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Factor H specifically capture novel Factor H-binding proteins of *Streptococcus suis* and contribute to the virulence of the bacteria

Quan Li, Caifeng Ma, Yang Fu, Yanan He, Yanfei Yu, Dechao Du, Huochun Yao, Chengping Lu, Wei Zhang*

Key Lab of Animal Bacteriology, OIE Reference Lab for Swine Streptococcosis, College of Veterinary Medicine, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

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ABSTRACT

Factor H (FH), a regulatory protein of the complement system, can bind specifically to factor H-binding proteins (FHBPs) of *Streptococcus suis* serotype 2 (SS2), which contribute to evasion of host innate immune defenses. In the present study, we aimed to identify novel FHBPs and characterize the biological functions of FH in SS2 pathogenesis. Here, a method that combined proteomics and Far-western blotting was developed to identify the surface FHBPs of SS2. With this method, fourteen potential novel FHBPs were identified among SS2 surface proteins. We selected eight newly identified proteins and further confirmed their binding activity to FH. The binding of SS2 to immobilized FH decreased dramatically after pre-incubation with anti-FHBPs polyclonal antibodies. We showed for the first time that SS2 also interact specifically with mouse FH. Furthermore, we found that FH play an important role in adherence and invasion of SS2 to HEp-2 cells. Additionally, using a mouse model of intraperitoneal challenge, we confirmed that SS2 pre-incubated with FH enhanced bacteremia and brain invasion, compared with SS2 not pretreated with FH. Taken together, this study provides a useful method to characterize the host-bacteria interactions. These results first indicated that binding of FH to the cell surface improved the adherence and invasion of SS2 to HEp-2 cells, promoting SS2 to resist killing and leading to enhance virulence.

1. Introduction

Streptococcus suis (SS) is a major invasive pathogen in swine and a zoonotic agent for humans that can cause meningitis, arthritis, and septicemia (Gottschalk and Segura, 2000; Touil et al., 1988). In contrast to other serotypes, SS serotype 2 (SS2) is the most virulent and is most commonly isolated from pigs (Gottschalk and Segura, 2000; Staats et al., 1997). Although some putative virulence factors of SS2 have been identified, such as muramidase-released protein (MRP) (Smith et al., 1992) extracellular protein factor (EF) (Smith et al., 1993) and suilysin (SLY) (Lun et al., 2003), the molecular mechanism of SS2 pathogenesis remains poorly understood (Feng et al., 2014; Fittipaldi et al., 2012; Staats et al., 1997). Bacterial pathogens need to evade the host innate immune mechanisms and maintain a high level of bacteremia to cause meningitis and septicemia. However, the mechanisms of SS survival in host blood and how resists complement-mediated phagocytosis remain unclear (Gottschalk and Segura, 2000; Liu et al., 2014).

Factor H (FH) is a regulatory protein of the complement system family and is a complement control protein of the alternative pathway (Morgan, 2000; Ormsby et al., 2006). Its main function is to protect host tissues from C3b complements by promptly inactivating C3b adhered on the surface of host cells (Pangburn et al., 2000; Zipfel and Skerka, 2009). However, many pathogenic streptococci have factor H-binding proteins (FHBPs) on their cell surface to recruit FH to evade complement-mediated phagocytosis. The surface FHBPs play critical roles in the ability of the pathogenic bacteria to avoid complement attack and invade the host (Pian et al., 2012; Welsch et al., 2008). Additionally, factor H was already been shown to play an important role in bacterial adherence to host epithelial and endothelial cells (Quin et al., 2007). Furthermore, a number of FHBPs in bacterial pathogens are important virulence factors, and play various roles in adherence, invasion, and virulence, including Fhb (Pian et al., 2012), PspC (Quin et al., 2005; Quin et al.,

* Corresponding author at: Key Lab of Animal Bacteriology of Ministry of Agriculture & OIE Swine Streptococcosis Diagnostic Laboratory, Nanjing Agricultural University, Nanjing 210095, China.
E-mail addresses: 2015207024@njau.edu.cn (Q. Li), 2014107080@njau.edu.cn (C. Ma), 2015107080@njau.edu.cn (Y. Fu), 625226335@qq.com (Y. He), 2013207020@njau.edu.cn (Y. Yu), 529429075@qq.com (D. Du), yaohch@njau.edu.cn (H. Yao), lucp@njau.edu.cn (C. Lu), vszw@njau.edu.cn (W. Zhang).

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2007), fHbp (Biagini et al., 2016), Scl1 (Caswell et al., 2008), and the Emm18 protein (Perez-Caballero et al., 2004). To date, two SS2 surface FHBPs Fhb (Pian et al., 2012) and SSU0186 (Vaillancourt et al., 2013) were reported to interact with FH. It has been demonstrated that Fhb was involved in the pathogenesis of SS2 (Pian et al., 2012). SSU0186 was also described, which may contribute to pathogenesis of SS2 during infection (Li et al., 2010; Vaillancourt et al., 2013). Thus, large-scale identification of the surface FHBPs of SS2 that interact with FH will increase our understanding of SS2 pathogenesis.

Generally, surface proteins are important for virulence, immune escape, physiology and pathogenesis of the pathogenic bacteria via different mechanisms. Many cell wall surface proteins of SS2 have been confirmed as adhesion molecules (Brassard et al., 2004; Chen et al., 2011; Ferrando et al., 2010; Li et al., 2015b; Li et al., 2007) and have been identified as contributing virulence and antiphagocytic activities (Liu et al., 2014) (Li et al., 2015a; Liu et al., 2014; Pian et al., 2012; Pian et al., 2015). In our previous study, a number of laminin- and fibronectin-binding surface proteins of SS2 were identified by 2D-Far-western blotting (Li et al., 2015b). Moreover, the putative laminin- and fibronectin-binding surface proteins were further confirmed by Far-western blotting and ELISA-binding assays respectively. These results suggested that proteomic analysis together with Far-western blotting could be developed to identify the surface FHBPs in SS2 on a large scale.

In this study, the capacity of eight important novel FHBPs to bind FH was confirmed by Far-western blotting, ELISA and the specific antibody blocking assay, including enolase, elongation factor Tu (EF-Tu), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-bisphosphate aldolase (FBA), fibronectin-binding protein (FBPS), 3-ketoacyl-ACP reductase (KAR), and Muramidase released protein (MRP). Fhb and SSU0186, two previously reported FHBPs of SS2, were used as positive control proteins in specific FH-binding assay. Far-western blotting and cell-based ELISA binding assays confirmed that SS2 has the ability to interact specifically with mouse FH. Our results indicated that pre-incubation of SS2 with FH (designated SS2-FH) significantly improved the adherence and invasion of SS2 to HEp-2 cells, promoting SS2 to resist killing.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Highly virulent SS2 sequence type 7 strain ZY05719, originally isolated from a diseased pig in an outbreak in Ziyang, China, was used in this study. The bacteria were maintained in Todd Hewitt Broth (THB; pH 7.8; BD) or agar medium at 37 °C and collected by centrifugation at the late exponential phase of growth. A total of 100 μg/ml gentamicin and 5 μg/ml penicillin G (Sigma, USA) were used for the invasion assay, and 50 μg/ml ampicillin (Amp; Sigma) was used for the *Escherichia coli* transformants. *E. coli* DH5α and BL21 (DE3) cells were cultured in LB broth or on LB agar plates. The pET-32a (+) and pMD18-T vectors (Takara) were used for protein expression.

2.2. Preparation of cell wall proteins

The cell wall proteins used for proteomics analysis were prepared as previously described (Ling et al., 2004; Pian et al., 2015; Wu et al., 2008; Zhang et al., 2011). Bacterial pellets were rinsed two times with PBS after centrifugation; resuspended in a 4 ml solution buffer containing 125 U/ml mutanolysin (Sigma), 3 mM $MgCl_2$, 25% sucrose, 30 mM Tris-HCl (pH 7.5); and incubated at 37 °C for 90 min. The cell lysate was separated by centrifugation at 12,000*g* for 10 min at 4 °C. Cell wall proteins in the supernatant were precipitated in 10% chilled TCA in ice-water. The proteins were rinsed twice with chilled acetone to remove the residual TCA, and air-dried.

2.3. Identification of FHBPs by 2D-Far-western blotting

The 2-DE Far-western blotting experiment was performed as described previously, with minor modifications (Li et al., 2015b; Zhang and Lu, 2007). Briefly, cell wall proteins were solubilized at 25 °C in 250 μl of solution buffer (2% w/v CHAPS, 7 M urea, 0.2% w/v DTT and 2 M thiourea) for 30 min to remove the insoluble components. Two equivalent samples were solubilized in 250 μl rehydration buffer containing 0.5% v/v IPG buffer, 2% w/v CHAPS, 7 M urea, 0.002% w/v bromophenol blue, 0.2% w/v DTT and 2 M thiourea after treatment with a 2-D Clean-up Kit (GE Healthcare; USA). Subsequently, 13 cm immobilized pH gradient strips (GE Healthcare, pH 4–7) were used for isoelectric focusing analysis. For Far-western blotting assays, the protein samples were separated by 12% SDS-PAGE, and then the 2-DE gels were transferred onto PVDF membranes (Merck Millipore) and blocked with 5% (w/v) skimmed milk diluted with TBST containing 0.05% Tween 20, 50 mM Tris-HCl buffer (pH 7.4) and 150 mM NaCl for 12 h at 4 °C. The membranes were then washed three times and incubated with 5 μg/ml human FH (Hycult) dissolved in TBST for 24 h at 4 °C. Meanwhile, the negative control membrane was incubated with 1% BSA, followed by three washes with TBST. The membranes were then incubated with rabbit anti-human FH polyclonal antibodies (Abcam; 1:2000) followed by HRP conjugated goat anti-rabbit polyclonal antibodies (Boster; 1:2000) at 37 °C for 1 h. After three washes, the positive spots were detected using 3,3′-diaminobenzidine (Tiangen, China).

2.4. Identification of protein spots by MALDI-TOF-MS

The positive spots in the 2-DE gel (by comparison with the Far-western blotting) were performed according to our previous report (Li et al., 2015b). Briefly, the individual spots were excised from the gel for digestion, followed by MALDI-TOF-MS measurement. Peptide mass fingerprints were carried out using the MASCOT Software (http://www.matrixscience.com). MASCOT Searching was carried out as previously described (Li et al., 2015b) to identify accurate peptides and to avoid inaccurate identification of proteins. The criteria used to accept protein spots as valid were: five or more tryptic peptides and more than 15% sequence coverage matched for the protein sequence.

2.5. Purification of recombinant potential FHBPs and preparation of polyclonal antibodies

To express recombinant FHBPs in *E. coli*, the genomic DNA of ZY05719 was used as template for PCR reactions using the primers described in Table 1 and Phanta HS Super-Fidelity DNA Polymerase (Vazyme). DNA encoding eight potential surface FHBPs and two previously reported FHBPs (Fhb and SSU0186) were cloned and expressed, nine of them expressed full-length proteins. MPR1 (aa 340–592) was the non-conserved region of MRP and MRP2 (aa 848–1222) was the conserved region of MRP (data not shown). The expression experiment was carried out as previously described (Li et al., 2015b; Pian et al., 2015). PCR products were cloned into the pET-32a vector and transformed into BL21 (DE3) for expression. The cloned sequences were verified by DNA sequencing. The bacteria were induced for 5 h at 37 °C by treatment with 1 mM IPTG after they grew to an OD_{600} of 0.6. Bacterial cells were separated by centrifugation at 12,000 × *g* for 10 min at 4 °C. Protein purification was carried out using Ni-chelating chromatography (GE Healthcare), according to the instruction manual.