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Multi-Immunogenic Outer Membrane Vesicles Derived from a MsbB-Deficient *Salmonella enterica* Serovar Typhimurium Mutant

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To develop low endotoxic and multi-immunogenic outer membrane vesicles (OMVs), a deletion mutant of the *msbB* gene in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) was used as a source of low endotoxic OMV, and an expression vector of the canine parvovirus (CPV) VP2 epitope fused to the bacterial OmpA protein was constructed and transformed into the *Salmonella* Δ*msbB* mutant. In a lethality test, BALB/c mice injected intraperitoneally with the *Salmonella* Δ*msbB* mutant survived for 7 days, whereas mice injected intraperitoneally with the wild type survived for 3 days. Moreover, all mice inoculated orally with the Δ*msbB* mutant survived for 30 days, but 80% of mice inoculated orally with the wild type survived. The OmpA::CPV VP2 epitope fusion protein was expressed successfully and associated with the outer membrane and OMV fractions from the mutant *S.* Typhimurium transformed with the fusion protein-expressing vector. In immunogenicity tests, sera obtained from the mice immunized with either the *Salmonella msbB* mutant or its OMVs containing the OmpA::CPV VP2 epitope showed bactericidal activities against wild-type *S.* Typhimurium and contained specific antibodies to the CPV VP2 epitope. In the hemagglutination inhibition (HI) assay as a measurement of CPV-neutralizing activity in the immune sera, there was an 8-fold increase of HI titer in the OMV-immunized group compared with the control. These results suggested that the CPV-neutralizing antibody response was raised by immunization with OMV containing the OmpA::CPV VP2 epitope, as well as the protective immune response against *S.* Typhimurium in BALB/c mice.

Keywords: Outer membrane vesicle, *S.* Typhimurium, low endotoxicity, multi-immunogenicity, canine parvovirus

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Outer membrane vesicles (OMVs) released from the outer membrane (OM) of Gram-negative bacteria are mainly constituted with major antigenic OM components and some periplasmic contents [2, 10]. OMVs are able to induce protective immunity *in vivo* [1], in addition to their utilization as a delivery vehicle for antibiotics and peptide vaccines [7]. Therefore, OMVs could be employed as a non-replicating vaccine against intracellular pathogens, such as *S.* Typhimurium, which is a zoonotic pathogen common to both humans and animals [1, 11, 15].

With respect to vaccine safety, endotoxicity of the bacterial lipopolysaccharide (LPS) is a critical issue and is known to be reduced by genetic mutation in the bacterial genome [12, 14]. For example, the LPS of an *msbB* knockout mutant showed a reduced ability to stimulate the production of proinflammatory cytokines associated with septic shock, and thereby increases the safety for the use of *Salmonella msbB* mutant in humans [12].

Canine parvovirus (CPV), which belongs to the genus *Parvovirus* of the family *Parvoviridae*, causes highly contagious and potentially fatal diseases, such as severe gastroenteritis in juvenile dogs and myocarditis in neonatal puppies [17]. Because VP2 is the most abundant capsid protein of CPV, this protein has been a target antigen to produce neutralizing antibodies against CPV [16, 17, 18].

OmpA is one of the most abundant OM proteins in the *Salmonella* bacteria and confers stability to the OM [22]. This property has been utilized as a fusion target to locate the fusion partner to the OM [19, 23]. Thus, OmpA is employed as a fusion target to incorporate the CPV VP2 epitope into the OMV in this study. To be incorporated, however, the fusion protein of the OmpA::CPV VP2 epitope has to be expressed and destined to the OM of the bacteria.

In this study, a recombinant vector derived from pBAD24 was constructed for expressing the OmpA::CPV VP2 epitope fusion protein in the *msbB* mutant of *S.* Typhimurium.

Next, the localization of the OmpA::CPV VP2 epitope protein was verified in the OM and OMV fractions from the *Salmonella* mutant. Finally, OMVs containing the OmpA::CPV VP2 epitope protein were used to immunize BALB/c mice, in order to verify their vaccine potential in eliciting the *S.* Typhimurium- and CPV VP2-specific antibody responses.

Materials and Methods

Bacteria, Plasmids, Virus, and Cell Culture

S. Typhimurium 40253 was obtained from the Korean Culture Center of Microorganisms (KCCM) and used for the creation of the *msbB* mutant. Plasmids of pKD46 and pKD3 were used for mutagenesis by homologous recombination as described by Datsenko and Wanner [5]. pBAD24 [6] and pGEX-4T-1 (Amersham Biosciences, U.K.) were used for constructions of the OmpA::CPV VP2 epitope and GST-fused CPV VP2 epitope-expression vectors, respectively. Crandell feline kidney (CRFK) cells (KCLB 10094) and CPV (ATCC 780916) were obtained from Korean Cell Line Bank (KCLB) and American Type Culture Collection (ATCC), respectively. The CRFK cells were cultured in DMEM (Gibco, U.S.A.) supplemented with 10% FBS (Gibco, U.S.A.) and 100 μg/ml streptomycin/penicillin (Sigma, U.S.A.) at 37°C in a 5% CO_2 incubator.

Construction of *msbB* Mutant of *S.* Typhimurium

An *msbB* deletion mutant of *S.* Typhimurium was generated by the method of Datsenko and Wanner [5] with modifications described in the 3-step PCR method of Serra-Moreno *et al.* [20] (Fig. 1). To place an antibiotic selection marker into the disrupted *msbB* gene, the primer pair of P1 (5'-GTGTAGGCTGGAGCTGCTTCGA-3') and P2 (5'-GTCGCTTTGTATGTCGCAAAG-3') was used for the amplification of the chloramphenicol (Cm)-resistant (*cat*) gene from pKD3, with the PCR conditions as follows: 94°C–30 sec for denaturation, 54°C–1 min for annealing, and 72°C–2 min for extension. A primer set of MsbB-F1 (5'-AGTGAGTATATCCCTGA ATTC-3') and MsbB-R1 (5'-CGAAGCAGCTCCAGCCTACACAG CGA-ACATCTCATCGACAAT-3') was used for the amplification of the 5'-fragment of the *msbB* gene, and the primers of MsbB-F2 (5'-

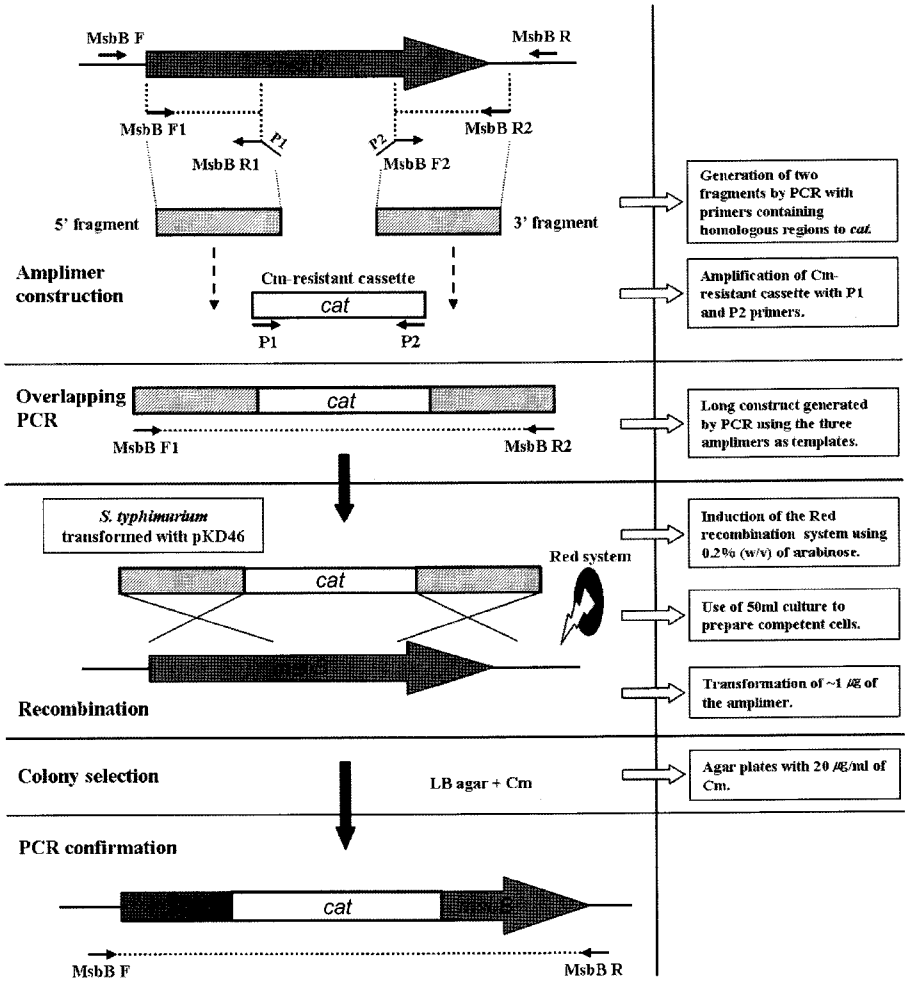

Fig. 1. Schematic illustration of the strategy used for the *Salmonella msbB* mutation.
The Cm-resistant gene (*cat*) was designed to be inserted in the middle of the *msbB* gene, as the *msbB* gene fragments at both ends enable homologous recombination by the pKD46-encoded Red-recombinase for creation of the *msbB* mutant of *S.* Typhimurium. The depiction was adapted from a previous report [20].

ATAGGAACTAAGGAGGATATTCATATGCTTTGCGACATACA AAGCGAC-3') and MsbB-R2 (5'-TTACGCTTATACGGCTGAAT-3') were used for the amplification of the 3'-fragment of the *msbB* gene (Fig. 1). The primer set of MsbB-F (5'-AGCAGACCCTGGA AAAGCATG-3') and MsbB-R (5'-TTATTTGATGGGATAAAG ATC-3') was used for the final PCR step to amplify the whole disrupted *msbB* allele with the insertion of the Cm-cassette. The PCR amplicon was used as the template DNA for the targeting of the *msbB* gene in *S.* Typhimurium. Next, preparation of electrocompetent cells with *S.* Typhimurium, the electroporation procedure, and mutant screening were performed as described in the manual of the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, U.S.A.). Finally, the *msbB* allelic replacement was confirmed by genomic PCR and DNA sequence analysis from the potential *msbB* mutants of *S.* Typhimurium.

Analysis of LPS and Lipid A Molecules

LPS samples were prepared by the whole-cell lysate method [8]. The LPS sample was then mixed with an equal volume of 3× SDS sample buffer, and boiled for 10 min prior to loading on a 16% Tricine SDS–PAGE gel (Novex, U.S.A.). The separated LPS bands were visualized by silver staining. Additionally, the *Salmonella* lipid A released by mild acid hydrolysis from ^{32}Pi-labeled LPS was analyzed by thin-layer chromatography (TLC), as described previously [21, 24]. Briefly, the purified lipid A sample was dissolved in 100 µl of chloroform/methanol (4:1, v/v), and approximately 1,000 cpm of the sample was applied to the origin of a Silica Gel 60 TLC plate. TLC was conducted in a developing thank in the solvent system consisting of chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). The TLC plate was dried and visualized with an imaging analyzer (FLA-7000, FUJIFILM).

Transmission Electron Microscopy (TEM)

To visualize OMV blebbing from the OM of the *Salmonella*, the colonies of wild type and the *msbB* mutant of *Salmonella* were prepared and observed with TEM. Briefly, after overnight growth of *S.* Typhimurium on the LB plate at 37°C, single colonies were picked and fixed for 2 h in a 2.5% paraformaldehyde–glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2). Next, the samples were post-fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812. Ultrathin sections, made with the ULTRACUT E ultramicrotome (Leica, Austria), were stained with uranyl acetate and lead citrate and examined under CM 20 TEM (Philips, The Netherlands).

In addition, OMVs collected from the liquid culture supernatant were negatively stained and observed with TEM. Briefly, a formvar-coated grid was floated on a sample droplet on parafilm for 1 min to permit adsorption of the specimen. The grid was then transferred onto a drop of negative stain (1% uranyl acetate) for 30 s, blotted with filter paper, and then air-dried before being examined.

Construction of the OmpA::CPV VP2 Expression Vector

A modified 3-step PCR method was exploited for the generation of DNA encoding the OmpA::CPV VP2 epitope, which resulted in protein fusion by the insertion of the CPV VP2 epitope into the periplasmic turn of the secondary OmpA structure between the 4th and 5th transmembrane region, as predicted by the OM topology of OmpA [22] (Fig. 2).

First, the primer pair of H1 (5'-TTGGGCTTACCACCATTTCT-3') and H2 (5'-TGGATATCTTCCTGTATC-3') was used for the amplification

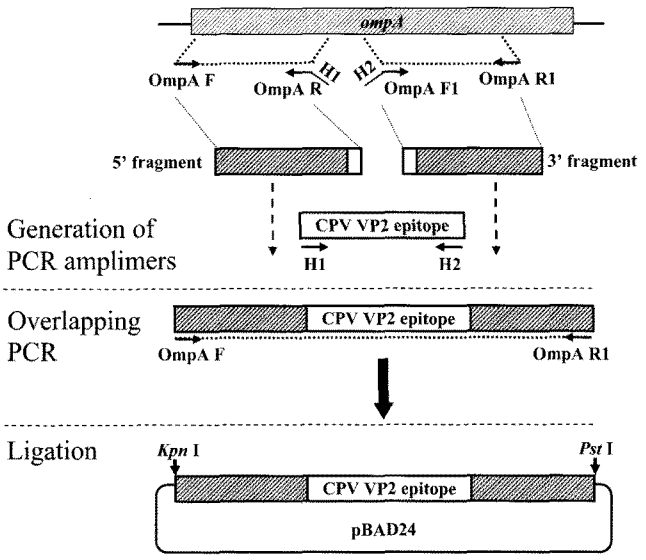

Fig. 2. Construction of an expression vector for the OmpA::CPV VP2 epitope fusion protein.

The 5'- and 3'-fragments of the *Salmonella ompA* gene were amplified by PCRs (362-bp and 337-bp products, respectively), and combined with the central DNA fragment encoding the CPV VP2 epitope by using an overlapping PCR method, as illustrated. The DNA cassette for the OmpA::CPV VP2-epitope was placed into the downstream of an arabinose-inducible promoter of pBAD24.

of the CPV VP2 epitope gene encoding the 3rd loop region of the CPV VP2 protein (J. W. Kim *et al.*, 2006. Korean Patent No. 10-0563197) by PCR using a CPV DNA template with the PCR condition as follows: 94°C– 30 sec for denaturation, 54°C– 1 min for annealing, and 2 min extension at 72°C. Second, the primer set of OmpA-F (5'-ATGGTACCCATGAAAAAGATAGCTATCGC-3') and OmpA-R (5'-AGTGATGGGATAACCCAGTT-3') was used for the amplification of the 5'-*ompA* fragment. OmpA-R1 (5'-ATCTGCAGTGAAGTTGA ACAGTACGTCA-3') and OmpA-F1 (5'-AACTGGGTTATCCCATC ACTTTGGGCTTACCACCATTTCT-3') were used for the amplification of the 3'-*ompA* fragment from the *S.* Typhimurium *ompA* gene. The primers of OmpA-R and OmpA-F1 contained the DNA sequences complementary to the H1 and H2 regions of the CPV VP2 epitope gene, respectively (Fig. 2). The PCR condition for the two amplifications was as follows: 94°C– 30 sec for denaturation, 52°C– 1 min for annealing, and 72°C– 2 min for elongation. Then, an overlapping PCR was conducted with OmpA-F and OmpA-R1 primers to construct a DNA cassette for the OmpA::CPV VP2 epitope, by using the amplified PCR products as 3-templates (the CPV VP2 epitope, the 5'-*ompA*, and the 3'-*ompA* DNA fragments). This final PCR product was then purified and followed by digestion with the restriction enzymes KpnI and PstI, and the fragment was used for ligation with pBAD24 digested with the same restriction enzymes (Fig. 2). Finally, the expression vector constructed for the OmpA::CPV VP2 epitope was introduced into the *msbB* mutant by electroporation.

Preparation of OMVs

The *Salmonella* was inoculated into 500 ml of LB broth and cultured until the OD_{600} reached 0.3. Then, 0.2% (w/v) arabinose (Sigma, U.S.A.) was added to the culture to induce the expression of the OmpA::CPV VP2 epitope. Once the culture was grown to an OD_{600}

of 0.8, it was pulsed with 50 μg/ml of gentamicin (Sigma, U.S.A.) for 30 min to increase OMV production, as described previously [9].

Western Blot Analysis

The protein quantity of OMVs recovered from the *Salmonella* was measured by the BCA Protein Assay Kit (PIERCE, U.S.A.). Approximately 20 μg of the protein portion of the OMVs was boiled in SDS–PAGE sample buffer and loaded onto a 16% SDS–PAGE gel. The separated protein bands were transferred onto nitrocellulose membranes (Invitrogen, U.S.A.) by using the XcellII blot apparatus (Invitrogen, U.S.A.). The monoclonal antibody against canine parvovirus (Santa Cruz Biotechnology, U.S.A.) and the peroxidase-conjugated rabbit anti-mouse IgG (Sigma, U.S.A.) were used to detect the OmpA::CPV VP2 epitope protein in the OMVs.

***In vivo* Virulence Assays and Immunization**

Eight-week-old male BALB/c mice (purchased from Koatech, Korea) were divided randomly to 7 groups (n=10 per group) for the lethality and immunization tests. Briefly, two groups of mice received an intraperitoneal (i.p.) injection with 10^8 CFU of wild-type *S.* Typhimurium or *msbB* mutant, and the other two groups were inoculated orally with 10^9 CFU of wild-type *S.* Typhimurium or *msbB* mutant, respectively. The survival of each mouse in all groups was recorded.

Another 2 groups of mice were immunized twice at 2-week intervals, intraperitoneally, with the *msbB* mutant of *S.* Typhimurium (0.2 ml of 5×10^5 CFU/ml in PBS) or 100 μg of the protein portion of the OMVs containing the OmpA::CPV VP2 epitope. Mice in the remaining group were the control group and received PBS. At 2 weeks after the second injection, blood was collected from the mice, allowed to clot at 4°C, and centrifuged at 3,500 rpm for 20 min. The serum samples were aliquoted and maintained at −70°C until use.

All animal housing and experiments were performed in accordance with Korea Research Institute of Bioscience and Biotechnology (KRIBB) Institutional Animal Care and Use Committee Guidelines (Accepted No. KRIBB-AEC-0801077).

Measurement of Serum IgG by ELISA

Measurements of serum antibody titers raised against *S.* Typhimurium and CPV VP2 epitope were conducted *via* ELISA in a 96-well plate coated with *S.* Typhimurium (2×10^7 CFU/well) or the GST–CPV VP2 fusion protein (10 μg/well) expressed by the pGEX-4T-1 vector-based construct [23]. Briefly, a 96-well microtiter plate (Nunc Maxi-Sorp, Denmark) was coated with the specified antigen suspended in 50 ml of PBS, overnight at 4°C. After being washed with PBS plus 0.5% Tween 20 and blocked with PBS plus 10% FBS, the plate was incubated with 100 ml/well of 3-fold serial dilutions of sera pooled from the same immunized group of mice, for 1 h at 37°C. HRP-conjugated rabbit anti-mouse IgG (Southern Biotechnology, U.S.A.) was used as a secondary antibody. The end-point serum IgG titer was determined from the highest serial dilution, using the mean plus 2× standard deviation of OD values (A_{450}) of the negative control serum as the cut-off.

Serum Bactericidal Assay

The serum bactericidal assay was performed by a modification of methods of Borrow *et al.* [3] and Martin *et al.* [13]. Briefly, serum samples were heated at 56°C for 30 min to inactive the complement system. An aliquot (20 ml) of 2-fold serially diluted sera was mixed with 10 ml of wild-type *S.* Typhimurium (8×10^4 CFU/ml) and with 10 μl of mouse complement (Pel/Freez Biological, U.S.A.) at 37°C for 1 h. Then, 30 ml of the mixture was plated on LB agar for viable counts. The number of colonies was counted and compared with the control bacteria, treated with mouse complement only. The percentage of serum bacterial killing was calculated by normalizing the mean of bacterial counts treated with the complement alone as 0% killing.

Hemagglutination Inhibition (HI) Test

For HI assay with the OMV-immunized sera, sera pooled from 5 mice were heated at 56°C for 30 min to inactivate the complement. To eliminate nonspecific reactions, the serum was adsorbed with 50% erythrocyte suspension at 4°C for 30 min. The mixture was centrifuged at 6,500 ×*g* for 2 min, and the supernatant was saved for the HI tests. In a 96-well V-shape bottom microtiter plate, 2-fold serial dilutions were made with the sera (50 μl per well), and an equal volume (50 μl) of CPV suspension (diluted to contain 3-HA units/50 μl) was added to each well. After incubation for 1 h, 50 μl of 0.5% swine erythrocyte suspension in VAD buffer (0.15 M NaCl, 0.3 M Na_2HPO_4, 0.15 M NaH_2PO_4, pH 6.0) was added and incubated overnight at 4°C. The HI titers were expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination (HA) and were presented as the geometric mean of three independent tests.

Statistical Analysis

Statistical analysis was performed by using the Student's *t*-test. The *p*-values of <0.05 were considered significant.

RESULTS

Confirmation of the *msbB* Inactivation in *S.* Typhimurium

Putative *msbB* mutants of *S.* Typhimurium were screened by genomic PCR to identify the desired mutation. The *msbB* mutation was confirmed by genomic PCR, detecting the DNA size increase due to the insertion of the *cat* cassette in the middle of the *msbB* gene (Fig. 3A). Compared with intact *msbB* (lane WT), the *msbB* mutant (lane MT) showed a 1.6-kb DNA band matching to the disrupted *msbB* allele with the insertion of the *cat* cassette as constructed.

LPS Alterations Associated with the *msbB* Mutation in *S.* Typhimurium

The whole LPS molecules and their lipid A portions were extracted and separated for the comparison between the wild type and the *msbB* mutant. The silver-stained LPS molecules of the *msbB* mutant differed from that of wild type in that the LPS bands of the *msbB* mutant migrated faster than the corresponding LPS band of wild-type control (Fig. 3B). This LPS alteration in the *Salmonella msbB* mutant was considered to be equivalent to the case of *E. coli* O157:H7 reported recently [21].

The lipid A spots separated by TLC clearly indicated that the *Salmonella msbB* mutant was genuine, because the

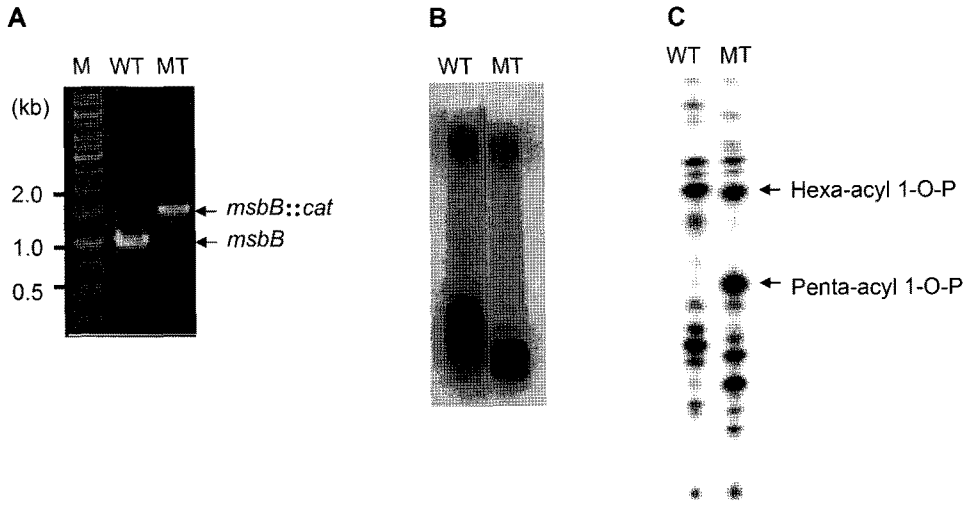

Fig. 3. Molecular characterization of the *msbB* mutant of *S.* Typhimurium.
A. The *msbB* mutation was confirmed genotypically by a genomic PCR with MsbB-F and MsbB-R primer set, in which the *cat* gene insertion in the middle of the *msbB* gene of the mutant (MT) resulted in greater DNA size (*msbB*::*cat*), compared with the wild type (WT). Lane M was loaded with a DNA size marker (1 kb ladder; Fermentas). **B.** Whole LPS molecules from the wild type (WT) and the mutant (MT) were visualized by silver-staining after separation on a 16% Tricine SDS–PAGE gel. **C.** ^{32}P-labeled lipid A molecules were separated by thin layer chromatography. The penta-acyl form of lipid A in the mutant (MT) was increased significantly, compared with wild type (WT).

MsbB deficiency in the mutant causes a lack of secondary acylated myristate in lipid A, thus forming the penta-acyl lipid A spot rather than the hexa-acylated molecules, as indicated in Fig. 3C. The remaining hexa-acyl lipid A molecules in the *msbB* mutant presumably correspond to the palmitoylated lipid A spot generated by the OM enzyme PagP on the penta-acyl lipid A-containing LPS accumulated in the OM of the *msbB* mutant [21].

OMVs of the *Salmonella msbB* Mutant

In order to observe the OMVs released from the OM of the *msbB* mutant and the wild-type *Salmonella*, a colony-lift method was employed to prepare the ultrathin sections of the bacterial colony for TEM observations (Figs. 4A and 4B). The results showed that the amount of OMV released from the *msbB* mutant (Fig. 4B) was greater than that of the wild-type *Salmonella* (Fig. 4A), presumably due to compromising the OM integrity by the MsbB deficiency. However, the shape and size of OMVs collected from the wild-type and the *msbB* mutant *Salmonella* were not different overall (Figs. 4C and 4D).

Virulence Attenuation Associated with the *msbB* Mutation

The mice injected i.p. with 10^8 CFU of wild-type *Salmonella* were all dead 4 days post injection, whereas all mice with the *msbB* mutant survived 5 days post i.p. injection, then the mice became gradually lethally affected until 8 days after challenge with the *msbB* mutant (Fig. 5A). In addition, the survival rate of the mice inoculated orally with 10^9 CFU of the wild type was 80% for 30 days, whereas all mice orally inoculated with the *msbB* mutant survived (Fig. 5B). The difference in mice survivals after oral challenge with wild-type *Salmonella* and the *msbB* mutant indicated that

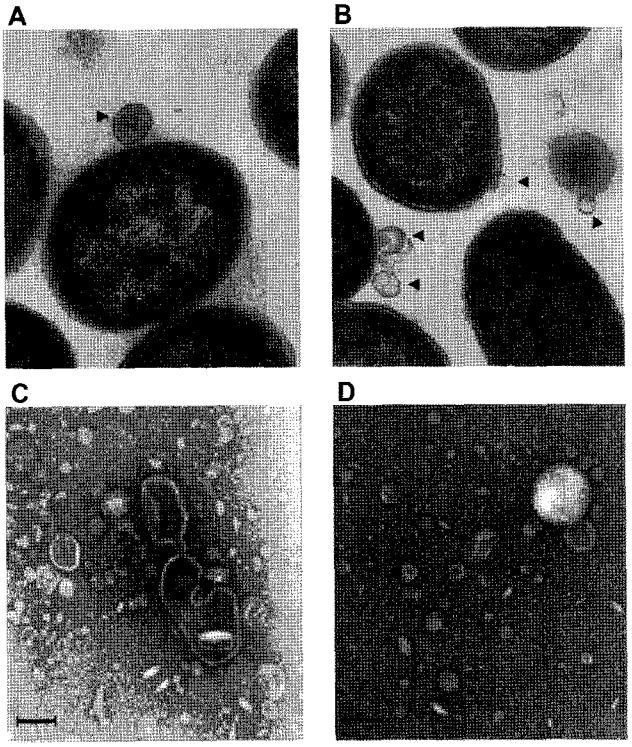

Fig. 4. TEM observations of the OMVs.
The OMVs released from the bacterial colonies of the wild type (**A**) and the *msbB* mutant (**B**) of *S.* Typhimurium grown on LB agar plate. The arrowheads indicate the OMVs. After gentamicin treatment, the OMVs were collected from the supernatant of LB broth cultures of the wild type (**C**) and the *msbB* mutant (**D**). The scale bar is 100 nm.

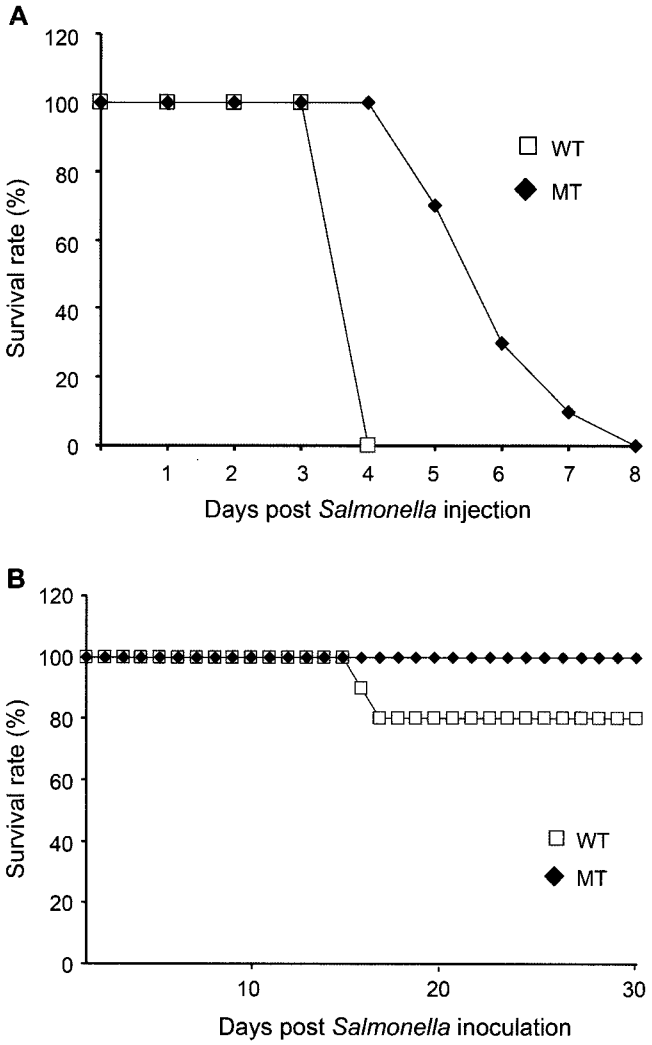

Fig. 5. Survival rates of mice in lethality tests.
A. Survival rate of mice determined after i.p. injection with 10^8 CFU of the wild type (WT) (n=10) and the *msbB* mutant (MT) of *S.* Typhimurium (n=10). **B.** Survival rate of mice calculated after oral inoculation with 10^9 CFU of the wild type (WT) (n=10) and the mutant (MT) (n=10).

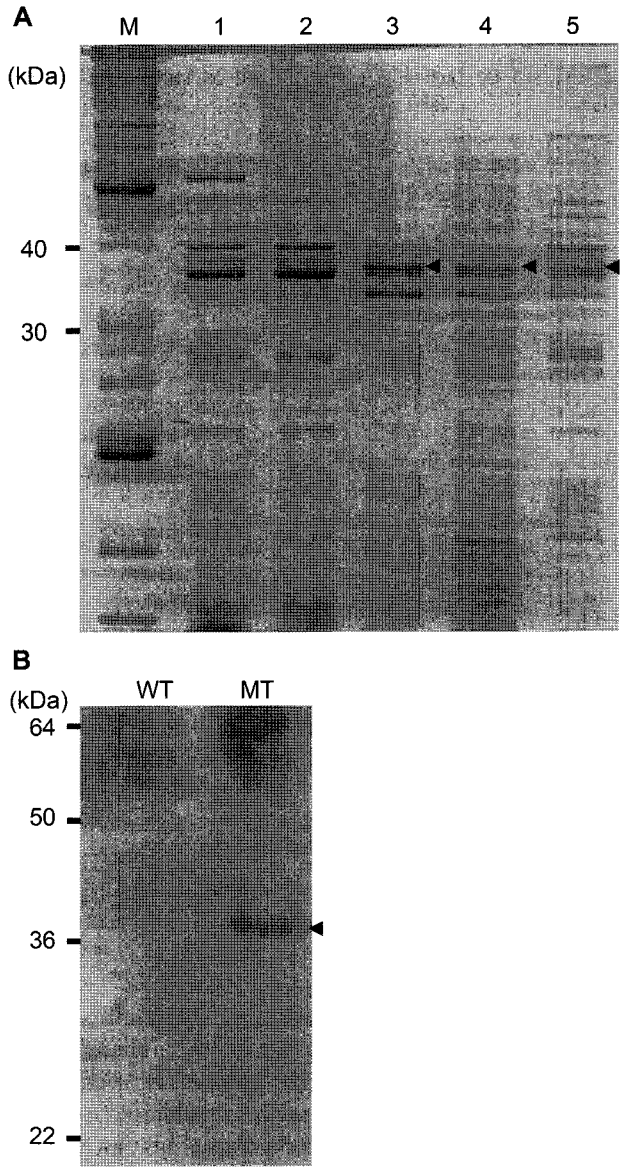

Fig. 6. Detection of the OmpA::CPV VP2 fusion protein in the OM and OMV fractions of *Salmonella msbB* mutant.
A. The OM proteins from wild-type *S.* Typhimurium (lane 1), the *msbB* mutant (lane 2), and the *Salmonella msbB* mutant transformed with the OmpA::CPV VP2-expressing vector (lane 3). Total membrane proteins of the *Salmonella* transformant were loaded on lane 4 in comparison with the OMVs from the same strain (lane 5). The arrowheads indicate the fusion protein of the OmpA::CPV VP2 epitope. A molecular mass standard (Invitrogen, U.S.A.) was used as a marker (M). **B.** Western blot analysis of the OMVs of the wild type *Salmonella* (lane 1) and the *Salmonella msbB* mutant induced expression of the OmpA::CPV VP2 (lane 2). The arrowhead indicates the 37 kDa protein band of the OmpA::CPV VP2 epitope reacted with anti-CPV monoclonal antibody (Santa Cruz Biotechnology, U.S.A.).

the inactivation of the *msbB* gene in *S.* Typhimurium is the cause of virulence attenuation.

Localization of the OmpA::CPV VP2 Epitope in the OM and OMV Fractions

The OmpA::CPV VP2 fusion protein was detected in the OM and OMV fractions of the *Salmonella msbB* mutant transformed with the OmpA::CPV VP2-expressing vector (Fig. 6). The OM proteins and OMVs derived from the *Salmonella msbB* mutant transformed with the OmpA::CPV VP2-expressing vector contained an approximately 37 kDa band corresponding to the calculated molecular mass of the OmpA::CPV VP2 fusion protein (lanes 3, 4, and 5 in Fig. 6A), but the wild type and the *msbB* mutant alone did not contain the fusion protein band (lanes 1 and 2, Fig. 6A). In the Western blot with anti-CPV monoclonal antibody, the OMV containing the OmpA::CPV VP2 epitope derived from the *Salmonella* transformant showed a positive band near 37 kDa (Fig. 6B). These results suggest that by using OmpA as a fusion partner, the CPV VP2 epitope tagged to the OmpA can be successfully targeted to the OM, and

subsequently incorporated into the OMVs during bacterial growth.

Induction of Antigen-Specific Antibodies in Mice by OMV Immunization

To measure the antibody responses in immunized mice, ELISA was performed as described above. Serum IgG titers against *S.* Typhimurium were 4.8-fold higher in the mutant *Salmonella*-immunized group and were 5.5-fold higher in the OMV-immunized group compared with the non-immunized control group (Fig. 7A). Moreover, there was no difference between the serum IgG titers of the mutant *S.* Typhimurium- and the OMV-immunized mice. In addition, the sera from the mutant *Salmonella*- and the OMV-immunized mice showed higher bactericidal activities (each<1:256) than the control (Table 1). These results indicated that the OMV immunization could induce bactericidal effects against *S.* Typhimurium in the serum.

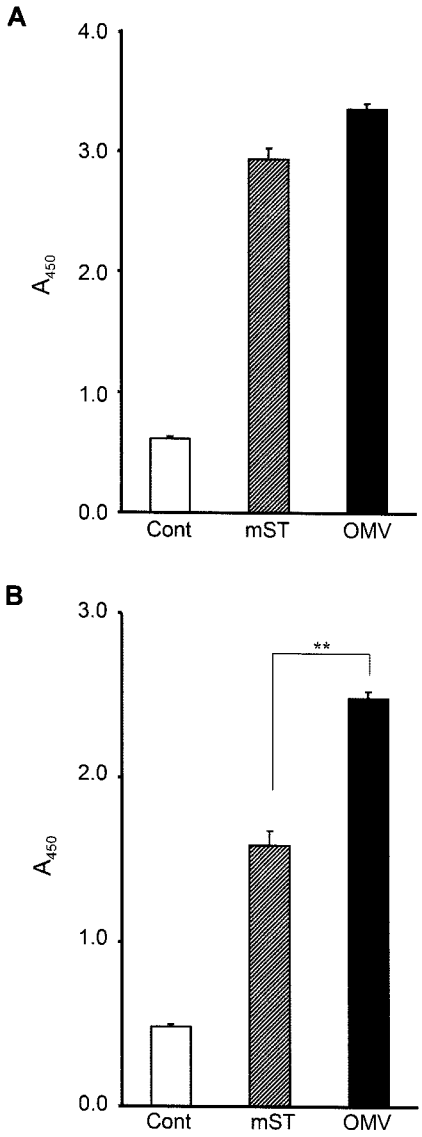

Fig. 7. Analysis of serum IgG antibody titers by ELISA.
ELISA detection of specific antibodies bound to wild-type *S.* Typhimurium (**A**) and the GST-fused CPV VP2 epitope (**B**) was conducted with sera from the mutant *S.* Typhimurium (mST), the OMVs containing the OmpA::CPV VP2 fusion protein (OMV), and the non-immunized mice (Cont). Sera were tested at a dilution of 1:810 and the values are the average of absorbance readings at 450 nm. Tests were carried out in duplicate in two separate assays. ** means $p<0.01$ by Student's *t*-test.

Table 1. Serum bactericidal assay.

Groups	Serum dilution resulting in 50% killing[a]
Non-immunized control	<1:8
The mutant *Salmonella*-immunized	<1:256
OMV-immunized	<1:256

[a]Sera obtained from bleedings at 2 weeks after the secondary immunization.

The serum IgG titer induced by the OMV containing the OmpA::CPV VP2 epitope was 5.1-fold higher than the non-immunized group against the GST-fused CPV VP2 epitope-coated plate (Fig. 7B). Although there was high cross-reactivity against the GST–CPV VP2 protein between the OMV-immunized and the *Salmonella* mutant-immunized groups, the OMV-immunized mice group showed a significantly higher titer of antibodies compared with the mutant-immunized group ($p<0.01$) (Fig. 7B). These results indicated that the immunization with the OMV containing the OmpA::CPV VP2 epitope could induce CPV VP2 epitope-specific antibody response in mice, as well as induction of the anti-*Salmonella* antibodies.

CPV-neutralizing Antibodies Raised by the OMV Immunization

HI tests were carried out to demonstrate the presence of CPV-neutralizing antibodies in the sera of mice immunized with the OMVs. The sera obtained from immunization with the OMVs containing OmpA::CPV VP2 epitope showed an HI titer of 2,560, whereas the *Salmonella*- and non-immunized sera showed an HI titer of 1,280 and 320, respectively (Table 2). Although there was cross-reactivity between OMV- and mutant *Salmonella*-immunized sera, an 8-fold increase in the neutralizing antibodies against CPV in the OMV-immunized sera versus the non-immunized control was shown. These findings indicate that immunization of the *Salmonella* OMVs containing the OmpA::CPV VP2 epitope may induce antibody-mediated protection, as assessed by an *in vitro* CPV neutralization assay.

Table 2. Serum hemagglutination inhibition (HI) activity.

Groups	Serum HI titers[a]
Non-immunized control	320
Mutant *Salmonella*-immunized	1,280
OMV-immunized	2,560

[a]Sera obtained from bleedings at 2 weeks after the secondary immunization.

Discussion

OMVs are proteoliposomal vesicles produced naturally from Gram-negative bacteria and have potential as delivery vehicles for heterologous antigens [10]. Foreign proteins can be spontaneously loaded within the OMVs if the foreign proteins are expressed and secreted into the periplasm or co-localized in the OM *via* translational fusion with OM proteins [10, 23]. Thus, the potential use of OMVs as vaccine delivery vehicles would broaden vaccine efficacy toward Gram-positive bacteria and viruses.

In this study, we tried to devise low endotoxic and multi-immunogenic OMVs containing a foreign epitope for an experimental vaccine trial. The platform technologies employed for such OMV vaccine development are 2-fold. The first was making an *msbB* mutant of *S.* Typhimurium in order to produce OMVs with the structurally modified (penta-acylated) LPS, which was known to be much less endotoxic to humans [12]. We employed a chromogenic *Limulus* amebocyte lysate (LAL) assay to differentiate the expected reduction of endotoxicity of the mainly penta-acylated LPS (the *msbB* mutant) from that of the hexa-acylated LPS (wild type). However, LAL assay could not differentiate the subtle change in the LPS of the *msbB* mutant (data not shown). Rather, the mouse lethality test shown in Fig. 5 revealed the difference in the overall virulence that attenuated in the *msbB* mutant, implying that the possible reduction of endotoxicity due to the LPS penta-acylation could be a factor that contributed to the virulence attenuation (Fig. 5). The second was the translational fusion technology involving expression of the foreign protein-bearing plasmid in the *Salmonella* mutant, leading to the OM localization of the foreign epitope *via* fusion with the OmpA protein. In this study, we have chosen the CPV VP2 epitope as a partner for the fusion with OmpA and confirmed that the OmpA::CPV VP2 epitope fusion protein was localized in the OM and OMV fractions of the *Salmonella msbB* mutant carrying the OmpA::CPV VP2-expressing vector (Fig. 6).

We have used a *Salmonella* OMV as a carrier of the OmpA::CPV VP2 epitope fusion protein. In the western blot assay with a monoclonal antibody against CPV chosen randomly, the positive band near 37 kDa showed weak reactivity (Fig. 6B). When HI tests were carried out with ≥4-HA units of CPV suspension, there was no difference between the serum neutralizing activities of the OMV-immunized and the *Salmonella* mutant-immunized groups (data not shown). These results have suggested that the initial amount of foreign antigen present in the OMV is an important factor for priming a good immune response [4], and studies to optimize the expression level of the fusion protein by using different promoters and also to integrate the fusion gene into the mutant *Salmonella* chromosome are needed.

Interestingly, a recent report suggested that the native OMVs released from *S.* Typhimurium were enough to elicit a similar level of immune response as the case of the immune response induced by live *Salmonella* [1]. In parallel with the previous report, our results (Fig. 7A) showed that the mice group immunized with the *Salmonella* OMVs containing the OmpA::CPV VP2 epitope could induce a similar level of immune response to the case of the live *Salmonella msbB* mutant bacteria in BABL/c mice. In addition, anti-CPV antibodies were raised in mice by immunization of the OMV containing the CPV VP2 epitope (Fig. 7B). These OMV-induced antibodies gave an HI titer representing the CPV-neutralization antibody, which was 8-fold higher than that of the non-immunized control group (Table 2). These results indicated that mice immunized by OMVs containing OmpA::CPV VP2 could induce protective immunity against *S.* Typhimurium, as well as inducing neutralizing antibodies production against CPV at the same time (Table 2).

Thus, the *Salmonella* OMV prepared to contain a foreign epitope may provide an alternative to the live *Salmonella* expressing the heterologous protein in the vaccine development. Although live-attenuated *Salmonella* vaccines were used successfully as carriers conveying heterologous antigens to the immune system [14], such live vaccines can always pose a risk to immunocompromised vaccine recipients [4]. By using OMV, we provided an example of a non-living vaccine candidate that can be successfully utilized in the presentation of a virus epitope to the host immune system. The strategy we used in OMV preparation will be applied to the development of multifunctional OMV vaccines in the near future.

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References

1. Alaniz, R. C., B. L. Deatherage, J. C. Lara, and B. T. Cookson. 2007. Membrane vesicles are immunogenic facsimiles of *Salmonella* Typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity *in vivo*. *J. Immunol.* **179:** 7692–7701.
2. Beveridge, T. J. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* **181:** 4725–4733.
3. Borrow, R., I. S. Aaberge, G. F. Santos, T. L. Endey, P. Oster, A. Glennie, *et al.* 2005. Interlaboratory standardization of the measurement of serum bactericidal activity by using human complement against meningococcal serogroup b, strain 44/76-

SL, before and after vaccination with the Norwegian MenBvac outer membrane vesicle vaccine. *Clin. Diagn. Lab. Immunol.* **12:** 970–976.
4. Cardenas, L. and J. D. Clements. 1992. Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. *Clin. Microbiol. Rev.* **5:** 328–342.
5. Datsenko, K. A. and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97:** 6640–6645.
6. Guzman, L., D. Belin, M. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177:** 4121–4130.
7. Henry, T., S. Pommier, L. Journet, A. Bernadac, J. P. Gorvel, and R. Lloubès. 2004. Improved methods for producing outer membrane vesicles in Gram-negative bacteria. *Res. Microbiol.* **155:** 437–446.
8. Hitchcock, P. J. and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154:** 269–277.
9. Kadurugamuwa, J. L. and T. J. Beveridge. 1997. Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. *J. Antimicrob. Chemother.* **40:** 615–621.
10. Kuehn, M. J. and N. C. Kesty. 2005. Bacterial outer membrane vesicles and the host pathogen interaction. *Genes Dev.* **19:** 2645–2655.
11. Liu, T., R. König, J. Sha, S. L. Agar, C. T. Tseng, G. R. Klimpel, and A. K. Chopra. 2008. Immunological responses against *Salmonella enterica* serovar Typhimurium Braun lipoprotein and lipid A mutant strains in Swiss-Webster mice: Potential use as live-attenuated vaccines. *Microb. Pathog.* **44:** 224–237.
12. Low, K. B., M. Ittensohn, T. Le, J. Platt, S. Sodi, M. Amoss, *et al.* 1999. Lipid A mutant *Salmonella* with suppressed virulence and TNF-alpha induction retain tumor-targeting *in vivo*. *Nat. Biotechnol.* **17:** 37–41.
13. Martin, D., L. McCallum, A. Glennie, N. Ruijne, P. Blatchford, J. O'Hallahan, and P. Oster. 2005. Validation of the serum bactericidal assay for measurement of functional antibodies against group B meningococci associated with vaccine trials. *Vaccine* **23:** 2218–2221.
14. Mastroeni, P., J. A. Chabalgoity, S. J. Dunstan, D. J. Maskell, and G. Dougan. 2001. *Salmonella*: Immune responses and vaccines. *Vet. J.* **161:** 132–164.
15. Mo, E., S. E. Peters, C. Willers, D. J. Maskell, and I. G. Charles. 2006. Single, double and triple mutants of *Salmonella enterica* serovar Typhimurium *degP (htrA), degQ (hhoA)* and *degS (hhoB)* have diverse phenotypes on exposure to elevated temperature and their growth *in vivo* is attenuated to different extents. *Microb. Pathog.* **41:** 174–182.
16. Park, J. S., B. K. Choi, L. S. Vijayachandran, V. Ayyappan, C. K. Chong, K. S. Lee, S. C. Kim, and C. W. Choi. 2007. Immunodetection of canine parvovirus (CPV) in clinical samples by polyclonal antisera against CPV-VP2 protein expressed in *Escherichia coli* as an antigen. *J. Virol. Methods* **146:** 281–287.
17. Patial, S., V. K. Chaturvedi, A. Rai, M. Saini, R. Chandra, Y. Saini, and P. K. Gupta. 2007. Virus neutralizing antibody response in mice and dogs with a bicistronic DNA vaccine encoding rabies virus glycoprotein and canine parvovirus VP2. *Vaccine* **25:** 4020–4028.
18. Pratelli, A., A. Cavalli, V. Martella, M. Tempesta, N. Decaro, L. E. Carmichael, and C. Buonavoglia. 2001. Canine parvovirus (CPV) vaccination: Comparison of neutralizing antibody responses in pups after inoculation with CPV2 or CPV2b modified live virus vaccine. *Clin. Diagn. Lab. Immunol.* **8:** 612–615.
19. Schorr, J., B. Knapp, E. Hundt, H. A. Kupper, and E. Amann. 1991. Surface expression of malarial antigens in *Salmonella* Typhimurium: Induction of serum antibody response upon oral vaccination of mice. *Vaccine* **9:** 675–681.
20. Serra-Moreno, R., S. Acosta, J. P. Hernalsteens, J. Jofre, and M. Muniesa. 2006. Use of the lambda Red recombinase system to produce recombinant prophages carrying antibiotic resistance genes. *BMC Mol. Biol.* **7:** 1–12.
21. Smith, A. E., S. H. Kim, F. Liu, W. Jia, E. Vinogradov, C. L. Gyles, and R. E. Bishop. 2008. PagP activation in the outer membrane triggers R3 core oligosaccharide truncation in the cytoplasm of *Escherichia coli* O157:H7. *J. Biol. Chem.* **283:** 4332–4343.
22. Vogel, H. and F. Jahnig. 1986. Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. *J. Mol. Biol.* **190:** 191–199.
23. Wang, L., J. A. Huang, H. S. Nagesha, S. C. Smith, A. Phelps, I. Holmes, J. C. Martyn, P. J. Coloe, and P. R. Reeves. 1999. Bacterial expression of the major antigenic regions of porcine rotavirus VP7 induces a neutralizing immune response in mice. *Vaccine* **17:** 2636–2645.
24. Zhou, Z., S. Lin, R. J. Cotter, and C. R. Raetz. 1999. Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH_4VO_3 in *Escherichia coli* K12. *J. Biol. Chem.* **274:** 18503–18514.