Microbial Pathogenesis 98 (2016) 149–154

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Formate-tetrahydrofolate ligase is involved in the virulence of *Streptococcus suis* serotype 2

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ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form
11 July 2016
Accepted 13 July 2016
Available online 15 July 2016

Keywords:
Streptococcus suis
Formate-tetrahydrofolate ligase
Virulence

ABSTRACT

Streptococcus suis is an emerging zoonotic pathogen that causes severe infections in pigs and humans. However, the pathogenesis of *S. suis* remains unclear. The present study targeted a putative virulence-associated factor (*fhs*, encoding the formate-tetrahydrofolate ligase) of *S. suis*. To investigate the role of *fhs* in the virulence potential of *S. suis* serotype 2, an *fhs* deletion mutant (*Δfhs*) and the corresponding complementation strain (C*Δfhs*) were generated. The *Δfhs* mutant displayed similar growth compared to that of the wild-type and complementation strains. Using murine and pig infection models, we demonstrated for the first time that the formate-tetrahydrofolate ligase is required for the full virulence of *S. suis* 2. Our findings provide a new insight into the pathogenesis of *S. suis* 2.

1. Introduction

Streptococcus suis is an important bacterial pathogen in the swine industry, and causes a wide variety of diseases in pigs, including meningitis, septicemia and endocarditis [1]. It is also a zoonotic agent responsible for human infections such as meningitis, septicemia and streptococcal toxic shock syndrome (STSS) [2]. Based on differences in capsular polysaccharides, 33 serotypes (types 1–31, 33, and 1/2) have been described for *S. suis* [3]. Among them, *S. suis* serotype 2 (*S. suis* 2) is considered the most virulent and the most frequently isolated serotype associated with disease in pigs and humans in most countries [4]. *S. suis* is a previously neglected, but reemerging human pathogen of increasing infective capacity [5,6]. Since the first case of *S. suis* infection in humans described in Denmark in 1968, approximate 1600 human cases had been reported as of 2012 [7]. Notably, two large-scale outbreaks of *S. suis* infection occurred in China in 1998 (Jiangsu Province) and in 2005 (Sichuan Province), which resulted in 240 human cases with 52 deaths in total [8]. Recently, human cases of *S. suis* infection have also been reported in the United States [9], Thailand [10,11], Greece [12], and Japan [13].

The severity of *S. suis* infection in pigs and humans emphasizes the urgent need for a better understanding of its pathogenesis. Over the past few years, great developments have been made in *S. suis* research. Comparative genomics analyses revealed that Chinese *S. suis* 2 virulent isolates harbor an 89 K pathogenicity island (PAI) [14], and that loss of this PAI might lead to the emergence of variants [15]. Recently, *N*-acetyl-galactosamine and biotin utilization pathways have been described in *S. suis* [16,17]. Although numerous virulence-associated factors have been identified in *S. suis* [6,18], it has still been difficult to establish their association with different steps in the infection process [18]. In addition, the absence of one or more of these factors does not always result in impaired virulence in *S. suis* [19], indicating the presence of other virulence determinants. To gain further insight into the pathogenesis of *S. suis*, the identification and characterization of more virulence-associated factors are required. One important strategy is targeting genes encoding homologues of known virulence-associated factors in other Gram-positive bacteria. Examples of virulence-associated factors identified using this method are Trigger factor [20], a type IV-Like secretion system [21], and LuxS [22]. Through this strategy, we also identified two Spx regulators that modulate stress tolerance and virulence in *S. suis* 2 [23].

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http://dx.doi.org/10.1016/j.micpath.2016.07.009
0882-4010/

In this study, we targeted the gene encoding formate-tetrahydrofolate ligase (Fhs, also known as formyltetrahydrofolate synthetase), as the homologue of this gene is required for full virulence of *Streptococcus pneumoniae* [24]. An *fhs* deletion mutant (*Δfhs*) and the corresponding complementation strain (C*Δfhs*) were constructed in *S. suis* 2 strain SC19. The virulence of the wild-type (WT), *Δfhs*, and C*Δfhs* strains was evaluated in both murine and piglet models of infection. Our findings revealed that the formate-tetrahydrofolate ligase is involved in virulence of *S. suis* 2.

2. Material and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were cultured in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI, USA) supplemented with 10% (vol/vol) newborn bovine serum (Zhejiang Tianhang Biotechnology Co., LTD., Hangzhou, China) at 37 °C. *Escherichia coli* strain DH5α was grown in Luria-Bertani (LB) broth or on LB agar at 37 °C. When required, spectinomycin (Sigma) was added at the following concentrations: 50 μg/ml for *E. coli* and 100 μg/ml for *S. suis*.

2.2. Deletion of the fhs gene and functional complementation

The thermosensitive suicide vector pSET4s [25] was used to delete the *fhs* gene in *S. suis* 2 strain SC19. Briefly, two DNA fragments (LA and RA) flanking *fhs* were amplified from the SC19 genome using the primers listed in Supplementary Table 1, with Phanta Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). The PCR products were purified, digested with the appropriate restriction enzymes, and simultaneously cloned into pSET4s. The resulting plasmid, pSET4s::*fhs*, was introduced into competent *S. suis* SC19. After two steps of allelic exchange, spectinomycin-sensitive clones were selected, and mutants were identified by PCR using two pairs of primers listed in Supplementary Table 1. The *fhs* gene deletion was confirmed by reverse transcription PCR (RT-PCR) and DNA sequencing analysis.

For complementation analysis, a DNA fragment containing the *fhs* gene and its predicted promoter was amplified from the SC19 genome, and cloned into pSET2 [26], to generate plasmid pSET2::*fhs*. This plasmid was electroporated into the *Δfhs* mutant. The complementation strain C*Δfhs* was selected with spectinomycin and verified by PCR, RT-PCR, and DNA sequencing.

2.3. Bacterial growth curves

Overnight cultures of the WT, *Δfhs* and C*Δfhs* strains were diluted 1:100 in fresh medium (TSB with 10% newborn bovine serum) and incubated at 37 °C under static conditions. Samples were taken from the cultures to measure the optical density at 600 nm (OD_{600}) every hour.

2.4. Experimental infection of mice

All animal studies were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei Province, China. A total of 32 female BALB/c mice (Five-week-old) were randomly divided into four groups with eight animals per group. *S. suis* strains were harvested at early stationary phase and resuspended to 1×10^9 CFU/ml. Mice in groups 1, 2 and 3 were challenged by intraperitoneal injection of 1×10^8 CFU of the WT, *Δfhs* and C*Δfhs* strains, respectively. Group 4 was injected with 100 μl PBS, and served as a control group. The infected mice were monitored twice daily during the first 2 days and daily during the next 5 days for clinical signs and survival.

To estimate the number of viable bacteria in the blood and organs, 12 female BALB/c mice were assigned to three groups (four animals per group) and inoculated intraperitoneally with 2×10^7 CFU of the WT, *Δfhs*, and C*Δfhs* strains, respectively. Mice were sacrificed 6 h after infection for collection of blood, brain, liver, and spleen samples. Blood samples were serially diluted for plating, whereas organs were weighed and homogenized in 1 ml PBS. Homogenates were then diluted and plated on TSA containing 10% newborn bovine serum to determine the number of viable bacteria.

2.5. Competitive-infection assay in the piglet infection model

A competitive-infection assay was performed to compare the abilities of the *Δfhs* mutant and the WT strain to colonize different tissues in piglets. A group of four piglets were inoculated intravenously with a mixture of the WT and mutant strains at a ratio of 1:1 (5×10^5 CFU for each strain). Before mixing, the suspension containing each strain was diluted and plated to determine the actual ratio of the inoculum. The infected piglets were sacrificed 24 h after challenge for colonization analysis. Briefly, samples from the heart, liver, spleen, lung, kidney, and brain tissue were collected, homogenized, serially diluted, and plated on TSA containing 10% newborn bovine serum. Blood and cerebrospinal fluid (CSF) samples were directly diluted for plating. The *Δfhs*:WT ratios in these

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
S. suis		
SC19	Virulent strain isolated from the brain of a dead pig; Serotype 2	Laboratory collection
Δfhs	*fhs* deletion mutant of strain SC19	This study
C*Δfhs*	Complemented strain *of Δfhs*; Spc^R	This study
E. coli		
DH5α	Cloning host for recombinant vector	TransGen
Plasmids		
pSET4s	*E. coli-S. suis* shuttle vector; Spc^R	25
pSET4s::*fhs*	Knockout vector designed for deletion of *fhs*	This study
pSET2	*E. coli-S. suis* shuttle vector; Spc^R	26
pSET2::*fhs*	pSET2 containing the *fhs* gene and its promoter	This study

[a] Spc^R, spectinomycin resistant.