChoP is due to phase variation of *lic1A*$_{Hs}$ and not to steric interference. The number of VNTR in strains 124P, 93, and 7735 *lic1A*$_{Hs}$ also correlated with the presence or absence of ChoP in their LOS, further supporting that phase variation of *lic1A*$_{Hs}$ in these strains was due to SSM of the 5′-AACC-3′ repeats, although truncation of their outer core may also be a contributing factor. Of interest was the higher number of VNTR and the polymorphism in the coding sequence of *lic1A*$_{Hs}$ of strain 7735, suggesting that this strain may have undergone some evolutionary divergence compared to the other strains.

In summary, we have identified the genes *lic1ABCD*$_{Hs}$ and *glpQ* that control expression of ChoP on *H. somni* LOS. A functional assay of *lic1A*$_{Hs}$ indicated that the gene encoded a choline kinase. Our results also showed that there are two possible mechanisms for antigenic variation of ChoP expression on *H. somni* LOS: phase variation of *lic1A*$_{Hs}$ expression through variation of the number of VNTR, and phase variable elongation/truncation of the LOS outer core beyond the ChoP-attached glycose. Further investigation is required to understand the interrelationship between LOS composition, phase variation of other LOS biosynthesis genes, and antigenic variation of the ChoP epitope.

4. Materials and methods

4.1. Bacterial strains and growth conditions

The *H. somni* strains used in this study have been described [44] and are listed in Table 1. *H. somni* strains were grown on Columbia agar base (Difco culture media, Becton Dickinson and Company, Franklin Lakes, NJ) supplemented with 5% ovine or bovine blood (CBA). CBA plates were incubated 16–24 h at 37°C in a candle extinction jar or in the presence of 5% CO_2 [45]. *E. coli* BL21DE3-pLysS (Invitrogen, Carlsbad, California) was grown on Luria Bertani (LB) agar plates or in LB broth supplemented with 100 μg ml^{-1} of ampicillin and 34 μg ml^{-1} of chloramphenicol. Stocks of all bacterial strains were maintained at −80°C in 10% skim milk.

4.2. Gene identification and sequence analysis

To identify putative coding sequences (CDS) in the *H. somni* genome, the sequences of *H. influenzae lic1* and *glpQ* were compared to the finished genome sequences of *H. somni* strain 2336 (Laboratory for Genomics and Bioinformatics [Microgen], University of Oklahoma Health Sciences Center at http://www.micro-gen.ouhsc.edu/index.html) and GenBank (NC_010519), and strain 129Pt (Department of Energy Joint Genome Institute (JGI) at http://genome.jgi-psf.org /finished_microbes/haeso/haeso.home.html) and GenBank (NC_008309) using the basic local alignment search tool (BLAST) [46]. Further examination of the sequences was performed on the National Center for Biotechnology Information (NCBI) server at http://www.ncbi.nlm.nih.gov/BLAST.

For analysis and manipulation of DNA sequences, restriction mapping, and designing plasmid constructs, the BioEdit Sequence Alignment Editor version 5.0.9 was used (Tom Hall, North Carolina State, University; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Lasergene DNA and protein sequence analysis and contig alignment software was used for designing PCR primers (DNASTAR molecular biology software, http://www.dnastar.com). The Artemis DNA sequence viewer and annotation software (Sanger institute, http://www.sanger.ac.uk/Software/Artemis) was used to identify ORFs and annotate sequences from the *H. somni* genome.

4.3. Polymerase chain reaction (PCR) and DNA sequencing

PCR and sequencing amplification reactions were performed in either a Mastercycler gradient (Eppendorf, Westbury, NY) or a PCRExpress (Hybaid Limited, Thermo Electron Corporation, Waltham, MA) thermocycler. PCR reactions were carried out in a volume of 25–50 μl and included 1–3 units of Taq polymerase (Eppendorf, Westbury, NY), 1.5 mM $MgCl_2$, 2 mM of dNTP, and 20 pM of primers. The primers used in this study and the corresponding PCR annealing temperatures are shown in Table 4. Genomic DNA was purified using the Puregene DNA purification kit (Gentra systems, Minneapolis, MN) according to the manufacturer's instructions and 10–200 ng was used in PCR reactions. Alternatively single colonies were boiled in distilled water, centrifuged, and the supernatant used as a template for PCR.

For analysis of the VNTR repeats in $lic1A_{Hs}$ or in *lob2A*, primers (Table 4) were used to amplify a region that contained the VNTR; one primerwas used for subsequent sequencing. The HslicA-F1 and HslicA-R1 primers were used to amplify $lic1A_{Hs}$ while the YWC and YWE primers [33] were used to amplify *lob2A* as a control. *lob2A* encodes for an GlcNAc transferase that attaches the GlcNAc of the terminal lacto-*N*-tetraose unit onto the outer core of the LOS [33]. The amplified products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and then sequenced. The BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) was used for preparing sequencing reactions with

the HslicA-F1 primer or the YWC primer (Table 4); extra nucleotides were removed from the PCR product. All sequencing was done at the DNA core sequencing facility at the Virginia Bioinformatics Institute, Virginia Tech.

4.4. Vector construction

The primers SE-Hs-lic1A-F-EcoRI and SE-Hs-lic1A-R-HindIII were used to amplify a 1-kb fragment, which contained *lic1A*$_{Hs}$ from an isolate of *H. somni* strain 738 that was reactive with MAb to ChoP (738^{+}). The amplified fragment was cloned into the *Eco*RI and *Hind*III sites of the inducible expression vector pRSET A (Invitrogen, Carlsbad, California). The resulting vector, designated pSE1, was linearized with *Hinc*II, which cut the plasmid immediately upstream of the VNTR (which were later determined to be 5′-AACC-3′) region of *lic1A*$_{Hs}$. The primers SE-pSE1-Forward-1 and SE-pSE1-Reverse-1 were used to amplify the sequence of the linearized plasmid without including the repeat region. The amplified product was self-ligated to obtain plasmid pSE3, which contained *lic1A*$_{Hs}$ missing the VNTR region (data not shown).

4.5. Biochemical enzyme assay

E. coli BL21DE3pLysS transformed with pSE3 (expressing *lic1A*$_{Hs}$) was grown to exponential phase at 37°C with shaking. The cells were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 4 hrs and washed with Tris buffered saline (TBS). The cells were lysed by use of a French Press in buffer containing 50 mM Tris–HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 M sucrose, 10 mM b-ME, and protease inhibitors. Unbroken cells were removed by centrifugation (30 min at 15,000 rpm) and the supernatant was used to assay for choline kinase activity. Yeast choline kinase was extracted from yeast strain KS106 (*eki1cki1*) that over-expressed wild type choline kinase using a multicopy vector. Yeast cells were grown to exponential phase in leucine synthetic media containing 100 mM choline, lysed using a bead beater, and unbroken cells were removed by centrifugation at 1500 × *g* for 10 minutes. Fifteen μg of cell extract was added to a reaction mixture containing 67 mM glycine–NaOH buffer (pH 9.5), 5 mM [^{14}C] choline (2000 cpm/nmol), 5 mM ATP, 1.3 mM DTT, and 10 mM $MgSO_4$ in a total volume of 30 μl, and the mixture incubated at 30°C for 20 min. Free choline was precipitated using Reinecke salt [47] and ChoP was measured using a Beckman LS 6500 scintillation counter. *E. coli* BL21DE3pLysS that did not contain any vectors was used as a negative control.

4.6. Colony immunoblotting

Detection of *H. somni* colonies expressing ChoP was performed by colony immunoblotting as previously described [6]. Blotted colonies were incubated with a 1:10 dilution of MAb 5F5.9 to ChoP [25] or a 1:4000 dilution of MAb TEPC-15 (Sigma–Aldrich, Saint Louis, MO) overnight at 4°C, and then washed with TBS. The specificity of IgG3 MAb 5F5.9 for ChoP was previously confirmed through immunoblotting, inhibition ELISA, and mass spectrometry [25]. The IgA MAb TEPC-15 is also specific for ChoP and has been used to study expression of ChoP on *H. influenzae* LOS [11,17]. The membranes were incubated with a 1:1000 dilution of horse radish peroxidase (HRP) conjugated to anti-mouse IgG or IgA (Jackson Immunoresearch Laboratories) for detection of MAb 5F5.9 or TEPC-15, respectively. Single ChoP^{+} or ChoP^{-} colonies were selected from the CBA plates and streaked onto new plates. The colony blotting was repeated to obtain colonies that were either predominantly positive (~95%/plate) or negative (>90%/plate) for ChoP.

4.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

E. coli BL21DE3pLysS cells transformed with pSE3 were induced with IPTG and grown in broth cultures. Bacterial samples were obtained from the broth 1, 2, and 3 h post-induction.

Samples were washed with PBS, suspended in loading buffer containing β–mercaptoethanol, boiled for 10 minutes, loaded onto NuPAGE 4–12% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, California), and subjected to electrophoresis at 200 volts for 35 minutes.

LOS for SDS-PAGE analysis was extracted using a micro-scale hot phenol/water method, as previously described [48]. For electrophoretic separation of LOS, a discontinuous 14% polyacrylamide gel was used [49]. After fixation and periodate oxidation, gels were stained with ammoniacal silver for visualization of LOS bands [50].

4.8. Electrospray mass spectrometry (ES-MS) analysis

LOS was O-deacylated by mild hydrazinolysis and treatment with 4 M KOH, as previously described [26]. After washing twice with cold acetone, O-deacylated LOS was redissolved in water and lyophilized. Deacylated samples were dissolved in an aqueous solvent containing 50% acetonitrile and 0.1% formic acid and analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments). The mass spectrometer was scanned from m/z 150 to 2,500 with a scan time of 10 s; the electrospray tip voltage was 2.5 kV [26]. Percentage distribution of glycoforms was determined by comparing the intensities of the doubly and triply charged ions corresponding to each glycoform in the mass spectra.

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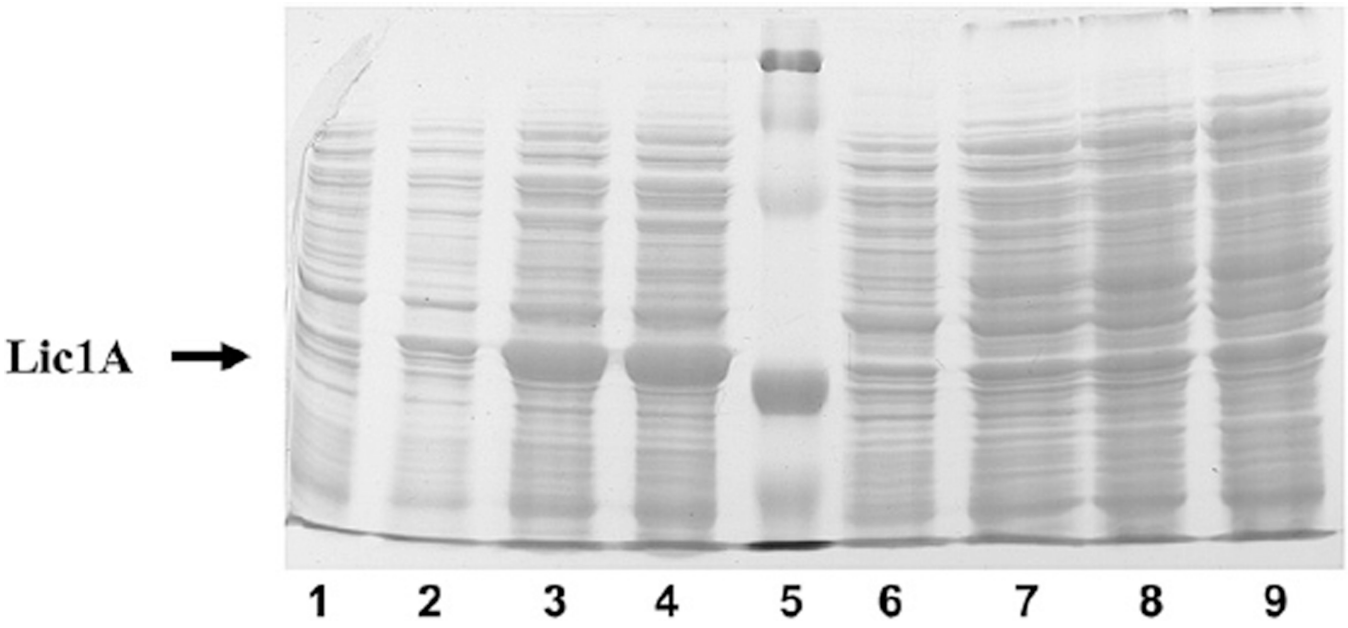

Fig. 1.

Electrophoretic profile of *E. coli* expressing the gene $lic1A_{Hs}$. The plasmid pSE1, which contained $lic1A_{Hs}$ was transformed into *E. coli*. Transformed cells expressed a protein of the approximate molecular size to that of the predicated *H. somni* choline kinase (*Lic1A*). Lanes: 1, *E. coli* containing pSE1 pre-induced with IPTG; 2–4, *E. coli* containing pSE1 induced with IPTG after 1, 2, and 3 h; 5, Molecular size marker; 6, *E. coli* control lacking pSE1 pre-induced with IPTG; 7–9, *E. coli* control lacking pSE1 induced with IPTG after 1, 2, and 3 h.

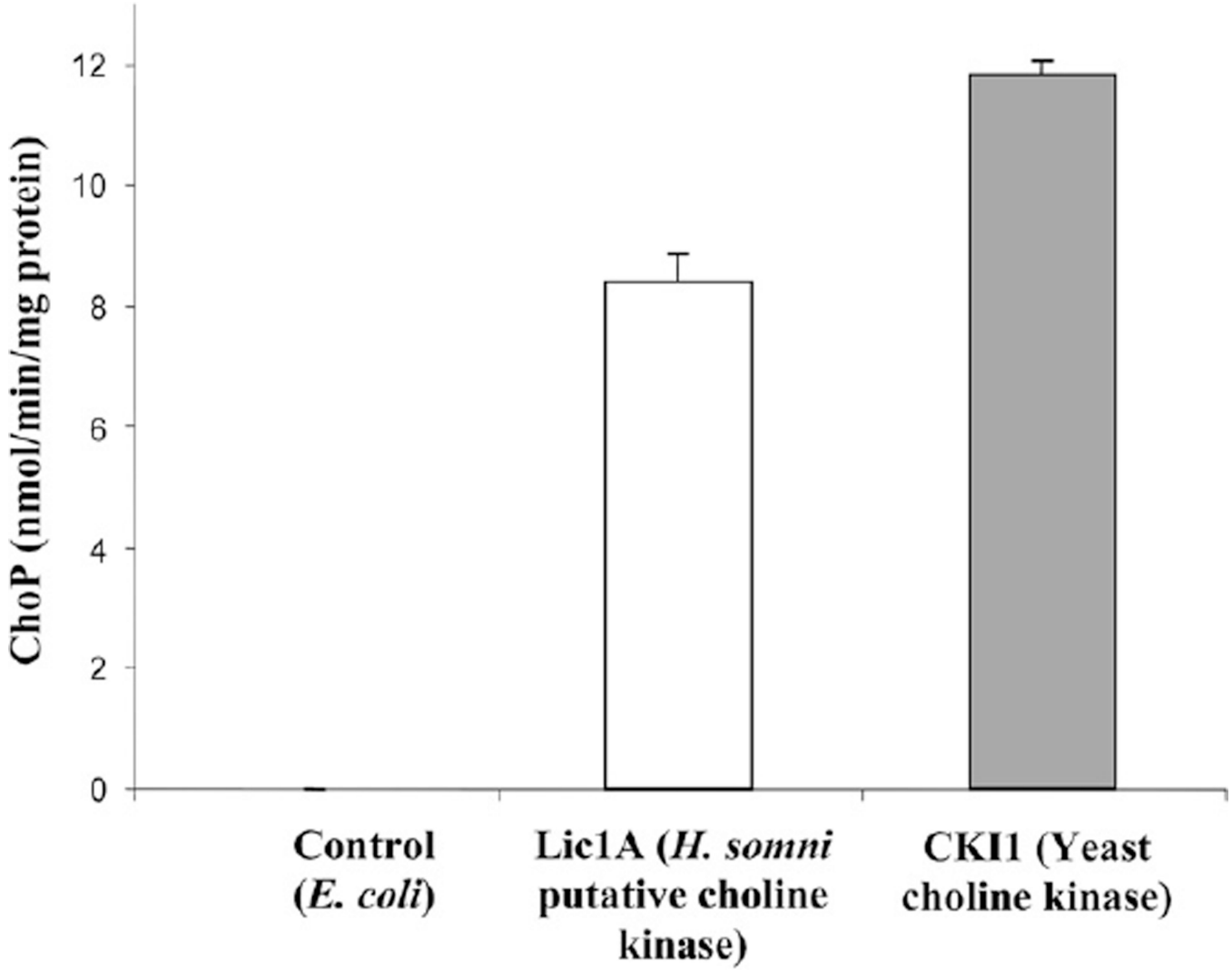

Fig. 2.

The choline kinase activity of *H. somni Lic1A* compared to the activity of yeast choline kinase CKI1. *Lic1A* catalyzed the production of 8.39 nmol ChoP/min/mg protein while CKI1 catalyzed the production of 11.86 mg ChoP/min/mg protein. The results are the average of three experiments.

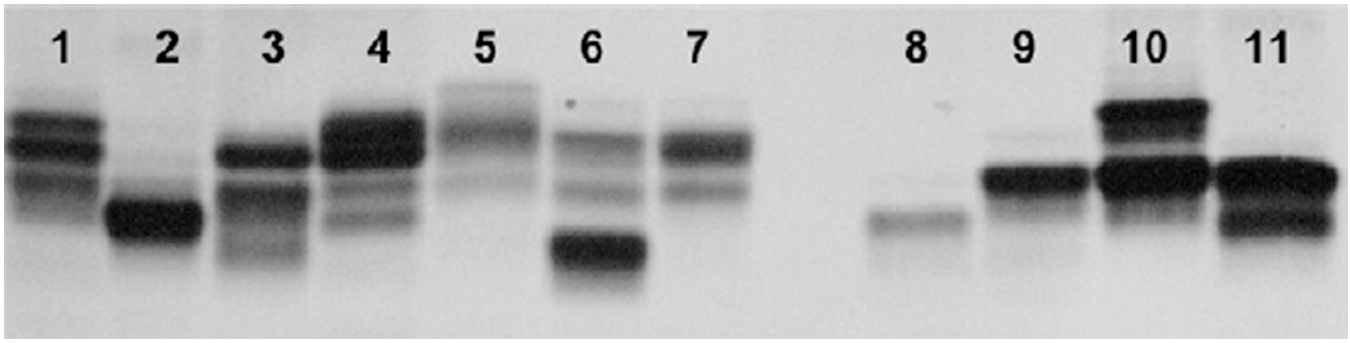

Fig. 3.

Electrophoretic profiles of LOS from ChoP$^+$ and ChoP$^-$ variants of *H. somni* strains. The ChoP$^+$ variants contain more of the lower molecular size bands than LOS from ChoP$^-$ variants and parent strains. Lanes: 1, parent strain 738; 2, 738P (a ChoP$^+$ isolate from a previous study [27]); 3, 738$^+$; 4, 738$^-$; 5, parent strain 7735; 6, 7735$^+$; 7, 7735$^-$; 8, 124P$^+$; 9, 124P$^-$; 10, 2336; 11, 129Pt.

Table 1

H. somni strains used in this study.

Strain	Source	Reference
2336	Pneumonic lung isolate	[44]
738	Clonal, calf-passaged isolate of 2336	[26]
738P	ChoP-positive clonal isolate of 738	[25,27]
738^{+}	ChoP-positive clonal isolate of strain 738	This work
738^{-}	ChoP-negative clonal isolate of strain 738	This work
7735	Pneumonic lung isolate	A Potter. Veterinary Infectious Disease Organization. University of Saskatchewan, Canada
7735^{+}	ChoP-positive clonal isolate of strain 7735	This work
7735^{-}	ChoP-negative clonal isolate of strain 7735	This work
93	Pneumonic lung isolate	A Potter. Veterinary Infectious Disease Organization. University of Saskatchewan, Canada
93+	ChoP-positive clonal isolate of strain 93	This work
93^{-}	ChoP-negative clonal isolate of strain 93	This work
124P	Normal prepuce	[44]
$124P^{+}$	ChoP-positive clonal isolate of strain 124P	This work
$124P^{-}$	ChoP-negative clonal isolate of strain 124P	This work
129Pt	Normal prepuce	[44]

Table 2

The number of VNTR in *lic1A*$_{Hs}$ and *lob2A* of ChoP$^+$ and ChoP$^-$ isolates of *H. somni* strains.

Strain[a]	Number of VNTR in *lic1A* (5′-AACC-3′)	Expression of ChoP	Number of VNTR in *lob2A* (5′-GA-3′)
738P[b]	24	Yes	20
738$^+$	24	Yes	21
738$^-$	24	No	21
7735$^+$	43	Yes[c]	21
7735$^-$	42	No	20
124$^+$	27	Yes	ND
124$^-$	29	Yes	ND
93$^+$	24	Yes	ND
93$^-$	23	Yes	ND
2336[d]	25	Yes	20

ND: Not determined.

[a] Strains designated with (+) or (−) are either reactive or non-reactive to anti-ChoP MAb, respectively.

[b] Strain 738P is a ChoP$^+$ derivative obtained from a previous study [30].

[c] The presence of two additional nucleotides downstream of the VNTR place the gene in frame when there are 43 repeats present rather than 42.

[d] Determined from the finished genome sequence of strain 2336.

Table 3

The proposed composition of LOS from ChoP$^+$ and ChoP$^-$ isolates of pathogenic strain 738 and commensal strain 124P and the corresponding number of VNTR in *lic1A*$_{Hs}$ of each isolate.

Clonal isolat	Molecular Mass (Da)	Percent Distribution of glycoforms	Proposed composition	Number of VNTR	Predicted expression of *Lic1A*
124P$^+$	2512	100	**ChoP,** 2Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH	27	Yes
124P$^-$	3004.0	45	Sial, HexNAc, 3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH	29	No
	2712.9	32	HexNAc, 3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH		
	2509.5	23	3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH		
738+	2755	38	**ChoP,** HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH	24	Yes
	2590	24	HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2390	38	**ChoP,** 2Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
738$^-$	2918	19	**ChoP,** HexNAc, 4Hex, EtnP, 2Hep, 2Kdo, LipA-OH	24	Yes
	2755	12	**ChoP,** HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2714	13	**ChoP,** 4Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2590	19	HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2552	10	**ChoP,** 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2389	17	**ChoP,** 2Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2224	10	2Hex, EtnP, 2Hep, 2Kdp, LipA-OH		

Kdo: 3-deoxy-D-*manno*-octulosonic acid. Hep: heptose. Hex: hexose. HexNAc: *N*-acetylhexosamine. ChoP: phosphorylcholine. EtnP: phosphoethanolamine. LipA-OH: deacylated lipid A.

Table 4

Primers used in this study and their corresponding annealing temperatures.

Primer	Sequence ($5' \rightarrow 3'$)[a]	Purpose	Annealing temperature
HslicA-F1	ATCGTTAAGCGGAAAATGACT	Amplification of *lic1A*$_{Hs}$ for sequencing	50°C
HslicA-R1	CTCCCAAAATCGCTAACAAA		
SE-Hs-lic1A-F-EcoRI	CATTGAATTCTTAGTGTAGTATGTGCGGAG	Amplification of *lic1A*$_{Hs}$ for cloning into pRSET A	48.8 °C
SE-Hs-lic1A-R-HindIII	CTAATCGTTAAGCTTCACTAAATAAACCCAT		
SE-pSE1-Forward-1	AAATGAACGTTTATTTTCCATAGTA	Amplification of pRSET A (*lic1A*) without 5′-5′-AACC-3′ repeats	50.4 °C
SE-pSE1-Reverse-1	AACATGATATTCTTCCTATTTCCAT		
YWC [49]	TATCCGGTTTATCAATGTG	Amplification of *lob2A* for sequencing	50 °C
YWE [49]	Cy5-GAGCCTGCCATTATTATTCA		

[a]The underlined sequence indicates the restriction endonuclease site designed into the primer.