CRUCIAL ROLE OF HISTOPHILUS SOMNI EXOPOLYSACCARIDE IN BIOFILM FORMATION

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ABSTRACT

Histophilus somni (Haemophilus somnus) is an obligate inhabitant of the mucosal surfaces of bovine and sheep and an opportunistic pathogen responsible for respiratory disease, meningoencephalitis, myocarditis, arthritis, and other systemic infections. Although exopolysaccharides (EPS) are a large component of bacterial biofilms, their contribution to biofilm structure and function has been examined for only a few organisms. Here, we undertook a genetic approach to examine the potential role of EPS of H.somni 2336 in biofilm formation. We conducted a transposon random gene knockout to study the genetic function of biofilm and EPS. A total of 96 real random transposon mutants were generated of H.somni 2336 using the EZ::TN™ <KAN-2>Tnp Transposome™. These mutants were confirmed by colony blot and PCR of kanamycin resistance gene. 25 mutants were confirmed to make less biofilm than the wild type strain (~30-80% decrease) By creation of mutations in the gene encoding for a homolog of filamentous haemagglutinin (FHA), predicted to be involved in attachment in biofilm process. We concluded that this gene might also play a role in EPS production and that EPS might be required for cellular attachment to abiotic surfaces.

INTRODUCTION

Histophilus somni (Haemophilus somnus) is a Gram-negative coccobacillus and member of the Pasteurellaceae **(Angen et al., 2003)**. This host specific opportunistic pathogen of cattle is one of the organisms responsible for bovine respiratory disease complex (BRDC), or shipping fever **(Corbeil et al., 1986)**. In addition to pneumonia **(Miller et al., 1983, Andrews et al., 1985)**, H.somni can cause meningoencephalitis, myocarditis, arthritis, septicemia, and other systemic infections **(Corbeil et al., 1986, Inzana and Corbeil, 2004)**. H. somni expresses a wide array of virulence factors, including phase variation of lipooligosaccharide epitopes **(Inzana et al., 1992, Howard et al., 2000, Inzana et al., 2002)**, decoration of lipooligosaccharide with sialic acid and phosphorylcholine **(Howard et al., 2000, Inzana et al., 2002, Siddaramppa and Inzana, 2004)**, expression of high-molecular-weight immunoglobulin- binding

proteins **(Corbeil et al., 1985, Widders et al., 1989 Corbeil et al., 1997, Sanders et al., 2003)**, intracellular survival in professional phagocytes **(Czuprynski and Hamilton, 1985, Lederer et al., 1987)**, inhibition of the oxidative burst of phagocytic cells **(Howard et al., 2004)**, cell adherence **(Corbeil et al., 1996)**, serum resistance **(Corbeil et al., 1985)** and induction of apoptosis to endothelial cells **(Sylte et al., 2005)**.

Biofilms are sessile communities of bacterial cells enclosed in a self-produced extracellular polysaccharide matrix and adherent to a biotic or abiotic surface. Biofilms play an important role in immune evasion and tolerance toward antimicrobial agents, leading to persistent and chronic infections **(Costerton et al., 1999, Hall-Stoodley et al., 2004)**.

A capsule has not been identified on the surface of H.somni. However, the production of an exopolysaccharide was described **(Inzana and Corbeil 2004)** which is a common component of bacterial biofilms. The functions of EPS in biofilm formation are not yet clear, but it appears to promote surface attachment and provide structural support **(Davies et al. 1993)**. It may also afford protection from external threats, such as antimicrobial compounds and predatory organisms **(Stewart 2002)**, or help secreting strains to grow toward nutrient-rich locations **(Foster and Xavier 2007)**.

MATERIALS AND METHODS

Bacteria and culture conditions :

H. somni strain 2336 (a pathogenic, pneumonia isolate) was used to study the role of EPS in biofilm formation. H. somni strain 2336 was selected for this study because the genome of this strain has been sequenced (http://microgen.ouhsc.edu/h_somnus/h_somnus_home.htm), and because this strain is the most mature biofilm forming strain **(Sandal et al., 2007)**. H. somni was propagated from _80ºC skim milk stocks onto Columbia agar containing 5% sheep blood (CBA). For planktonic growth in broth medium, colonies from 24-h cultures were inoculated into brain heart infusion broth supplemented with 0.1% Trizma base (Sigma, St. Louis, MO) and 0.01% thiamine monophosphate (Sigma, St. Louis, MO) **(Asmussen and Baugh, 1981)**, and shaken at 200 rpm at 37ºC to late-log phase (5_109 colony forming units (CFU)/ml), determined spectrophotometrically. For topo cloning of the inverse PCR products, Escherichia coli DH5™-T1R Competent cells (Invitrogen, Carlsbad, CA) were used as commercially chemically competent cells. Cells were grown on LB agar plates supplemented with 50 _g/ml kanamycin.

Scanning electron microscopy :

The lower portion of the flow cell containing the coverslip was washed with PBS three times and fixed using 2.5% glutaraldehyde in PBS for 60 min. The cover slips were then rinsed twice for 10 min each in PBS and processed for scanning electron microscopy (SEM) by using a graded acetone dehydration series, followed by the addition of osmium tetraoxide **(Hong et al., 2007)**. The samples were subjected to critical drying, mounted onto stubs, and coated with palladium. The specimens were viewed on a Phillips SEM-515 scanning electron microscope, and images were obtained at various levels of magnification.

Transposon mutagenesis :

Transposon mutants were generated with EZ::TN™ <KAN-2>Tnp Transposome™ **(Epicentre, Madison, WI)**. Preparation of electrocompetent cells was performed as described by **Wu et al., (2000)**. The bacteria were chilled on ice for 30 min and harvested by centrifugation at 5000 ^{x}g for 5 min. The cells were washed twice with 272 mM sucrose, and sedimented after each wash by centrifugation at 5000 ^{x}g for 10 min. The bacteria were suspended in 272 mM sucrose to 1% of the original volume, and aliquots of 39μl were used for electroporation after the addition of one μl of transposome. Electroporation was performed as described **(Sandal et al., 2008)**.

DNA manipulations, sequencing and sequence analysis

All DNA manipulations, including plasmid purifications, restriction digestions, ligations and gel electrophoresis were performed according to the established protocols **(Sambrook et al., 1989)**. All genomic DNA purifications, PCR amplifications were performed as described by **(Sandal et al., 2008)**. The sequence flanking transposon mutants were determined by inverse PCR as described by **(Martin and Mohn, 1999)**. It was important to use frequent cutting restriction enzymes, because smaller fragment are more easily amplified in subsequent PCR reactions. The restriction enzyme PstI (New England Biolabs, Ipswich, MA) that cut the transposome once, but have frequent restriction sites at H.somni 2336 genome was chosen to completely digest genomic DNA. The kanamycin resistance primers and the primers used to amplify both Transposome flanking sequences are included in table 1. PCR products were purified using the PCR purification kit (Qiagen, Valencia, CA) and topocloned using Topo TA cloning kit (Invitrogen, Carlsbad, CA). Sequencing was performed at core laboratory facilities at the Virginia Bioinformatics Institute (VBI), Virginia Tech using universal primers M13 (Invitrogen, Carlsbad, CA) and transposome primers. Flanking sequences of insertion sites were analyzed by comparison to public translated DNA sequence database using BLAST software **(Altschul et al., 1997)**.

EPS purification :

The bacteria were also grown in filled, 1-L bottles containing Terrific broth supplemented with Trizma base and thiamine monophosphate with shaking at 75 rpm at 37^{o}C for 4-5 days. The clear supernatant was carefully removed and the cells and biofilm that settled to the bottom of the flask were extracted with hot, 45% aqueous phenol, digested with DNase, RNase, and Proteinase K, and subjected to ultracentrifugation at 125,000 x g at 4^{o}C, as described for purification of H. somni lipooligosaccharide (LOS) **(Inzana et al., 1988)**. The supernatant from the ultracentrifugation step was precipitated by the addition of 30 mM sodium acetate (final) and 5 volumes of 95% ethanol after incubation at -20^{o}C for at least 4 hours. The pellet obtained by centrifugation was suspended in distilled water and eluted through a sephacryl S-400 column (2.5 x 50 cm) with distilled water as eluent. The first fractions containing carbohydrate (determined by phenol-sulfuric acid assay) were pooled and lyophilized.

Polyacrylamide gel electrophoresis and alcian blue silver staining

Polyacrylamide gel electrophoresis (PAGE)

for polysaccharides followed by alcian blue and silver staining was done as described by **(Pelkonen et al., 1988)** and **(Min and Cowman, 1986)**, respectively.

RESULTS

Isolation of mutants defective in biofilm formation

A total of 96 real random transposon mutants were generated of H.somni 2336 using the EZ::TN™ <KAN-2>Tnp Transposome™. These mutants were confirmed by colony blot and PCR of kanamycin resistance gene. By PCR, an approximately 900 bp product could be amplified from all the mutants. The results indicated the kan resistant gene was integrated into the bacterial genome (Fig. 1). Of the 96 mutants screened, 31 mutants unable to form such a biofilm were isolated. Scanning electron microscope (SEM) was used to confirm the alteration in biofilm formation in these mutants. By SEM, 25 mutants were confirmed to make less biofilm than the wild type strain (~30-80% decrease) as shown in Fig.2.

Some biofilm mutants are defective in EPS production

A preliminary Alcian Blue silver stain was performed with the exopolysaccharide extracted from mutants that diminish biofilm formation to determine the correlation between biofilm formation and EPS production. Of interest that 3 of these biofilm deficient mutants were making very faint material in contrast with H.somni 2336 wild type that was observed on the gel as typical high molecular weight polysaccharides (Fig. 3). To confirm these results, ELISA was conducted to test the purified EPS from the mutants and the wild type using polyclonal antirabbit serum. Absorbance of mutant strains was much less than wild type 2336. This might explain some of the contribution of EPS to biofilm structure and function as well as cellular attachment to surfaces. Of interest also, that the total EPS amount of these mutants was much less than the wild type (~ 4-6 mg for mutants and ~14 mg for the wild type).

IPCR followed by topo cloning and sequencing allowed transposome insertion site determination.

We analyzed the DNA sequence flanking the transposon inserts of these three mutants that are both biofilm and EPS defective. IPCR of the 3 mutants that are both biofilm and EPS defective revealed amplification of ~1.3 kb of the flanking region. Topocloning of the inverse PCR products and sequencing of the plasmid containing the PCR product verified a mutation in the gene encoding for a homolog of filamentous haemagglutinin (FHA), predicted to be involved in attachment (Locus Tag HSM_1090) which play a role in Bordetella pertussis as the attachment bacteria to cells of the respiratory epithelium in whooping-cough infections **(Nicholson et al., 2009)** as indicated by the BLAST software analysis.

DISCUSSION

EPS is one of the main constituents of the biofilm matrix **(Watnick and Kolter, 2000)**. The exopolysaccharides (EPS) synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties. There is limited information about the bacterial mechanisms involved in the biosynthesis and modification of EPS components. Specifically, we lack knowledge about

how the structure of EPS influences the virulence of animal pathogens. Previous work with Pseudomonas aeruginosa and with Escherichia coli has shown that EPS (alginate and colanic acid, respectively) synthesis is induced upon attachment of the bacteria to a surface **(Davies et al., 1993, Davies and Geesey, 1995, Prigent-Combaret et al., 1999**). Although the association of exopolysaccharide (EPS) with attached bacteria has been demonstrated by both electron microscopy **(Geesey et al., 1977)** and light microscopy **(Allison and Sutherland., 1984)**, there is little direct evidence to suggest that EPS participates in the initial stages of adhesion, despite its synthesis by many species in the adherent population. It is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms **(Allison and Sutherland, 1987, Watnick and Kolter, 1999)**.

H.somni EPS production appeared to be upregulated during stress-like conditions that favours biofilm formation. Furthermore, maximum amounts of EPS were obtained under growth conditions in broth that stimulated biofilm formation. H. somni produces an excellent biofilm under most growth conditions other than rapid shaking and high aeration **(Sandal et al., 2007)**. Therefore, it is highly likely that this EPS is a component of the biofilm matrix. We were interested to role played by H.somni 2336 EPS in biofilm formation.

In this study, the role of EPS in biofilm formation of H.somni 2336 was investigated by creating random transposon knockouts using the EZ::TN™ <KAN-2>Tnp Transposome™. The mutants were then screened phenotypically for mutants that are biofilm deficient using the crystal violet assay and SEM. We screened these biofilm deficient mutants for mutants that are deficient in EPS by loading the purified EPS from these mutants and the parent stain as well onto 25% polyacrylamide gels, followed by electrophoresis and staining with Alcian blue and silver stain. Our results showed 3 mutants that are biofilm and EPS deficient Sequence and BLAST analysis revealed that these mutants have a mutation in the gene encoding for a homolog of filamentous haemagglutinin (FHA) **(Locus Tag HSM-1090)**.

The filamentous hemagglutinin (FHA) which is a fimbrial-like structure on the bacterial surface, and cell-bound pertussis toxin (PTx) is one of two hemagglutinins produced by phase I strains of Bordetella pertussis. It is an adhesin that binds the bacteria to cells of the respiratory epithelium in whooping-cough infections. It also plays critical roles in the adhesion process and is necessary for colonization Bordetella bronchiseptica to the swine respiratory tract **(Nicholson et al., 2009)**. Earlier studies reported the isolation and characterization of mutants of Pseudomonas aeruginosa PA14 defective in the initiation of biofilm formation that are designated surface attachment defective (sad). These mutants were defective in biogenesis of the polar-localized type IV pili. Competition studies between wild-type V. cholera and pilus mutants show that these structures provide a great advantage in surface colonization. Finally, exopolysaccharide production is necessary to stabilize the pillars of the biofilm **(Watnick and Kolter, 1999)**.

Table 1 : PCR primers used in this study.

Name	Primer sequence
KAN-2 FP-1	5' - ACCTACAACAAAGCTCTCATCAACC - 3' Forward primer used for DNA sequencing
KAN-2 FP-1	5' - GCAATGTAACATCAGAGATTTTGAG - 3' Reverse primer used for DNA sequencing
Kan_CHK_F	5'- AAGTTGGGTAACGCCAGGGTTTTCC -3' Forward PCR primer for kanamycin resistant gene amplification
Kan_CHK_R	5'- AAGTTGGGTAACGCCAGGGTTTTCC -3' Forward PCR primer for kanamycin resistant gene amplification
M13 F	5´-GTAAAACGACGGCCAG-3´ Forward primer used for DNA sequencing
M13 R	5´-CAGGAAACAGCTATGAC-3´ Reverse primer used for DNA sequencing

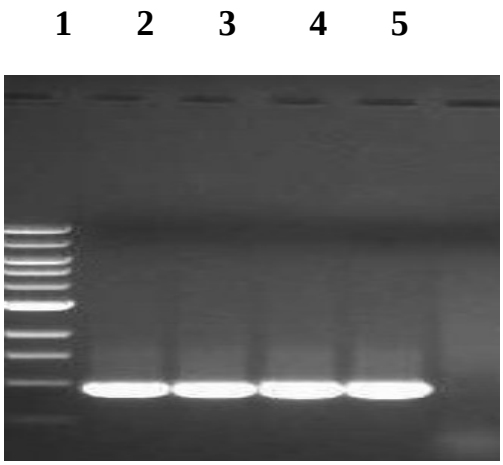

Fig. 1 : Mutant confirmation by PCR. The kanamycin résistance gene could be amplified from all mutants (lanes 1-4, ~0.9 kb). No amplification in the *H.somni* 2336 wild type (lane 5).

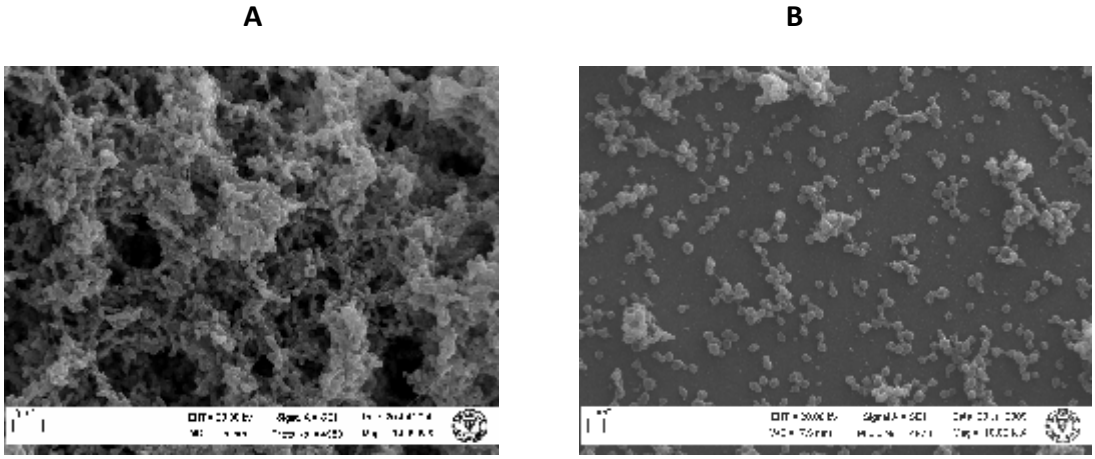

Fig. 2 : Scanning electron microscope showing the architecture of the biofilm mass of biofilm deficient mutant versus the wild type *H.somni* 2336. A is showing the aggregated cells in the biofilm of the parent strain with magnification 10000 X. B is showing the significant decrease in the aggregated cells of the bofilm deficient mutant

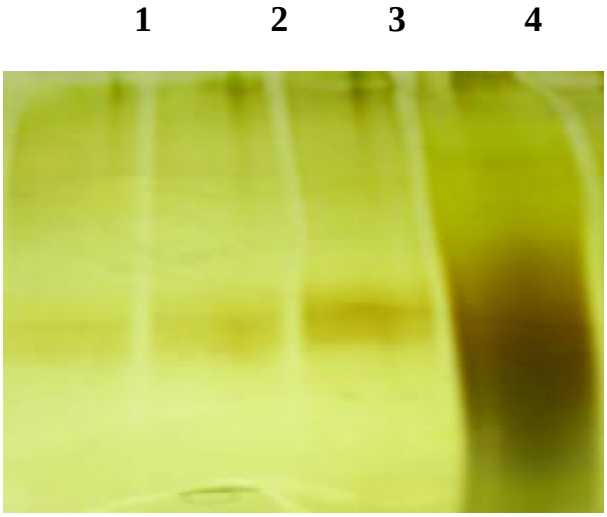

Fig. 3 : Preliminary Alcian Blue silver stain of purified EPS Purified EPS from transposon mutants waere loaded onto 25% polyacrylamide gels, followed by electrophoresis and staining with Alcian blue and silver stain. Lanes: 1 to 3, 30 µg of purified EPS from EPS deficient mutants.Lane:4 purified EPS from wild type *H.somni* 2336 showing high molecular weight material that is typical of bacterial capsules.

Fig. 4. : EPS extraction from by *H.somni* 2336 biofilm after 5 days of growth in supplemented Terrific broth. Bacteria were grown as planktonic cells in a starter culture of 50 ml and then transferred to 1 L of filtered Terrific broth supplemented with TMP, Trizma base, and glucose in a 1 L bottle. Maximum amounts of EPS could be isolated from this biofilm after at least 5 days growth

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الملخص العربى

النقاط الدقيقة (قسمى البحث العلمى) واستكشافه فى تعليمات البيوفيلم

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الهيموفلس سومنس (الهيستوفلس سومنى) من الميكروبات السالبة الجرام الانتهازية المسئولة عن الأمراض التنفسية المعقدة فى الأبقار أو حمى الشحن، إلتهاب السحايا والدماغ التخثرى، إلتهاب عضلة القلب، إلتهاب المفاصل، الإجهاض، تسمم الدم وغيرها من الأمراض الجهازية، أحد العوامل الهامة لضراوة الهيستوفلس سومنى هى تكوين الغشاء الحيوى (البيوفيلم) وإنتاج السكاريد الخارجى (أكسوبولى سكاريد).

يكون الهيستوفلس سومنى غشاء حيوى بكمية كبيرة سواء داخل الجسم أو فى المختبر وبالتالى يكون تكوين الغشاء الحيوى مهم لاستمرار الهيستوفلس سومنى داخل الجسم، ينتج الهيستوفلس سومنى أيضاً سكاريد خارجى الذى يسيل من على سطح الخلايا المناعية على النحو الذى حدده المجهر المناعى الإلكترونى المتنقل إلا أن البيانات التى لدينا على تلك عوامل الضراوة تفتقر إلى معلومات عن الوظائف الچينية التى تنظم تكوين السكاريد الخارجى، لذلك فقد تم إنشاء طفرات چينية باستخدام الترانسبوسون EZ::Tn5TM<KAN-2>Tnp Transposome وفرزهم للوصول لطفرات غير مكونة للغشاء الحيوى، تم تحديد ٢٥ طفرة تكون غشاء حيوى أقل كثيراً من السلالة الأم (٥٠-٧٠٪ أقل) وهو ماأكده المجهر الإلكترونى الماسح.

تم التعرف على مواقع إدراج الترانسبوسون فى هذه الطفرات التى تكون الغشاء الحيوى بدرجة أقل عن طريق تسلسل الحامض النووى لمنتجات إختبار البلمرة المتسلسل المعكوس وبعض هذه الطفرات يقع الترانسبوسون فى الچين الهيماغلوتين الخيطى (FHA) والذى يتوقع أن يكون مسئولاً عن إلتصاق الغشاء الحيوى.

لوحظ أيضاً أن بعض الطفرات فى الچين (FHA) تنتج قليلاً من السكاريد الخارجى عن السلالة الأم وذلك عن طريق إختبارات الأليزا، صبغة الألشيان الأزرق الفضى وكذلك عن طريق وزن كمية السكاريد الخارج، هذه النتائج قد تفسر الدور الذى يلعبه السكاريد الخارجى فى عملية إلتصاق الغشاء الحيوى على السطوح، لقد تم استخلاص السكاريد الخارجى من أكبر كمية من الرسوب الخلوية التى نمت فى إنخفاض الأوكسجين لمدة خمسة أيام وعلاوة على ذلك تم إنتاج مقادير الحد الأقصى من السكاريد الخارجى خلال المرحلة الثابتة، فى الظروف اللاهوائية وخلال نمو فى ٢٪ كلوريد الصوديوم وهذه المؤثرات تعتبر مؤثرات إجهاد التى تحفز الغشاء الحيوى، هذا يعنى أن الحد الأقصى للسكاريد الخارجى يمكن الحصول عليه فى ظل الظروف التى حفزت نمو الغشاء الحيوى والتى هى عنصر من نسيج الغشاء الحيوى.