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Engineering of a live *Salmonella enterica* serovar Choleraesuis negative-marker strain that allows serological differentiation between immunised and infected animals

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ABSTRACT

The usefulness of *Salmonella* vaccine vehicles is limited by the fact that control programmes relying on *Salmonella* bacteriology and serology cannot differentiate infected animals from vaccinated ones, an ability referred to as DIVA (differentiating infected from vaccinated animals). As a first step towards *Salmonella*-based DIVA vaccines, the *ompA* gene was deleted in live attenuated Δ*phoP* and Δ*rpoS* vaccine strains. The *ompA* gene is present in all *Salmonella enterica* serovars and it encodes an abundant, highly immunogenic outer membrane protein.

The double mutant Δ*phoP* Δ*ompA* and Δ*rpoS* Δ*ompA* strains showed similar virulence attenuation, safety and immunogenicity in a mouse model of infection as the parental Δ*phoP* and Δ*rpoS* strains. Sera from mice inoculated with the double mutant strains failed to recognise OmpA in Western blots of outer membrane extracts, whereas the protein was recognised by sera from mice inoculated with wild-type *Salmonella* or a mixture of double mutant and parental strains. These data suggest that OmpA can be a suitable negative marker for DIVA vaccines.

Introduction

Salmonella enterica presents several advantages as a basis for live recombinant vaccines; it is a gastrointestinal facultative intracellular pathogen, it can easily be grown in large quantities, and it can infect the host efficiently through the mucosa (Medina and Guzman, 2001).

Attenuated strains of *Salmonella enterica* subspecies *enterica* serovar Choleraesuis (*Salmonella* Choleraesuis) have attracted particular interest as potential live oral vaccine carriers for delivering antigens to pigs (Mastroeni et al., 2001; Mollenkopf et al., 2001; Ku et al., 2005; Dominguez-Bernal et al., 2008; Bartolome et al., 2010). For example, our group has constructed two highly attenuated strains of *Salmonella* Choleraesuis, Δ*rpoS* and Δ*phoP*, which show good promise as bactofection vaccine vectors (Bartolome et al., 2010). However, the usefulness of *Salmonella* for constructing vaccines for livestock is limited, at least within the European Union (EU), because such vaccines could interfere with surveillance systems monitoring the pork production chain (European Food Safety Authority, 2015). This highlights the need for DIVA (differentiating infected from vaccinated animals) vaccines for livestock, particularly pigs (Capua et al., 2004). Serovars of Typhimurium (Selke et al., 2007; Leyman et al., 2011) and Enteritidis (Adriaensen et al., 2007) with wide host ranges have been developed for use in DIVA vaccines. These DIVA-compatible Typhimurium strains present significant disadvantages as delivery vehicles; immunising mice with one strain allowed colonisation by wild-type pathogen upon oral challenge (Selke et al., 2007), while the Δ*rfaJ* and Δ*rfaL* strains must be inactivated before use (Leyman et al., 2011). In contrast to previous efforts, we aimed to examine the feasibility of generating a DIVA-compatible, live attenuated vaccine vector based on the host-adapted *Salmonella* Choleraesuis.

We sought to generate a so-called negative-marker vaccine vector strain that could be differentiated from field strains serologically, based on the absence of a wild-type protein. We selected the *Salmonella* outer membrane protein OmpA, which belongs to a class of proteins highly conserved in the *Enterobacteriaceae* family and which elicits a strong immune response in mice (Smith et al., 2007). Therefore, we deleted the *ompA* gene in the background of the *rpoS* or *phoP* deletions in our well-studied attenuated *Salmonella* Choleraesuis strains (Bartolome et al., 2010). We compared the safety and immunogenicity of the resulting double mutants (Δ*rpoS* Δ*ompA* and Δ*phoP*Δ*ompA)* and the parental strains (Δ*rpoS*

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Table 1
Bacterial strains and plasmids.

Strain or plasmid	Description and relevant characteristics	Source (Reference)
Salmonella enterica serovar Choleraesuis		
CECT[a] 915	Wild-type	Spanish Culture Type Collection
SCL[b] 00027	XL1-Blue, Tc^R	Stratagene
SCL 00031	Δ*rpoS* from CECT 915	Dominguez-Bernal et al. (2008)
SCL 00032	Δ*phoP* from CECT 915	Dominguez-Bernal et al. (2008)
SC-54	Commercial live vaccine strain	Boehringer Ingelheim (Roof and Doitchinoff, 1995)
SCL 00021	Wild-Type/pKD46, Amp^R	This work
SCL 00062	Δ*rpoS*::Km/pKD46, Amp^R Km^R	This work
SCL 00063	Δ*phoP*::Km/pKD46, Amp^R Km^R	This work
SCL 00061	Δ*rpoS*::Km Δ*ompA*::Cm, Cm^R	This work
SCL 00085	Δ*phoP*::Km Δ*ompA*::Cm, Cm^R	This work
SCL 00086	Δ*rpoS* Δ*ompA* from SCL 00031	This work
SCL 00088	Δ*phoP* Δ*ompA* from SCL 00032	This work
Plasmids		
pKD46	Expressing Red, ts; Amp^R	Datsenko and Wanner (2000); Bauer et al. (2005)
pCP20	Expressing FLP, ts; Amp^R	Datsenko and Wanner (2000)
pKD3	Cm^R	Datsenko and Wanner (2000)
pKD4	Km^R	Datsenko and Wanner (2000)

CECT, Spanish collection of Type Cultures, Universidad de Valencia, Burjassot (Valencia), Spain; SCL, *Salmonella* Choleraesuis collection from the INBAVET group; Amp, Ampicillin; Cm, Chloramphenicol; Km, kanamycin.
[a] CECT.
[b] SCL.

and Δ*phoP)*, as well as our ability to distinguish them by Western blotting.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Salmonella* Choleraesuis CECT 915 (ATCC 13312) was used as the wild-type (WT) strain. Bacteria were routinely cultured in Luria–Bertani (LB) broth and on LB agar plates. Ampicillin (Amp) (100 μg/mL) was added to medium when required for selecting recombinants or maintaining plasmids. The reference vaccine was a commercially available live avirulent culture (Enterisol SC-54, Boehringer Ingelheim).

Construction of ompA deletion mutants

The *ompA* gene (GenBank: NC_006905.1) was specifically disrupted as described (Datsenko and Wanner, 2000) with minor modifications (Dominguez-Bernal et al., 2008). All PCR procedures were carried out according to standard protocols (Ausubel et al., 1992). Bacteria were concentrated by centrifugation, and then spread onto LB-chloramphenicol (Cm) agar plates to select Cm^R transformants. When necessary, the xxx (FRT)-flanked antibiotic resistance cassette was eliminated after transformation with pCP20, as described (Datsenko and Wanner, 2000). PCR products containing the Cm-resistance gene flanked by sequences upstream and downstream of the disrupted genes were obtained using primers OmpAD-3D and OmpAD-3R (Table 2). To confirm disruption of the *ompA* gene, PCR products obtained by using primers OmpA-3D and OmpA-2R, which bind flanking the deleted gene, were sequenced on both strands.

Virulence and safety in mice

Eight-week-old female BALB/c mice were purchased (Harlan Interfauna Ibérica). All mice were treated in accordance with the institutional guidelines for treatment of animals approved by the Ethical Committee for Animal Experimentation of Complutense University of Madrid (reference number 022010 approval date 26 January 2007).

Four groups, each of three mice (Table 3), were co-inoculated intraperitoneally with 10^6 (WT) and 10^7 (SC-54 or mutant strains) colony-forming units (CFU), suspended in 0.2 mL of phosphate-buffered saline (PBS, pH 7.4). Mice were euthanased at 3 days post-infection or when observed to be moribund. Spleens were aseptically removed then teased apart and homogenised in 5 mL of cold PBS. Homogenate (100 μL) was serially diluted in PBS and plated on LB and LB with antibiotics for counting. The competitive index (CI) is an alternative measure that uses mixed infections to determine the degree of virulence attenuation caused by a given mutation (Beuzon and Holden, 2001). CI for each mutant was calculated by dividing the ratio of the mutant to the wild-type strain in the output (bacteria recovered from the host after infection) by the ratio of these strains in the input (initial inoculum) as previously described (Dominguez-Bernal et al., 2008).

Immunisation of animals

WT and mutant *Salmonella* strains were grown under static conditions to an optical density at 600 nm (OD_{600}) of approximately 0.5 (18 h). BALB/c mice were immunised as described (Bartolome et al., 2010). Mice were divided into six groups ($n = 5$ each), and groups were orally dosed with 200 μL of the following inoculums containing 1×10^{10} CFU on days 0, 21 and 36: Δ*rpoS*, Δ*rpoS* Δ*ompA*, Δ*phoP*, Δ*phoP* Δ*ompA*, *E. coli*; or just 200 μL of PBS (negative control). All mice were bled on days

Table 2
Primers used.

Primer	Sequence (5′→ 3′)	Orientation	Template plasmid/Gene target
OmpAD-3D[a]	TTAACCGTGTTATCTCGTTGGAGATATTCATGGCGTATTTTGGATGATAACGAGGCGCAAAAA*GTGTAGGCTGGAGCTGCTTC*	Forward	pKD3
OmpAD-3R[a]	ACCCAGACCAGAGCAAAAAACCCCGCGACGCGGGGTTTTTTATCAGACGGAAACTTA*CATATGAATATCCTCCTTAG*	Reverse	pKD3
OmpA 3D	TAAATGATGTGTATATCCGTC	Forward	*ompA*
OmpA 2R	AGAGTTTATGGTCTGGCAGCG	Reverse	*ompA*
PhoP N1D	AGAGGGTGACTATTTGTCTGG	Forward	*phoP*
PhoP C1R	CAGGTTGCTTTCGCCGCGCAG	Reverse	*phoP*
RpoS N1D	GCACCGGCACCAGCTCTACAC	Forward	*rpoS*
RpoS C1R	CCAGCCGCGAACACTATCCAC	Reverse	*rpoS*
Cat 1R	CTTGTTACACCGTTTTCCATG	Reverse	Cm cassette

The specific plasmid sequences are depicted in italics.
[a] Primers were designed according to (Datsenko and Wanner, 2000). 3′ regions marked in bold anneal to the pKD3 plasmid template. Extensions of their 5′ regions are homologous to the target sequences for recombination.