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Streptococcus suis small RNA rss04 contributes to the induction of meningitis by regulating capsule synthesis and by inducing biofilm formation in a mouse infection model

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ABSTRACT

Streptococcus suis (SS) is an important pathogen for pigs, and it is also considered as a zoonotic agent for humans. Meningitis is one of the most common features of the infection caused by SS, but little is known about the mechanisms of SS meningitis. Recent studies have revealed that small RNAs (sRNAs) have emerged as key regulators of the virulence in several bacteria. In the previous study, we reported that SS sRNA rss04 was up-regulated in pig cerebrospinal fluid and contributes to SS virulence in a zebrafish infection model. Here, we show that rss04 facilitates SS invasion of mouse brain and lung in vivo. Label-free quantitation mass spectrometry analysis revealed that rss04 regulates transcriptional regulator CcpA and several virulence factors including LuxS. Transmission electron microscope and Dot-blot analyses indicated that rss04 represses capsular polysaccharide (CPS) production, which in turn facilitates SS adherence and invasion of mouse brain microvascular endothelial cells bEnd.3 in vitro and activates the mRNA expression of TLR2, CCL2, IL-6 and TNF-α in mouse brain in vivo at 12 h post-infection. In addition, rss04 positively regulates SS biofilm formation. Survival analysis of infected mice showed that biofilm state in brain contributes to SS virulence by intracranial subarachnoidal route of infection. Together, our data reveal that SS sRNA rss04 contributes to the induction of meningitis by regulating the CPS synthesis and by inducing biofilm formation, thereby increasing the virulence in a mouse infection model. To our knowledge, rss04 represents the first bacterial sRNA that plays definitive roles in bacterial meningitis.

1. Introduction

Streptococcus suis (SS) is not only a major bacterial pathogen for pigs in the world, but also an important zoonotic agent for humans who have contacted with infected pigs or pork products. Among the 33 serotypes based on capsular polysaccharide (CPS), SS serotype 2 (SS2) is the most virulent and prevalent serotype frequently associated with several diseases including meningitis, endocarditis, septicemia, arthritis, and pneumonia (Hill et al., 2005). Among those diseases caused by SS, meningitis is the most common clinical symptom in pigs. In addition, SS2 is also an important pathogen for human meningitis in Vietnam, Thailand and Hong Kong (Segura, 2015). However, the mechanism of meningitis caused by SS is poorly understood.

SS uses global regulators to coordinate and orchestrate virulence programs for infection. Catabolite control protein A (CcpA) is an important transcriptional regulator involved in carbohydrate metabolism regulation, CPS production, and virulence (Tang et al., 2012; Willenborg et al., 2014, 2011). In addition to protein regulators, small RNAs (sRNAs) have emerged as key regulators for bacterial virulence (Toledo-Arana et al., 2009). For examples, *Staphylococcus aureus* sRNA RsaA promotes bacterial persistence and decreases virulence by regulating regulator MgrA (Romilly et al., 2014); *Streptococcus pneumoniae* sRNA F20 contributes to adhesion and invasion of host nasopharyngeal cells (Mann et al., 2012); *Streptococcus pyogenes* sRNA FasX controls the transition from colonization to dissemination during infection by regulating streptokinase and pilus expression (Liu et al., 2012).

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However, so far there have been no reports of bacterial sRNAs playing definitive roles in bacterial meningitis.

In our previous study, 29 sRNAs have been identified by differential RNA-sequencing approach in different infection conditions (Wu et al., 2014). One of them, rss04, located between genes *SSU0908* and *SSU0909* in SS virulent strain P1/7, contributes to SS virulence in a zebrafish infection model (Wu et al., 2014). Since rss04 was significantly upregulated in pig cerebrospinal fluid (CSF) compared with control condition (Wu et al., 2014), it is interesting to hypothesize that rss04 may be involved in SS meningitis. In the present study, we show that rss04 facilitates SS invasion of mouse brain and lung, promotes SS adherence and invasion of mouse brain microvascular endothelial cells (BMEC), and activates the expression of TLR2, CCL2, IL-6 and TNF-α in mouse brain by regulating capsule production and biofilm formation. These findings have deepened the understanding of SS meningitis mechanism.

2. Materials and methods

2.1. Bacterial strains and culture conditions

SS strains were grown in Todd-Hewitt broth (THB, Difco Laboratories) and plated on Todd-Hewitt agar (THA) at 37 °C. SS virulent strain P1/7 was isolated from a disease pig with meningitis (Holden et al., 2009). *Escherichia coli* strains XL10 and BL21(DE3) were cultured in Luri Bertani Broth (LB, Sigma-Aldrich) at 37 °C. Differential concentrations of antibiotics (Sigma-Aldrich) were added into medium when necessary as following: for SS, penicillin at 5 μg/mL, spectinomycin at 100 μg/mL; for *E. coli*, spectinomycin at 50 μg/mL, ampicillin at 100 μg/mL, kanamycin at 30 μg/mL.

2.2. Ethics statement

All the animal experiments were performed in the Laboratory Animal Center of Nanjing Agricultural University with the approval of the Laboratory Animal Monitoring Committee of Jiangsu Province (Permit number: SYXK (Su) 2011-0036).

2.3. Determination of viable bacteria in organs of infected mice

The virulence of wild type (WT) strain P1/7, rss04 mutant (Δrss04), and rss04 complemented strain (C-rss04) was evaluated in BALB/c mouse infection model, according to our previous report (Wu et al., 2016). The BALB/c mice (female, 6-weeks-old) were obtained from Yangzhou Laboratory Animal Research Center, Yangzhou, China. The Δrss04 and C-rss04 were constructed in our previous research (Wu et al., 2014). Bacteria were grown to mid-log phase, harvested, washed twice in PBS, and then adjusted to the appropriate doses. Five mice per group were intraperitoneally injected with the dose of 2.1×10^8 CFU of WT strain P1/7, Δrss04, or C-rss04. Bacterial CFUs of injected inoculum were confirmed by plating on THA. All the mice were euthanized at 24 h post-infection. Brain, lung, heart, liver, spleen, and kidney samples from infected mice were weighed and homogenized in PBS (pH 7.4). All the samples were diluted with appropriate dilutions and plated onto THA. Statistical analyses were performed by Graphpad Prism 6 software using one-way ANOVA and Dunnett's multiple comparisons test.

2.4. Quantification of CPS production

Transmission electron microscope analysis was performed to compare the CPS thickness of P1/7, Δrss04, and C-rss04. Bacteria grown in THB to mid-log phase were collected. The transmission electron microscope analysis was performed using HITACHI H-7650 system (HITACHI) according to the manufacturer's instruction.

The CPS production was further validated by Dot-blot analysis according to our previous report (Wu et al., 2014). Five microliter of two-fold serially diluted bacteria in PBS were spotted onto the nitrocellulose membrane. The samples were then fixed with 70% ethanol for 5 min, air-dried, and blocked with 5% w/v skim milk in PBST buffer (PBS containing 0.05% Tween 20) for 2 h. The nitrocellulose membrane spotted with bacteria was probed using the specific antibody against SS serotype 2 CPS (Wu et al., 2014) as a primary antibody (with a dilution 1:500); after washing with PBST buffer, the membrane was then incubated with HRP-conjugated anti-rabbit (Boster) as a secondary antibody (with a dilution 1:2000). The Dot-blot signal was detected according to the protocol of Tanon ™ High-sig ECL Western Blotting kit (Tanon) using Tanon 5100 (Tanon). The average gray value was quantified by using Image J 1.50 software (National Institutes of Health). The assay was repeated for three times. Statistical analyses were performed by Graphpad Prism 6 software using one-way ANOVA and Dunnett's multiple comparisons test.

2.5. Adherence and invasion of mouse BMEC bEnd.3

The ability of SS adhere to and invade mouse BMEC bEnd.3 was evaluated using WT strain, Δrss04, or complemented strain according to our previous report (Pan et al., 2015). In briefly, Corning Costar 24-well cell culture plates were pre-coated overnight at 4 °C with 1% bovine serum albumin (Sigma) solution in PBS and washed twice with PBS before use. bEnd.3 cells were grown at 37 °C and 5% CO_2 in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), washed twice with PBS, and maintained in RPMI 1640 culture medium. The monolayers, grown to the number of 1×10^5 cells/well, were infected with mid-log growth phase bacteria at a multiplicity of infection of 10 (bacteria: bEnd.3 cells). Then, the infected monolayers were centrifuged for 10 min at 800 $\times g$ and incubated for 1 h at 37 °C and 5% CO_2. The wells only containing bacteria without the bEnd.3 cells were served as a control. For adhesion, monolayers were washed six times with PBS, lysed with 100 μL 0.05% Triton X-100, and disrupted by mild pipetting. Appropriate dilutions were plated onto THA plates to determine the viable bacterial cells. For the invasion assay, the infected cells were incubated for 1 h at 37 °C and 5% CO_2, washed three times with PBS, and then incubated for another 1 h in RPMI 1640 culture medium containing penicillin (5 μg/mL) and gentamicin (100 μg/mL) to kill extracellular bacteria. Monolayers were washed six times with PBS, and then the viable intracellular bacteria were determined as described in adhesion assay. The adherence rate was calculated by dividing the number of bacteria remaining attached to cells after the incubation period by total bacterial inoculation number. The invasion rate was calculated by dividing the number of invaded bacteria by total bacterial inoculation number. The assay was performed in triplicate. Statistical analyses were performed by Graphpad Prism 6 software using one-way ANOVA and Dunnett's multiple comparisons test.

2.6. Purification of recombinant CcpA and preparation of mouse polyclonal antisera against CcpA

The recombinant plasmid pET-30a-ccpA for expressing and purifying CcpA was kindly provided by Prof. Weihuan Fang from Zhejiang University, China. The protocol for purifying recombinant CcpA has been described in details in the previous report (Tang et al., 2012). Specific mouse polyclonal antisera again CcpA were obtained by immunizing ICR mice (Yangzhou Laboratory Animal Research Center) with purified recombinant protein. Briefly,