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Multifaceted immune responses and protective efficacy elicited by a recombinant autolyzed *Salmonella* expressing FliC flagellar antigen of F18$^+$ *Escherichia coli*

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ABSTRACT

Porcine edema disease (ED) caused by F18$^+$ Shiga toxin 2e-producing *Escherichia coli* (STEC) has imposed significant economic losses in the swine industry worldwide, resulting in sudden deaths in post-weaned piglets. The flagellin protein of F18$^+$ STEC, a structural component of the flagellar filament, is a known virulence factor that mediates adhesion and invasion to porcine epithelial cells. In this study, *Salmonella* inactivated by the *E* lysis gene and expressing the flagellin (*fliC*) antigen was genetically engineered utilizing a plasmid (pMMP184) carrying an efficient heterologous antigen delivery system. The resulting strain JOL1485 producing FliC was successfully inactivated by the *E* lysis gene cassette. Following the lysis procedure, FliC secretion and production of JOL1485 was validated by immunoblot analysis. To evaluate protective immunogenicity elicited by the constructed strain, BALB/c mice were injected with 1×10^8 lysed cells via the intramuscular route. The markedly elevated titers of FliC-specific IgG, IgG1 and sIgA antibodies were observed, indicating a robust Th2-associated humoral immune response was raised in the immunized mice. The proportion of CD3$^+$ CD4$^+$ splenic T cells and proliferative activity were also elevated in *in vivo* and *in vitro* stimulated mice splenocytes. Further, JOL1485 successfully elicited upregulated gene expression of cytokines IL-6, IL-8, IL17, IL-21, IFN-γ and TNF-α in naïve porcine peripheral blood mononuclear cells (PBMCs). The overall immune response elicited by JOL1485 conferred a significant rise of protection against a lethal virulent F18$^+$ STEC challenge whereas all non-immunized mice died following the challenge. Our results demonstrate that *fliC* efficiently expressed in the genetically inactivated *Salmonella* strain has immunostimulatory and protective effects against a F18$^+$ STEC lethal challenge, and may be promising as a potential vaccine candidate against ED infection.

1. Introduction

Porcine edema disease (ED) caused by Shiga toxin 2e-producing *Escherichia coli* (STEC) occurs in post-weaned piglets worldwide [1]. Due to high mortality from sudden death of the diseased piglets, development of efficient vaccine candidates against ED has been required in swine industries. The STEC strains adhere to and colonize small-intestinal epithelial cells via F18 fimbrial adhesins in piglets [2]. Following colonization by the STEC, Shiga toxin 2e excreted into the vascular endothelium of small intestine results in subcutaneous edema and neurological disruption in the piglets [3]. Given the pathogenicity mechanism of ED, the gene clusters encoding F18$^+$ adhesins and Shiga toxin 2e have been used as attractive vaccine candidates to protect piglets against ED [4–6].

E. coli flagella are an assembly of approximately 60 proteins that play an important role in motility, biofilm formation, and adhesion by the bacteria, which are crucially important for virulence [7]. A recent study determined the role of flagella in F18$^+$ *E. coli* adhesion and invasion into intestinal cells [8]. In the study, a flagellin-deficient mutant F18 fimbriae carrying STEC strain (F18$^+$ STEC) showed significantly decreased ability of adhesion and invasion into IPEC-1 porcine epithelial cells. They also demonstrated that a cell line pre-incubated with the purified flagella inhibited F18$^+$ STEC adherence to the cell line, which indicated that flagellin, a principal structural component of flagella, is involved with virulence in F18$^+$ STEC [9]. The structural components of bacterial flagellin can activate the innate immune response via binding to Toll like receptor (TLR)-5 since the conserved flagellin domains are recognized as a pathogen-associated molecular pattern (PAMP) [10]. The binding of TLR5 with flagellin activates the canonical nuclear factor NF-κB path-

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way, resulting in the activation of genes associated with bacterial clearance and anti-apoptotic function [10]. The innate immune system stimulated by flagellin triggers the humoral and cellular immune responses via activation of antigen presenting cells and the subsequent production of cytokines. This immunogenic property of flagellin has also led for its potential as an adjuvant [11]. Given the immunomodulatory properties and virulence characteristics of flagellin related to ED occurrence, the *fliC* gene encoding flagellin from F18$^+$ STEC could be a novel target for a vaccine candidate against ED.

In this study, we developed a genetically inactivated *Salmonella* ghost produced by lysis gene *E* that can express the *fliC* gene of F18$^+$ STEC to produce FliC protein on the cell surface. The *Salmonella* ghost inactivated by a single lysis gene *E* of bacteriophage ϕX 174 has been applied as a homologous vaccine [12] and as a heterologous target antigen expression platform [13]. Protein *E* mediated lysis induces formation of a transmembrane pore on the surface of the *Salmonella* cell [14]. Despite the cytoplasmic contents being expelled through the pore, the lysed *Salmonella* conserves the entire surface antigenic components in their native form [14]. In the present study, *fliC*-encoding flagellin from F18$^+$STEC was subcloned into the antigen delivery site of the pMMP184 plasmid harboring the lysis gene cassette [15]. The resultant plasmid was introduced into a *cpxR*, *lon*, and *asd* deleted *S. typhimurium* mutant. The lysis efficiency of the *Salmonella* strain carrying the *E* gene lysis cassette was assessed under optimal conditions. The immunogenicity and protective efficacy against a lethal challenge was also evaluated in the mice injected with inactivated *S. typhimurium* expressing the flagellin antigen.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth media

The strains and plasmids utilized in this study are listed in Table 1. The aspartate-semialdehyde dehydrogenase (*asd*) gene deleted mutants of *S. typhimurium* JOL912 and *Escherichia coli* χ6212 (JOL232) were grown in either Luria-Bertani broth (LB) or LB agar containing 50 μg/ml of diaminopimelic acid (DAP) at 37 °C. Nutrient broth or agar supplemented with 0.2% L-arabinose was used to culture bacterial strains harboring the *E* lysis gene cassette. All bacteria strains and plasmids were kept at −80 °C until the use.

2.2. Construction of S. typhimurium expressing flagellin antigen

The *fliC* gene was amplified by polymerase chain reaction (PCR) from the wild type F18$^+$ STEC strain JOL500 using the following oligonucleotides: FC_F:5′-ccgcgaattcgcacaagtcattaataccaacagc-3′ and FC_R:5′- ccgcaagctttaaccctgcagcagagacagaac-3′, containing *Eco*RI and *Hin*dIII (underlined) restriction enzyme sites at the 5′ and 3′ ends, respectively. The resultant PCR product was digested with *Eco*RI and *Hin*dIII to yield a DNA fragment that was ligated into the pET28a plasmid. The recombinant plasmid pET28a-*fliC* was used to transform *E. coli* BL21 (DE3) pLysS, a host strain utilized for the overexpression of the target proteins to purify the flagellin protein [16]. The pMMP184 plasmid carrying the *E* lysis gene cassette and a heterologous antigen delivery site [15] was applied in this study. The transcription and expression of the *E* lysis gene was regulated by the temperature inducible expression system, the lambda P_R cI857 and the arabinose-activated promoter systems [17]. The conditions affecting expression of the *E* gene include growth temperature shift from 28 °C to 42 °C for P_R cI857 system induction and removal of arabinose for P_{araBAD} system inactivation [17]. The 1638-bp DNA fragment containing the *fliC* gene amplified by PCR was inserted into the *Eco*RI/*Hin*dIII digested antigen delivery site of pMMP184, resulting in pMMP190. The *asd*, *lon,* and *cpxR* deleted *S. typhimurium* JOL912 mutant was created using a method of allele-coupled exchange as previously described [18]. The recombinant pMMP190 plasmid was initially transformed into a Δ*asd E. coli* χ6212 (JOL232) strain to maintain stability of the plasmid and then the plasmid was introduced into JOL912 by electroporation. The resulting strain was designated as JOL1485. JOL1400, the JOL912 strain harboring only pMMP184, was also prepared for the negative control.

2.3. Generation of Salmonella activated by the E gene and in vitro flagellin expression

The JOL1485 strain was cultivated in LB supplemented with 0.2% L-arabinose with a stirring rate of 80 rpm at 28 °C to reach mid-logarithmic phase. The bacterial cells were harvested and washed twice using LB broth to remove residual arabinose in the culture. The washed cells were placed in 50 ml LB broth and grown at 42 °C for 48 h to initiate transcription of the *E* lysis gene. To monitor the extent of the cell lysis, 100 μl of the culture was collected at the end of the lysis procedure and plated onto LB agar. After overnight incubation, the number of colonies present on the plate determined cell viability. The LB medium from the

Table 1
Bacterial strains and plasmids used in this study.

Strain, plasmid	Description	Reference/source
Bacterial strains		
E. coli		
BL21(DE3) pLysS	F$^-$ *ompT hsdSB* (rB$^-$ mB$^-$) *dcm gal*λ(DE3) pLysS Cmr	Promega
χ6212 (JOL232)	F$^-$ λ$^-$ ϕ80 Δ(*lacZYA-argF*) *endA1 recA1 hadR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4*	Lab stock
JOL500	Wild-type LT$^+$, K99$^+$, F6$^+$, F18$^+$, stx_2^+, stx_{2e}^+ STEC isolate from pig	Lab stock
JOL654	Wild-type LT$^+$, F18$^+$, STa$^+$, stx_2^+, stx_{2e}^+ STEC isolate from pig	Lab stock
S. typhimurium		
JOL912	Δ*lon* Δ*cpxR* Δ*asd*, a derivative of *S. typhimurium*	[16]
JOL 1400	JOL912 harboring pMMP184	This study
JOL 1485	JOL912 harboring pMMP190	This study
Plasmids		
pET28a	IPTG-inducible expression vector; Km^r	Novagen
pET28a-*fliC*	pET28a derivative containing *fliC*	This study
pMMP184	*asd*$^+$ vector, pBR ori plasmid carrying ss *ompA*/His_6, multiple cloning site, cI857/λPR promoter, araC P_{araBAD}, *phi*X174 lysis gene *E*	[20]
pMMP190	pMMP184 harboring *fliC* gene	This study