Received: 1 March 2022 | Revised: 30 May 2022 | Accepted: 14 July 2022

DOI: 10.1111/jam.15720

ORIGINAL ARTICLE

A SARS-CoV-2 oral vaccine development strategy based on the attenuated *Salmonella* type III secretion system

Leyang Wu[1,2] | Lin Li[1] | Xingpeng Yin[1] | Chenyang Li[1] | Wenjie Xin[1] | Lina Liu[1] | Zichun Hua[1,2,3]

[1]The State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu, China

[2]Changzhou High-Tech Research Institute of Nanjing University and Jiangsu TargetPharma Laboratories Inc., Changzhou, Jiangsu, China

[3]School of Biopharmacy, China Pharmaceutical University, Nanjing, Jiangsu, China

Correspondence
Zichun Hua, The State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, Jiangsu, China.
Email: zchua@nju.edu.cn

Funding information
Jiangsu Provincial Department of Science and Technology, Grant/Award Number: BK20192005; National Natural Science Foundation of China, Grant/Award Number: 81630092 and 82130106

Abstract

Aims: This study aimed to provide a safe, stable and efficient SARS-CoV-2 oral vaccine development strategy based on the type III secretion system of attenuated *Salmonella* and a reference for the development of a SARS-CoV-2 vaccine.

Methods and Results: The attenuated *Salmonella* mutant Δ*htrA*-VNP was used as a vector to secrete the antigen SARS-CoV-2 based on the type III secretion system (T3SS). The *Salmonella* pathogenicity island 2 (SPI-2)-encoded T3SS promoter (sifB) was screened to express heterologous antigens (RBD, NTD, S2), and the SPI-2-encoded secretion system (sseJ) was employed to secrete this molecule (psifB-sseJ-antigen, abbreviated BJ-antigen). Both immunoblotting and fluorescence microscopy revealed effective expression and secretion of the antigen into the cytosol of macrophages in vitro. The mixture of the three strains (BJ-RBD/NTD/S2, named AisVax) elicited a marked increase in the induction of IgA or IgG S-protein Abs after oral gavage, intraperitoneal and subcutaneous administration. Flow cytometric analysis proved that AisVax caused T-cell activation, as shown by a significant increase in CD44 and CD69 expression. Significant production of IgA or IgG N-protein Abs was also detected by using psifB-sseJ-N(FL), indicating the universality of this strategy.

Conclusions: Delivery of multiple SARS-CoV-2 antigens using the type III secretion system of attenuated *Salmonella* Δ*htrA*-VNP is a potential COVID-19 vaccine strategy.

Significance and Impact of the Study: The attenuated *Salmonella* strain Δ*htrA*-VNP showed excellent performance as a vaccine vector. The *Salmonella* SPI-2-encoded T3SS showed highly efficient delivery of SARS-COV-2 antigens. Anti-loss elements integrated into the plasmid stabilized the phenotype of the vaccine strain. Mixed administration of antigen-expressing strains improved antibody induction.

KEYWORDS
attenuated *Salmonella* Δ*htrA*-VNP, mixed bacterial preparations, SARS-CoV-2, SPI-2, T3SS

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the most serious challenge humanity has faced in a century. The spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins are the four structural proteins of SARS-CoV-2. This novel coronavirus shares approximately 82% genomic similarity with the SARS coronavirus (SARS-CoV) from the 2003 outbreak (Lan

Leyang Wu and Lin Li contributed equally.

© 2022 Society for Applied Microbiology.

et al., 2020). During the early development of vaccines against SARS-CoV, researchers found that antibodies against the S protein were efficient in neutralizing the virus and preventing infection (Buchholz et al., 2004), making the S protein of SARS-CoV-2 a prime target for current vaccine development (Chen et al., 2021; Nguyen et al., 2020).

Several research institutes and pharmaceutical companies are rapidly developing vaccines against SARS-CoV-2, including live attenuated vaccines, recombinant virus vector vaccines, inactivated virus vaccines, protein subunit vaccines, virus-like particle vaccines and nucleic acid vaccines (Jeyanathan et al., 2020; Wang et al., 2021). However, the current vaccines are still not sufficient to meet the global demand due to various factors, such as low production and high price (Choi, 2021). The detection of SARS-CoV-2 antibodies in a wide range of wild white-tailed deer (Chandler et al., 2021) and virus fragments in wastewater that may have originated from nonhuman hosts (Smyth et al., 2021) suggest the possibility of viral transmission between wild animals and humans. Therefore, there is an urgent need for an immunization strategy that can be applied to large numbers of animals. Oral delivery is a major advantage of attenuated strain vector-based vaccines, which have received much attention (Sears et al., 2021). Strain-vectored vaccines can exploit the immune response of the mucosal system. Vaccines delivered via the mucosal route are designed to stimulate both local and systemic immune responses, whereas vaccines using other injection methods mainly stimulate systemic immunity (Lin et al., 2015; Zhang et al., 2021). This immunization route is usually associated with fewer side effects and can lower the threshold and cost of vaccination (Yang et al., 2021). Attenuated *Salmonella* is a widely used oral vaccine vector. After oral administration, the strain targets M cells in the Peyer's patches of the intestine (Jensen et al., 1998) and delivers antigens directly to antigen-presenting cells (APCs), eliciting mucosal and systemic immune responses (Mei et al., 2017).

The type III secretion system (T3SS) is a complex with a needle tubing structure (Bai et al., 2018). This system allows direct injection of virulence proteins expressed by several Gram-negative bacteria (e.g. *Salmonella*) into the cytoplasm of the host cell (dos Santos et al., 2019; Sears et al., 2021). Some T3SS effector proteins are encoded by *Salmonella* pathogenicity islands (SPIs) and are expressed only when *Salmonella* is inside APCs (Abrahams & Hensel, 2006). This phenomenon enables the use of live attenuated *Salmonella* vectors for delivery of the heterologous antigens of interest to the class-I antigen presentation pathway of intact professional APCs. SPIs are chromosomal regions that carry virulence genes. To date, five SPIs have been identified in *Salmonella*, and SPI-1 and SPI-2 have been the most intensively studied (Pinaud et al., 2018). The T3SS encoded by SPI-1 functions mainly in contact with host cells and transports bacterial effector proteins across the cytosolic membrane (T3SS-1). In contrast, SPI-2 encodes a T3SS that is expressed within the phagosome and transports effector proteins across the vesicle membrane (T3SS-2) (Pinaud et al., 2018). Homeostatic conditions, for example, low oxygen, in the intestine lead to the expression of the SPI-1 and T3SS-1 related genes (Thompson et al., 2006). Then, T3SS-1 injects effector proteins encoded by SPI-1 into the host cell to facilitate bacterial invasion. After these molecules enter epithelial cells or macrophages, metabolic signals such as low pH and low phosphate trigger the expression of the bacterial SPI-2 and T3SS-2 genes (Garmendia et al., 2003).

VNP20009 (abbreviated as VNP), a widely studied attenuated *Salmonella*, has been validated for safety in phase I clinical trials (Toso et al., 2002). Our preliminary study identified attenuated *Salmonella* VNP20009 as an effective plasmid delivery vector (Cheng et al., 2014) as well as a heterologous protein expression vector (Liu et al., 2015), and our previous research also successfully achieved a schistosome vaccine candidate based on an attenuated *Salmonella* vector (Chen et al., 2011). We also obtained an attenuated *Salmonella* mutant, Δ*htrA*-VNP, with a good safety profile, which has significantly reduced splenic and hepatic virulence compared with wild-type VNP, making it suitable for vaccine or drug delivery vectors (Zhang et al., 2016). Here, we attempted to engineer Δ*htrA*-VNP as the SARS-CoV-2 antigen expression vector. We constructed a constitutive expression system of the J23100 promoter with the pelB signal peptide and four inducible expression systems of the hypoxia-inducible promoter (pNirB), SPI-1 promoter (psopE) and SPI-2 promoter (psseJ and psifB) with the sseJ signal peptide to express the SARS-CoV-2 RBD domain. The expression system equipped with the sifB promoter and sseJ signal peptide (psifB-sseJ) showed optimal antigen delivery and efficient induction of specific antibodies. We then used this system to construct engineered bacteria expressing multiple domains of the S protein, including the RBD, NTD and S2. Higher levels of IgA or IgG antibodies against the S protein were induced in mice immunized with the hybrid strains after oral gavage, intraperitoneal or subcutaneous administration. The bacterial vector vaccine based on the psifB-sseJ secretion expression system and the immune strategy of the bacterial hybrid formulation are expected to provide a reference for the development of vaccines for COVID-19 or other diseases.

MATERIALS AND METHODS

Cells, strains and plasmids

RAW264.7 macrophages were preserved in our laboratory. RAW264.7 cells were induced using 100 ng/mL LPS (Beyotime, S1732, China) for 24 h to obtain RAW264.7 (M1-like) cells. The Δ*htrA*-VNP strain used in this article was obtained from a previous construction and preserved in our laboratory (Zhang et al., 2016). The constitutive promoter J23100, hypoxia promoter NirB and secretion signal peptide PelB were described previously (Liu et al., 2015; Zhang et al., 2016). The SPI-1-related promoter sopE and SPI-2-related promoters sseJ and sifB and their corresponding signal peptide sseJ were obtained by PCR amplification using the VNP20009 genome as a template. The RBD, NTD and S2 domains in the S protein and N(FL) of SARS-CoV-2 were all based on related plasmids (pUC57-2019-nCOV-S (*Escherichia coli*), GenScript, China; pET28a-N (SARS-CoV-2), Miaoling, China) as a template for PCR amplification (Vazyme, P131-01, China), and the related sequences are shown in Table S1. The N-terminus of each signal peptide was coupled with all or part of the sequence of the RBD, NTD, S2 and N (FL) antigen molecules through Linker to realize its secretion. The *asd* gene (ASD), Alp7 *hok/sok* (AHS) and *Axe/Txe* (AT) were synthesized as described previously (Faridani et al., 2006; Fedorec et al., 2019; Yan et al., 2013). The target protein was fused to the HA tag to facilitate subsequent detection. The plasmid pQE30, preserved in our laboratory, was inserted with the AT element as a vector template. The above corresponding fragments were assembled by homologous recombination (Vazyme, C113-01/02, China), and the correctness of the plasmid was verified.

Electroporation transformation

The preparation of *Salmonella* competent cells was carried out in accordance with the following procedure. A single colony selected from the plate of activated attenuated *Salmonella* strains was inoculated in LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) and cultured at 37°C to $OD_{600} = 0.4–0.6$. After incubation in an ice bath for 15–30 min, the bacterial liquid was centrifuged at 4°C and 5000 rev min^{-1} for 5 min to collect the bacteria. The bacteria were washed once with precooled sterile double-distilled water and collected by centrifugation at 4°C and 5000 rev min^{-1} for 5 min. The bacteria were washed with precooled 10% glycerol and centrifuged at 5000 rpm at 4°C for 5 min to collect the bacteria. The bacteria were then washed with 10% glycerol, and the operation was repeated three to four times. The bacteria were resuspended in 10% ice-cold glycerol to a concentration of 3.5×10^8 CFU (colony forming unit)/mL or more for electroporation. The recombinant plasmid was transformed into attenuated *Salmonella* Δ*htrA*-VNP by electroporation according to the scheme described above (Chen et al., 2011). All the electroporated bacterial liquid was spread evenly on agar plates containing kanamycin, and the final obtained colonies were the recombinant bacteria.

Plasmid stability and strain growth curves

We constructed recombinant attenuated *Salmonella* Δ*htrA*-VNP strains containing red fluorescent protein (RFP) expression plasmids with different loss-resistant elements and measured the expression of RFP to determine the stability of the plasmids. For assays in vitro, the different strains were serially passaged and diluted onto LB plates without antibiotics. For assays in vivo, mice were orally administered 1×10^9 CFU of the different engineered strains per mouse, and spleens were collected and weighed at 3, 6, and 9 days postinoculation. Tissue samples were homogenized by punching with 0.5% Triton X-100 (Sigma, 9002-93-1) in PBS at room temperature and adding 0.5% Triton X-100 at a ratio of 2:1 (0.5% Triton X-100 volume: organ mass). The homogenate was diluted in a 10-fold gradient with PBS, 50–100 μl of the diluted homogenate was applied to an agar plate without antibiotics, and the plasmid loss of the strain was calculated based on the ratio between the number of colonies with red fluorescence that grew and the total number of colonies. For the detection of the growth curve of the strain, individual colonies of each strain were selected from agar plates and cultured in LB medium overnight. Inoculation with 1×10^4 colony-forming units (CFUs) of strains was used to start up cultures (six independent cultures for the same strain) in liquid LB medium (200 μl) and then placed in a microtiter plate. The cultures were grown for 36 h at 37°C using the 'medium' speed and shaking amplitude parameters, with optical density measurements (OD_{600}) every 15 min. The data were tested and recorded using a microplate reader (BioTek).

Protein collection and Western blot assay

The engineered strains were incubated in 40 ml of LB medium with kanamycin until the OD_{600} was between 0.6 and 1.0. For the induction of the hypoxia promoter NirB, the pNirB-sseJ-RBD (NJ-RBD) strain was

cultured in screw-capped glass flasks filled with LB medium. For the induction of the SPI-I promoter sopE, the psopE-sseJ-RBD (EJ-RBD) strain was inoculated in LB medium supplemented with 0.3 M NaCl. Then, the solution was centrifuged at 5000 rpm and 4°C for 10 min, and the supernatant and precipitate were collected. The precipitate bacterial cells were resuspended in 5 ml of PBS, broken ultrasonically at 300 W on ice for 15 min (5-s sonication; 5-s interval) to collect the bacterial total protein, and then centrifuged at 13,000 rpm for 10 min, and total protein in the precipitated bacteria was contained in the supernatant. Proteins were collected from the supernatant obtained in the first step according to the trichloroacetic acid protein precipitation protocol (Wu, Li, et al., 2022). The method for collecting proteins expressed by engineered attenuated *Salmonella* strains induced in cells is described below. The strain obtained by amplification in LB medium was centrifuged at 12,000 rev min^{-1} for 10 min, and then, the bacterial cells were adjusted to OD_{600} = 1.0 with PBS. Subsequently, the bacteria and RAW264.7 cells (M1) were cocultured for 90 min at MOI = 10:1, washed with PBS 3–4 times and cultured in cell medium supplemented with 50 μg/ml gentamicin and 10% serum. After 6 h, the total intracellular protein was collected for subsequent detection. The concentration of total protein in both the precipitate and supernatant was determined by a BCA kit (Beyotime, P0012, China) and adjusted to the same concentration. The protein solution was mixed with 5× SDS loading (Beyotime, P0015, China) and heated in a dry bath incubator at 95°C. A total of 20 μl of solution (approximately 15 μg of protein) was loaded for the Western blot assay. An HA-tagged antibody (Sigma, F1804, USA) was used to plot the expression and secretion of recombinant protein, and a secondary anti-mouse IgG antibody (CST, 7076, USA) was used.

Immunofluorescence analysis

The expression and secretion of recombinant protein by BJ-RBD, BJ-NTD, and BJ-S2 recombinant bacteria in cells were analysed with immunofluorescence. After we obtained slides with RAW264.7 (M1-like) cells that had engulfed the engineered bacteria according to the above-mentioned method, they were washed three times with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and washed three times with PBS. Triton X-100 (0.5%, Sigma, 9002-93-1, USA) in PBS was used to perforate the cells at room temperature for 30 min, and the cells were washed three times with PBS. Subsequently, the punched cells were blocked with 3% BSA at room temperature for 30 min and then washed with PBS three times. Rabbit monoclonal HA (CST, C29F4, USA) tag antibody was used as the primary antibody to incubate the slides overnight at 4°C. After three washes with PBST, monkey anti-rabbit IgG fluorescent secondary antibody (Absin, abs20021, China) and *Salmonella* fluorescent antibody (Jiangsu TargetPharma Laboratories, Inc.) were incubated at room temperature for 1 h in the dark. After three washes with PBST, the nuclear dye DAPI (Beyotime, C1002, China) was added, and then, a fluorescence microscope (Carl Zeiss Axioplan 2, Germany) was used to observe and photograph the cells.

Immunization of mice

All procedures were conducted in compliance with all the relevant ethical regulations and were approved by the Nanjing University Institutional Animal Care and Use Committee. BALB/c (7–8 weeks, female) mice were purchased from Changzhou Cavens Animals Corporation. Immunization methods for the mice included oral gavage (o.g.) and subcutaneous (s.c.) and intraperitoneal (i.p.) injection. After the engineered strains were incubated overnight on a shaker at 37°C, they were adjusted to a uniform OD_{600} value with PBS and diluted to the same concentration. Subsequently, 1×10^9 CFU per mouse was immunized by o.g., 1×10^7 CFU per mouse was immunized by s.c. injection, and 1×10^6 CFU per mouse was immunized by i.p. injection. The mice were immunized at 7-day intervals for 3 consecutive weeks. For the IgG antibody assay, blood was taken from the ocular venous plexus at different time points. The obtained blood was incubated at room temperature for 2–4 h, and after centrifugation at 3000 rpm at 4°C for 15 min, the upper layer of serum was drawn and stored at −80°C for subsequent experimentation. For the IgA antibody assay, faecal samples were collected from each mouse at different time points as described previously (Gao et al., 2021). Briefly, 30 mg per mouse fresh faecal sample was frozen, lyophilized overnight, and then homogenized in 300 μl of PBS. The resuspended faecal material was centrifuged at 24,000 g for 8 min, and each supernatant was then transferred to a clean tube and stored at −20 °C until assayed.

Flow cytometry assay

The mouse spleens, collected 1 week after three immunizations (once a week), were ground and passed through a cell sieve to obtain a single-cell suspension. After red blood

cells were lysed with red blood cell lysis buffer (Solarbio, R1010, China), 2×10^6 cells per well were plated in a 24-well plate in RPMI 1640 containing antibiotics and 10% FBS. After the separated cells were stimulated with 20 μg/ml S protein for 16 h, anti-CD4-APC (BD, 553051, USA), anti-CD8-PE (BD, 553033, USA) and anti-CD69-FITC (BD, 557392, USA) antibodies were used for cell surface marker staining, and the stained cells were incubated in the dark for 20 min at room temperature. The cells were washed and resuspended with 3% BSA and then analysed by flow cytometry. For detection of CD44 expression, spleen cells collected above were directly stained with the anti-CD44-FITC (BD, 553133, USA) antibody without S protein stimulation and analysed.

Enzyme-linked immunosorbent assay

ELISAs were performed to detect the total IgG antibody titre. The recombinant RBD protein (Vazyme, CG201-00, China), S protein (Vazyme, CG202-01, China) or N protein (Vazyme, CG101-00, China) of SARS-CoV-2 was diluted to 10 μg/ml using 50 mM carbonate buffer (pH 9.6), and the diluent was coated onto separate 96-well plates with 100 μl per well and left overnight at 4°C. The next day, after three washes with PBST (once every 5 min), 300 μl of 3% BSA was added to each well, and the plate was closed for 3 h at 37°C. After three washes with PBST (once every 5 min), 100 μl of serum or faecal sample diluted in a PBS gradient was added to the wells. Next, HRP (horseradish peroxidase)-coupled sheep anti-mouse IgG secondary antibody or sheep anti-mouse IgA secondary antibody (1:5000 dilution in PBST) was added to each well. After 1 h of reaction at 37°C, the wells were washed three times (once every 5 min) with PBST. Then, 100 μl of TMB substrate solution was added to develop colour at room temperature for 5–10 min. When the colour turned blue, 100 μl of 2 M concentrated sulfuric acid was added to each well to stop, and then, the 450 nM light absorption of each well was measured. The endpoint titers were defined as the result of the dilution that gave an absorbance over the cutoff value (0.1), which was calculated based on the mean optical density of empty vector strain-treated mouse serum.

Statistical analysis

Data were analysed with GraphPad Prism software version 5 (GraphPad Software) and are expressed as the mean ± standard deviation (SD). Student's t test was performed to assess statistical significance. p values <0.05 were considered significant ($^*p<0.05$; $^{**}p<0.01$; and $^{***}p<0.001$).

RESULTS

Attenuated *Salmonella* Δhtr*A*-VNP effectively secretes RBD protein through the SPI-2-encoded T3SS

The SARS-CoV-2 spike protein (S) is mainly composed of three key domains, RBD, NTD and S2, among which the RBD domain is frequently used as an immune induction protein because it is the main binding site for viral receptor (Nguyen et al., 2020) (Figure 1a,b). To identify the best secretory expression system for recombinant protein with attenuated *Salmonella* Δ*htrA*-VNP, we preferentially selected RBD protein as the effector antigen. Recombinant antigens are expressed constitutively or inducibly by strains after introduction into a host, and then, they are taken up by APCs, which induce antibody production and T-cell responses (Sizemore et al., 2006). Therefore, a stable and efficient expression delivery system is crucial for the presentation of heterologous antigens. The J23100 promoter is a strong constitutive promoter that can achieve the continuous expression and secretion of the recombinant protein when paired with the pelB signal peptide. Based on the above elements, we constructed the pJ23100-pelB-RBD expression system (JB-RBD) as a baseline for the strain's ability to express heterologous proteins. NirB is a typical hypoxic promoter that can achieve controlled protein expression in cells (Everest et al., 1995). SopE is a widely studied SPI-1 promoter, while psseJ and psifB are SPI-2 promoters (Xu et al., 2010). With the above promoters and the classic SPI-2-type signal peptide (sseJ), we constructed the inducible expression systems pNirB-sseJ-RBD (NJ-RBD), psopE-sseJ-RBD (EJ-RBD), psseJ-sseJ-RBD (JJ-RBD) and psifB-sseJ-RBD (BJ-RBD) (Figure 1c). The above plasmids had HA tags attached at the C-terminus of the recombinant protein for subsequent detection.

A balanced lethal system based on nutrient deficiency (Yan et al., 2013) or virulent gene elements (anti-loss element) (Fedorec et al., 2019) is an effective strategy to prevent plasmid loss and maintain the phenotype of recombinant strains. To prevent the weakened immune effect caused by plasmid loss, an anti-loss element is recommended, and we first evaluated the effects of different anti-loss elements (Figure S1). The results showed that the AHS, AT and ASD elements all significantly prevented plasmid loss. Of these, the AT element, which had better retention of the plasmid without an obvious effect on strain growth (Figure S2), was selected for insertion into the related plasmids. The novel attenuated *Salmonella* mutant, Δ*htrA*-VNP, which we described before, was used as an expression vector, given its superior safety (Zhang et al., 2016). A major advantage of using *Salmonella* as a vaccine carrier is that it can survive and proliferate in macrophages for a period of time and maintain

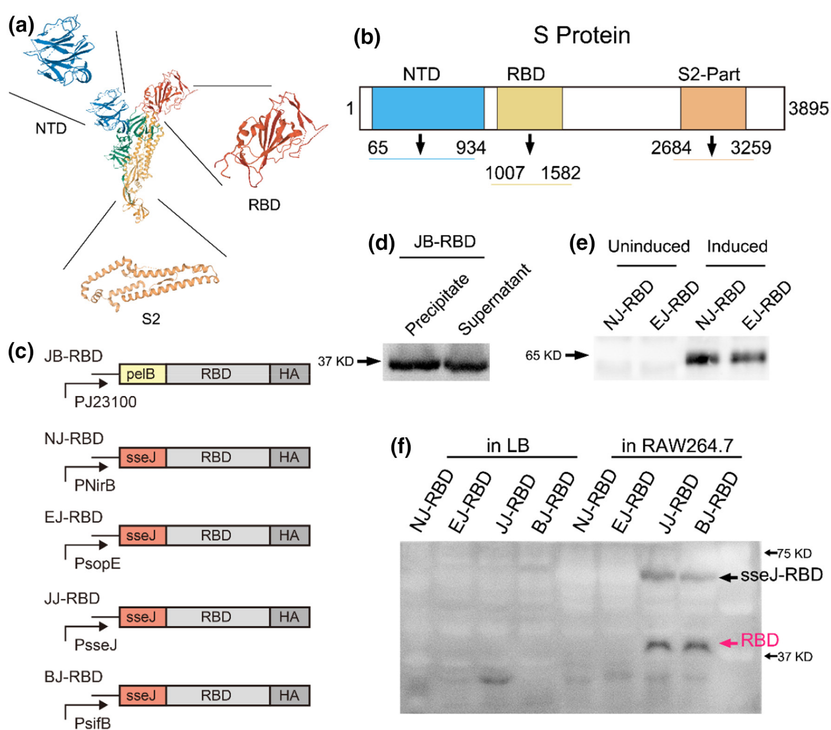

FIGURE 1 Engineered attenuated *Salmonella* Δ*htrA*-VNP effectively expresses and secretes recombinant RBD protein. (a) Schematic diagram of the structure of the SARS-CoV-2 spike protein (PDB ID: 6ZWV), which is composed of three main domains, RBD, NTD and S2. The 3D structures of the complete S protein and each structural domain were visualized using Mol* viewer. (b) Schematic diagrams of regions of each part of the S protein selected for strain expression, including the RBD, NTD and part of the S2 domain. (c) Schematic diagram of the secretory expression of RBD by means of an expression and secretion system composed of different promoters and signal peptides, including the constitutive expression system JB-RBD and NJ-RBD, EJ-RBD, JJ-RBD and BJ-RBD based on the SPI-2-encoded T3SS expression and secretion system. (d) The expression of the RBD protein in the precipitate and medium supernatant of the JB-RBD strain described in **c** was detected after culture in LB medium for more than 12 h. (e) The expression of the RBD protein in the NJ-RBD and EJ-RBD strains described in **c** was detected after culture in hypoxia or medium supplemented with 0.3 M NaCl for more than 12 h. (f) The four kinds of inducible expression engineered strains described in (c) were cultured in LB medium or cocultured with RAW264.7 cells for 6 h. RBD protein expression in the bacteria cultured in LB medium and the macrophages containing the strain was detected. The black arrow indicates the larger molecular weight sseJ-RBD protein expressed by the intracellular environment-induced strain. The red arrow indicates the RBD protein with a smaller molecular weight, which was secreted into the cytoplasm by the bacteria via the T3SS and then cleaved at the signal peptide by the enzymes. All data are representative of two independent experiments.

metabolic activity to achieve more precise and effective delivery of target antigens to macrophages (Rao et al., 2020; Wu, Li, et al., 2022). To determine whether Δ*htrA*-VNP has similar effects, we examined the proliferation and survival of wild-type VNP (wt-VNP) and Δ*htrA*-VNP in macrophages. The results indicated that the Δ*htrA*-VNP mutant exhibited a similar ability to invade macrophages as wt-VNP, as well as a certain degree of intramacrophage survival and replication (Figure S3), consistent with the results of previous reports (Phillips & Roop 2nd, 2001; Zhang et al., 2016). Therefore, we hypothesize that Δ*htrA*-VNP can survive and replicate at low rates after the invasion of macrophages, resulting in efficient antigen presentation, while low systemic toxicity makes it a safer vector for heterologous antigen presentation. To verify this finding, we obtained five kinds of different engineered strains by transferring the above five plasmids into Δ*htrA*-VNP, and the expression and secretion effects of effector proteins were examined under different conditions. Immunoblotting demonstrated that the constitutive secretion system JB-RBD can express the correct recombinant RBD protein and secrete it into the supernatant of the culture medium (Figure 1d). None of the four strains with the inducible secretion system could express recombinant RBD proteins when cultured in LB, but when the strains were coincubated with RAW264.7 macrophages, high levels of recombinant RBD proteins were detected in the lysates of macrophages invaded by the JJ-RBD and BJ-RBD strains (Figure 1f). Notably, NJ-RBD and EJ-RBD strains, which have been confirmed to express the target protein under appropriate induction conditions, failed to achieve protein expression after coincubation with macrophages (Figure 1e,f). This may result from the failure to meet the effective induction conditions. Based on the above results, we evaluated the immune effect of the JJ-RBD and

BJ-RBD systems in subsequent experiments and used JB-RBD as a control.

The secretory expression of RBD protein by attenuated *Salmonella* Δ*htrA*-VNP through the psifB-sseJ system has a more efficient IgG antibody induction effect

Strains with constitutive expression and a secretion system can continuously express and secrete recombinant RBD protein, which is then phagocytosed, recognized and presented by APCs. In contrast, the strain with SPI-2-encoded T3SS was activated in the *Salmonella*-containing vacuole after phagocytosis by APCs and initiated the expression of recombinant RBD proteins. The heterologous protein would be directly "injected" into the cells through the T3SS of *Salmonella* with the assistance of the sseJ signal peptide and then efficiently recognized, degraded and presented (Figure 2a) (Walker et al., 2017). There was no significant difference in growth between the three selected engineered strains (JB-RBD, JJ-RBD, BJ-RBD) and the empty-vector strain (Δ*htrA*-VNP strain loaded with plasmid but with no protein expression function) in vitro. These strains all reached a growth plateau after 27 h of culture in LB medium (Figure 2c). The four strains were immunized by oral gavage (o.g.) administration to wild-type Balb/c mice, and the blood was obtained by retro-orbital puncture from anaesthetised mice 1 week after the third vaccination to detect the production of specific IgG antibodies against RBD protein (Figure 2b). The results showed that the three kinds of engineered strains we constructed could effectively induce mice to produce specific IgG antibodies against the RBD protein. Notably, systems with promoters and signal peptides of the SPI-2-encoded T3SS (JJ-RBD, 0.4862 ± 0.6186; BJ-RBD, 0.8042 ± 0.2033; mean ± SD) showed higher humoral immune activation than the constitutive expression and secretion system (JB-RBD, 0.2928 ± 0.0645), representing the OD_{450} value of serum IgG, while the system regulated by the sifB promoter showed the best results, with a 2.75-fold increase compared with JB-RBD and a 1.65-fold increase compared with JJ-RBD (Figure 2d). Therefore, the psifB-sseJ system was chosen for the follow-up study.

Attenuated *Salmonella* ΔhtrA-VNP effectively secretes the RBD, NTD and S2 proteins through the psifB-sseJ system

Both the NTD and S2 domains are key components of the S protein and can also induce the production of specific neutralizing antibodies (Figure 1a) (Wang et al., 2019). Therefore, we tried to use the screened psifB-sseJ system to express and secrete the NTD and S2 proteins by attenuated *Salmonella* Δ*htrA*-VNP (Figure 3a). The final expression and secretion effect of the bacteria on the protein are related to the size of the protein, especially with the aid of T3SS (Van Engelenburg & Palmer, 2010). Based on the predicted antigenic determinant regions on the NTD and S2 regions (Lin et al., 2020), we selected the full-length NTD domain and part of the S2 domain for expression and secretion by the BJ-NTD and BJ-S2 strains (Figure 1b). After coculture of the engineered strains with RAW264.7 cells for 90 min, the extracellular strains were removed, and the cells were cultured for another 6 h. The protein expression and secretion by the strains were detected by Western blotting. The results showed that the psifB-sseJ system could effectively induce the expression of the RBD, NTD, and S2 proteins after coculture of the strains and macrophages. Significantly cleaved bands, indicating that the target protein was secreted into the cytoplasm by the strain, were observed (Figure 1f and 3c). Immunofluorescence images revealed that all four strains (green) could be phagocytosed by macrophages after coculture. Colocalization of fluorescently labelled HA tags reveals that the intracellular strains, including BJ-RBD, BJ-NTD, and BJ-S2, achieved high efficiency and induced protein expression (red). The expressed protein diffused into the cytoplasm of macrophages, indicating the effective secretion of the protein into the cytoplasm by the strain (Figure 3d). To test the timeliness of this intracellularly induced secretory expression system, we cocultured the BJ-RBD strain with macrophages following the above method and then continued to coculture for 2, 6 and 12 h. The results of immunoblotting showed that the expression and secretion of the RBD protein by intracellular strains could be detected within 2 h, and the levels of recombinant protein were close among the three different time points, indicating the rapid responsiveness and long-term stability of this system (Figure 3e).

A hybrid formulation of multiple strains expressing different domains of the S protein effectively induces specific IgA and IgG antibodies and T-cell activation

Cocktail treatments combining neutralizing antibodies (nAbs) that recognize epitopes on the NTD, RBD or S2 or different epitopes on the same segments have been found to have synergistic effects and minimize the occurrence of antibody escape mutants, potentially enhancing nAb efficacy in preventing COVID-19 (Suryadevara et al., 2021). To test the efficacy of the BJ-RBD-, BJ-NTD- and BJ-S2-engineered bacterial vector vaccines and to identify a more efficient vaccine, we matched and mixed

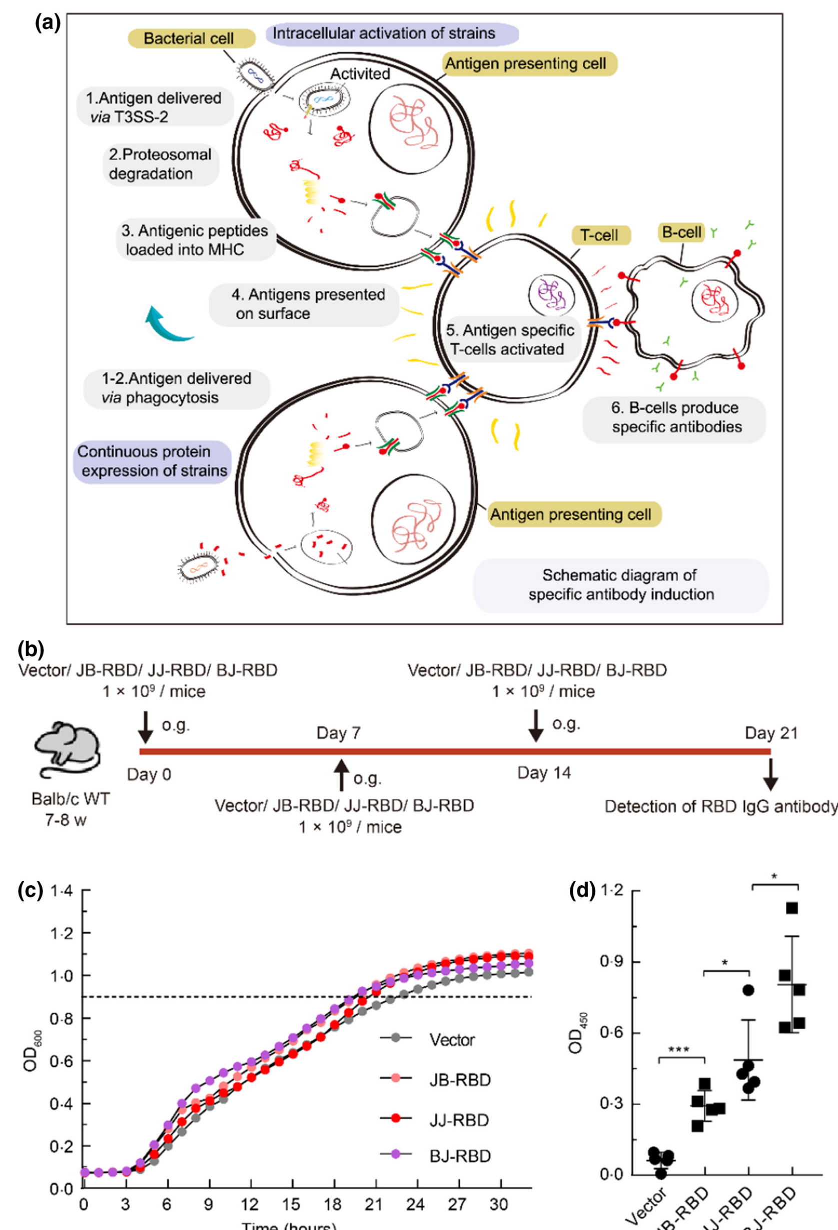

FIGURE 2 The RBD protein secreted by engineered attenuated *Salmonella* Δ*htrA*-VNP effectively induced specific IgG antibodies. (a) Diagram of the mechanism of inducing 918 specific antibodies by using attenuated Salmonella ΔhtrA-VNP as a carrier. This 919 method is different from the constitutive expression and secretion of RBD protein, 920 which is then recognized, processed and transmitted by antigen-presenting cells (APCs). 921 After the strain is recognized and phagocytosed by APCs, it will be activated inside the 922 cell and express and secrete the RBD protein into the cytoplasm with the aid of the 923 T3SS. The heterologous protein is then cleaved and presented by APCs and eventually 924 induces T-cell activation and B cells to produce antibodies. (b) Mice were immunized 925 with four kinds of engineered strains by oral gavage (o.g.) administration, once a week, 926 and the serum was collected to detect specific IgG antibodies one week after the third 927 immunization. (c) Detection of the growth curves of four kinds of engineered bacteria 928 over 36 h ($n = 6$). (d) Comparison of four kinds of strains induced specific IgG antibody Only 929 against RBD protein determined by ELISAs in (b). The immunized mouse serum was 930 tested after 100-fold dilution, and the strain carrying the empty plasmid was used as the 931 normal control ($n = 5$). All data are representative of two independent experiments. 932 Data in d are reported as the mean ± SD. Statistics were calculated using a two-tailed, 933 unpaired Student's t test with Welch's correction. *$p < 0.05$, ***$p < 0.001$.

the above engineered and empty vector strains (BJ-RBD, BJ-NTD, BJ-S2 and EV) to obtain eight different sets of hybrid bacterial formulations and separately immunized the mice by o.g. administration (Figure 4a,b). To exclude the interference of the protein quantity on the results, we mixed the strains in equal proportions. A consistent total dose of bacteria was maintained, and the necessary quantity was filled with empty strains. IgG antibodies against S

FIGURE 3 The psifB-sseJ system allows efficient expression and secretion of the RBD/NTD/S2 proteins by attenuated *Salmonella* Δ*htrA*-VNP. (a) Schematic diagram of the SPI-2-encorded T3SS expressing recombinant RBD, NTD, and S2 proteins. Through electroporation of the above plasmids into Δ*htrA*-VNP, three kinds of engineered Δ*htrA*-VNP strains, BJ-RBD, BJ-NTD, and BJ-S2, were obtained. (b) Schematic diagram of the activation and expression of the three engineered Δ*htrA*-VNP strains to secrete antigen proteins. The strain was activated to express and secrete heterologous protein after being engulfed by APCs. (c) Western blotting was used to detect the expression and secretion of recombinant protein by the BJ-NTD and BJ-S2 strains. After the two strains were cocultured with RAW264.7 cells at 10:1 for 90 min, 50 μg/ml gentamicin was used to kill the free extracellular strains. After culture for another 6 h, the recombinant protein in the total protein of the cell lysate was detected. (d) After RAW264.7 macrophages were cocultured with the strain for 90 min and the extracellular bacteria were removed and incubated for another 6 h, the expression and secretion of the recombinant protein by the three strains in the cell were observed by immunofluorescence microscopy. The white arrow indicates the attenuated *Salmonella* Δ*htrA*-VNP that expressed and secreted the recombinant protein, bar = 10 μm. (e) The BJ-RBD strain and RAW264.7 macrophages were cocultured at 10:1 for 90 min. After the free extracellular strains were removed, the coculture was continued for 2, 6, and 12 h, and the levels of the intracellular recombinant protein were detected by immunoblotting. All data are representative of three independent experiments.

protein in the serum were detected 1 week after the third immunization (Figure 4a,b). ELISAs demonstrated that the presence of any BJ-RBD, BJ-NTD or BJ-S2 strain in the hybrid bacterial formulation could induce the production of IgG S-protein Abs. Notably, formulations containing BJ-RBD and BJ-NTD or three strains (BJ-RBD, BJ-NTD and BJ-S2) showed higher induction of S protein IgG antibodies than other formulations (Figure 4c). The formulation of three kinds of strains, BJ-RBD, BJ-NTD and BJ-S2 (subsequently referred to as AisVax), was selected for the subsequent experiments because it had the highest ability to stimulate antibody production (Figure 4d). To assess systemic immune activation and proprietary intestinal mucosal immune activation by AisVax, we immunized mice with saline (control), empty vector strain (vector) or AisVax weekly via o.g. administration (1×10^9 CFU per mouse), and serum and faecal samples were harvested at 1, 2, and 3 weeks (Figure 4e). The results showed that o.g. vaccination with AisVax could induce the body to produce IgA and IgG Abs against the S-protein 1 week after immunization, and the antibody titers were positively correlated with the immunization time, indicating the efficient activation of the mucosal and systemic immune systems (Figure 4f,g). There was no significant production of special Abs over time in the vector and control groups, neither difference between these two groups (data not shown). We also monitored mouse body weight before and after vaccination and found no significant differences (Figure 4h). In addition, other routes of administration, including intraperitoneal (i.p., 1×10^6 CFU per mice) and subcutaneous (s.c., 1×10^7 CFU per mice) routes, were effective in activating the systemic immune system and inducing specific IgG antibody titers similar to those of o.g. administration 1 week after the second immunization (Figure 4i). The three different doses were selected based on effective doses within the tolerated dose range for different

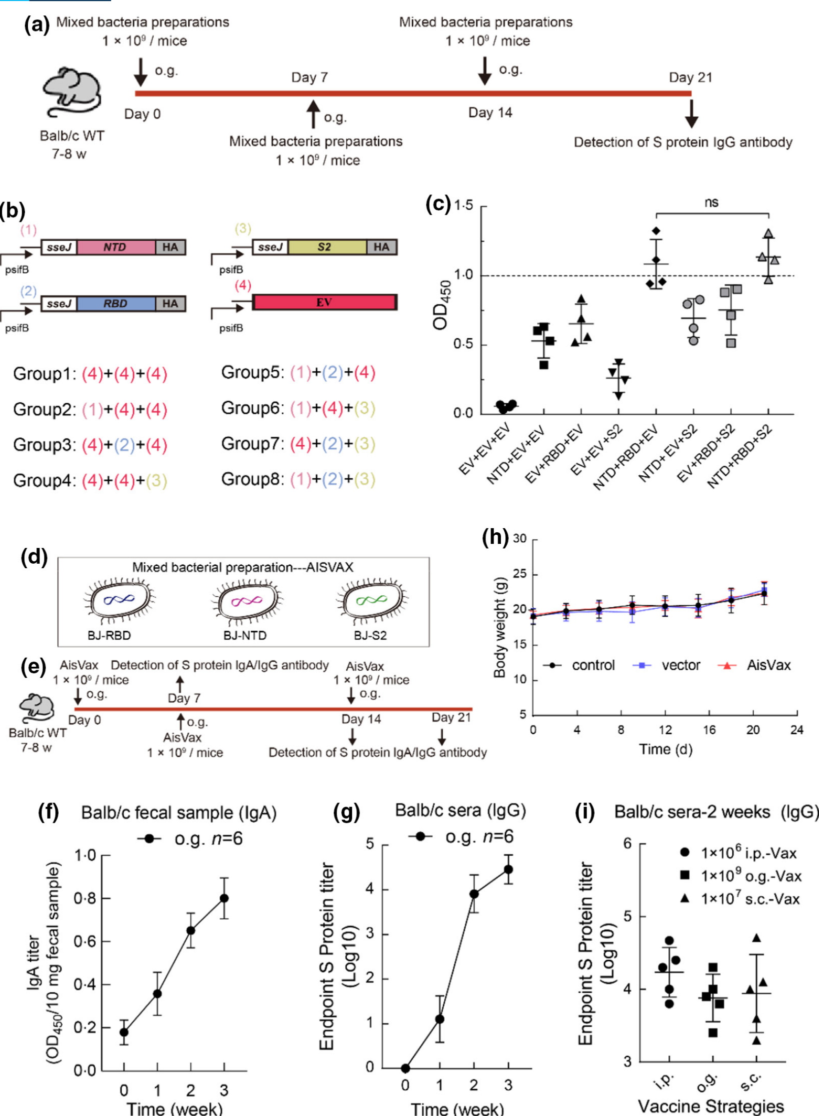

FIGURE 4 The hybrid bacterial formulation AisVax effectively induced specific IgA and IgG antibodies. (a) Operation diagram of mice immunized with different combination schemes by oral gavage (o.g.) administration. Serum was collected 1 week after the third immunization to detect the production of specific IgG antibodies against the S protein. (b) Schematic diagram of the structure of the plasmids carried by the four engineered bacteria and the immunization combination scheme. With Δ*htrA*-VNP strains carrying an empty plasmid as an empty vector (EV) control, the three engineered bacteria expressing heterologous proteins were immunized individually or combined in two or three and filled with EV strains to ensure the same number of bacteria. The total amount of bacteria administered was 1×10^9 CFU per mouse per dose. (c) Mice were immunized with a total of eight different combinations in (b). Serum was collected 1 week after the third immunization and diluted 100 times, and then, ELISAs were performed to detect the production of IgG Abs against the S protein ($n = 4$). (d) Schematic diagram of strain mixing. The mixed bacterial preparation vaccine obtained by mixing 3 different antigen-expressing engineered bacteria is named AisVax. (e) Diagram of AisVax immunization of mice followed by detection of specific antibodies at different times. (f, g) Comparison of IgA in (f) and IgG in (g) Abs against S protein production in fecal samples or serum before AisVax immunization and 1, 2, and 3 weeks after o.g. administration ($n = 6$). (h) Body weight changes of mice treated with saline (control), empty vector strain (vector) or AisVax ($n = 6$). (i) AisVax was injected intraperitoneally, orally (gavage), and subcutaneously to immunize mice for 2 weeks (once per week). Comparison of IgG S-protein Abs in mouse serum ($n = 5$). Data in (c), (f) and (g) are reported as the mean ± SD. All data are representative of three independent experiments. Statistics were calculated using the two-tailed, unpaired Student's *t* test with Welch's correction. ns, no significance.

routes of administration identified in previous studies and are typical values for a variety of applications, including vaccines and tumour treatments (Chen et al., 2011; DiGiandomenico et al., 2004; Lalsiamthara et al., 2018).

CD44 is a classic activation and memory T-cell marker, and its expression level is often used as an indicator to evaluate the activation state of T cells, which can produce long-term immune effects (Sommer et al., 1995). Flow

cytometry was used to detect the changes in the content of $CD44^+$ cells in the mouse spleens after immunization with AisVax. The results showed that compared with vector immunization (5.6 ± 0.2%), AisVax immunization effectively increased the percentage of $CD44^+$ cells in the spleen (9.3 ± 0.3%) (Figure 5a,b). CD69 is an early activation marker for mature T cells stimulated by a specific antigen (Testi et al., 1989). We compared CD69 expression on the surface of $CD4^+$ T cells and $CD8^+$ T cells in the mouse spleens of the saline group (control), the empty vector group (vector) and the AisVax group (AisVax) by flow cytometry. The results showed that AisVax immunization significantly increased the percentage of $CD69^+$ $CD4^+$ T cells (1.9 ± 0.1%) and $CD69^+$ $CD8^+$ T cells (1.1 ± 0.1%) compared with those of the empty vector group (Figure 5c–f).

Expression and secretion of the N protein through the psifB-sseJ system by attenuated *Salmonella* ΔhtrA-VNP effectively induces specific IgA and IgG Abs in vivo

We obtained an efficient antigen expression system, psifB-sseJ, through the screening of promoters and signal peptides and successfully obtained a hybrid bacterial formulation vaccine, AisVax, based on this system. However, SARS-CoV-2 is highly mutagenic, similar to other viruses. Once a large mutation occurs in some antigens of the pathogen, a vaccine developed based on this antigen may show a substantially reduced immune effect or even fail (Wall et al., 2021). Therefore, a good vaccine vector system should be able to rapidly switch to express other

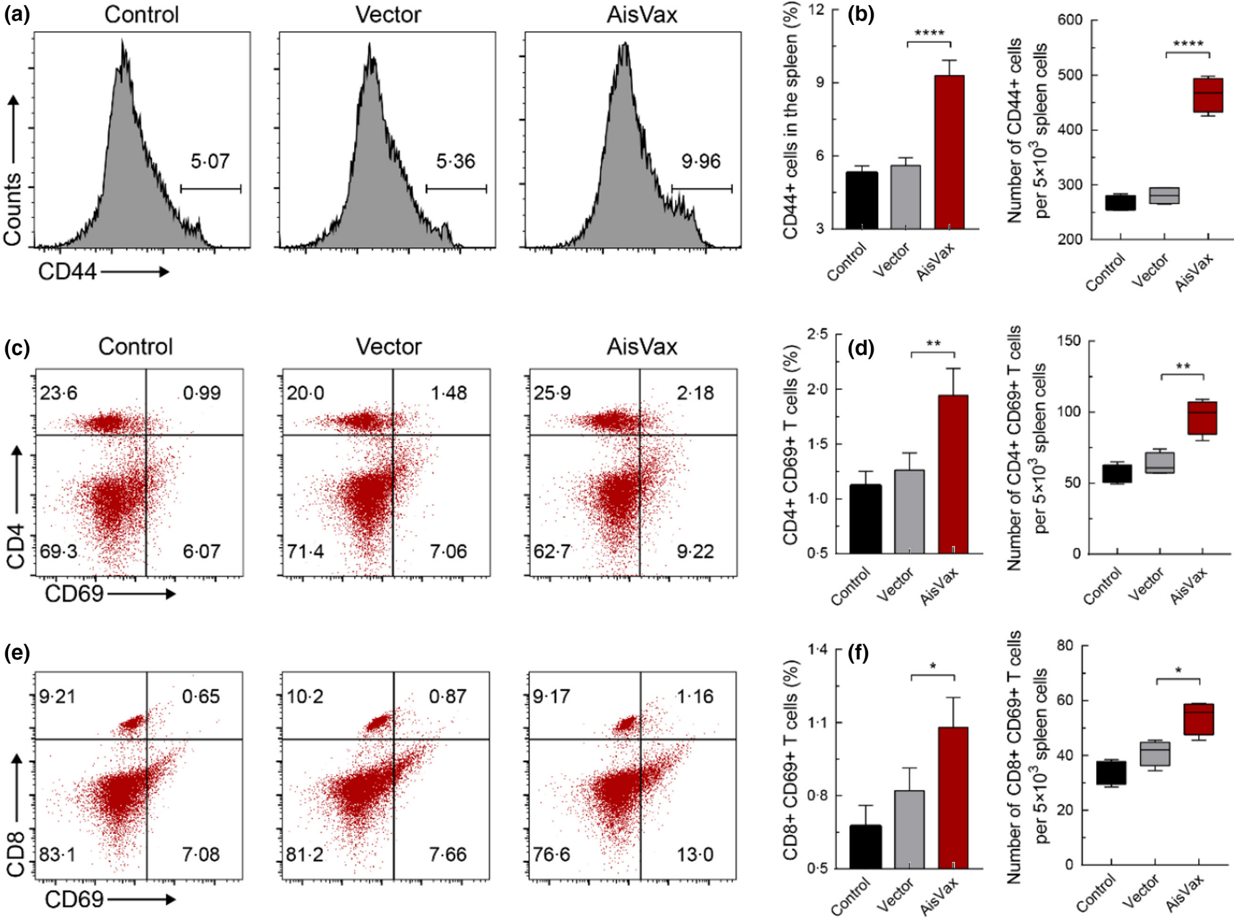

FIGURE 5 Orally administered AisVax promotes $CD44^+$ cell proliferation and $CD4^+$ and $CD8^+$ T-cell activation in vivo. Mice were immunized three times with o.g. administration, once per week, with saline (control), empty vector strain (vector) or AisVax. The spleen cells of the immunized mice were collected one week after the third immunization ($n = 4$). (**a, b**) Spleen cells were labeled with anti-CD44-FITC antibodies and detected by flow cytometry. Flow cytometry plots (**a**) and quantitation with the percentages (**b, left**) and numbers (**b, right**) are shown. (**c-f**) Spleen cells were collected and stimulated in vitro with heterologously expressed S protein for 16 h. Then, the cells were labeled with anti-CD69-FITC antibodies, anti-CD4-APC antibodies, and anti-CD8-PE antibodies and detected by flow cytometry. Dot plots assayed by flow cytometry (**c, e**) and the percentage and numbers of $CD4^+CD69^+$ double-positive cells (**d**) and $CD8^+CD69^+$ double-positive cells (**f**) are shown. Data in **b, d** and **f** are reported as the mean ± SD. All data are representative of two independent experiments. Statistics were calculated using a two-tailed, unpaired Student's t test with Welch's correction. *, $P < 0.05$; **, $P < 0.01$; * * * *, $P < 0.0001$.

key antigens or new mutant antigens. The rapid mutation of the S protein during virus transmission has led to a decrease in the protective efficacy of developed vaccines (Wall et al., 2021), whereas vaccine strategies designed on the basis of the nucleocapsid (N) protein, which is more conserved than the S protein and highly immunogenic, have proven to be effective (Matchett et al., 2021). Given this finding, we tried to use psifB-sseJ to express the nucleocapsid (N) protein, another widely used antigen of SARS-CoV-2, to assess the universality of the system. We connected the N-terminus of the N full-length protein sequence to the sseJ signal peptide, while the C-terminus was connected to the HA tag and expressed with the help of the SPI-2 promoter sifB. Finally, we obtained the BJ-N(FL) strain (Figure 6a). The immunoblot results indicated that the BJ-N(FL) strain could express and secrete

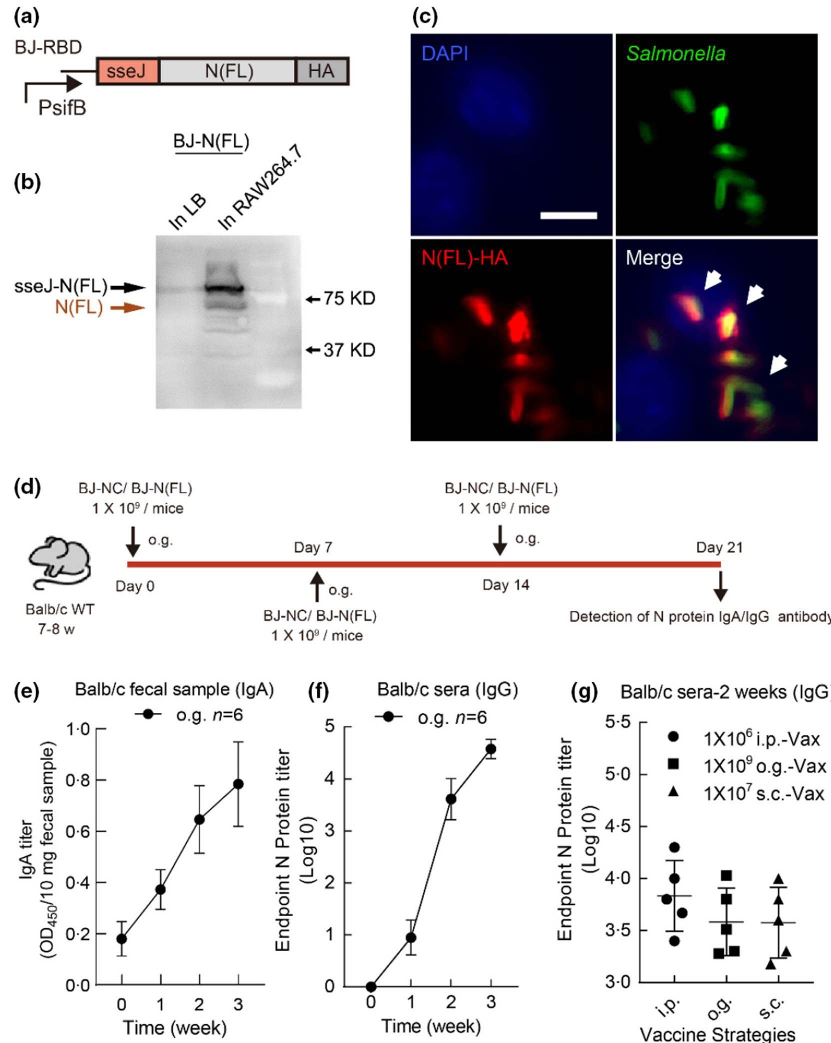

FIGURE 6 Attenuated *Salmonella* Δ*htrA*-VNP uses the psifB-sseJ system to efficiently express and secrete N proteins and induce specific antibodies in vivo. (**a**) Schematic diagram of the psifB-sseJ-N(FL) system structure. (**b**) Western blotting was used to detect the expression and secretion of recombinant protein by the BJ-N (FL) strain. After the strain was cocultured with RAW264.7 cells at 10:1 for 90 min, 50 μg/mL gentamicin was used to kill the free extracellular strain. After culture for 6 h, the recombinant protein in the total protein of the cell lysate was detected. (**c**) After the macrophages were cocultured with the strain for 90 min and the extracellular bacteria were removed and incubated for another 6 h, the expression and secretion of the heterologous protein by the three strains in the cell were observed by immunofluorescence microscopy. The white arrow indicates the engineered Δ*htrA*-VNP strains that expressed and secreted the heterologous protein, bar = 10 μm. (**d**) Operation diagram of oral gavage (o.g.) immunization of mice with the BJ-N(FL) strain followed by the detection of specific antibodies at different times. (**e, f**) Comparison of IgA in (**e**) and IgG in (**f**) Abs against N protein production in fecal samples or serum before BJ-N(FL) strain immunization and 1, 2, and 3 weeks after o.g. administration ($n = 6$). (**g**) The BJ-N(FL) strain was injected intraperitoneally, orally (gavage), and subcutaneously to immunize mice for 2 weeks (once per week). Comparison of the IgG Abs against the N protein in the mouse serum ($n = 5$). Data in **e, f** and **g** are reported as the mean ± SD. Data in **b** and **c** are representative of three independent experiments, and data in **e, f** and **g** are representative of two independent experiments. Statistics were calculated using the two-tailed, unpaired Student's t test with Welch's correction. ns, no significance.

the recombinant N(FL) protein after coculture with macrophages but not in normal LB medium (Figure 6b). Immunofluorescence images also showed that the recombinant N(FL) protein was effectively expressed and secreted in macrophages by the engineered strain after incubation for 6 h (Figure 6c). To assess the immune induction effect, we immunized the BJ-N(FL) engineered strain into mice weekly via o.g. administration while saline (control) and the empty vector strain BJ-NC (vector) were used as control. The results showed that, similar to AisVax, BJ-N(FL) could induce IgA and IgG Abs against N protein in vivo within 1 week, and the titre of specific antibody increased with the duration and frequency of immunization (Figure 6d–f). There was no significant difference between the vector and control groups in the specific Abs content (data not shown). Additionally, IgG-Abs against the N protein could be induced rapidly by different routes of administration, including the o.g., i.p. and s.c. route within 2 weeks (Figure 6g).

DISCUSSION

The use of live bacteria carrying an antigenic protein expression plasmid is a common bacterial vector vaccine strategy. This type of bacterial vector vaccine is widely used, and several clinical trials have been conducted (da Silva et al., 2014). The immunostimulatory capacity of live bacteria further makes them effective immune adjuvants to induce long-term specific immune responses against heterologous antigens (da Silva et al., 2014). Despite the multiple advantages of a bacterial vector vaccine system delivering heterologous molecules (Wu, Bao, et al., 2022), the development of a high efficiency and applicable bacteria-vector-dependent pathway for novel COVID-19 vaccines still faces some technical challenges, for example, (a) maintaining the phenotypic stability of recombinant bacteria and (b) the effectiveness of recombinant protein secretion into the cell via the bacteria. Most bacteria are encapsulated by a membrane-encapsulated vesicle after entering the APCs, and this structure would largely limit the effective delivery of antigenic molecules (Zhang et al., 2008). Therefore, the development of efficient expression systems and new immunization strategies will be the key directions for the commercialization and development of live bacterial vector vaccines in the future.

One viable immunization strategy is to express heterologous proteins on the outer surface of bacteria (Plavec & Berlec, 2019) or to achieve rapid and substantial release by regulating the cleavage of bacteria (Chung et al., 2015). Here, we chose a more effective and natural immune strategy, that is, direct injection of SARS-CoV-2 antigen proteins into the cytoplasm of host cells by the unique T3SS of *Salmonella*. In fact, the effectiveness of this strategy has been extensively demonstrated since 1998, when Rüssmann et al. (1998) first demonstrated that the T3SS of *S. typhimurium* was capable of triggering $CD4^+$ and $CD8^+$ T-cell responses to specific antigens by delivering heterologous proteins into host cells. For example, secreting the *Listeria monocytogenes* P60 protein through this system in vivo effectively protected mice from p60-transfected invasive fibrosarcoma (Panthel et al., 2008). Monkey and human immunodeficiency virus Gag proteins delivered via T3SS induced a significant immune response that provided strong protection against the corresponding wilt-type pathogen (Chen et al., 2006). To our knowledge, this is the first report to explore the feasibility and effectiveness of a bacterial vector vaccine for SARS-CoV-2 based on the SPI-2-encoded T3SS. In addition, we used attenuated *Salmonella* Δ*htrA*-VNP rather than wt-VNP as a vector to express the heterologous antigen because the former possesses better biosafety (Zhang et al., 2016). However, considering that wt-VNP can infect macrophages better than Δ*htrA*-VNP, wt-VNP may theoretically perform better as a vector in inducing antigen-specific antibody production, which warrants further investigation.

Viruses are highly susceptible to mutations during transmission, which may lead to greater virulence or infectivity. More seriously, the effectiveness of vaccines originally developed based on such sites may be greatly reduced or even lost when some of the viral epitope sites are mutated. For example, the neutralizing antibody produced by the vaccine BNT162b2 was 5.8-fold less efficient against the SARS-CoV-2 variant (Delta), which has the S protein P681R variant (Wall et al., 2021). Therefore, we explored the development of an antigen-expressing secretory system with the help of *Salmonella*, which revealed rapid substitution based on different protein sequences to obtain a more time-efficient vaccine. For example, BJ-N(FL) strain obtained by changing the recombinant protein sequence in the psifB-sseJ system induced IgA and IgG N-protein Abs, which proved the effectiveness of the rapid transformation of this system. Previous studies have shown that felines, dogs, and ferrets are also susceptible to SARS-CoV-2, accelerating the mutation and transmission of the virus (Shi et al., 2020). The cross-species transmission of these variant strains to humans would undoubtedly have a negative impact on the protective efficacy of vaccines. Therefore, a novel SARS-CoV-2 vaccine suitable for use in animals is also urgently needed. Bacterial vector vaccines are the preferred choice for animal vaccines due to the advantages of low cost, short production cycle and good efficacy. Our study showed that AisVax could induce IgA and IgG S-protein-Abs in mice by oral gavage administration, indicating the safety and feasibility of using

bacterial vector vaccines as animal-available COVID-19 preventive vaccines.

Considering the complex structure of S proteins, total expression with the help of T3SS may lead to structural interference, as well as difficulties in secretion (Van Engelenburg & Palmer, 2010). In addition, the non-RBD domains included in the S protein, such as NTD and S2, can all induce neutralizing antibodies (Wang et al., 2019), while some other regions of the protein may induce the production of nonneutralizing antibodies, leading to antibody-dependent enhancement effects, which not only fail to destroy the virus but may also enhance its ability to invade the cells (Dai et al., 2020). Therefore, we tried to use different strains to express different regions of the S protein and mix them proportionally, which effectively avoided these problems. The strategy we described was effective in inducing specific IgG antibodies, and oral gavage vaccination can activate mucosal immunity and induce secretion-specific IgA. Effective viral prophylaxis or treatment with bacterial vector vaccines has been widely reported (Ding et al., 2018). However, as heterologous antigens are typically synthesized in mammalian cells through viral replication, a main problem in bacteria-based vaccines is that antigens synthesized in the prokaryotic expression system of bacteria may experience incorrect folding, which results in incorrect structures (Sahdev et al., 2008) or a lack of appropriate post-translational modifications, for example, glycosylation, phosphorylation and myristoylation. Although the produced antibodies can bind to viruses, they have no neutralizing ability (Khow & Suntrarachun, 2012). Therefore, it is necessary to conduct a more in-depth evaluation of the true virus preventive effect of the system we describe, which will also be the goal of our follow-up research. To the best of our knowledge, this is the first SARS-CoV-2 bacterial vector vaccine strategy using a combination formulation with administration of a strain mixture. Several studies to enhance vaccine efficacy through combination immunization have been reported. Vance et al. (2015) for example, reported a vaccine combining the ricin subunit antigen RiVax with anthrax and found improved protective immune responses against ricin and anthrax lethal toxin. A combination of *Schistosoma mansoni* antigen Sm14 and Sm29-related vaccines also achieved more efficient deworming and prophylaxis (Ewaisha et al., 2014). This method is also an effective strategy to induce a stronger immune response by fusing multiple antigenic proteins of the virus and then expressing them in the strain (Jia et al., 2020). All these studies also demonstrate the importance of using multiantigen vaccines as an effective and simple approach to enhance protection against different infectious diseases.

Preexisting immunity has a complex effect on a variety of bacterial vector vaccines, including *Salmonella*, as well as viral vector vaccines and is a common issue in the development of vector vaccines. Previous studies have shown that preexisting immunity may enhance the effect of *Salmonella* vector vaccines or may counteract their efficacy, depending on the type of disease, but the negative effects can also be offset by increasing the number of immunization doses (Saxena et al., 2013). In addition, the excellent performance of various vector vaccines, including *Salmonella* vectors, has been widely demonstrated, providing new avenues for vaccine development. In the future, engineering modified bacterial surfaces to alter their immunogenicity may become the optimal solution for preexisting immunity.

In conclusion, we screened a controlled secretory expression system based on the unique T3SS of *Salmonella*, integrated loss prevention elements into it, and then loaded it with the novel attenuated *Salmonella* Δ*htrA*-VNP. We used this system for the first time to express multiple SARS-CoV-2 key antigenic proteins and prepare a bacterial formulation, AisVax, which was shown to activate mucosal and systemic immunity by oral gavage administration and to be equally effective for other routes of administration, including intraperitoneal and subcutaneous administration. The global spread of COVID-19 has exposed a loophole in cross-species transmission blockage. The novel bacterial vector COVID-19 vaccine and its hybrid formulation AisVax reported in this study have advantages in terms of ease of vaccination and low production cost, which are suitable for veterinary vaccine development. This novel system provides an option for blocking virus transmission in intermediate hosts and will also provide an important reference for vaccine development for other diseases.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the National Natural Sciences Foundation of China (82130106), Nanjing Special Fund for Life and Health Science and Technology (202110016, China), Changzhou Bureau of Science and Technology (CJ20210024, CZ20210010, CJ20220019, China) and Jiangsu TargetPharma Laboratories Inc., China.

CONFLICT OF INTEREST

The authors have applied for patents related to this study.

ORCID

Leyang Wu https://orcid.org/0000-0002-0864-9168
Zichun Hua https://orcid.org/0000-0001-7962-0402

REFERENCES

Abrahams, G.L. & Hensel, M. (2006) Manipulating cellular transport and immune responses: dynamic interactions between

intracellular *Salmonella enterica* and its host cells. *Cellular Microbiology*, 8, 728–737.

Bai, F., Li, Z., Umezawa, A., Terada, N. & Jin, S. (2018) Bacterial type III secretion system as a protein delivery tool for a broad range of biomedical applications. *Biotechnology Advances*, 36, 482–493.

Buchholz, U.J., Bukreyev, A., Yang, L., Lamirande, E.W., Murphy, B.R., Subbarao, K. et al. (2004) Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 9804–9809.

Chandler, J.C., Bevins, S.N., Ellis, J.W., Linder, T.J., Tell, R.M., Jenkins-Moore, M., et al. (2021) SARS-CoV-2 exposure in wild white-tailed deer (*Odocoileus virginianus*). *Proceedings of the National Academy of Sciences of the United States of America*, 118, 47.

Chen, G., Dai, Y., Chen, J., Wang, X., Tang, B., Zhu, Y. et al. (2011) Oral delivery of the Sj23LHD-GST antigen by *Salmonella typhimurium* type III secretion system protects against *Schistosoma japonicum* infection in mice. *Plos Neglected Trop Dis*, 5, 9.

Chen, L.M., Briones, G., Donis, R.O. & Galán, J.E. (2006) Optimization of the delivery of heterologous proteins by the *Salmonella enterica* serovar Typhimurium type III secretion system for vaccine development. *Infection and Immunity*, 74, 5826–5833.

Chen, Z., Du, R., Galvan Achi, J.M., Rong, L. & Cui, Q. (2021) SARS-CoV-2 cell entry and targeted antiviral development. *Acta Pharmaceutica Sinica B*, 11, 3879–3888.

Cheng, X., Zhang, X., Zhou, Y., Zhang, C. & Hua, Z.-C. (2014) A Salmonella Typhimurium mutant strain capable of RNAi delivery Higher tumor-targeting and lower toxicity. *Cancer Biology & Therapy*, 15, 1068–1076.

Choi, E.M. (2021) COVID-19 vaccines for low- and middle-income countries. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 115, 447–456.

Chung, T.-C., Jones, C.H., Gollakota, A., Kamal Ahmadi, M., Rane, S., Zhang, G. et al. (2015) Improved *Escherichia coli* bactofection and cytotoxicity by heterologous expression of bacteriophage ΦX174 lysis gene E. *Molecular Pharmaceutics*, 12, 1691–1700.

da Silva, A.J., Zangirolami, T.C., Novo-Mansur, M.T.M., Giordano, R.C. & Martins, E.A.L. (2014) Live bacterial vaccine vectors: an overview. *Brazilian Journal of Microbiology*, 45, 1117–1129.

Dai, L., Zheng, T., Xu, K., Han, Y., Xu, L., Huang, E., et al. (2020) A universal design of betacoronavirus vaccines against COVID-19, MERS, and SARS. *Cell* 182, 722-733.e711.

DiGiandomenico, A., Rao, J. & Goldberg, J.B. (2004) Oral vaccination of BALB/c mice with *Salmonella enterica* serovar Typhimurium expressing *Pseudomonas aeruginosa* O antigen promotes increased survival in an acute fatal pneumonia model. *Infection and Immunity*, 72, 7012–7021.

Ding, C., Ma, J., Dong, Q. & Liu, Q. (2018) Live bacterial vaccine vector and delivery strategies of heterologous antigen: a review. *Immunology Letters*, 197, 70–77.

dos Santos, A.M.P., Ferrari, R.G. & Conte-Junior, C.A. (2019) Virulence factors in Salmonella Typhimurium: the sagacity of a bacterium. *Current Microbiology*, 76, 762–773.

Everest, P., Frankel, G., Li, J., Lund, P., Chatfield, S. & Dougan, G. (1995) Expression of LacZ from the htrA, nirB and groE promoters in a Salmonella vaccine strain: influence of growth in mammalian cells. *FEMS Microbiology Letters*, 126, 97–101.

Ewaisha, R.E., Bahey-El-Din, M., Mossallam, S.F., Amer, E.I., Aboushleib, H.M. & Khalil, A.M. (2014) Combination of the two schistosomal antigens Sm14 and Sm29 elicits significant protection against experimental *Schistosoma mansoni* infection. *Experimental Parasitology*, 145, 51–60.

Faridani, O.R., Nikravesh, A., Pandey, D.P., Gerdes, K. & Good, L. (2006) Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*. *Nucleic Acids Research*, 34, 5915–5922.

Fedorec, A.J.H., Ozdemir, T., Doshi, A., Ho, Y.-K., Rosa, L., Rutter, J. et al. (2019) Two new plasmid post-segregational killing mechanisms for the implementation of synthetic gene networks in *Escherichia coli*+. *Iscience*, 14, 323.

Gao, T., Ren, Y., Li, S., Lu, X. & Lei, H. (2021) Immune response induced by oral administration with a *Saccharomyces cerevisiae*-based SARS-CoV-2 vaccine in mice. *Microbial Cell Factories*, 20, 95.

Garmendia, J., Beuzon, C.R., Ruiz-Albert, J. & Holden, D.W. (2003) The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella typhimurium* SPI-2 type III secretion system. *Microbiology*, 149, 2385–2396.

Jensen, V.B., Harty, J.T. & Jones, B.D. (1998) Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. *Infection and Immunity*, 66, 3758–3766.

Jeyanathan, M., Afkhami, S., Smaill, F., Miller, M.S., Lichty, B.D. & Xing, Z. (2020) Immunological considerations for COVID-19 vaccine strategies. *Nature Reviews Immunology*, 20, 615–632.

Jia, Q., Bielefeldt-Ohmann, H., Maison, R., Masleša-Galić, S., Bowen, R. & Horwitz, M.A. (2020) Replicating bacterium-vectored vaccine expressing SARS-CoV-2 membrane and nucleocapsid proteins protects against severe COVID-19 disease in hamsters. *NPJ Vaccines*, 6, 47.

Khow, O. & Suntrarachun, S. (2012) Strategies for production of active eukaryotic proteins in bacterial expression system. *Asian Pacific Journal of Tropical Biomedicine*, 2, 159–162.

Lalsiamthara, J., Senevirathne, A., So, M.Y. & Lee, J.H. (2018) Safety implication of Salmonella based Brucella vaccine candidate in mice and in vitro human cell culture. *Vaccine*, 36, 1837–1845.

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S. et al. (2020) Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*, 581, 215.

Lin, I.Y.C., Thi Thu Hao, V. & Smooker, P.M. (2015) Live-attenuated bacterial vectors: tools for vaccine and therapeutic agent delivery. *Vaccine*, 3, 940–972.

Lin, L., Ting, S., Yufei, H., Wendong, L., Yubo, F. & Jing, Z. (2020) Epitope-based peptide vaccines predicted against novel coronavirus disease caused by SARS-CoV-2. *Virus Research*, 288, 198082.

Liu, Z., Liu, X., Cao, W. & Hua, Z.-C. (2015) Tumor-specifically hypoxia-induced therapy of SPRY1/2 displayed differential therapeutic efficacy for melanoma. *American Journal of Cancer Research*, 5, 792–801.

Matchett, W.E., Joag, V., Stolley, J.M., Shepherd, F.K., Quarnstrom, C.F., Mickelson, C.K. et al. (2021) Cutting edge: nucleocapsid vaccine elicits spike-independent SARS-CoV-2 protective immunity. *Journal of Immunology*, 207, 376–379.

Mei, Y., Zhao, L., Liu, Y., Gong, H., Song, Y., Lei, L. et al. (2017) Combining DNA vaccine and AIDA-1 in attenuated salmonella activates tumor-specific CD4(+) and CD8(+) T-cell responses. *Cancer Immunology Research*, 5, 503–514.

Nguyen, H.L., Lan, P.D., Thai, N.Q., Nissley, D.A., O'Brien, E.P. & Li, M.S. (2020) Does SARS-CoV-2 bind to human ACE2 more strongly than does SARS-CoV? *The Journal of Physical Chemistry B*, 124, 7336–7347.

Panthel, K., Meinel, K.M., Sevil Domènech, V.E., Trülzsch, K. & Rüssmann, H. (2008) Salmonella type III-mediated heterologous antigen delivery: a versatile oral vaccination strategy to induce cellular immunity against infectious agents and tumors. *International Journal of Medical Microbiology*, 298, 99–103.

Phillips, R.W. & Roop, R.M., II. (2001) Brucella abortus HtrA functions as an authentic stress response protease but is not required for wild-type virulence in BALB/c mice. *Infection and Immunity*, 69, 5911–5913.

Pinaud, L., Sansonetti, P.J. & Phalipon, A. (2018) Host cell targeting by enteropathogenic bacteria T3SS effectors. *Trends in Microbiology*, 26, 266–283.

Plavec, T.V. & Berlec, A. (2019) Engineering of lactic acid bacteria for delivery of therapeutic proteins and peptides. *Applied Microbiology and Biotechnology*, 103, 2053–2066.

Rao, S., Xu, T., Xia, Y. & Zhang, H. (2020) Salmonella and *S. aureus* escape from the clearance of macrophages via controlling TFEB. *Frontiers in Microbiology*, 11, 573844.

Rüssmann, H., Shams, H., Poblete, F., Fu, Y., Galán, J.E. & Donis, R.O. (1998) Delivery of epitopes by the Salmonella type III secretion system for vaccine development. *Science*, 281, 565–568.

Sahdev, S., Khattar, S.K. & Saini, K.S. (2008) Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Molecular and Cellular Biochemistry*, 307, 249–264.

Saxena, M., Van, T.T.H., Baird, F.J., Coloe, P.J. & Smooker, P.M. (2013) Pre-existing immunity against vaccine vectors—friend or foe? *Microbiology*, 159, 1–11.

Sears, K.T., Galen, J.E. & Tennant, S.M. (2021) Advances in the development of Salmonella-based vaccine strategies for protection against Salmonellosis in humans. *Journal of Applied Microbiology*, 131, 2640–2658.

Shi, J., Wen, Z., Zhong, G., Yang, H., Wang, C., Huang, B. et al. (2020) Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science*, 368, 1016.

Sizemore, D.R., Warner, B., Lawrence, J., Jones, A. & Killeen, K.P. (2006) Live, attenuated *Salmonella typhimurium* vectoring Campylobacter antigens. *Vaccine*, 24, 3793–3803.

Smyth, D.S., Trujillo, M., Gregory, D.A., Cheung, K., Gao, A., Graham, M., et al. (2022) Tracking cryptic SARS-CoV-2 lineages detected in NYC wastewater. *Nature communications*. 13, 635.

Sommer, F., Huber, M., Röllinghoff, M. & Lohoff, M. (1995) CD44 plays a co-stimulatory role in murine T cell activation: ligation of CD44 selectively co-stimulates IL-2 production, but not proliferation in TCR-stimulated murine Th1 cells. *International Immunology*, 7, 1779–1786.

Suryadevara, N., Shrihari, S., Gilchuk, P., VanBlargan, L.A., Binshtein, E., Zost, S.J., et al. (2021) Neutralizing and protective human monoclonal antibodies recognizing the N-terminal domain of the SARS-CoV-2 spike protein. *Cell* 184, 2316-2331. e2315.

Testi, R., Phillips, J.H. & Lanier, L.L. (1989) T cell activation via Leu-23 (CD69). *Journal of Immunology*, 143, 1123–1128.

Thompson, A., Rolfe, M.D., Lucchini, S., Schwerk, P., Hinton, J.C. & Tedin, K. (2006) The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of Salmonella. *The Journal of Biological Chemistry*, 281, 30112–30121.

Toso, J.F., Gill, V.J., Hwu, P., Marincola, F.M., Restifo, N.P., Schwartzentruber, D.J. et al. (2002) Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. *Journal of Clinical Oncology*, 20, 142–152.

Van Engelenburg, S.B. & Palmer, A.E. (2010) Imaging type-III secretion reveals dynamics and spatial segregation of Salmonella effectors. *Nature Methods*, 7, 325–330.

Vance, D.J., Rong, Y., Brey, R.N., III & Mantis, N.J. (2015) Combination of two candidate subunit vaccine antigens elicits protective immunity to ricin and anthrax toxin in mice. *Vaccine*, 33, 417–421.

Walker, B.J., Stan, G.V. & Polizzi, K.M. (2017) Intracellular delivery of biologic therapeutics by bacterial secretion systems. *Expert Reviews in Molecular Medicine*, 19, e6.

Wall, E.C., Wu, M., Harvey, R., Kelly, G., Warchal, S., Sawyer, C. et al. (2021) Neutralising antibody activity against SARS-CoV-2 VOCs B.1.617.2 and B.1.351 by BNT162b2 vaccination. *Lancet (London, England)*, 397, 2331–2333.

Wang, L., Wang, X., Yang, F., Liu, Y., Meng, L., Pang, Y. et al. (2021) Systemic antiviral immunization by virus-mimicking nanoparticles-decorated erythrocytes. *Nano Today*, 40, 101280.

Wang, N., Rosen, O., Wang, L., Turner, H.L., Stevens, L.J., Corbett, K.S., et al. (2019) Structural definition of a neutralization-sensitive epitope on the MERS-CoV S1-NTD. *Cell Reports* 28, 3395-3405.e3396

Wu, L., Bao, F., Li, L., Yin, X. & Hua, Z. (2022) Bacterially mediated drug delivery and therapeutics: strategies and advancements. *Advanced Drug Delivery Reviews*, 187, 114363.

Wu, L., Li, L., Li, S., Liu, L., Xin, W., Li, C., et al. (2022) Macrophage-mediated tumor-targeted delivery of engineered *Salmonella typhimurium* VNP20009 in anti-PD1 therapy against melanoma. *Acta Pharmaceutica Sinica B*. https://doi.org/10.1016/j.apsb.2022.05.006.

Xu, X., Husseiny, M.I., Goldwich, A. & Hensel, M. (2010) Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines. *Infection and Immunity*, 78, 4828–4838.

Yan, Y., Mu, W., Zhang, L., Guan, L., Liu, Q. & Zhang, Y. (2013) Asd-based balanced-lethal system in attenuated Edwardsiella tarda to express a heterologous antigen for a multivalent bacterial vaccine. *Fish & Shellfish Immunology*, 34, 1188–1194.

Yang, F., Meng, L., Lin, S., Wu, F. & Liu, J. (2021) Polyethyleneimine-complexed charge-reversed yeast cell walls for the enhanced oral delivery of pseudovirus-based antigens. *Chemical Communications*, 57, 12768–12771.

Zhang, X., Xu, Q., Yang, L., Lai, Y., Zhang, Z., Han, C. et al. (2016) The genes slyA, STM3120 and htrA are required for the anticancer ability of VNP20009. *Oncotarget*, 7, 81187–81196.

Zhang, X.-L., Jeza, V.T. & Pan, Q. (2008) Salmonella TYPHI: from a human pathogen to a vaccine vector. *Cellular & Molecular Immunology*, 5, 91–97.

Zhang, Y., Li, M., Du, G., Chen, X. & Sun, X. (2021) Advancedoral vaccine delivery strategies for improving the immunity. *Advanced Drug Delivery Reviews*, 177, 113928.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wu, L., Li, L., Yin, X., Li, C., Xin, W. & Liu, L. et al. (2022) A SARS-CoV-2 oral vaccine development strategy based on the attenuated *Salmonella* type III secretion system. *Journal of Applied Microbiology*, 00, 1–17. Available from: https://doi.org/10.1111/jam.15720