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Short Communication

Signature-tagged mutagenesis screening revealed the role of lipopolysaccharide biosynthesis gene *rfbH* in smooth-to-rough transition in *Salmonella* Enteritidis

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ABSTRACT

Salmonella enterica serovar Enteritidis (*S. Enteritidis*, SE) is a major cause of foodborne diseases for humans. The completeness of the O-chain antigen of Lipopolysaccharide (LPS) determines whether a *S. Enteritidis* strain is smooth or rough. However, genes that are involved in the synthesis of LPS and rough-smooth variation are not completely understood. In this study, we used monoclonal antibody against O-antigens (O_9 mAb) to identify novel factors that are involved in LPS synthesis and rough variation in *S. Enteritidis* by using signature-tagged mutagenesis (STM) technique. Our results showed that transposon insertion in the gene *rfbH* led to different LPS phenotype, auto-aggregation characteristic, motility and resistance to environmental stress compared with SE wild-type strain C50041. In addition, sera tests showed that *rfbH* mutant does not elicit specific antibodies against O-antigens in vaccinated animals. Taken together, the *S. Enteritidis rfbH* gene is implicated in LPS biosynthesis, rough variation, sera distinguishable reaction, motility and stress resistance. The *rfbH* mutant strain could be potentially used as a distinguishable vaccine or a live vector to deliver drugs and antibodies *in vivo*.

Salmonella enterica serovar Enteritidis (*S. Enteritidis*, SE) is Gram-negative, facultative anaerobes, and intracellular pathogens that pose a major cause of diarrhoea and systemic infections for humans (Mishu et al., 1994; Angulo and Swerdlow, 1998; Patrick et al., 2004). *Salmonella* possess protective outer membrane, with its external leaflet composed of lipopolysaccharides (LPS) (Nikaido, 2003; Nobre et al., 2015). Recently studies show that LPS is responsible for smoothness, virulence, flagellar assembly and for mounting cross reactivity (Deditius et al., 2015; Lalsiamthara et al., 2015; Jiao et al., 2017). Signature-tagged mutagenesis (STM) is a genome-wide functional screening assay based on an insertional mutation technique to identify gene locus that affects certain phenotypes, *e.g.*, virulence, stress resistance and phase variation. (Shea et al., 1996; Geng et al., 2014; Kukavica-Ibrulj and Levesque, 2014).

In this study, STM technique was used to discover novel factors that are essential for LPS synthesis and rough variation in *S. Enteritidis*, and we found that a previously uncharacterized gene *rfbH* (also called *ddhC*) was involved in this process. We further investigated the role of *rfbH* gene in LPS phenotype variation, sera distinguishable reaction, *in vitro* growth, biochemical characteristic, motility, stress resistance and some other biological characteristics in *S. Enteritidis*. These data expanded our knowledge of the effect of *rfbH* gene, even *rfb* gene cluster to LPS biosynthesis and flagellar assembly in *S. Enteritidis*.

The gene screening and identifying were performed according to the

Abbreviations: S. Enteritidis, SE, *Salmonella enterica* serovar Enteritidis; STM, signature-tagged mutagenesis; O_9 mAb, monoclonal antibody against *Salmonella* O:9 antigen; LPS, lipopolysaccharide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; LB, Luria-Bertani; Amp, ampicillin; Km, kanamycin; Cm, chloromycetin

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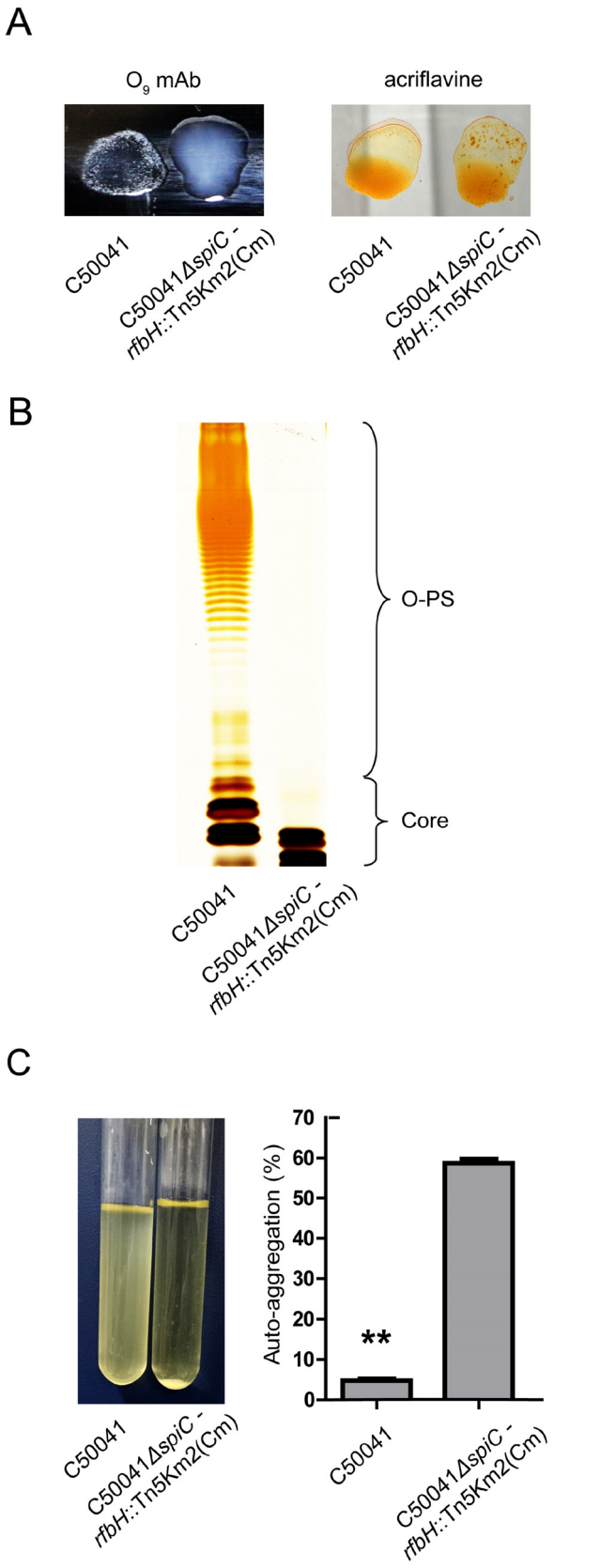

Fig. 1. LPS shortage of *rfbH* mutant strain. (A) O_9 mAb and acriflavine were used to do agglutination assay. Pictures were taken within 5 min. (B) SDS-PAGE with silver staining of LPS from *rfbH* mutant strain compared to SE C50041. (C) Visual auto-aggregation and auto-aggregation percentage of *rfbH* mutants strain and SE C50041, which culture grown statically for 16 h at 37 °C. ** $p \leq 0.01$ by Student's *t*-test. Two technical replicates were performed in two independent experiments.

method as described previously (Jiao et al., 2017). The homemade O_9 mAb was used to screen rough strains from *S. Enteritidis* signature-tagged transposon mutants by agglutination assay on glass plate. And we found that O_9 mAb has no reaction with a new mutant in the STM library (Fig. 1A). To confirm this mutant was a rough strain, acriflavine was used to perform agglutination assay. The results demonstrated that the agglutination reaction was obvious between this mutant strain and acriflavine (Fig. 1A). By a PCR-based method for specific amplification of transposon-flanking sequences (Kwon and Ricke, 2000; Geng et al., 2014), the inactivated gene was identified as *rfbH*, which encodes a CDP-4-keto-6-deoxy-D-glucose-3-dehydrase *RfbH*. Interestingly, previous study in our lab showed that *rfbH* gene was associated with *S. Enteritidis* biofilm formation, which is also a kind of rough phenotype (Dong et al., 2008). Therefore, we called this rough mutant strain SE C50041△*spiC* – *rfbH*::Tn5Km2(Cm), or *rfbH* mutant strain for short. And *spiC* deletion in *S. Enteritidis* was determined as no effect to LPS phenotype, sera distinguishable reaction, *in vitro* growth, biochemical characteristic and motility in previous studies (Jiao et al., 2017).

SDS-PAGE and silver staining were used to check the LPS phenotype of mutant strain (Leyman et al., 2011). LPS patterns obtained from SE C50041 and SE C50041△*spiC* – *rfbH*::Tn5Km2(Cm) are showed in Fig. 1B. The results showed an absence of core units and O-antigens for *rfbH* mutant strain compared to SE wild-type. Furthermore, the auto-aggregation phenomenon was different between the two strains in LB broth (the method followed (Zhou et al., 2014)). Fig. 1C showed the visual auto-aggregation and auto-aggregation percentage (AAg%) of SE C50041 and *rfbH* mutants strain. The AAg% of SE C50041 was 4.8%, while the SE C50041△*spiC* – *rfbH*::Tn5Km2(Cm) demonstrated 58.7% auto-aggregation. It has been reported that, as a part of O-antigen biosynthesis related genes, the *rfb* gene cluster of group B *Salmonella* (*e.g.* S. Typhimurium) are involved in the biosynthesis of abequose, mannose and rhamnose. Among them, the *rfbH* gene, which encodes a CDP-4-keto-6-deoxy-D-glucose-3-dehydrase, is a component of abequose biosynthetic pathway in S. Typhimurium (Yuasa et al., 1969; Wyk and Reeves, 1989; Jiang et al., 1991). However, in group D *Salmonella*, such as *S. Enteritidis*, the CDP-paratose is itself converted to CDP-tyvelose, resulting in paratose and tyvelose replacing abequose in the O-antigens of group D strain (Jiang et al., 1991). Currently, the function of *rfbH* gene in *S. Enteritidis* is unclear. Therefore, our results of agglutination assay, LPS staining and auto-aggregation test showed that *S. Enteritidis rfbH* mutant strain has typical rough characteristics, and *rfbH* mutation results in deep loss of LPS synthesis in *S. Enteritidis* (Fig. 1). This data suggested that the role of *rfbH* in LPS biosynthesis in *S. Enteritidis* needs to be further investigated.

Sera tests were done according to the method as described previously (Jiao et al., 2017). In brief, SPF chickens (n = 6/each group) were immunized with 1×10^8 CFU of each *Salmonella* (100 µl bacterial suspension in PBS) or treated with sterile PBS (control) by intramuscular injection. 14 days later the second immunization were done *via* the same way. Sera were collected and determined by both agglutination assay and ELISA test on days 14 after the boost immunization. The results showed that blood collected from *rfbH* mutant strain group (n = 6) and control group (n = 6) were considered *Salmonella* negative by both ways. In contrast, sera collected from the SE C50041 immunization group (n = 6) showed obvious reaction by agglutination assay, while were considered 5 seropositive and 1 doubtful for *Salmonella* by ELISA test (Table 1). Taken together, *rfbH* mutant strain do not stimulate the animals to produce specific antibodies against O-antigens, which can be agglutinated with SE C50041 culture or detected by ELISA test. It is known that LPS-deficient strains (*e.g. rfc*, *rfaJ* and *rfaL* mutants) were used in distinguishable vaccine study (Kong et al., 2011; Kwon and Cho, 2011; Leyman et al., 2011). Because these LPS-deficient strains are lack of O-antigens, they can not elicit specific antibodies against O-antigens in vaccinated animals. Samples from those animals can not be detected by serologically diagnostic procedures, which were designed based on O-antigens of *Salmonella*. These