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A bivalent vaccine derived from attenuated *Salmonella* expressing O-antigen polysaccharide provides protection against avian pathogenic *Escherichia coli* O1 and O2 infection

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC), a leading cause of avian airsacculitis and colibacillosis, is responsible for significant economic loss in the poultry industry. APEC serogroups O1, O2, and O78 are predominantly associated with disease. Lipopolysaccharide (LPS) O-antigen has been shown to be a potent antigen for inducing specific protective immune responses. Therefore, we sought to develop a multivalent polysaccharide vaccine to prevent most APEC infections. We previously reported the stable expression of plasmid pSS27 encoding the APEC O1 O-antigen gene cluster (10.8 kb) in attenuated *Salmonella enterica* serovar Typhimurium S740 provided excellent protection against APEC O1 challenge. In this study, the plasmid pSS28 harboring the APEC O2 O-antigen polysaccharide gene cluster (15.5 kb) was constructed. Biosynthesis of pSS28-encoded APEC O2 O-antigen in *Salmonella* vaccine strain S740 was validated by Western blot. The recombinant *Salmonella* vaccine strain S740 (pSS28) elicited homologous protection against virulent wild-type APEC O2 challenge in a chicken model. Furthermore, through equal-volume mixing the two monovalent vaccine strains S740 (pSS27) and S740 (pSS28), a bivalent vaccine candidate against both APEC O1 and O2 was developed. Immunization of chickens with the bivalent vaccine elicited production of serum IgG and mucosal sIgA antibodies against the LPS of both APEC O1 and O2. Moreover, antibodies induced by the bivalent vaccine promoted opsonization, provoked complement-mediated bactericidal activity, and elicited protection against lethal challenge with both virulent APEC O1 and O2 strains. These results demonstrate that the bivalent vaccine comprised of S740 (pSS27) and S740 (pSS28) is a promising vaccine candidate against APEC O1 and O2 infection.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) infects a wide diversity of bird species, causing avian colibacillosis [1,2]. APEC is the most common bacterial pathogen infecting chickens, resulting in substantial economic losses in the poultry industry worldwide [2]. Recent reports indicate that *E. coli* infection accounts for 50% of layer flock mortality during the first week post-hatch [3], and 45.2% of contaminated poultry carcasses are infected with APEC strains [4]. Multiple virulence factors characteristic of extraintestinal pathogenic *E. coli* (ExPEC) strains that cause human disease have been detected in APEC, suggestive of zoonotic potential [5,6]. Additionally, multi-drug resistant *E. coli* strains have been isolated from infected birds, raising concern regarding current antibiotic-dependent control measures [7–9]. Therefore, it is imperative to develop an efficacious vaccine to control APEC in reservoir hosts.

Lipopolysaccharide is a crucial component of the Gram-negative bacterial outer membrane. The externally exposed O-antigen is a highly immunogenic and variable domain, conferring antigen specificity and serving as a biomarker to distinguish *E. coli* serogroups [10]. Over 180 *E. coli* serogroups have been classified based upon distinct O-antigen species; of which, serogroups O1, O2, and O78 are most frequently isolated from worldwide chicken colibacillosis infections [1,11,12]. It has been well-established that immune responses generated against the surface-localized O-antigen are capable of conferring protection against homologous APEC infection

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[13,14]. Thus, in accordance with the objective of this study, we seek to develop an O-antigen based vaccine candidate which elicits protective immunity against the most prevalent APEC serogroups [15].

Immunization with live-attenuated *Salmonella* has been demonstrated to elicit a broad range of humoral, mucosal and cell-mediated immune responses [16,17]. Live-attenuated vaccine vectors present many advantages including intrinsic adjuvant properties, affordable manufacturing, and ease of administration [18,19]. Prior studies have shown that recombinant *Salmonella* is capable biosynthesizing O-antigen polysaccharides of many bacterial species including *Pseudomonas aeruginosa*, *Burkholderia mallei*, *Shigella dysenteriae*, and *Escherichia coli*. Moreover, immunization with live-attenuated *Salmonella* vaccines recombinantly expressing O-antigen polysaccharides elicits robust protective immune responses [13,20–23].

In our previous study, we have utilized an innovative yeast/bacterial system to express the 10.8 kb operon synthesizing the APEC O1 O-antigen polysaccharide in attenuated *Salmonella* strain S740 [14]. *Salmonella* vaccine strain S740, characterized by Δ*cya* Δ*crp* mutations, is isogenic to a commercially available vaccine utilized to reduce *Salmonella* infection in young chickens within the USA (Megan Vac, Elanco Animal Health, Greenfield, USA) [24]. Herein, we applied the same strategy to assemble the APEC O2 O-antigen operon (15.5 kb) to yield plasmid pSS28. Recombinant expression of pSS28 in *Salmonella* strain S740 was confirmed to biosynthesize the APEC O2 O-antigen, *in vitro*. Immunization of Lohmann hens with S740 (pSS28) induced protection against lethal APEC O2 challenge. Furthermore, co-immunization with equal-volume mixed S740 (pSS27) and S740 (pSS28) afforded significant protection against both APEC O1 and APEC O2 infection.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. APEC O1 C24-2 and O2 CE37 were kindly provided by Dr. Shengqing Yu. Mav203 yeast cells were grown in Yeast Peptone Dextrose (YPD) (Sigma-Aldrich, St. Louis, USA, Y0626-250G) broth or on agar plates at 30 °C. *S.* Typhimurium and APEC strains were grown in Luria-Bertani (LB) broth or on agar plates at 37 °C. Diaminopimelic acid (DAP) was added (50 μg/ml) for the growth of the *asd* mutant strains in the absence of plasmid complementation. Synthetic Dropout-Tryptophan (SD-Trp) media was prepared by adding Yeast Synthetic Drop-out Medium Supplements (Sigma-Aldrich, St. Louis, USA, Y1876-20G) to SD Agar (Sigma-Aldrich, St. Louis, USA, 84605-500G), which was used for the selection of Mav203 yeast cells harboring plasmids encoding the putative *E. coli* O1 or O2 O-antigen gene clusters. The antibiotic spectinomycin (50 μg/ml) was used for plasmid selection in *E. coli* TOP10 cells.

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Description	Source
Strains		
S100	*S.* Typhimurium	[34]
S184	Δasd-66	[47]
S185	Δasd-66 ΔrfbP45	This study
S740	Δasd-66 Δcrp-24 Δcya-25 ΔrfbP45	[14]
C24-2	APEC O1 serotype, isolated from chicken	[14]
CE37	APEC O2 serotype, isolated from chicken	Shengqing Yu
Mav203	Yeast	Life Technology
Plasmids		
pSS26	pSC101 ori, Asd$^+$, ARS4/CEN5, TRP1	[14]
pSS27	Heterologous O1 O-antigen gene cluster into pSS26	[14]
pSS28	Heterologous O2 O-antigen gene cluster into pSS26	This study

2.2. Assembling the gene cluster encoding APEC O2 O-antigen into plasmid pSS26

The primers used in this study are listed in supplementary Table 1. The APEC O2 O-antigen polysaccharide gene cluster was assembled into the shuttle plasmid pSS26 via homologous recombination in yeast as described previously [14].

2.3. Plasmid stability in attenuated S. Typhimurium

Plasmid pSS28 was electrotransformed into attenuated *S.* Typhimurium strain S740 (Δ*asd*-66 Δ*crp*-24 Δ*cya*-25 Δ*rfbP45*). Plasmid stability was determined through antibiotic-independent maintenance as previously described [14,25].

2.4. Analysis of LPS synthesizing in Salmonella

The biosynthesis of the O2 O-antigen afforded by pSS28 in S740 was validated by silver staining and Western blotting as described previously [26]. The LPS profile was resolved by silver staining. The synthesis of O2 O-antigen in *Salmonella* was detected by reaction with rabbit antiserum against serotype O2 APEC (Tianjin Biochip Corporation, Tianjin, China) and *Salmonella* LPS was detected by rabbit antiserum against *Salmonella* serotype O4 (BD Biosciences. NJ, USA). The secondary antibody was an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, USA). Immunoreactive bands were detected by the addition of BCIP (5-Bromo-4-chloro-3-indolyphosphate)-nitroblue tetrazolium solution (Sigma-Aldrich, St. Louis, USA).

2.5. Growth kinetics, sensitivity, and attachment/invasion assays

The *in vitro* growth kinetics, sensitivity assays to polymyxin B and sodium deoxycholate (DOC), and bacterial attachment/invasion ability of *S.* Typhimurium strains were determined as described in previous reports [27,28].

2.6. Virulence and colonization determination

All animal assays were conducted according to the Animal Welfare Act and regulations and Sichuan Agricultural University guidelines. The animal care protocols were approved by Sichuan Agricultural University (Ya'an, China; Approval no. 2011-028). Female BALB/c mice were purchased from Dashuo Biotechnology Co., Ltd. (Chengdu, China) and Lohmann hens were purchased from Chengdu Muxing poultry LLC. (Chengdu, China).

The oral LD_{50} values of *S.* Typhimurium S184 (pSS26), S184 (pSS28) and S185 (pSS28) in 7-weeks-old female BALB/c mice, and the colonization of S185 with or without plasmids in both 7-weeks-old female BALB/c mice and 7-days-old Lohmann hens were determined as previously described [29].

2.7. Immunization and challenge

One-day-old Lohmann hens were randomly divided into 4 groups. After acclimation for 10 days, all of the birds were immunized via oral gavage with 10^9 CFU/100 μl of designated vaccine strain(s) or negative control. Hens were boosted with 5×10^7 CFU/100 μl by intramuscular injection two weeks after the initial oral immunization. Experimental groups consisted of the following: Group A (n = 25) was inoculated with buffered saline with gelatin (BSG); Group B (n = 33) was immunized with S740 (pSS26, vector control); Group C (n = 19) was immunized with S740 (pSS28);