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Regulated delayed attenuation enhances the immunogenicity and protection provided by recombinant *Salmonella enterica* serovar Typhimurium vaccines expressing serovar Choleraesuis O-polysaccharides

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ABSTRACT

Regulated delayed attenuation is a well-studied strategy for retaining the immunogenicity of *Salmonella*-vectored vaccines. In this study, this strategy was used to optimize two previously constructed recombinant *Salmonella enterica* serovar Typhimurium vaccines expressing *S.* Choleraesuis O-polysaccharides (OPS). The novel vaccine strains SLT31 (Δ*asd* Δ*rmlB-rfbP* ΔP_{crp}::T *araC* P_{BAD}) and SLT33 (Δ*asd* Δ*rfbP* Δ*pagL*::T *araC* P_{BAD} *rfbP* ΔP_{crp}::T *araC* P_{BAD}) were constructed by replacement of the native *crp* promoter with the arabinose-dependent *araC* P_{BAD} promoter. As controls, two vaccine strains with direct *crp* mutations were also constructed, namely, SLT30 (Δ*asd* Δ*rmlB-rfbP* Δ*crp*) and SLT32 (Δ*asd* Δ*rfbP* Δ*pagL*::T *araC* P_{BAD} *rfbP* Δ*crp*). Then, the ability to deliver the heterologous *S.* Choleraesuis OPS on the Asd^+ plasmid pCZ1 to the mouse immune system was evaluated in the strains with or without regulated delayed attenuation. The SLT30 (pCZ1) and SLT31 (pCZ1) strains expressed only the heterologous OPS, while the SLT32 (pCZ1) and SLT33 (pCZ1) strains co-expressed the homologous and heterologous OPS. The strain SLT31 (pCZ1) or SLT33 (pCZ1), which exhibited regulated delayed attenuation, colonized mouse tissues significantly better and stimulated stronger antibody responses against *S.* Choleraesuis LPS post immunization than the SLT30 (pCZ1) or SLT32 (pCZ1) strain. Immunization with SLT31 (pCZ1) or SLT33 (pCZ1) resulted in a significant reduction in bacterial loads in mouse tissues and a greater degree of protection against a lethal *S.* Choleraesuis dose compared with the effects observed after SLT30 (pCZ1) or SLT32 (pCZ1) immunization (100% vs. 80% or 70% vs. 50%, respectively). In addition, all four vaccines conferred complete protection against *S.* Typhimurium challenge. Overall, our study demonstrates that regulated delayed attenuation via an *araC* P_{BAD}-regulated *crp* gene can enhance the cross-protection by *Salmonella*-vectored vaccines expressing heterologous OPS, and strain SLT31 (pCZ1) is a good candidate vaccine for preventing both *S.* Typhimurium and *S.* Choleraesuis infections.

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1. Introduction

Serovars of non-typhoidal *Salmonella* (NTS) are important causes of gastrointestinal disease in humans and a variety of animals [1] and often cause severe invasive diseases in children, the elderly and immunocompromised individuals [2–4]. Poultry and other livestock are common reservoirs of NTS [5,6], and transmission from animals to humans occurs predominantly via consumption of animal products [7,8]. NTS comprise thousands of serovars;

Abbreviations: OPS, O-polysaccharides; NTS, non-typhoidal *Salmonella*; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; LPS, lipopolysaccharides; CVCC, China Veterinary Culture Collection Center; LB, Luria-Bertani; LD_{50}, median lethal dose; BSG, buffered saline with gelatin.

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however, certain serovars belonging to serogroups B1, C1, C2 and D1 are most frequently identified [9–11]. In addition to the most common serovars *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium, Group B1) and *S.* Enteritidis (Group D1), group C serovars such as Choleraesuis (Group C1) and Newport (Group C2) are emerging as dominant epidemic strains that lead to salmonellosis outbreaks in some regions [12,13]. *S.* Choleraesuis is also one of the serovars most frequently associated with bacteremia [14]. There is limited cross-protection from *Salmonella* strains in different serogroups. Thus, multivalent NTS vaccines are urgently needed to prevent a variety of epidemic serovar infections.

To date, there have been no specific commercial NTS vaccines for humans. Although a majority of NTS vaccines in development are restricted to a single serovar [15], several vaccines with cross-protection have been explored. Live attenuated vaccines constructed by the introduction of mutations in the global regulator gene *dam* [16,17] or genes required for lipopolysaccharide (LPS) and enterobacterial common antigen synthesis [18,19] in *S.* Typhimurium strains elicit full protective immunity to homologous challenge and a degree of heterologous protection. Highly conserved surface proteins such as OmpD and OmpL have potential for the development of broadly protective subunit vaccines. Immunization with these proteins has been shown to protect mice during homologous challenge [20–22], while the potency against heterologous challenge has not been fully investigated. Recently, we constructed several bivalent vaccines by heterologous expression of LPS O-polysaccharides (OPS) from Group C1 or C2 in attenuated *S.* Typhimurium strains [23,24]. The OPS are important protective antigens as demonstrated by the success of OPS-based conjugate vaccines [25,26]. Bivalent vaccines that express Group C2 OPS stimulate high levels of serum IgG specific to heterologous LPS and provide full protection against lethal *S.* Newport challenge in mice [24]. In addition, immunization with another two bivalent vaccines that express Group C1 OPS induces strong antibody responses against heterologous LPS but results in only moderate protection in mice against lethal *S.* Choleraesuis challenge [23]. As strains expressing more heterologous OPS stimulate stronger antibody responses [23], we hypothesized that the amount of Group C1 OPS antigen was a critical factor influencing the efficacy of protection.

Attenuated *Salmonella* strains are excellent vaccine vehicles for the delivery of exogenous antigens to the host immune system [27]. Generating an attenuated vaccine vector with the characteristics of both avirulence and ideal immunogenicity is very challenging. To address this issue, regulated delayed attenuation technology was developed several years ago to maintain the immunogenicity of attenuated *S.* Typhimurium strains [28]. This goal can be achieved by a smooth-to-rough LPS phenotypic transition via deletion of the *pmi* gene or regulated expression of virulence-related genes such as *crp* and *fur* by replacing the promoters of these genes with the arabinose-dependent promoter *araC* P_{BAD} [29]. Vaccine strains with regulated delayed attenuation exhibit wild-type-like ability to invade and colonize lymphoid tissues during the initial stage of infection due to the sugar provided *in vitro* and become attenuated after cell division because of the unavailability of the sugar *in vivo* [28]. Recombinant *S.* Typhimurium vaccine strains with regulated delayed attenuation due to *araC* P_{BAD}-regulated *rfbB* gene expression [18] or *araC* P_{BAD}-regulated *fur* gene expression [30] exhibit better colonization of mouse tissues and stronger immune responses against *Salmonella* antigens or heterologous antigens than control strains with direct attenuation [31]. Additionally, a *S.* Typhimurium strain with an *araC* P_{BAD}-regulated *crp* gene has been proven to be both highly attenuated and immunogenic [28], whereas a comparative study is lacking between a strain with a regulated *crp* gene and a strain with a Δ*crp* mutation.

In this study, we introduced regulated delayed attenuation to optimize our previously constructed bivalent vaccines with heterologous expression of Group C1 OPS via *crp* gene regulation by the arabinose-dependent *ara*C P_{BAD} promoter and evaluated whether this strategy could enhance heterologous OPS-mediated antibody responses and the consequent protection efficacy. Vaccine strains with or without regulated delayed attenuation were constructed via unmarked chromosomal deletion-insertion mutations. The plasmid pCZ1 with the cloned OPS gene cluster of *S.* Choleraesuis was transformed into each strain, and then, the immunogenicity and protection efficacy of each recombinant vaccine were evaluated in BALB/c mice.

2. Materials and methods

2.1. Ethics statement

All animal procedures were approved by the Animal Ethics Committee of Sichuan Agricultural University and the Sichuan Administration Committee of Laboratory Animals under protocol number SYXK2014-187.

2.2. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 1. The recombinant *S.* Typhimurium strains were derived from the highly virulent ATCC14028 strain. The wild-type *S.* Choleraesuis

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Description	Source
Plasmids		
pCZ1	Asd$^+$, pSC101 origin, Kan^r, Ptrc-OPS gene cluster of *S.* Choleraesuis	[23]
pRE112	*sacB* mobRP4 R6K ori Cm$^+$	[33]
BBa_J72113-BBa_J72152	*araC* P_{BAD} p15a origin, Cm^r Amp^r	[34]
pCZ18	pRE112-ΔP_{crp}	This work
pCZ19	pRE112-ΔP_{crp}::T *araC* P_{BAD}	This work
Strains		
ATCC14028	Wild-type *S.* Typhimurium	ATCC
CVCC2139	Wild-type *S.* Choleraesuis	CVCC
SLT28	ATCC14028 Δ*asd* Δ(*rmlB-rfbP*)	This work
SLT29	ATCC14028 Δ*asd* Δ*rfbP* Δ*pagL*::T *araC* P_{BAD} *rfbP*	This work
SLT30	ATCC14028 Δ*asd* Δ(*rmlB-rfbP*) Δ*crp*	This work
SLT31	ATCC14028 Δ*asd* Δ(*rmlB-rfbP*) ΔP_{crp}::T *araC* P_{BAD}	This work
SLT32	ATCC14028 Δ*asd* Δ*rfbP* Δ*pagL*::T *araC* P_{BAD} *rfbP* Δ*crp*	This work
SLT33	ATCC14028 Δ*asd* Δ*rfbP* Δ*pagL*::T *araC* P_{BAD} *rfbP* ΔP_{crp}::T *araC* P_{BAD}	This work

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