J. Microbiol. Biotechnol. (2017), 27(8), 1519–1528
https://doi.org/10.4014/jmb.1705.05028

jmb

Outer Membrane Vesicles Derived from *Salmonella* Enteritidis Protect against the Virulent Wild-Type Strain Infection in a Mouse Model

Qiong Liu[1,2†], Jie Yi[2†], Kang Liang[2], Xiangmin Zhang[3], and Qing Liu[4*]

[1]*Department of Medical Microbiology, School of Medicine, Nanchang University, Nanchang 330006, P.R. China*
[2]*Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, P.R. China*
[3]*Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy/Health Sciences, Wayne State University, Detroit, MI 48202, USA*
[4]*College of Animal Science and Technology, Southwest University, Chongqing 400715, P.R. China*

Received: May 10, 2017
Revised: May 23, 2017
Accepted: May 24, 2017

First published online
May 25, 2017

**Corresponding author*
Phone: +86-13699555282;
Fax: +0791-83969069;
E-mail: qingliu1123@126.com

†These authors contributed equally to this work.

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Foodborne contamination and salmonellosis caused by *Salmonella* Enteritidis (*S.* Enteritidis) are a significant threat to human health and poultry enterprises. Outer membrane vesicles (OMVs), which are naturally secreted by gram-negative bacteria, could be a good vaccine option because they have many biologically active substances, including lipopolysaccharides (LPS), outer membrane proteins (OMPs), and phospholipids, as well as periplasmic components. In the present study, we purified OMVs derived from *S.* Enteritidis and analyzed their characteristics through silver staining and sodium dodecyl sulfate polyacrylamide gel electrophoresis. In total, 108 proteins were identified in *S.* Enteritidis OMVs through liquid chromatography tandem mass spectrometry analysis, and OMPs, periplasmic proteins, and extracellular proteins (49.9% of total proteins) were found to be enriched in the OMVs compared with bacterial cells. Furthermore, native OMVs used in immunizations by either the intranasal route or the intraperitoneal route could elicit significant humoral and mucosal immune responses and provide strong protective efficiency against a lethal dose (~100-fold LD_{50}) of the wild-type *S.* Enteritidis infection. These results indicated that *S.* Enteritidis OMVs might be an ideal vaccine strategy for preventing *S.* Enteritidis diseases.

Keywords: Outer membrane vesicles, *Salmonella* Enteritidis, protection

Introduction

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) can infect chickens, including laying hens and broilers, during the production period and post-harvest, and it may result in persistent infections, even with low numbers of *Salmonella* [1]. *S.* Enteritidis is also the human pathogen that infects routinely by contaminated food, such as eggs and chicken meat [2]. In the past decade, an increase in the number of foodborne infections of *S.* Enteritidis has frequently caused outbreaks in many countries, and it has become the top serotype among all nontyphoidal salmonellae serotype strains [3, 4]. *S.* Enteritidis causes large economic losses in the poultry industry, and poses a significant global health threat to humans [5, 6].

Although antibiotics are commonly used to prevent *S.* Enteritidis infections in chicken farms of developing countries [7], strains of multiple antibiotic resistance have emerged before being transmitted to humans through the food chain [8, 9]. Therefore, vaccination should become the most effective method of reducing the prevalence of *S.* Enteritidis in poultry, ultimately resulting in a lower incidence of human salmonellosis in the post-antibiotic era [10]. Current candidate vaccines include live-attenuated *Salmonella* vaccines [11], killed vaccines, and protein subunit vaccines [12], but they are not always effective. It is well known that among live oral attenuated *Salmonella* vaccines, the Aro$^-$ strain has been reported to protect against virulent *S.* Enteritidis infection [13]; however, there are safety concerns that the attenuated vaccine strains may exhibit

reversion to virulence [10]. In addition, the whole-cell killed vaccines and protein subunit vaccines are capable of inducing humoral immune responses, but they confer a weak cellular immune response and short-term immunity [10, 14].

Outer membrane vesicles (OMVs) might be the ideal vaccine option to control *Salmonella* infection. OMVs are vesicle-like structures that are widely present in gram-negative bacteria [15]. OMVs contain biologically active substances, and their sizes are normally between 20 and 250 nm [16]. The components of OMVs include lipopolysaccharides (LPS), outer membrane proteins (OMPs), phospholipids, as well as the periplasmic components produced during their formation [17]. OMVs promote multiple physiological activities, including bacterial growth, survival, and toxicity, and assistance with biofilm formation, transfer of biomolecules between cells, killing of competing microbial cells, responses to physical and chemical stress, nutrition, defense, and resistance [18–21].

Many factors could contribute to OMVs becoming a popular choice for vaccine development. One is that OMVs are nano-scale spherical vesicles that contain multiple antigenic proteins from the bacterial outer membrane, and they are capable of activating the immune system [17]. Another is that OMVs are safe owing to their non-replicative acellular characteristics [22]. Finally, LPS, a major component of OMVs, could be used as an adjuvant [18]. Furthermore, many research studies of vaccines based on OMVs to prevent infectious diseases have been reported [23, 24], where the *Neisseria meningitidis* serogroup B OMV vaccine is the most successful example [25]. In this study, we identified and analyzed the components of *S.* Enteritidis OMVs, and we also investigated whether immunization with OMVs that were spontaneously released from *S.* Enteritidis can provide significant protection against lethal challenge in a mouse model.

Materials and Methods

Bacterial Strains and Isolation of OMVs

OMVs were isolated from the *Salmonella* Enteritidis χ3744 strain, which was obtained from Dr. Roy Curtiss' laboratory, and the genetic background of this strain is clear. The clinical strain *S.* Enteritidis S246, which was used to infect the mice, was isolated from chicken by the Institute of Preventive Veterinary Medicine [26], and this isolate had no flagellae, which means that it did not contain a functional FliC gene. The LD_{50} of these two strains has been determined in a mouse model and both were 1×10^5 CFU. All strains were grown at 37°C in Luria-Bertani medium (Difco, USA). A 2 L *S.* Enteritidis culture in the logarithmic phase (approximately 2×10^{12} CFU bacteria) was used to purify OMVs, and the methods of OMV isolation were as according to those previously described [27].

LPS and Protein Assay

LPS profiles of *S.* Enteritidis OMVs were examined using a modified version of the standard method [28]. Briefly, 50 µl of OMV sample was mixed with 50 µl of Dissociation Buffer A (0.5 M Tris-Cl, pH 6.8, 10% glycerol, 10% sodium dodecyl sulfate (SDS), 5% beta-mercaptoethanol), boiled for 10 min, and air-cooled to room temperature. To remove insoluble substances, the samples were centrifuged at 12,000 ×*g* for 10 min. Then, the supernatant was diluted at 1:10 (10 µl into 90 µl) into Dissociation Buffer B (0.5 M Tris-Cl, pH 6.8, 10% glycerol, 0.05% Bromophenol blue), adding 1 µl of 20 mg/ml Proteinase K (Sigma-Aldrich, USA) to digest for 1 h at 37°C. After these preparations were complete, the samples were separated onto 12.5% gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained.

The protein contents of the OMVs were analyzed by 12% SDS-PAGE, according to Laemmli [29]. The concentration of *S.* Enteritidis OMVs (based on the total protein content) was measured with a BCA Protein Assay Kit (Thermo Pierce, USA). Then, a 10 µg OMV sample was uploaded and separated on a SDS-PAGE gel, and Coomassie blue staining was performed using Page Blue Protein Staining Solution (Thermo Pierce), according to the manufacturer's instructions.

Liquid Chromatography-Coupled Tandem Mass Spectrometry (LC-MS/MS) Analysis of OMVs

The purified vesicles were boiled in SDS loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% Bromophenol blue, 10% glycerol) for 10 min, and 20 µg of OMVs was separated by 12% SDS-PAGE. Each lane was cut into five slices and then further cut into 1 mm cubes. The gel cubes were destained in a buffer containing 50% acetonitrile (ACN) and 40 mM ammonium bicarbonate and then dehydrated in 100% ACN. The dried gel cubes were re-hydrated in 12.5 ng/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-trypsin, and incubated at 37°C overnight. The peptides were extracted by incubating the gel cubes two times in 0.5% formic acid solution at 37°C. The pooled peptides were dried using a SpeedVac and reconstituted with 0.1% trifluoroacetic acid. The peptides were purified with a C18 ZipTip (Millipore, USA), according to the manufacturer's manual. The purified peptides were subjected to high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-MS/MS analysis using a 230 min gradient of ACN in 0.1% formic acid. The on-line HPLC was composed of an Easy-nanoLC II HPLC, with a C18-reversed phase column (75 µm ID, 15 cm length), with the Thermo Finnigan LTQ-Orbitrap Elite fitted with the Nanospray Flex Ion Source. The data were acquired in a "top-20" manner.

Database Search and Bioinformatic Analysis

Raw MS files were searched against a UniProt database of

S. Enteritidis PT-4, strain P125109 (downloaded on February 14, 2016) using MaxQuant software [30, 31]. The precursor mass tolerance was set to 7 ppm. The fragment ion mass tolerance was set to 0.5 Da. Two missing trypsin cleavage sites were allowed. Methionine and protein N-terminal acetylation oxidation were defined as variable modifications. At least two unique peptides were required for the protein identification. The subcellular location of proteins was predicted by PSORTb (ver. 3.0.2), and all identified proteins were classified according to Gene Ontology (GO)-based biological processes and molecular functions, based on the databases provided by STRAP software (Boston University, USA).

Animals

Experiments involving mice were approved by the Committee on Ethics and Experimentation at Sichuan Agricultural University (IACUC number: SCU-2016023). Female BALB/c mice (6 weeks of age) were purchased from the Dashuo Biotechnology Co., Ltd. (China) and maintained at 5 (control) or 6 (vaccine) per cage under pathogen-free conditions. The animals were fed sterile rodent chow and water ad libitum, and were allowed to acclimate for 1 week prior to use.

Vaccination and Challenge

For immunization, OMVs isolated from the *S.* Enteritidis strain were resuspended in PBS. Six mice of the vaccinated group were immunized with 20 μg of OMVs in 10 μl of PBS or 5 μg of OMVs in 100 μl of PBS buffer on days 0 and 30, intranasally or intraperitoneally, respectively. Five mice of the mock-vaccinated group (10 μl or 100 μl PBS) that were housed in parallel with the vaccinated mice served as the negative control. Serum samples were collected at weeks 4 and 8 for sera antibody determination, and at the same time points, vaginal secretions were collected through repeated flushing using 0.1 ml of PBS buffer to determine the secretory IgA (S-IgA) concentrations. Five weeks after the booster immunization, the mice were challenged orally with 10^7 CFU of the wild-type *S.* Enteritidis (S246) in 20 μl of PBS with 0.01% gelatin (BSG buffer). The 50% lethal dose (LD_{50}) of S246 was 1×10^5 CFU when administered orally. Survival was monitored for up to 21 days post-infection. The animal experiments were performed twice, and the data were combined for analysis.

Quantitative Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the concentrations of IgG and secretory IgA (S-IgA) in mice immunized with OMVs, a quantitative ELISA was performed, as described below. OMPs and LPS (as the coating antigen) were isolated from *Salmonella*, as previously described [26], and purified OMPs (2 μg per well) and LPS (2 μg per well) were resuspended in 100 μl of sodium carbonate-bicarbonate buffer (pH 9.6) and added to polystyrene 96-well flat-bottom microtiter plates. To generate standard curves for each antibody isotype, the plates were coated in triplicates with 2-fold dilutions of the appropriate purified mouse Ig isotype, standard IgA or IgG (BD Biosciences, USA), starting at 0.5 μg/μl. Then, the plates were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) to prevent nonspecific binding of the proteins to the plates. The samples were diluted (1:100) in 1% BSA and then incubated in duplicate wells for 1 h at room temperature. After washing three times in PBS-0.05% Tween-20 to remove excess primary antibodies, the plates were incubated with biotinylated goat anti-mouse IgG or IgA (Southern Biotechnology Inc., USA) antibodies at a 1:5,000 dilution for 4 h at room temperature. Then, the plates were washed with 1 PBS-0.05% Tween 20 and further incubated with a 1:5,000 dilution of streptavidin-alkaline phosphatase conjugate (Southern Biotechnology, Inc.) for 1 h at room temperature. Alkaline phosphatase activity was detected by incubating the plates with *p*-nitrophenyl phosphate (Sigma-Aldrich) for 20 min at room temperature. After developing a yellow color, the plates were read at 405 nm. The final Ig isotype concentrations in the samples were calculated using appropriate standard curves; a log-log regression curve was calculated from at least four dilutions of the isotype standard.

Statistical Analysis

GraphPad Prism (ver. 5.01) was used for all statistical analyses. One-way analysis of variance was performed to determine the significance of the differences between the mean values of the various experimental and control groups. Data were expressed as the means ± standard deviation. The means were compared using the least significant difference test, and $p < 0.05$ was considered to indicate a significant difference.

Results

Purification and Characterization of *S.* Enteritidis OMVs

As shown in Fig. 1A, the presence of the LPS in *S.* Enteritidis OMVs was characterized by silver staining. The LPS profile analysis showed that the LPS in the *S.* Enteritidis OMVs was identical to that of the wild-type *S.* Enteritidis strain. Furthermore, the protein contents of OMVs isolated from *S.* Enteritidis were detected by 12% SDS-PAGE. The results showed that the flagellin protein was observed in the protein profile, while the common protein bands were also observed (Fig. 1B).

Identification and Subcellular Distribution of OMV Proteins

The OMVs derived from *S.* Enteritidis were subjected to LC-MS/MS analysis to identify their protein components. To avoid the identification of false positive contaminating proteins, the proteins with an intensity of more than 5.0E + 07 and unique matches of two or more peptide were screened. A total of 108 proteins were identified in *S.* Enteritidis

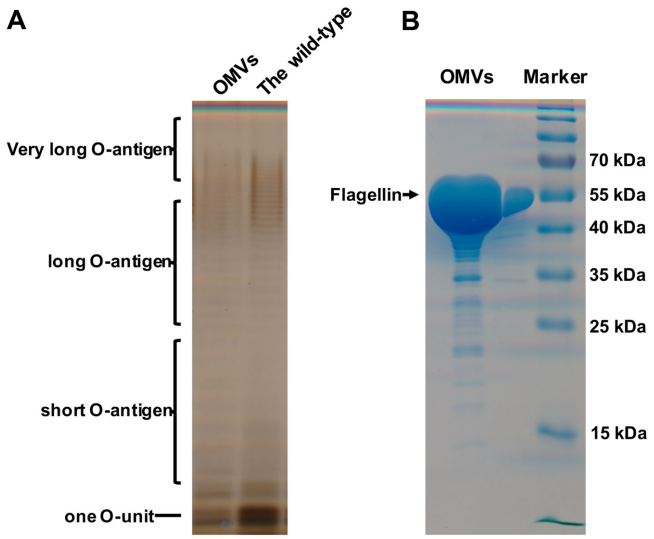

Fig. 1. Characterization of *S.* Enteritidis outer membrane vesicles (OMVs).

(**A**) LPS samples from OMVs and the wild-type strain of *S.* Enteritidis separated on a 12% SDS-PAGE gel and visible by silver staining. (**B**) The protein components of OMVs derived from the wild-type *S.* Enteritidis, resolved using 12% SDS-PAGE and stained with Page Blue Protein Staining Solution.

OMVs (Tables 1 and S1). OMPs (OmpA, OmpC, and NmpC), flagellin (FljB, FliD, FlgK, and FlgE), and *Salmonella* pathogenicity island 1 effector proteins (SipA, SipB, and SipC) were the most abundant OMV proteins (Table 1). The subcellular locations of the identified proteins were predicted using PSORTb software (Fig. 2A), and all selected proteins were classified according to their GO-based biological processes and molecular functions (Fig. 2B), based on the STRAP software databases (Boston University). *S.* Enteritidis OMVs were enriched for OMPs, periplasmic proteins, and extracellular proteins (total 49.9% in all); however, periplasmic proteins and OMPs accounted for only 8% of the total *Salmonella* bacterial proteins [32], which means that periplasmic proteins and OMPs were enriched in these products we purified, and were consistent with the characteristics of OMVs. Therefore, the subcellular location results indicated that OMVs derived from *S.* Enteritidis in this study were successfully purified. Notably, a large number of cytoplasmic proteins were also detected in the proteomic data sets, similar to the previously reported OMVs of other bacteria [33, 34]. This might be due to the inclusion of these cytoplasmic proteins in the formation process of OMVs, or their entry into OMVs in their synthesis

Table 1. Major proteins identified from wild-type OMVs.

UniProt accession	MW (kDa)[a]	Protein name	Subcellular localization	Intensity[b]	Matched peptides	Coverage (%)[c]
B5R0Z9	52.981	Flagellin (FljB)	Extracellular	2.07E+10	61	95.4
B5QVD2	42.983	Pathogenicity island 1 effector protein (SipC)	Extracellular	7.18E+09	44	93.9
B5QVD0	73.971	Uncharacterized protein (SipA)	Extracellular	2.94E+09	61	83.4
B5R028	61.988	Cell invasion protein (SopB)	Periplasmic	2.64E+09	43	75.8
B5QVD3	62.45	Pathogenicity island 1 effector protein (SipB)	Extracellular	2.05E+09	41	65.6
B5R1Z8	26.781	Type III secretion system, secreted effector protein (SopE)	Extracellular	1.09E+09	16	62.5
B5R0Z8	49.956	Flagellar hook-associated protein (FliD)	Extracellular	7.68E+08	32	79.9
B5QXY2	59.109	Flagellar hook-associated protein 1 (FlgK)	Extracellular	5.51E+08	33	73.1
B5R218	26.52	Invasion-associated secreted effector protein (SopE2)	Extracellular	3.28E+08	14	63.8
B5QVD1	37.103	Pathogenicity island 1 effector protein (SipD)	Extracellular	2.36E+08	20	71.1
B5QYQ2	37.785	Putative sopD2 type III secretion system effector protein (SEN0876)	Cytoplasmic	1.96E+08	15	44.2
B5R241	41.336	Outer membrane protein C (OmpC)	Outer membrane	1.48E+08	17	56.1
B5QXY8	42.21	Flagellar hook protein (FlgE)	Extracellular	9.99E+07	17	65
B5QZG0	37.515	Outer membrane protein A (OmpA)	Outer membrane	7.75E+07	14	46.6
B5R1L5	18.22	Fimbrial protein (SEN4247)	Extracellular	6.48E+07	5	56.2
B5QZK6	86.691	E3 ubiquitin-protein ligase (SopA)	Periplasmic	5.32E+07	24	38
B5QTN0	39.695	Outer membrane porin protein (NmpC)	Outer membrane	4.98E+07	14	53.3
B5QW44	98.641	Outer membrane usher protein (SEN2795)	Outer membrane	3.97E+07	2	3.4
B5QW33	36.151	Possible secreted protein (SopD)	Cytoplasmic	3.07E+07	18	58.4
B5QXR6	43.283	Elongation factor Tu (TufA)	Cytoplasmic	2.85E+07	18	64.5

[a]The values represented as MW (kDa) indicate the calculated molecular weight of the identified proteins.

[b]This table lists the 20 proteins with the highest abundance that were identified in the wild-type OMVs in the final arrangement.

[c]Coverage of the protein sequence by the peptides used for spot identification.

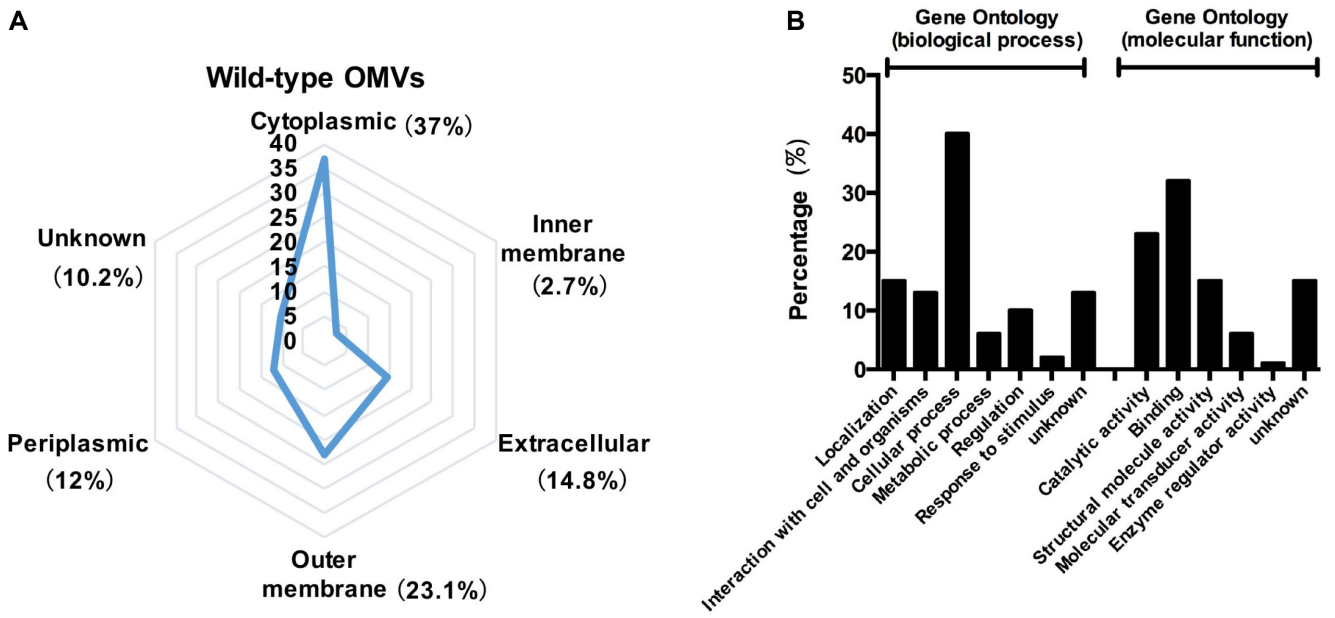

Fig. 2. Subcellular distribution and functional classification of the identified proteins in *S.* Enteritidis outer membrane vesicles (OMVs) revealed through one dimensional gel separation and LC-MS/MS.

(**A**) The proteins identified from OMV proteomics profiles were classified according to their subcellular distribution. The proteins identified through LC-MS/MS profiling were processed using PSORTb (http://www.psort.org/psortb) to predict their primary locations in the cell, in addition to the OMVs. The proteins in *S.* Enteritidis OMVs were predicted, and the numbers indicate the amounts of the proteins in each fraction. (**B**) The identified proteins were classified according to their functional classification. The OMV proteins were classified using GO-based functions. The proteins in the *S.* Enteritidis OMVs were grouped on the basis of their biological processes and molecular functions, and their proportions were plotted.

pathway, or the residues in the purification process of OMVs [35, 36].

Furthermore, we classified the selected OMV proteins according to their GO-based biological processes and molecular functions [37] (Fig. 2B). Based on the biological process classification, the proteins associated with cellular processes were frequently sorted into OMVs, and the OMVs were enriched with proteins involved in catalytic activities and binding, based on their molecular function classifications.

Antibody Response to Immunization with *S.* Enteritidis OMVs

To explore the immunogenicity of OMVs derived from the *S.* Enteritidis strain, groups of 12 mice each were immunized with the OMVs intranasally or intraperitoneally (20 μg in 10 μl of PBS or 5 μg in 100 μl of PBS). The control mice of 10 were administered PBS at a dose of 10 μl or 100 μl, and the immunizations were administered at 4-week intervals. All animals immunized intranasally or intraperitoneally remained in good health, and they exhibited no abnormal behavior.

As discussed above, OMVs consist of outer membrane and soluble periplasmic components shed from bacteria; as major components of the outer membrane, LPS and OMPs could play important roles as antigens in the immune response [38–40]. Therefore, we chose LPS and OMPs isolated from *S.* Enteritidis as immunogens to determine the antibody levels in the immunized mice. The quantitative ELISA data showed that the level of IgG against OMPs and LPS was significantly higher than that in the PBS control group, in which no responses were detected. The anti-OMP and anti-LPS IgG levels of the mice immunized intraperitoneally were relatively high compared with those of the mice immunized intranasally (Figs. 3A and 3B). Furthermore, the S-IgA concentrations of the mice immunized with OMVs were also measured. *S.* Enteritidis OMVs could induce significantly higher mucosal immune response levels ($p < 0.01$) when administered either intranasally or intraperitoneally; however, S-IgA in mice immunized intraperitoneally had a significantly lower level than mice immunized intranasally ($p < 0.05$) (Figs. 3C and 3D).

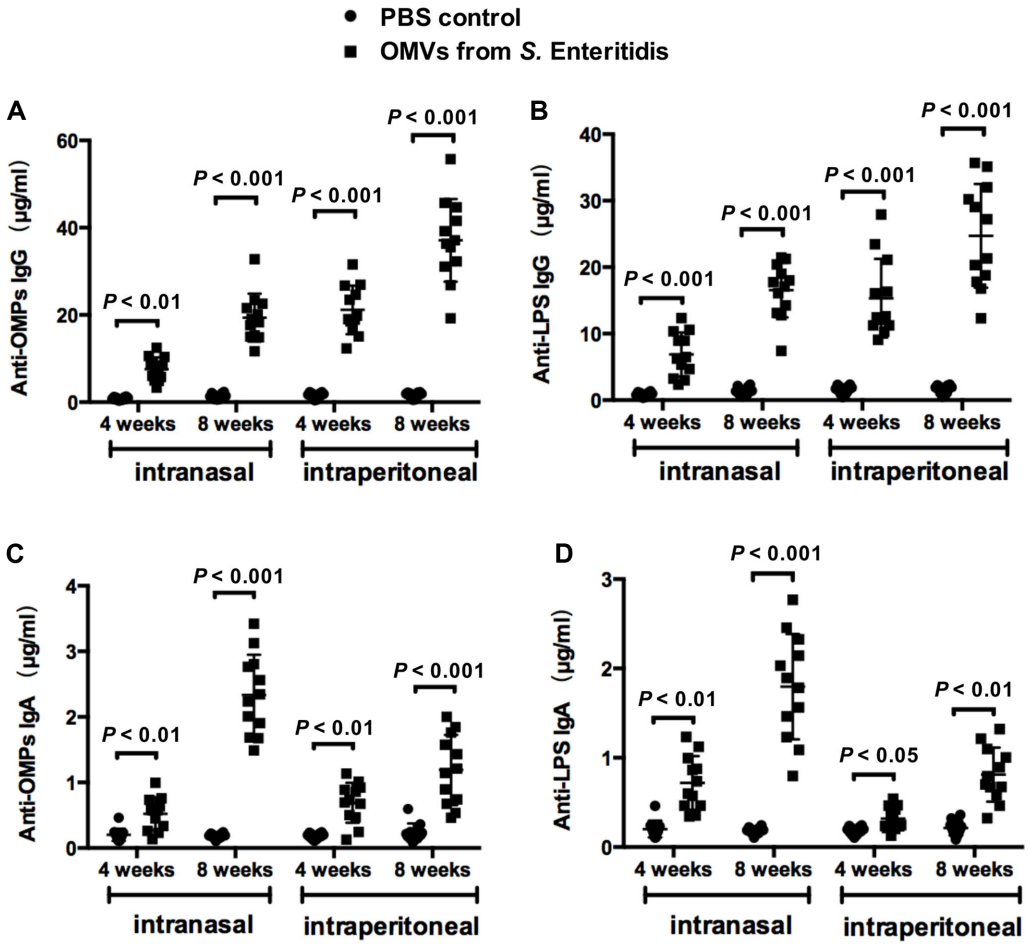

Fig. 3. Intranasally or intraperitoneally administered outer membrane vesicle (OMV) immunization elicits immune responses. Total serum IgG specific to outer membrane proteins (OMPs) (**A**) or LPS (**B**) and secretory IgA (S-IgA) specific to OMPs (**C**) or LPS (**D**) were measured by quantitative ELISA. Each group had 10 or 12 mice. The data show the concentrations of IgG or S-IgA antibodies quantified by a corresponding standard curve in the samples from mice immunized with OMVs derived from *S.* Enteritidis. The mice were boosted at week 4, and samples were collected at 4 and 8 weeks after the first immunization. The PBS-vaccinated mice were the controls. The error bars represent variations among all mice in each group.

Evaluation of Protective Efficiency

To determine whether the antibodies induced by *S.* Enteritidis OMVs could provide protective effects in mice, the mice immunized with wild-type *S.* Enteritidis OMVs were challenged by a lethal oral dose of the wild-type *S.* Enteritidis, and the mice were observed for 3 weeks. The results showed that 83.3% and 91% of the mice immunized intranasally or intraperitoneally, respectively, demonstrated significant protection compared with the PBS control group ($p < 0.01$) (Fig. 4). Together, these data demonstrate that *S.* Enteritidis OMVs are highly immunogenic and are capable of providing strong protection against wild-type *S.* Enteritidis infection in a mouse model.

Discussion

Salmonella is the most frequently reported causative agent of foodborne outbreaks worldwide; it affects an estimated 93.8 million persons annually and poses a significant health burden [5]. *S.* Enteritidis and *Salmonella* Typhimurium are the prevailing serotypes [41]. In China, *Salmonella* causes an estimated 22.2% of foodborne diseases [42]. The consumption of chicken products and eggs contaminated by *S.* Enteritidis is the primary transmission route [43]. Therefore, the treatment of *S.* Enteritidis has become urgent in countries with a high incidence of infections.

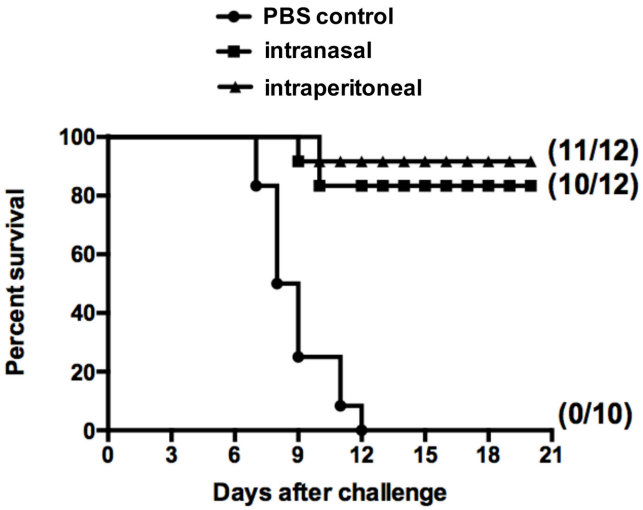

Fig. 4. Immunizing BALB/c mice with outer membrane vesicles (OMVs) derived from *S*. Enteritidis protected against oral challenge with the wild-type *S*. Enteritidis.

Ten (control) or 12 (vaccine) mice per group were immunized with OMVs twice at 4-week intervals. The mice were challenged with 10^7 CFU (~100-fold LD_{50}) of *S*. Enteritidis at 4 weeks after the booster immunization. Mortality was monitored for 21 days after the challenge. The numbers in parentheses refer to the numbers of surviving mice and the total number of mice per group. All vaccine groups differed significantly from the PBS control group ($p < 0.01$).

In this study, we successfully obtained natural OMVs derived from *S.* Enteritidis, which we confirmed through LC-MS/MS analysis. The LC-MS/MS analysis identified specific components of *S*. Enteritidis OMVs. The identified antigenic OMPs, such as OmpC and NmpC, revealed the immunogenicity of *S.* Enteritidis OMVs [44, 45]. Furthermore, the identified proteins, including SipA, SipB, and SipC, which were associated with the type III secretion system encoded by *Salmonella* pathogenicity island 1 (SPI-1), have been proved to induce a strong immune response, especially a mucosal immune response [46]. These identified components might explain why OMVs could elicit strong S-IgA (Fig. 3). Therefore, these data indicated that OMVs might play an important role in pathogenicity and interaction with the host environment [47]. However, we still do not understand the mechanism of OMV biogenesis or its genetic basis, which could be the target of our future research.

In this study, a mouse model was used to assess the immunogenicity and efficacy of candidate vaccines. Previous studies have demonstrated that oral challenge of mice with *S.* Enteritidis could generate an invasive, generalized infection of the gut-associated lymphoid tissue [48], and the challenge strain *S.* Enteritidis S246 was isolated in chicken by our laboratory and it could infect mice in the laboratory period. Moreover, naive mice immunized with PBS succumbed to oral infection from this virulent *S.* Enteritidis (Fig. 4). Therefore, the mouse model could play an invaluable role in accelerating the development of a safe and effective vaccine against *S.* Enteritidis infections in humans or poultry.

Although proof-of-principle studies have demonstrated the efficacy of live-attenuated and subunit vaccines targeting O-antigens, OMPs, and other virulent factors of *S.* Enteritidis in an animal model [49], safety concerns and short-term immunity still block the clinical applications of a vaccine [10, 14]. However, vesicles derived from pathogens might provide the ideal strategy by which to resolve these shortcomings, and they have become popular vaccine candidates that prevent the corresponding infection [23, 24, 50]. In this study, we evaluated the vaccine potential of OMVs isolated from *S*. Enteritidis in a mouse model. We demonstrated that immunization with *S*. Enteritidis OMVs produced a significantly higher level of humoral responses and protected against *Salmonella* infection compared with the control ($p < 0.01$). In addition, the quantitative ELISA data also showed that the antibodies induced by OMVs could react with bacterial antigens, such as OMPs and LPS, which are located in the bacterial outer membrane [51]. Furthermore, as a first line of defense, mucosal IgA plays an important role in protecting against oral infection with *Salmonella* by inhibiting bacterial penetration into the Peyer's patches [52]. Therefore, we also analyzed secretory IgA in vaginal secretions of immunized mice, and the data showed that *S.* Enteritidis OMVs could induce strong mucosal responses in mice immunized intranasally (Fig. 3). The concentration of S-IgA in the sera of mice immunized intranasally was significantly higher than that in mice immunized intraperitoneally or in the control group ($p < 0.01$), indicating that the level of mucosal immune responses was associated with the immunization administration; however, because the protection results showed that 83.3% and 91% of the mice immunized intranasally or intraperitoneally, respectively, survived after the wild-type *S.* Enteritidis challenge, the mucosal response level did not directly represent the protective efficacy of OMVs [53].

However, naturally released OMVs are slightly toxic because of endotoxin activity. Detergent-treated OMVs might be used to reduce this activity, but detergent has been shown to deplete OMVs of important detergent-soluble antigens [54]. Some researchers have noted that deletion or overexpression of specific genes involved in the LPS

biosynthesis pathway (to reduce endotoxin activity) could be a better solution [55, 56]. In a future study, we will focus on modification of LPS in *S.* Enteritidis to develop a safer and more efficient OMV-based vaccine, and target the cross-protection of OMVs based on genetic manipulation of LPS.

This report is the first to describe the protein components of *S.* Enteritidis OMVs and their vaccine potential in a mouse model. This strategy could elicit robust, protective immune responses, suggesting that the use of OMVs to immunize against *S.* Enteritidis might be a viable approach to reduce the morbidity and mortality caused by the virulent *S.* Enteritidis strain.

Abbreviations

OMVs, outer membrane vesicles; LPS, lipopolysaccharide; OMPs, outer membrane proteins; ELISA, Enzyme-linked immunosorbent assay

Acknowledgments

We thank Kenneth L. Roland (Biodesign Institute at Arizona State University) for editing this manuscript. This study was supported by the National Natural Science Foundation of China (31472179).

Conflict of Interest

The authors declare no conflict of interest.

References

1. Desmidt M, Ducatelle R, Haesebrouck F. 1997. Pathogenesis of *Salmonella* Enteritidis phage type four after experimental infection of young chickens. *Vet. Microbiol.* **56:** 99-109.
2. Guard-Petter J. 2001. The chicken, the egg and *Salmonella enteritidis*. *Environ. Microbiol.* **3:** 421-430.
3. Chai SJ, White PL, Lathrop SL, Solghan SM, Medus C, McGlinchey BM, *et al.* 2012. *Salmonella enterica* serotype Enteritidis: increasing incidence of domestically acquired infections. *Clin. Infect. Dis.* **54:** S488-S497.
4. Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, *et al.* 2010. Food-borne diseases — the challenges of 20 years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* **139:** S3-S15.
5. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, *et al.* 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* **50:** 882-889.
6. Van Immerseel F, De Buck J, Pasmans F, Bohez L, Boyen F, Haesebrouck F, *et al*. 2004. Intermittent long-term shedding and induction of carrier birds after infection of chickens early posthatch with a low or high dose of *Salmonella* Enteritidis. *Poult. Sci.* **83:** 1911-1916.
7. Seo KH, Holt PS, Gast RK, Hofacre CL. 2000. Combined effect of antibiotic and competitive exclusion treatment on *Salmonella* Enteritidis fecal shedding in molted laying hens. *J. Food Protect.* **63:** 545-548.
8. Molbak K, Gerner-Smidt P, Wegener HC. 2002. Increasing quinolone resistance in *Salmonella enterica* serotype Enteritidis. *Emerg. Infect. Dis.* **8:** 514-515.
9. Threlfall EJ. 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiol. Rev.* **26:** 141-148.
10. Mastroeni P, Chabalgoity J, Dunstan S, Maskell D, Dougan G. 2001. Salmonella: immune responses and vaccines. *Vet. J.* **161:** 132-164.
11. De Cort W, Geeraerts S, Balan V, Elroy M, Haesebrouck F, Ducatelle R, *et al*. 2013. A *Salmonella* Enteritidis hilAssrAfliG deletion mutant is a safe live vaccine strain that confers protection against colonization by *Salmonella* Enteritidis in broilers. *Vaccine* **31:** 5104-5110.
12. Toyota-Hanatani Y, Kyoumoto Y, Baba E, Ekawa T, Ohta H, Tani H, *et al*. 2009. Importance of subunit vaccine antigen of major Fli C antigenic site of *Salmonella* Enteritidis II: a challenge trial. *Vaccine* **27:** 1680-1684.
13. Hormaeche CE, Mastroeni P, Harrison JA, de Hormaeche RD, Svenson S, Stocker BA. 1996. Protection against oral challenge three months after i.v. immunization of BALBc mice with live Aro *Salmonella typhimurium* and *Salmonella enteritidis* vaccines is serotype (species)-dependent and only partially determined by the main LPS O antigen. *Vaccine* **14:** 251-259.
14. Okamura M, Lillehoj HS, Raybourne RB, Babu U, Heckert R. 2003. Antigen-specific lymphocyte proliferation and interleukin production in chickens immunized with killed *Salmonella enteritidis* vaccine or experimental subunit vaccines. *Avian Dis.* **47:** 1331-1338.
15. Bonnington KE, Kuehn MJ. 2014. Protein selection and export via outer membrane vesicles. *Biochim. Biophys. Acta* **1843:** 1612-1619.
16. Deatherage BL, Cookson BT. 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect. Immun.* **80:** 1948-1957.
17. Ellis TN, Kuehn MJ. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **74:** 81-94.
18. Kulkarni HM, Jagannadham MV. 2014. Biogenesis and multifaceted roles of outer membrane vesicles from gram-negative bacteria. *Microbiology* **160:** 2109-2121.
19. Bomberger JM, MacEachran DP, Coutermarsh BA, Ye S, O'Toole GA, Stanton BA. 2009. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer

membrane vesicles. *PLoS Pathog.* **5:** e1000382.
20. Yonezawa H, Osaki T, Woo T, Kurata S, Zaman C, Hojo F, *et al.* 2011. Analysis of outer membrane vesicle protein involved in biofilm formation of *Helicobacter pylori*. *Anaerobe* **17:** 388-390.
21. Elhenawy W, Debelyy MO, Feldman MF. 2014. Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles. *mBio* **5:** e00909-00914.
22. Collins BS. 2011. Gram-negative outer membrane vesicles in vaccine development. *Discov. Med.* **12:** 7-15.
23. McConnell MJ, Rumbo C, Bou G, Pachon J. 2011. Outer membrane vesicles as an acellular vaccine against *Acinetobacter baumannii*. *Vaccine* **29:** 5705-5710.
24. Petersen H, Nieves W, Russell-Lodrigue K, Roy CJ, Morici LA. 2014. Evaluation of a *Burkholderia pseudomallei* outer membrane vesicle vaccine in nonhuman primates. *Procedia Vaccinol.* **8:** 38-42.
25. Acevedo R, Fernández S, Zayas C, Acosta A, Sarmiento ME, Ferro VA, *et al.* 2014. Bacterial outer membrane vesicles and vaccine applications. *Front. Immunol.* **5:** 121.
26. Liu Q, Liu Q, Zhao X, Liu T, Yi J, Liang K, *et al*. 2016. Immunogenicity and cross-protective efficacy induced by outer membrane proteins from *Salmonella* Typhimurium mutants with truncated LPS in mice. *Int. J. Mol. Sci.* **17:** 416.
27. Liu Q, Liu Q, Jie Y, Kang L, Bo H, Zhang X, *et al.* 2016. Outer membrane vesicles from flagellin-deficient *Salmonella enterica* serovar Typhimurium induce cross-reactive immunity and provide cross-protection against heterologous *Salmonella* challenge. *Sci. Rep.* **6:** 34776.
28. Hitchcock P, Brown T. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154:** 269-277.
29. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
30. Cox J, Matic I, Hilger M, Nagaraj N, Selbach M, Olsen JV, *et al.* 2009. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat. Protoc.* **4:** 698-705.
31. de Godoy LM, Olsen JV, Cox J, Nielsen ML, Hubner NC, Frohlich F, *et al.* 2008. Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* **455:** 1251-1254.
32. Lee EY, Choi DS, Kim KP, Gho YS. 2008. Proteomics in gram-negative bacterial outer membrane vesicles. *Mass Spectrom. Rev.* **27:** 535-555.
33. Choi DS, Kim DK, Choi SJ, Lee J, Choi JP, Rho S, *et al.* 2011. Proteomic analysis of outer membrane vesicles derived from *Pseudomonas aeruginosa*. *Proteomics* **11:** 3424-3429.
34. Lee EY, Bang JY, Park GW, Choi DS, Kang JS, Kim HJ, *et al.* 2007. Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics* **7:** 3143-3153.
35. Lee J, Kim OY, Gho YS. 2016. Proteomic profiling of gram-negative bacterial outer membrane vesicles: current perspectives. *Proteomics Clin. Appl.* **10:** 897-909.
36. Wurpel DJ, Moriel DG, Totsika M, Easton DM, Schembri MA. 2015. Comparative analysis of the uropathogenic *Escherichia coli* surface proteome by tandem mass-spectrometry of artificially induced outer membrane vesicles. *J. Proteomics* **115:** 93-106.
37. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, *et al.* 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25:** 25-29.
38. Bjerre A, Brusletto B, Mollnes TE, Fritzsønn E, Rosenqvist E, Wedege E, *et al.* 2002. Complement activation induced by purified *Neisseria meningitidis* lipopolysaccharide (LPS), outer membrane vesicles, whole bacteria, and an LPS-free mutant. *J. Infect. Dis.* **185:** 220-228.
39. Beveridge TJ. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* **181:** 4725-4733.
40. Voulhoux R, Bos MP, Geurtsen J, Mols M, Tommassen J. 2003. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299:** 262-265.
41. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. 2012. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *Lancet* **379:** 2489-2499.
42. Wang S, Duan H, Zhang W, Li JW. 2007. Analysis of bacterial foodborne disease outbreaks in China between 1994 and 2005. *FEMS Immunol. Med. Microbiol.* **51:** 8-13.
43. Taylor M, Leslie M, Ritson M, Stone J, Cox W, Hoang L, *et al.* 2012. Investigation of the concurrent emergence of *Salmonella* Enteritidis in humans and poultry in British Columbia, Canada, 2008-2010. *Zoonoses Public Health* **59:** 584-592.
44. Jawad AA, Al-Charrakh AH. 2016. Outer membrane protein C (*ompC*) gene as the target for diagnosis of *Salmonella* species isolated from human and animal sources. *Avicenna J. Med. Biotechnol.* **8:** 42-45.
45. Cho Y, Park S, Barate AK, Truong QL, Han JH, Jung C-H, *et al.* 2015. Proteomic analysis of outer membrane proteins in *Salmonella enterica* Enteritidis. *J. Microbiol. Biotechnol.* **25:** 288-295.
46. Durand D, Ochoa TJ, Bellomo SM, Contreras CA, Bustamante VH, Ruiz J, Cleary TG. 2013. Detection of secretory immunoglobulin A in human colostrum as mucosal immune response against proteins of the type three secretion system of *Salmonella, Shigella* and Enteropathogenic *Escherichia coli*. *Pediatr. Infect. Dis. J.* **32:** 1122-1126.
47. Kuehn MJ, Kesty NC. 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev.* **19:** 2645-2655.
48. Collins F. 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in nonvaccinated mice challenged by various routes. *J. Bacteriol.* **97:** 667-675.
49. MacLennan CA, Martin LB, Micoli F. 2014. Vaccines against invasive *Salmonella* disease: current status and future

directions. *Hum. Vaccin. Immunother.* **10:** 1478-1493.
50. Kuipers K, Daleke-Schermerhorn MH, Jong WSP, Hagen-Jongman CMT, Opzeeland FV, Simonetti E, *et al.* 2015. *Salmonella* outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. *Vaccine* **33:** 2022-2029.
51. Ernst RK, Guina T, Miller SI. 2001. *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microb. Infect.* **3:** 1327-1334.
52. McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10:** 75-88.
53. Kraehenbuhl J-P, Neutra MR. 1992. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol. Rev.* **72:** 853-879.
54. Quakyi EK, Frasch CE, Buller N, Tsai CM. 1999. Immunization with meningococcal outer-membrane protein vesicles containing lipooligosaccharide protects mice against lethal experimental group B *Neisseria meningitidis* infection and septic shock. *J. Infect. Dis.* **180:** 747-754.
55. Koeberling O, Seubert A, Granoff DM. 2008. Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin. *J. Infect. Dis.* **198:** 262-270.
56. Kong Q, Yang J, Liu Q, Alamuri P, Roland KL, Curtiss R. 2011. Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **79:** 4227-4239.