FULL PAPER *Bacteriology*

Evaluation of the live vaccine efficacy of virulence plasmid-cured, and *phoP*- or *aroA*-deficient *Salmonella enterica* serovar Typhimurium in mice

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ABSTRACT. We evaluated the protective efficacy of 94-kb virulence plasmid-cured, and *phoP*- or *aroA*-deficient strains of *Salmonella enterica* serovar Typhimurium (Δ*phoP* or *ΔaroA S*. Typhimurium) as oral vaccine candidates in BALB/c mice. Two weeks after the completion of 3 oral immunizations with 1×10^8 colony-forming units (CFU) of virulence plasmid-cured, and Δ*phoP* or *ΔaroA S.* Typhimurium at 10-day intervals, *S*. Typhimurium lipopolysaccharide (LPS)-specific mucosal secretory immunoglobulin A (s-IgA) antibody titers were detected in the cecal homogenate, bile and lung lavage fluid, but not in the intestinal lavage fluid. In addition, the increases in *S*. Typhimurium LPS-specific immunoglobulin G (IgG) and IgA antibody titers in the serum were also observed 2 weeks after completing 3 oral immunizations with virulence plasmid-cured, and Δ*phoP* or *ΔaroA S.* Typhimurium. The series of 3 oral immunizations protected the mice against an oral challenge with 5×10^8 CFU of the virulent strain of *S.* Typhimurium, suggesting that both the virulence plasmid-cured, and Δ*phoP* and *ΔaroA S.* Typhimurium strains are promising candidates for safe and effective live *S*. Typhimurium vaccines.
KEY WORDS: *aroA*, BALB/c mouse, oral vaccine, *phoP*, *Salmonella enterica* serovar Typhimurium

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Nontyphoidal *Salmonella* (NTS) is a major cause of food-borne diarrheal illness in humans and is frequently acquired from contaminated livestock products. As such, vaccinations of livestock against NTS are an important step in preventing the spread of infection to humans. Mouse models of *S. enterica* serovars Typhimurium (*S*. Typhimurium) and Enteritidis (*S*. Enteritidis) invasive disease can accelerate the development of NTS vaccines [4, 33]. In mouse models of orally infected *S*. Typhimurium, the bacterium initially attaches to enterocytes and M-cells in Peyer's patches on the surface of gut-associated lymphoid tissue (GALT) and then invades the mucosa before colonizing deeper tissues, such the spleen and liver [13]. Delivery of an antigen to the GALT elicits generalized secretory, humoral and cellular immune responses in experimental animals [10].

NTS harbors numerous virulence plasmids (50–100 kb in size) [14, 29], all of which encode *spvRABCD* genes involved in the intra-macrophage survival of the bacterium [15, 17, 24]. PhoP (a transcriptional regulator)/PhoQ (an environmental sensor) is a two-component regulatory system that allows the expression of at least 40 *Salmonella* genes in response to low pH and a magnesium limitation *in vitro* [11, 31]. The PhoP/PhoQ system has also been shown to play a role in the response of *Salmonella* to host signals by modulating the expression of genes that are required for entry or survival within host cells [2, 3, 23]. By contrast, *aro* genes regulate the synthesis of aromatic amino acid metabolites that are normally unavailable in mammalian hosts. The inactivation of *aro* genes has most frequently been used for the construction of attenuated live *Salmonella* vaccines [1, 5, 18, 19].

It has been reported that the oral administrations of virulence plasmid-cured, Δ*phoP* and *ΔaroA* strains of *S.* Typhimurium promote different immune responses in the host, and these mutants show different susceptibilities to a variety of host defenses [34]. Viable attenuated *Salmonella* vaccines associated with single-gene deletion lines bear the intrinsic risk of causing disease in immunocompromised hosts. Therefore, in the present study, we used combined virulence plasmid-cured, and Δ*phoP* or Δ*aroA* strains of *S.* Typhimurium as oral vaccine candidates. We present data showing the protective efficacy of *Salmonella* oral vaccine candidates in a BALB/c mouse model.

MATERIALS AND METHODS

Vaccine strains, media and growth conditions: The construction of the vaccine strains of 94-kb virulence plasmid-cured, and Δ*phoP* or Δ*aroA S.* Typhimurium SR-11 (χ3337*phoP*, *phoP*::*aphT* or UF21, *aroA*::*tet*, respectively) was described previously [15, 26]. Both strains were routinely grown at 37°C in L-agar or L-broth (Difco and BBL, Detroit, MI, U.S.A.) supplemented with antibiotics at the

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following concentrations as appropriate: kanamycin (40 μg/m*l*), nalidixic acid (25 μg/m*l*) and tetracycline (15 μg/m*l*).

Immunization and subsequent challenge: Seven-week-old female BALB/c mice (Charles River Japan, Yokohama, Japan) were orally administered vaccine strains at doses of 1×10^8 colony-forming units (CFU) of exponential-growth-phase salmonellae concentrated in 20 μl doses mixed with phosphate-buffered saline, containing 0.01% (wt/vol) gelatin (BSG), pH 7.4 [22, 25]. The mice were harvested, and the following tissue and fluid samples were removed: blood, liver, spleen, mesenteric lymph nodes (MLNs), Peyer's patches (PP), gallbladder, cecum, intestine and lungs. The liver, spleen, MLNs and PP were homogenized in BSG and plated on L-agar containing the relevant antibiotics in order to enumerate CFU of vaccine strains [20, 21, 27]. Serum was prepared from the blood. Bile (2–10 μl) was collected from the gallbladder. The cecum was homogenized with 1 m*l* of solution A (0.1 mg/m*l* soybean trypsin inhibitor [Sigma, St. Louis, MO, U.S.A.], 1 mM freshly prepared phenylmethylsulfonyl fluoride [Sigma], 50 mM EDTA, and 0.1% bovine serum albumin [BSA; Fraction V, Sigma] in phosphate-buffered saline, pH 7.4), and the supernatant was pooled after centrifugation for 15 min at 12,000 rpm. Lung and intestinal secretions were extracted with 3 m*l* of solution A, and the supernatants were pooled after centrifugation for 15 min at 12,000 rpm. Immunized and nonimmunized (naïve) mice were orally challenged with a virulent strain of *S.* Typhimurium SR-11 (χ3456) at doses of 5×10^8 CFU (1,600 times the LD_{50} [lethal dose, 50%] value) [8, 9, 20, 21, 27]. Mortality was recorded daily for two weeks post-infection. All mice were bred at the animal facility of the Kitasato Institute, and all mouse experiments were performed in accordance with institutional guidelines under an approved protocol.

ELISA: An enzyme-linked immunosorbent assay (ELISA) was used to measure the anti-*S.* Typhimurium lipopolysaccharide (LPS) IgG and IgA concentrations in the serum and the anti-*S.* Typhimurium LPS s-IgA levels in the intestinal lavage fluid, cecal homogenate, bile and lung lavage fluid. Age-matched naïve mice were used as a negative control. Each value was obtained by subtracting the average value of naive mice (n=5/group) from that of immunized mice. The procedures were described in more detail in the previous report [27].

Statistics: The results were combined from two independent experiments with 5 mice per group (total n=10/group). Survival was analyzed using the Kaplan-Meier log-rank test. The mean plus or minus standard deviation (SD) between the groups was examined using the two-tailed Student's *t*-test. Values of $P<0.05$ were regarded as statistically significant.

RESULTS

*Efficacy of a single oral immunization with virulence plasmid-cured, and Δ*phoP *or Δ*aroA S. *Typhimurium*: Neither χ3337*phoP* nor UF21 was recovered from the liver, spleen, MLNs or PP at day 5 after oral immunization with 1×10^8 CFU. Unfortunately, we did not detect *S.* Typhimurium LPS-specific s-IgA antibody in the intestinal lavage fluid, cecal homogenate, bile or lung lavage fluid by ELISA at weeks 2, 4 and 6 after a single oral immunization. However, low levels of *S.* Typhimurium LPS-specific IgA antibody were detected in the serum at weeks 4 and 6 after a single oral immunization with χ3337*phoP* (Fig. 1). We did not carry out the challenge experiments with χ3456 after an oral immunization due to the lack of *S.* Typhimurium LPS-specific serum and mucosal antibodies in immunized mice.

*Efficacy of 2 oral immunizations with virulence plasmid-cured, and Δ*phoP *or Δ*aroA S. *Typhimurium*: Neither χ3337*phoP* nor UF21 was recovered from the liver, spleen, MLNs or PP at day 5 after the second oral immunization with 1×10^8 CFU. We detected higher levels of *S.* Typhimurium LPS-specific IgA and IgG antibodies in the serum at weeks 2 and 3 after 2 oral immunizations (i.e., IgA, 4.5 ± 5.5 μg/m*l* and 5.5 ± 2.7 μg/m*l* at weeks 2 and 3 after immunization with 3337*phoP*, respectively or 1.2 ± 2.6 μg/m*l* and 1.5 ± 2.7 μg/m*l* at weeks 2 and 3 after immunization with UF21, respectively; IgG, 4.9 ± 3.3 μg/m*l* and 11.6 ± 8.2 μg/m*l* at weeks 2 and 3 after immunization with χ3337*phoP*, respectively or 2.1 ± 1.9 μg/m*l* and 3.2 ± 2.2 μg/m*l* at weeks 2 and 3 after immunization with UF21, respectively), while *S.* Typhimurium LPS-specific s-IgA antibody was undetectable in the intestinal lavage fluid, cecal homogenate, bile and lung lavage fluid at weeks 2 and 3 after 2 oral immunizations (Fig. 2). We did not carry out the challenge experiments with χ3456 after 2 oral immunizations due to the lack of *S.* Typhimurium LPS-specific mucosal s-IgA in immunized mice.

*Efficacy of 3 oral immunizations with virulence plasmid-cured, and Δ*phoP *or Δ*aroA S. *Typhimurium*: Neither χ3337*phoP* nor UF21 was recovered from the liver, spleen, MLNs or PP at day 5 after the third oral immunization with 1×10^8 CFU. We detected the highest levels of *S.* Typhimurium LPS-specific IgA and IgG antibodies in the serum at week 2 after 3 oral immunizations (i.e., IgA, 9.9 ± 6.3 μg/m*l* and 5.2 ± 7.0 μg/m*l* of χ3337*phoP* and UF21, respectively; IgG, 42.2 ± 40.0 μg/m*l* and 10.4 ± 10.6 μg/m*l* of χ3337*phoP* and UF21, respectively). We also detected high levels of *S.* Typhimurium LPS-specific s-IgA antibody in the bile, cecal homogenate and lung lavage fluid, though not in the intestinal lavage fluid (Fig. 3). Subsequently, the immunized and naïve mice were orally challenged with 5×10^8 CFU of χ3456 at week 2 after final immunization. Although all naïve mice died by day 10 after challenge, mice immunized with either χ3337*phoP* or UF21 survived for up to 2 weeks after challenge (Fig. 4).

DISCUSSION

We have previously shown that ATP-dependent protease-deficient *S.* Typhimurium persistently resides in the spleen, Peyer's patches, mesenteric lymph nodes and cecum after a single oral immunization in mice, and such immunization can elicit *S.* Typhimurium LPS-specific serum IgG and mucosal s-IgA responses [20, 27], T cell-mediated immunity [8] and down-regulation of cell surface Toll-like receptors 4 and 2 (TLR4 and TLR2) [9] for the protection of mice against

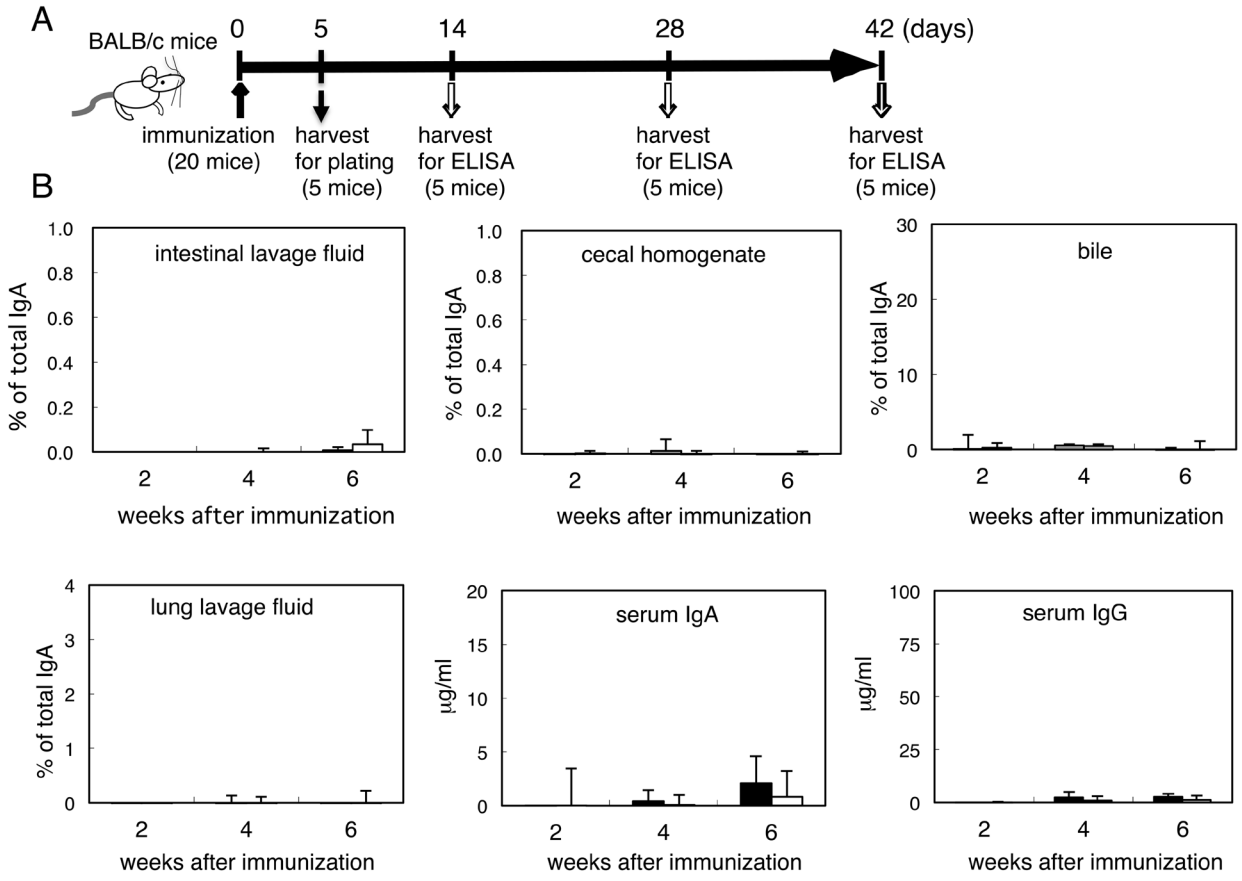

Fig. 1. Efficacy of a single oral immunization with virulence plasmid-cured, and Δ*phoP* or Δ*aroA S.* Typhimurium. (A) The immunization schedule. (B) Anti-*S.* Typhimurium s-IgA antibody in the intestinal lavage fluid, cecal homogenate, bile and lung lavage fluid in addition to anti-*S.* Typhimurium IgA and IgG antibodies in serum at weeks 2, 4 and 6 after oral immunization with χ3337*phoP* (black columns) or UF21 (white columns). The data are combined from two independent experiments (n=10/group).

subsequent oral challenge with χ3456. Furthermore, the vaccine-elicited humoral immunity facilitated the apoptosis of macrophages through enhancement of bacterial uptake, which led to establishment of protective immunity against virulent *S.* Typhimurium in mice [7]. Thus, in the present study, we attempted to confirm the vaccine-induced antibody titers in order to estimate the protective efficacy of the vaccine candidates.

We have also demonstrated that *S.* Typhimurium can reside and proliferate within phagocytes in deeper tissues, such as the liver and spleen [17, 23, 24], and induce macrophage death by necrosis [16]. The *S.* Typhimurium-infected oncotic macrophages are often packed with motile salmonellae, and some of these flagellated salmonellae intermittently escape from oncotic macrophages, which then undergo necrotic cell death [30]. In the present study, 1 or 2 oral immunizations with χ3337*phoP* or UF21 could not elicit *S.* Typhimurium LPS-specific mucosal s-IgA in immunized mice (Figs. 1 and 2). s-IgA was probably absent in the mucosa, because highly attenuated χ3337*phoP* or UF21 could not persistently infect the mucosal tissues after oral immunization, based on the finding that neither χ3337*phoP* nor UF21 was recovered from the mouse tissues at day 5 after oral immunization. Furthermore, it has previously been demonstrated that the mutation combinations of virulence plasmid-cured, and Δ*phoP* or Δ*aroA S.* Typhimurium result in a lack of recovery of splenic CFU, in contrast to a large number (around $\log_{10}5$ CFU per spleen) of recovery of the wild-type strain, at day 5 after oral infection in BALB/c mice [15, 26].

We chose the 94-kb virulence plasmid-cured, and Δ*phoP* or Δ*aroA S.* Typhimurium rather than the 94-kb virulence plasmid-carrying *S.* Typhimurium as the live vaccine strains. The virulence plasmid plays a role in increasing the growth rate of salmonellae within phagocytes of deeper tissues [15]. By contrast, the *phoP* controls the gene expression that promotes macrophage death [6], and the mutation of *aro* genes of *S.* Typhimurium decreases resistance to components of innate response [32]. Various researchers have reported that Δ*phoP S.* Typhimurium is avirulent in mice [12, 26, 28] and that it fails to replicate in mouse macrophage RAW264.7 cell lines [28]. In this study, following the 3 oral immunizations with χ3337*phoP* or UF21 in BALB/c mice, we did not detect significant differences in the susceptibility to subsequent challenge with χ3456 (Fig. 4), indicating that both strains

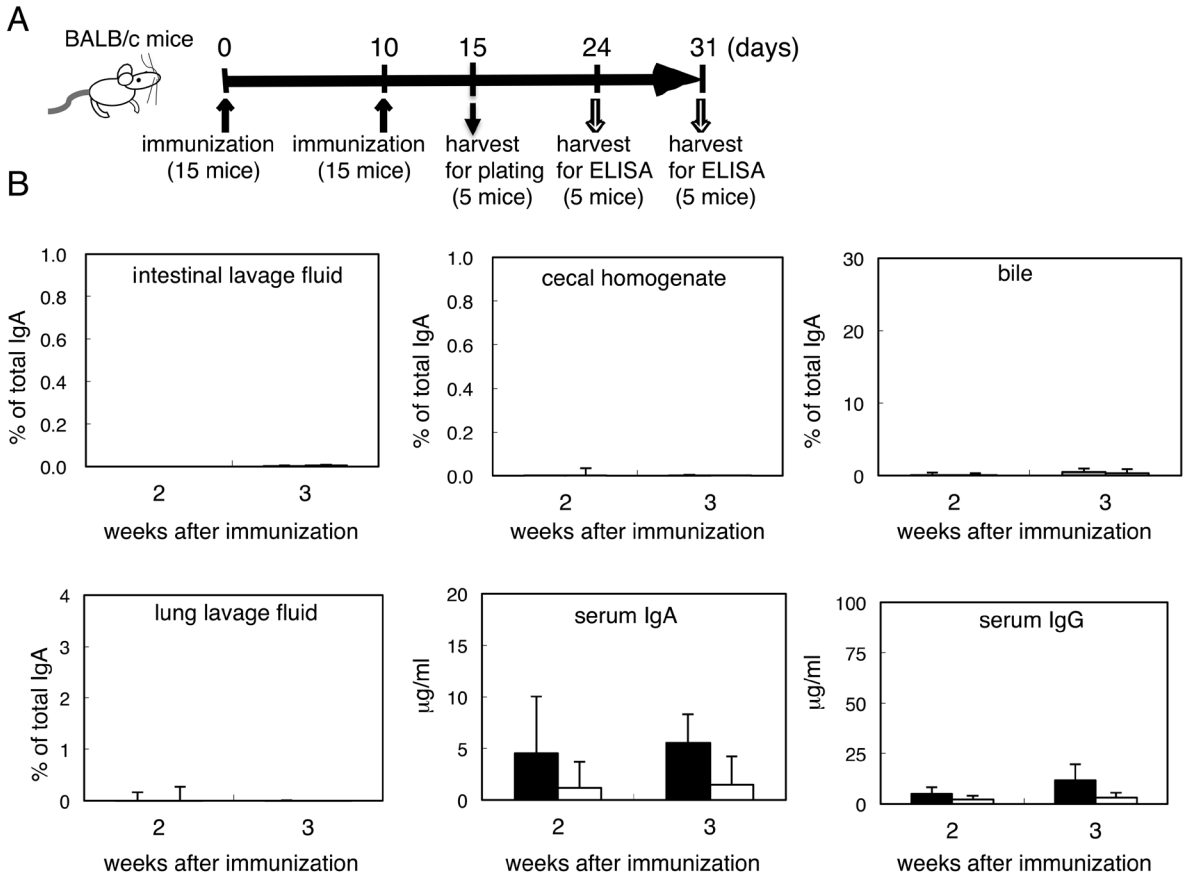

Fig. 2. Efficacy of 2 oral immunizations with virulence plasmid-cured, and Δ*phoP* or Δ*aroA S.* Typhimurium. (A) The immunization schedule. (B) Anti-*S.* Typhimurium s-IgA antibody in the intestinal lavage fluid, cecal homogenate, bile and lavage fluid in addition to anti-*S.* Typhimurium IgA and IgG antibodies in serum at weeks 2 and 3 after final oral immunization with χ3337*phoP* (black columns) or UF21 (white columns). The data are combined from two independent experiments (n=10/group).

were promising oral *Salmonella* vaccine candidates. Presumably, since χ3337*phoP* and UF21 were more attenuated than Δ*phoP* or Δ*aroA S.* Typhimurium in mice, the deletion of the virulence plasmid reduced the difference in immune responses induced by oral immunization with Δ*phoP* or Δ*aroA S.* Typhimurium.

As we did not carry out a challenge experiment after 2 oral immunizations, there was no firm proof that 2 oral immunizations would fail to induce protective immunity in mice. However, it is clear that a multiple oral immunization with χ3337*phoP* or UF21 was necessary to induce the protective immunity in mice. The ideal vaccines for livestock should be non-virulent to humans as well as host animals. After that, strong protection against pathogens would be an additional requirement. Both χ3337*phoP* and UF21 appear to satisfy these requirements, even though the immunization protocol requires a multiple oral immunization.

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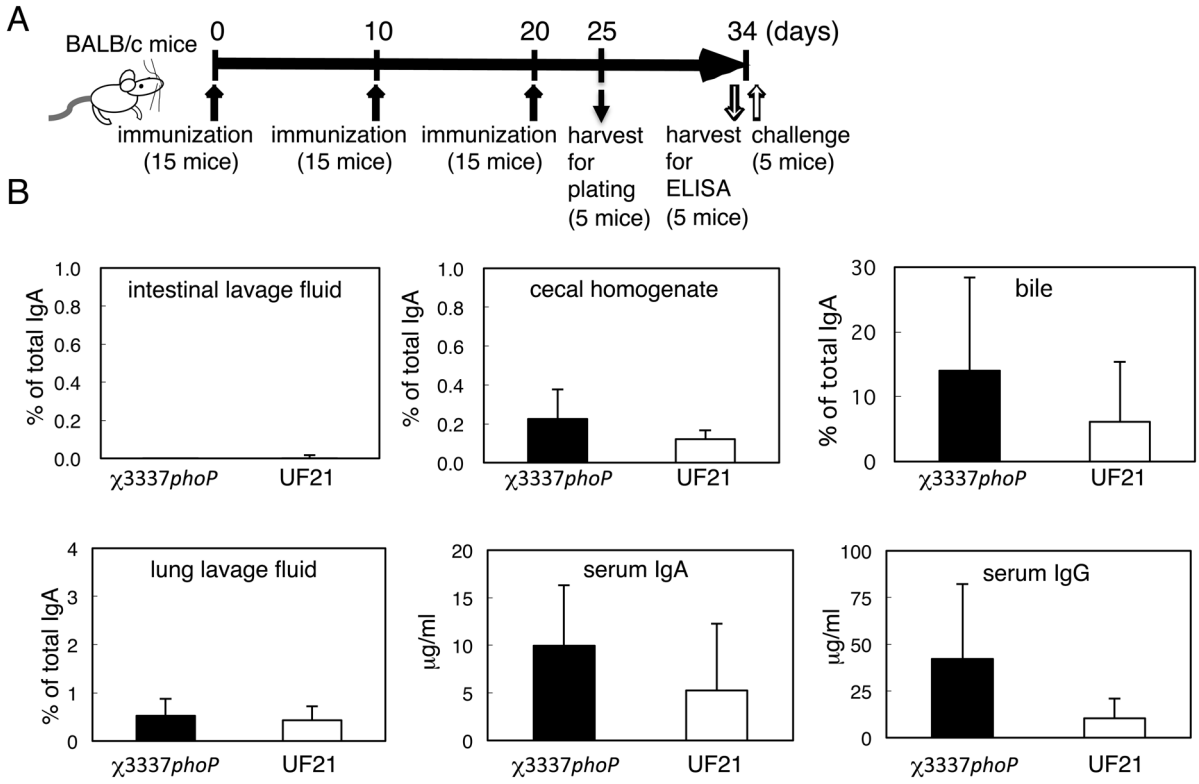

Fig. 3. Efficacy of 3 oral immunizations with virulence plasmid-cured and Δ*phoP* or Δ*aroA S.* Typhimurium. (A) The immunization schedule. (B) Anti-*S.* Typhimurium s-IgA in the intestinal lavage fluid, cecal homogenate, bile and lavage fluid in addition to anti-*S.* Typhimurium IgA and IgG antibodies in the serum at week 2 after final oral immunization with χ3337*phoP* (black columns) or UF21 (white columns). The data are combined from two independent experiments (n=10/group).

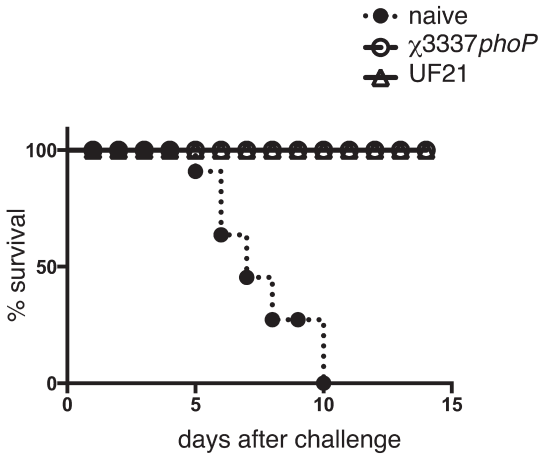

Fig. 4. Survival rates of mice after oral challenge with virulent *S.* Typhimurium. Two weeks after final oral immunization with $1x10^8$ CFU of χ3337*phoP* or UF21, the mice were orally challenged with $5x10^8$ CFU of χ3456 as illustrated in Fig. 3A. The age-matched naïve mice were also orally challenged with $5x10^8$ CFU of χ3456. $P<0.0001$ naïve mice vs. mice immunized with χ3337*phoP* or UF21 (n=10/group, from two experiments).

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