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Prevalent distribution and conservation of *Streptococcus suis* Lmb protein and its protective capacity against the Chinese highly virulent strain infection

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ABSTRACT

Streptococcus suis (*S. suis*) is an important zoonotic pathogen that causes multiple diseases in both pigs and humans. Many studies suggest that *Streptococcus* utilizes host extracellular matrix proteins, including laminin, for adhesion and invasion of host cells. Recently, we identified a putative Lmb protein (CDS 0330) of a highly virulent strain of *S. suis* (serotype 2). In this study, we characterized the ability of CDS 0330 to bind human laminin, and evaluated the protective efficacy of a recombinant protein vaccine. Bioinformatic analysis revealed that both the amino acid sequence and tertiary structure of CDS 0330 were similar to Lmb proteins in other *Streptococcus*. In addition, the sequence of *CDS 0330* was present in the genomes of 26 of the 38 sequenced streptococci species, indicating an early origin and conservation of this gene. Particularly, all 17 sequenced *S. suis* genomes, regardless of serotype or geographic origin, contained *CDS 0330* gene in their genome with a minimum pair-wise amino acid identity of 92%. PCR amplification revealed that *CDS 0330* gene is distributed throughout 35 *S. suis* serotypes in the *lmb-htp* format. Flow cytometry analysis confirmed that *CDS 0330* was expressed on the cell surface of *S. suis*, and ELISA revealed the recombinant CDS 0330 protein could bind laminin *in vitro*. Finally, vaccinating mice with recombinant CDS 0330 protein significantly prolonged survival after *S. suis* infection. Together, these data reveal that CDS 0330 is a laminin binding protein of *S. suis* 2, and open new avenues for preventing *S. suis* 2 infection.

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1. Introduction

Streptococcus suis (*S. suis*) is an important Gram-positive pathogen that is responsible for a wide range of serious diseases in pigs (Feng et al. 2010; Gottschalk et al. 2010). However, this zoonotic pathogen also infects human and induces endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis, endophthalmitis and purulent meningitis (Gottschalk et al. 2010; Choi et al. 2012). To date, thirty-five serotypes (types 1/2, and 1–34) of *S. suis* have been described. Among them, only a limited number of serotypes have been frequently isolated from diseased pigs and human, of which *S. suis* serotype 2 (*S. suis* 2) is the most frequent (Higgins and Gottschalk 1995; Messier et al. 2008; Gottschalk et al. 2010; Schultsz et al. 2012).

Cases of *S. suis* infections in human have been reported in many countries with varying impacts (Watkins et al. 2001; Rusmeechan and Sribusara 2008; Taipa et al. 2008; van de Beek et al. 2008; Fittipaldi et al. 2009). Two outbreaks that were particularly severe occurred in China in 1998 and 2005, and each was characterized by the high fatal streptococcal toxic shock syndrome (STSS) (Tang et al. 2006). To understand the pathogenic mechanism, and explore the potential virulence factors of these S. *suis* outbreaks, two highly virulent strains from Chinese patients with STSS were sequenced in 2007 (Chen et al. 2007). The sequence of these *S. suis* strains has made genome-wide screens for potential protective antigens possible. For instance, a collection of structural and enzymatic proteins that are associated with the bacterial cell wall have recently been identified (Feng et al. 2009; Liu et al. 2009; Shao et al. 2011). Vaccination against some of these proteins, including Sao, HtpS and Enolase, exhibit a distinctive protective effect against *S. suis* infection.

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Laminin-binding protein (Lmb) is an extracellular protein that was first identified in *S. agalactiae* (Spellerberg et al. 1999). Later, homologs of this protein were reported in *S. pyogenes* and *S. pneumonia* and termed Lbp and AdcAII, respectively (Terao et al. 2002; Loisel et al. 2008). Collectively, studies reveal that Lmb plays an important role in the physiology and pathogenicity of these *Streptococci*, and demonstrate that Lmb proteins may have distinctive protective abilities against *Streptococcus* infection (Spellerberg et al. 1999; Terao et al. 2002; Wahid et al. 2005; Loisel et al. 2008; Linke et al. 2009; Ragunathan et al. 2009). However, to date no Lmb protein has been identified in *S. suis* 2.

A previous study revealed that *S. suis* 2 can bind human laminin and other extracellular matrix proteins (Esgleas et al. 2005). These data suggest that a laminin-binding protein is present in this bacterium. Thus, we sought to identify if CDS 0330 was the Lmb of *S. suis* 2, and evaluate its use as a vaccine.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Reference *S. suis* strains (serotypes 1/2 and 1–34) were kindly provided by Dr. Marcelo Gottschalk at the University of Montreal, Canada. The highly virulent *S. suis* 2 strain 05ZYH33 was previously isolated from an infected Chinese patient (Tang et al. 2006). Eleven *S. suis* 2 wild isolates used in this study were kept in our lab. All *S. suis* strains were cultured at 37 °C in Todd–Hewitt (TH) broth (Difco Laboratories, U.S.A) or TH containing 1.5% (wt/vol) agar and 6% (vol/vol) sheep blood. *E. coli* strains DH5α and BL21 (DE3) were grown in Luria Bertani (LB) broth (Oxoid, Germany) medium or LB containing 1.5% (wt/vol) agar. Two vectors, pEASY-T1 (Transgene, China) and pET32a (Novagen, U.S.A.) were used for gene cloning and protein expression. Transformed cells were selected by adding ampicillin (Sigma, U.S.A.) to the media at a final concentration of 50 μg/ml.

2.2. Sequence analysis and structural modeling of CDS 0330

Conservation of the CDS 0330 amino acid sequence and structure was confirmed by aligning it with the Genebank amino acid sequence of Lmb proteins from three previously identified strains (*S. pyogenes*, *S. pneumonia* and *S. agalactiae*). Multiple sequence alignments were conducted by using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) was used to process the output. Using the structure of *S. agalactiae* Lmb as a template, a model of the CDS 0330 protein structure was generated by submitting the amino acid sequence to SWISS-MODEL Server (http://swissmodel.expasy.org). The tertiary structure of this model was visualized using the Jmol-13.0.6 software (http://www.jmol.org/), and then compared with Lmb protein structures from *S. pyogenes* (PDB ID: 3GI1), *S. pneumonia* (PDB ID: 3CX3) and *S. agalactiae* (PDB ID: 3HJT) downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Transmembrane region analysis and signal peptide prediction of CDS 0330 were performed using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP version 4.1 (http://www.cbs.dtu.dk/services/SignalP/).

2.3. Occurrence of lmb genes in Streptococcus genus

Genomic sequences and annotation information of 38 *Streptococcus* isolates were downloaded from Pathosystems Resouce Integration Center (PATRIC, http://www.patricbrc.org) on March 8, 2012 (Gillespie et al. 2011). Detailed information regarding these genomes is listed in Table S1. First, annotation information was used to retrieve *lmb* genes. Then, to account for homologs missed by auto-annotation, all *lmb* genes obtained were used as query sequences to perform a blast search against 38 streptococcal genomes. The *Streptococcus* phylogenetic tree used in this study was modified from our previously published version (Shao et al. 2013).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2013.09.007.

To detect the distribution of the *lmb-htp* gene context in different serotypes of *S. suis*, forward (5′-GATATCGAAGCGAGTCAAGTACAGCCAG-3′) and reverse (5′-CTCGAGTGGGTCAAATACCAATCCATC-3′) primers were designed. These primers targeted the *CDS 0330* gene sequence from the N-terminus to the middle of the downstream *htp* gene.

2.4. Cloning, expression and purification of recombinant CDS 0330 protein

Expression and purification of CDS 0330 was performed as previously described (Pan et al. 2011). Specific primers were designed to amplify the *CD0330* gene sequence of the *S. suis* 2 Chinese strain 05ZYH33. The primer sequences were as follows: forward, 5′-GATATCGAAGCGAGTCAAGTACAGCCAG-3′ and reverse, 5′-CTCGAGCGGTCGATAGAATTCGGCCTTTTA-3′. These two primers also contained *Eco*RV or *Xho*I recognition sequences, respectively. After cloning the *CDS 0330* gene into pEASY-T1 vector and verifying the insertion, double digestion *via Eco*RV and *Xho*I was used to insert this gene into the pET32a vector to generate the pET32a-*lmb* recombinant vector. The pET32a-*lmb* recombinant vector was then transformed into *E. coli* BL21 for over-expression. When *E. coli* BL21 cells containing pET32a-*lmb* were growing at log phase, they were induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h and then harvested by centrifugation. These bacteria were lysed by sonication, and the lysate was centrifuged to remove insoluble pellets. The supernatant was filtered through a 0.22 μm pore-size filter (Millipore, U.S.A) and purified using a Ni-NTA column (Novagen, U.S.A). Purified CDS 0330 protein was eluted with 100 mM imidazole and stored at −20 °C. The recombinant protein was confirmed by western blot with an anti-his-tag monoclonal antibody.

2.5. Preparation of mouse CDS 0330 antiserum

Polyclonal antibodies against CDS 0330 were obtained by subcutaneously immunizing mice in four to six different sites with approximately 25 μg of purified protein diluted in PBS and emulsified with Freund Complete Adjuvant (Sigma, U.S.A). Animals were boosted twice by the same route at 2-week intervals using 25 μg of purified protein diluted in PBS and emulsified with Freund Incomplete Adjuvant (Sigma, U.S.A). A week after the last immunization, blood samples were collected and sera were isolated for biological activity assays. Pre-immune mouse serum was collected before the first injection.

2.6. Analyzing binding of CDS 0330 to human laminin

An enzyme-linked immunosorbent assay (ELISA) was used to evaluate binding of CDS 0330 to immobilized human laminin. Human laminin (Sigma, U.S.A) was diluted to 40 μg/ml in carbonate buffer, pH 9.6, and coated on 96-well ELISA plate (Greiner Bio-One) overnight at 4 °C. Carbonate buffer with 1% BSA (Sigma, U.S.A), pH 9.6, was used as a control. The following day, ELISA plate wells were washed three times with PBST and blocked with 1% BSA for 1 h at room temperature. Recombinant CDS 0330 (0.2–1 μg) diluted in PBST–1% BSA was then added to wells and incubated for 2 h at room temperature. Wells were then washed three times with 100 μl PBST

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