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Immunization with *Salmonella enterica* Serovar Typhimurium-Derived Outer Membrane Vesicles Delivering the Pneumococcal Protein PspA Confers Protection against Challenge with *Streptococcus pneumoniae*[▽]

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Gram-negative bacteria produce outer membrane vesicles (OMVs) that serve a variety of functions related to survival and pathogenicity. Periplasmic and outer membrane proteins are naturally captured during vesicle formation. This property has been exploited as a method to derive immunogenic vesicle preparations for use as vaccines. In this work, we constructed a *Salmonella enterica* serovar Typhimurium strain that synthesized a derivative of the pneumococcal protein PspA engineered to be secreted into the periplasmic space. Vesicles isolated from this strain contained PspA in the lumen. Mice intranasally immunized with the vesicle preparation developed serum antibody responses against vesicle components that included PspA and *Salmonella*-derived lipopolysaccharide and outer membrane proteins, while no detectable responses developed in mice immunized with an equivalent dose of purified PspA. Mucosal IgA responses developed against the *Salmonella* components, while the response to PspA was less apparent in most mice. Mice immunized with the vesicle preparation were completely protected against a 10× 50% lethal dose (LD_{50}) challenge of *Streptococcus pneumoniae* and significantly protected against a 200× LD_{50} challenge, while control mice immunized with purified PspA or empty vesicles were not protected. These results establish that vesicles can be used to mucosally deliver an antigen from a Gram-positive organism and induce a protective immune response.

Outer membrane vesicles (OMVs) are released by most Gram-negative bacteria into the surrounding environment during growth (20). OMVs are formed by blebbing and pinching off segments of the bacterial outer membrane (21, 25). During this process, some of the underlying periplasmic components are entrapped, while components from the inner membrane and cytoplasm are excluded (3, 25). Considering that OMVs are formed by bacteria growing in diverse environments (3), it is likely that they can serve many biological functions. OMVs produced by nonpathogenic bacteria have been implicated in contributing to bacterial survival by serving as an efflux mechanism, thereby reducing the levels of toxic compounds (18). OMV production has also been considered an alternate secretion pathway capable of directing bacterial products (enzymes, toxins, and DNA) to both prokaryotic and eukaryotic cells (16, 19, 23, 28). OMVs from pathogenic bacteria are associated with secretion of virulence factors (see Table 1 in reference 7), likely contributing to their pathogenicity *in vivo*.

Several studies have demonstrated OMVs as immunogenic entities that are capable of eliciting stable immune responses and conferring protection against a homologous challenge when used to immunize mice (1, 29, 31). Intraperitoneal immunization with *Salmonella enterica* serovar Typhimurium-derived OMVs stimulates proinflammatory responses from professional antigen-presenting cells in addition to priming *Salmonella*-specific adaptive immunity *in vivo* (1). More importantly, the OMV-immunized mice were protected against *Salmonella* infection (1). Immunization with *Vibrio cholerae*-derived OMVs induces immunity in female mice and protects their offspring against *V. cholerae* colonization in an infant mouse model (31).

Heterologous proteins can be incorporated into OMVs (15, 27). In one study, the *Escherichia coli* PhoA protein synthesized by an engineered *V. cholerae* strain was packaged into vesicles (30). Mice intranasally immunized with vesicles purified from that strain developed anti-PhoA serum antibodies. In another study, NspA, an outer membrane protein from *Neisseria meningitidis*, was synthesized in a recombinant strain of *Neisseria flavescens*. Mice immunized with OMVs derived from the recombinant *N. flavescens* strain developed opsonizing antibodies against *N. meningitidis* (27).

In this work, we explore the feasibility of producing *Salmonella enterica* serovar Typhimurium-derived OMVs containing PspA, a surface protein present on all strains of the Gram-positive bacterium *Streptococcus pneumoniae* (26). PspA has been shown to be an immunogenic, protective pneumococcal antigen in animals and is also immunogenic in humans (4, 39). We examined the ability of PspA packaged in OMVs to elicit immune responses against PspA and whether these responses are protective against *S. pneumoniae* challenge.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid-containing derivatives of *Salmonella enterica* serovar Typhimurium strain χ9281 were grown in LB broth (2) or on LB agar plates. Diaminopimelic acid (50 μg/ml) was added when necessary to support the growth of strains with a Δ*asdA* mutation that was

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
S. Typhimurium		
χ9241	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT	38
χ9281	χ9241 Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT Δ*fliC2426* Δ*fljB217*	
S. pneumoniae WU2	Wild-type virulent, encapsulated, type 3	4
Plasmids		
pYA3620	Asd^+ vector *bla* SS+CT-based secretion periplasmic plasmid pBR*ori*	6
pYA3802	0.8-kb DNA encoding the α-helical region of PspA (aa 3 to 285) in pYA3620	38
pYA3493	Asd^+ vector *bla* SS-based secretion periplasmic plasmid	14
pYA4088	0.8-kb DNA encoding the α-helical region of PspA (aa 3 to 285) in pYA3493	38

[a] *lacI* TT, *lacI* transcriptional terminator; *bla* SS+CT, β-lactamase signal sequence and C-terminal sequence; aa, amino acids.

not complemented with a plasmid. Plasmids pYA3802 and pYA4088 carry nearly identical truncated *pspA* genes fused to the type 2 secretion signal sequence derived from the β-lactamase gene (38). The protein products translated from these genes are secreted into the periplasm (14).

OMV isolation and purification. Outer membrane vesicles (OMVs) were isolated from *S.* Typhimurium strains essentially as described previously (16). Briefly, strains were grown overnight at 37°C in LB broth, and bacteria were pelleted by centrifugation (10 min, 10,000 × *g*, 4°C). The supernatant was filtered (0.45 μm; Millipore), and vesicles were pelleted from the supernatant (1 h, 40,000 × *g*, 4°C). Vesicles were resuspended in 1× Dulbecco's phosphate-buffered saline (DPBS) (Mediatech, Manassas, VA) and filter sterilized (0.45 μm; Millipore, Bedford, MA). Vesicles were checked for live-cell contamination by plating 20 μl of each 200-μl preparation onto LB agar plates. No colonies were detected in any of the preparations. Vesicles were purified by density gradient centrifugation (overnight, 150,000 × *g*, 4°C) on a discontinuous Optiprep (Greiner Bio-One, Monroe, NC) gradient (2 ml each of 20 to 45% Optiprep in 10 mM HEPES [pH 6.8] containing 0.85% NaCl). Vesicle fractions were pooled, and vesicles were pelleted (1 h, 40,000 × *g*, 4°C) and resuspended in DPBS (Mediatech).

SDS-PAGE and immunoblot analysis. OMV samples were mixed with 100 μl of 2× SDS loading buffer, boiled for 10 min, and resolved on a 12% SDS-polyacrylamide gel at 180 V for 1 h. Protein bands were visualized by SYPRO ruby (Sigma-Aldrich, St. Louis, MO) staining per the manufacturer's instructions. For immunoblotting, proteins resolved on a 12% SDS-polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane (1 h, 20 V). Membranes were blocked with 3% skim milk in 20 mM Tris HCl (pH 7.8) containing 36 mM NaCl and incubated with 1:10,000 dilution of primary antibodies (rabbit anti-PspA-RX1 antibody or rabbit anti-OMP antibody) followed by alkaline phosphatase-conjugated anti-rabbit IgG antibodies (1:10,000; Sigma-Aldrich). Blots were developed by the addition of nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma Aldrich).

Proteinase K assay. Vesicles containing PspA were treated with 0.5 μg/ml proteinase K for 30 min at 37°C in the presence or absence of 1% SDS. In some experiments, the proteinase K inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM) was added. The effects of proteinase K and SDS treatments on PspA-loaded vesicles were determined by Western blotting.

Vesicle and PspA quantitation. The total protein content in each vesicle preparation was determined by measuring the optical density at 280 nm. To quantify the amount of vesicle-associated PspA, vesicles and known amounts of purified PspA-RX1 protein (6.25, 12.5, 25, and 50 ng) were separated by SDS-PAGE and subjected to immunoblot analysis using anti-PspA antibodies. The vesicle-associated PspA was quantified by densitometric analysis of the immunoblot using Alphaease spot densitometry software (Alpha Innotech, San Leandro, CA), and the results were compared to PspA standards.

Immunization and challenge of mice. Six- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were immunized intranasally with 10 μl of PBS, purified PspA protein (350 ng and 10 μg), OMVs (50 μg), or OMVs (50 μg) containing 350 ng PspA. A total of four immunizations were administered at weekly intervals. Sera were collected 3 weeks after the final immunization. At 8 weeks after the first immunization, mice were challenged with either 10× LD_{50} or 200× LD_{50} of *Streptococcus pneumoniae* WU2 via the intraperitoneal (i.p.) route. The LD_{50} of *S. pneumoniae* WU2 when administered i.p. is 200 CFU. In the three challenge experiments performed, the doses of *S. pneumoniae* WU2 ranged from 2.46×10^3 CFU/100 μl to 4.6×10^3 CFU/100 μl for the 10× LD_{50} groups and 3.0×10^4 CFU/100 μl to 4×10^4 CFU/100 μl for the 200× LD_{50} groups. Five mice per group were used in the first experiment, and 10 mice per group were used in the second and third experiments, for a total of 25 mice per treatment group. The mice were monitored for mortality for 2 weeks after the challenge.

Measurement of antibody responses. Blood and vaginal lavage fluid samples were collected 6 weeks after the first immunization. IgG and IgA responses were measured by enzyme-linked immunosorbent assays (ELISAs). The wells on microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 ng/well of *S.* Typhimurium lipopolysaccharide (LPS) (Sigma), *Salmonella* outer membrane protein (SOMP), or purified PspA protein. SOMPs and PspA were purified as described previously (14, 38). When outer membrane vesicles were used to coat wells, 200 ng/well of vesicles (with or without PspA) was used. Antigens were suspended in coating buffer (0.016 M anhydrous sodium carbonate, 0.034 M sodium bicarbonate [pH 9.6]) and applied in 100-μl volumes to each well. The plates were incubated overnight at 4°C, washed with 1× PBS containing 0.05% Tween 20 (1× PBS–0.05% Tween 20) and dried. Free antigen binding sites were blocked with 1× PBS–3% skim milk overnight at 4°C to prevent nonspecific binding of proteins to the plate. Test samples (serum samples for measuring IgG responses and vaginal lavage fluid samples for measuring IgA responses) were diluted 1:100 (IgG) or 1:10 (IgA) in 1× PBS–3% skim milk and incubated in duplicate wells for 1 h at room temperature. The wells were washed with 1× PBS–0.05% Tween 20 and dried to remove excess primary antibody. The plates were incubated with 1:10,000 dilution of anti-mouse IgG–alkaline phosphatase conjugate or 1:5,000 dilution of anti-mouse IgA–biotin conjugate for 1 h at room temperature. For measurement of IgA responses, the plates were washed with 1× PBS–0.05% Tween 20 and further incubated with 1:3,000 dilution of streptavidin-alkaline phosphatase conjugate for 1 h at room temperature. Alkaline phosphatase activity was detected by incubating plates with *p*-nitrophenyl phosphate for 1 h at room temperature. After the development of yellow color, the plates were read at 405 nm.

Statistical analysis. Analysis of variance (ANOVA) (GraphPad Prism; GraphPad Software) followed by application of Tukey's method to calculate statistically significant means ($P < 0.05$) was used to evaluate differences in antibody responses between the different immunization groups. Data were expressed as means ± standard errors. Survival analysis, following an i.p. challenge of intranasally immunized mice, was determined by the Kaplan-Meier method (GraphPad Prism; GraphPad Software).

RESULTS

Characterization of OMVs from *Salmonella*. We used *S. enterica* serovar Typhimurium strain χ9281 for generation of OMVs. Strain χ9281 was chosen because it carries Δ*fliC* and Δ*fljB* deletions, rendering it incapable of producing intact flagella (data not shown), thus precluding contamination of the

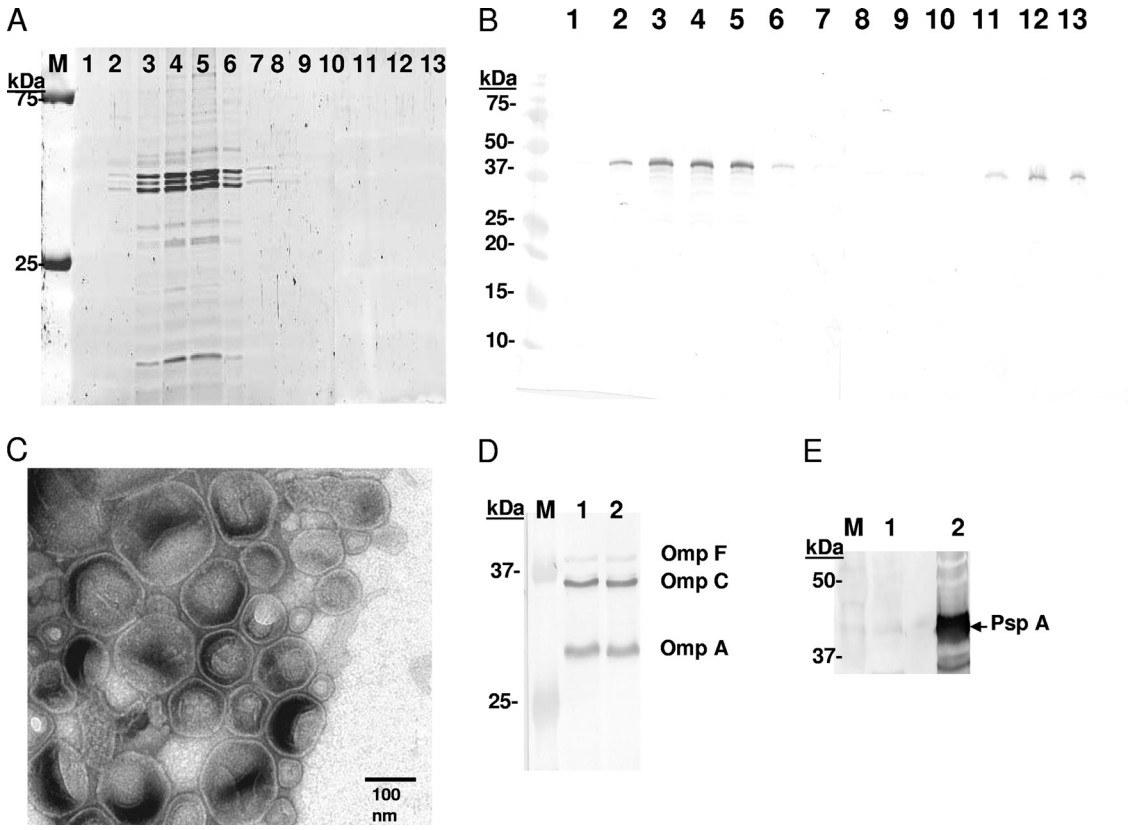

FIG. 1. Velocity gradient centrifugation-based purification of vesicles. (A) *S. enterica* serovar Typhimurium vesicles were loaded on the bottom of an Optiprep gradient. After centrifugation, fractions 1 to 13 (equal-volume fractions) were separated from the top to bottom and analyzed by SDS-PAGE and SYPRO ruby staining. Lane M, molecular mass markers; lanes 1 to 13, gradient fractions 1 to 13, respectively. (B) Gradient fractions from panel A were subjected to Western blotting and probed with anti-PspA antibodies. (C) Gradient-purified fractions 3 to 5 were pooled and analyzed by transmission electron microscopy. Outer membrane vesicles (OMVs) were visualized by negative staining and measured. (D) Pooled OMV preparations were subjected to immunoblotting using anti-outer membrane protein (anti-OMP) antibodies. The antibodies were raised against *E. coli* OMPs, so the *Salmonella*-specific OmpD band that runs between OmpC/F and OmpA was not recognized by the antisera. Lane M, molecular mass markers; lane 1, strain χ9281(pYA3620); lane 2, strain χ9281(pYA3802). (E) OMV-containing fractions 3, 4, and 5 were pooled and subjected to immunoblot analysis using anti-PspA and anti-OMP antibodies. Lane 1, OMVs from control strain χ9281(pYA3620); lane 2, OMVs from χ9281(pYA3802) (*pspA*).

OMVs with flagella. The strain also carries a Δ*asdA* mutation, allowing us to utilize plasmids pYA3802 and pYA4088, previously characterized Asd^+ plasmids carrying similar *pspA* derivatives (38). Preliminary analysis of strains χ9281(pYA3802) (*pspA*) and χ9281(pYA3620) (control) by Western blotting confirmed that strain χ9281(pYA3802) produced the expected 37-kDa band that reacted with rabbit anti-PspA antibody (data not shown). Control strain χ9281(pYA3620) did not produce PspA, as expected.

OMVs were isolated from strains χ9281(pYA3802) and χ9281(pYA3620) as described in Materials and Methods. Isolated vesicles were loaded on the bottom of an Optiprep column and subjected to velocity density gradient centrifugation. By this method, only proteins tightly associated with the vesicles copurify with OMVs. Equal fractions (1 ml) from top to bottom of the gradient were analyzed by SDS-PAGE followed by staining using SYPRO ruby protein gel stain. The resulting profile for strain χ9281(pYA3802) is shown in Fig. 1A. Bands corresponding to the expected sizes of the major *Salmonella* outer membrane proteins (OMPs) OmpA (37 kDa), OmpD (34 kDa), and OmpF/OmpC (35/36 kDa) (22), typically seen as a tightly grouped triplet of bands, copurified in fractions 3, 4, 5, and 6 (Fig. 1A), in addition to a number of lower-molecular-mass OMPs that we did not identify. While low levels of OMPs could be detected in fractions 2, 7, and 8, no OMPs could be detected in fractions 9 through 13.

Heterologous protein PspA directed to the periplasm of *S.* Typhimurium is incorporated into OMVs. Previous studies have shown that the pneumococcal protein PspA fused to the N-terminal β-lactamase signal sequence (*bla* SS-*psp*A) is transported to the periplasmic space of *S.* Typhimurium by the type II secretion system and is subsequently released into the surrounding medium (14). When used to immunize mice, *S.* Typhimurium vaccine strains synthesizing this secreted PspA elicit higher PspA-specific immune responses and increased protection against pneumococcal challenge than an identical strain synthesizing PspA without the signal sequence (13). To investigate whether the PspA directed to the periplasm is incorporated into outer membrane vesicles, OMVs from a culture of *S.* Typhimurium χ9281(pYA3802) were subjected to either electron microscopic (EM) analysis or SDS-PAGE followed by immunoblot analysis using anti-PspA antibodies. The

fractions containing OMVs were identified by EM analysis, and the presence of outer membrane proteins was detected by SYPRO ruby staining of SDS-polyacrylamide gels (data not shown), as shown in Fig. 1A. The OMV fractions contained a 37-kDa protein, the expected size of PspA, that reacted in Western blots probed with anti-PspA antibodies (Fig. 1B). This demonstrated that when PspA was directed to the periplasm, it was packaged into outer membrane vesicles and released into the surrounding medium. While PspA could be detected in the vesicle fraction, some PspA was also detected in the last three fractions (fractions 11 to 13) obtained from velocity gradient centrifugation of OMVs (Fig. 1B). Since no OMVs were detected in these last few fractions by EM analysis, we infer that this PspA may be free protein that has aggregated into higher-order structures which precipitated out during centrifugation. In this regard, native PspA has been shown to form dimers and aggregates (33).

Fractions 3, 4, and 5 were pooled. The absence of bacterial contamination was verified by plating an aliquot representing 10% of the preparation onto LB agar plates. Pooled fractions were examined by negative staining transmission EM for the presence of OMVs (Fig. 1C). OMVs could be detected in the pooled fraction as intact, spherical, bilayered structures ranging from 50 to 250 nm in diameter, as previously described (17, 24). Velocity gradient centrifugation of OMVs from strain χ9281(pYA3620) yielded a similar fractionation profile (data not shown). Immunoblot analysis using antibodies prepared against outer membrane proteins confirmed the presence of OMPs F/C and A in the pooled OMVs from both strains (Fig. 1D), reflecting their outer membrane composition. In gradients from both strains, fractions 9 to 13 did not react with the anti-OMP antisera, as expected (data not shown).

The average yields of purified vesicles were 918 μg/liter for strain χ9281(pYA4088) and 985 μg/liter for control strain χ9281(pYA3620). Similar results were obtained for three independent preparations. The amount of PspA copurified with vesicles from strain χ9281(pYA4088) was 7.2 μg, indicating that PspA constituted approximately 0.8% of the total protein in the vesicle preparations.

PspA is associated with the lumens of OMVs. To study the association between PspA and OMVs, proteinase protection assays were performed. OMVs were treated with proteinase K either in the presence or absence of 1% SDS. The quantity of OMV-associated PspA was not altered by the presence of proteinase K (Fig. 2, lanes OMV-PspA and OMV-PspA/ PK). Proteolytic degradation of PspA was detected only when vesicles were treated with proteinase K in the presence of the membrane-disrupting agent SDS (Fig. 2, OMV-PspA/ PK/ SDS), indicating that PspA was protected from digestion because of its localization inside the vesicle. Inhibition of proteinase K with the proteinase inhibitor PMSF prevented PspA degradation (Fig. 2, OMV-PspA/ PK/SDS/PMSF).

Evaluation of the immunogenicity of OMVs containing PspA. Groups of 5 to 10 mice were immunized intranasally with purified PspA antigen (350 ng or 10 μg), empty OMVs derived from strain χ9281(pYA3493), or OMVs containing 350 ng PspA derived from strain χ9281(pYA4088). A group of control mice were administered PBS intranasally. Four immunizations were given at 1-week intervals. Antibody responses to OMVs and PspA were measured in the sera collected from

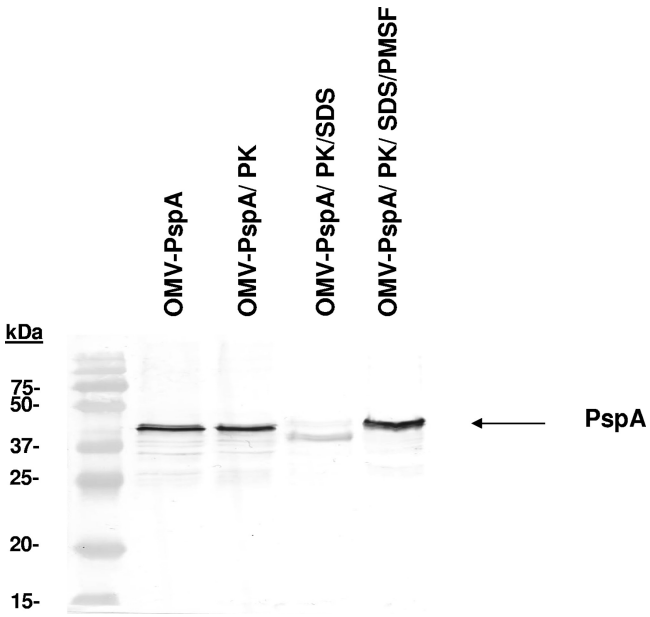

FIG. 2. PspA is associated with the lumens of OMVs. Purified *S. enterica* serovar Typhimurium vesicles (OMV-PspA) were incubated with proteinase K (PK) and without proteinase K for 30 min at 37°C. Some vesicle preparations were lysed with 1% SDS, in the absence or presence of PMSF, prior to treatment with proteinase K. After proteinase K treatment, vesicles were subjected to SDS-PAGE and immunoblot analysis using anti-PspA antibodies. The leftmost lane contains molecular mass markers.

immunized animals 3 weeks after the final immunization. Serum IgG responses against OMVs from three individual experiments were determined simultaneously to reduce experimental variation. Since all the experiments gave similar results, the data were pooled for analysis. Serum IgG responses against intact OMVs (Fig. 3A), *Salmonella* LPS (Fig. 3B), and *Salmonella* outer membrane proteins (Fig. 3C) in groups that received OMVs and OMVs containing PspA were significantly higher than the control or PspA-immunized groups ($P < 0.05$), where no responses were detected. Only mice immunized with OMVs containing PspA developed significant anti-PspA immune responses compared to other groups ($P < 0.05$) (Fig. 3D). Of the mice that received PspA protein intranasally, only one of 25 mice immunized with 10 μg of PspA showed a positive immune response to PspA, while none of the mice receiving 350 ng of PspA seroconverted.

Analysis of secretory IgA responses against OMVs revealed a pattern similar to the pattern of serum IgG responses (Fig. 4). Both groups that received OMVs developed significantly higher IgA responses against OMVs than the control or PspA-immunized groups (Fig. 4A). While significant IgG responses to PspA were detected in the sera of OMV-PspA-immunized animals, mucosal IgA responses to PspA were very weak and were not significantly different from those of control mice (Fig. 4B). This is surprising, since mucosal IgA responses to OMVs in mice immunized with OMVs containing PspA were significantly higher than those of the control mice. The sensitivity of detection for IgA responses may be lower than that for IgG responses, and hence, the IgA responses to PspA may be too low to be detected.

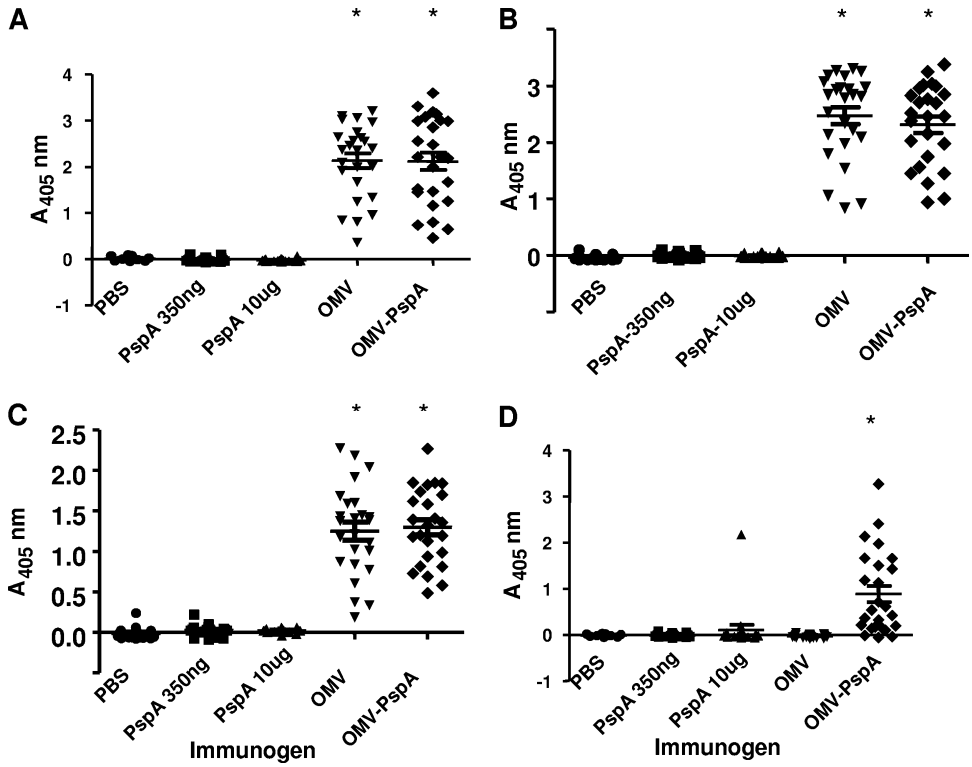

FIG. 3. Serum IgG responses in mice immunized with PspA, OMVs, or OMVs containing PspA (OMV-PspA). Groups of mice were immunized intranasally four times at weekly intervals with 350 ng PspA, 10 μg PspA, OMVs, OMV-PspA (an amount equivalent to 350 ng PspA), or PBS. OMVs and OMV-PspA were isolated from strain χ9281(pYA3493) and χ9281(pYA4088), respectively. Sera were collected 3 weeks after the final immunization. Sera were diluted 1:100 and analyzed by ELISAs for IgG responses against OMV (A), *S.* Typhimurium LPS (B) *Salmonella* OMPs (SOMPs) (C), and PspA (D). Combined data from three independent experiments are shown. The means ± standard errors (error bars) for the groups from triplicate experiments are shown. Each symbol represents the value for an individual mouse. IgG responses in mice immunized with OMV or OMV-PspA were significantly different from the responses of the control mice given PBS and other groups given OMVs, LPS, and SOMPs (*, $P < 0.05$). The anti-PspA responses in mice immunized with OMV-PspA were significantly different ($P < 0.05$) from those of all other groups (*).

Protection against challenge with *S. pneumoniae*. To determine whether the antibody responses obtained upon immunizations with OMVs containing PspA correlated with protection, the immunized mice were challenged against a pneumococcal infection in a sepsis model. Mice were challenged via the intraperitoneal route with 2×10^3 CFU (10× LD_{50}s) or 4×10^4 CFU (200× LD_{50}s) of *S. pneumoniae* WU2 5 weeks after the final immunization. We found that immuni-

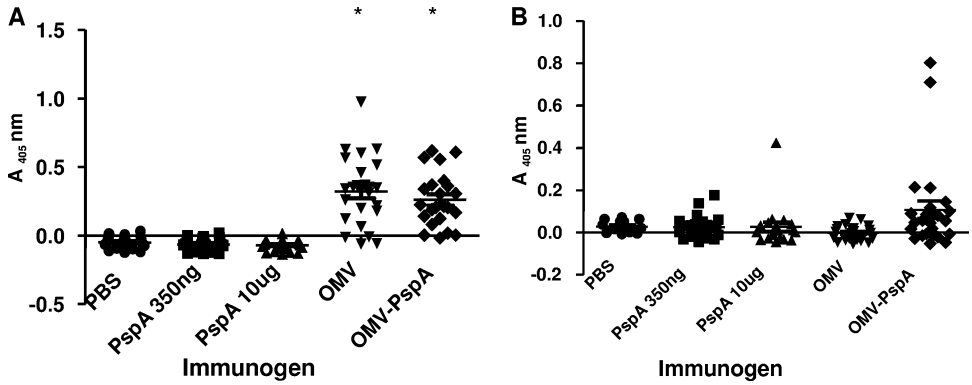

FIG. 4. Secretory IgA responses to OMVs in vaginal secretions. Vaginal secretions were collected from immunized mice 3 weeks after the final immunization, and the mucosal IgA responses against OMV(A) and PspA (B) were determined by ELISAs. The vaginal lavage fluid samples were diluted 1:10 and assayed. Combined data from three independent experiments are shown. The means ± standard errors (error bars) for the groups from triplicate experiments are shown. Each symbol represents the value for an individual mouse. OMV-specific IgA responses in OMV- and OMV-PspA-immunized groups were significantly different from the control group given PBS (*, $P < 0.05$). There were no differences in the anti-PspA responses between groups.

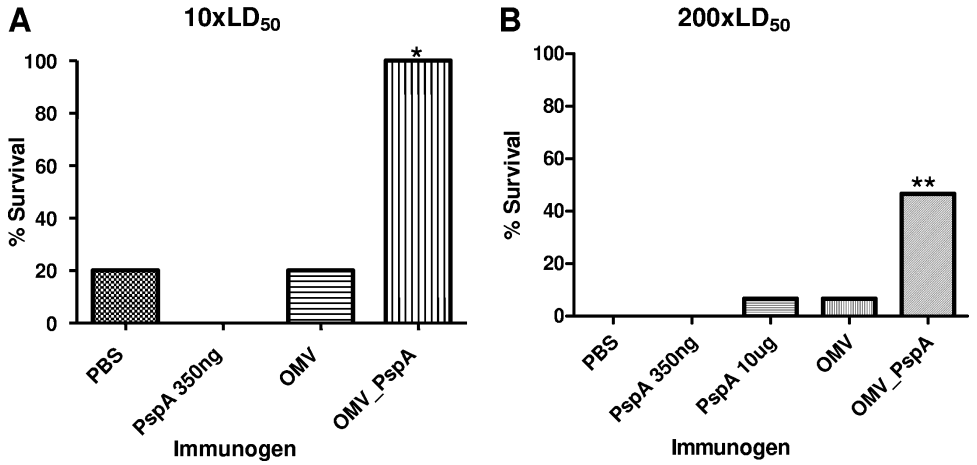

FIG. 5. Protective immunity against *S. pneumoniae* WU2 challenge. Groups of mice were immunized intranasally four times at weekly intervals with 350 ng PspA, 10 μg PspA, OMVs, OMVs containing PspA (OMV_PspA) (an amount equivalent to 350 ng PspA), or PBS. Five weeks after the final immunization, all mice were challenged with either 2,000 CFU (A) or 40,000 CFU (B) of *S. pneumoniae* WU2. Mortality was monitored for 2 weeks after the challenge. The values for the OMV_PspA groups were significantly different from those for the control groups given PBS (*, $P < 0.05$; **, $P < 0.0001$).

zation with OMVs containing PspA provided complete protection against the low-dose *S. pneumoniae* challenge, which was significantly greater than for any of the other treatment groups (Fig. 5) ($P < 0.05$). Immunization with OMVs containing PspA also provided significant protection against the high-dose pneumococcal challenge compared with nonvaccinated mice that received PBS intranasally ($P < 0.0001$). In the group that received 10 μg of PspA, one mouse survived the challenge. This was the same mouse that developed an anti-PspA serum IgG response (Fig. 3A). All mice in the nonvaccinated control groups succumbed to the infection. The experiments using a high dose of the challenge strain was repeated two times, confirming our results.

DISCUSSION

There have been a number of reports describing the packaging of heterologous proteins into OMVs (5, 15, 27, 35). In one study, OMVs were prepared from *Salmonella* containing one of two tested *Leishmania* antigens (32). The OMVs were used to subcutaneously boost mice orally immunized with a live *Salmonella* vaccine strain synthesizing the same antigen. The results from that study demonstrated that immunization with OMVs elicits a significant boost in the immune responses to both the *Salmonella* carrier and the vectored antigen.

The use of vesicles as vaccines has been extensively explored in *Vibrio cholerae* (30, 31) and *Neisseria* species (10, 34, 40, 41). In one study, female mice were immunized with OMVs from *Vibrio cholerae*, and their offspring were protected against subsequent challenge by *V. cholerae*. This result established that the intranasal (mucosal) route of immunization leads to a strong immune response against both the OMV components and a vectored antigen (30).

Our study expands upon these previous studies in several important areas. The construction of ClyA fusions has been favored by several groups as an effective way to direct heterologous antigens into vesicles (5, 8). Other studies have shown that heterologous proteins from other Gram-negative bacteria can be packaged into OMVs by directing their secretion into the periplasm or making fusions with other OMV proteins (15, 17, 30). Our data demonstrate that localization of a protein from a Gram-positive organism, pneumococcal PspA, into the *Salmonella* periplasm by fusion to the β-lactamase secretion signal leads to its packaging and release in outer membrane vesicles (Fig. 1), further establishing the utility and versatility of the system for delivery of a variety of antigens. Furthermore, PspA is incorporated into the lumens of OMVs as demonstrated by proteinase protection assays (Fig. 2).

In a recent study, OMVs containing a ClyA-green fluorescent protein (GFP) fusion protein were prepared from *E. coli* and used to subcutaneously immunize mice, inducing an anti-ClyA-GFP serum response similar to that of control mice immunized with ClyA-GFP mixed with an alum adjuvant (5). The ability of OMVs to act as a mucosal adjuvant is evident from our results. No anti-PspA antibody responses were observed in mice immunized with 350 ng of purified protein, whereas mice immunized with an equivalent amount of PspA delivered in OMVs developed a significant anti-PspA serum IgG response (Fig. 3D). Upon further analysis, we demonstrated that the responses to native OMV components included responses to both LPS and *Salmonella* outer membrane proteins (Fig. 3B and C).

One mouse immunized with 10 μg PspA developed a strong anti-PspA antibody response. This was the only mouse given either dose of purified protein to survive *S. pneumoniae* challenge (Fig. 5). In contrast, immunization with OMVs containing PspA (350 ng) led to complete protection against a 10× LD_{50} dose challenge and nearly 50% protection against the 200× LD_{50} challenge. These results are comparable with previous results obtained after giving a single dose of a live attenuated *Salmonella* vaccine strain carrying pYA4088 (38).

The IgA responses to PspA in vaginal secretions were lower than corresponding IgA responses seen against native OMV components. This may be a reflection of the reduced quantities of PspA associated with OMVs and also its location inside the

lumens of OMVs rather than on the surfaces of the OMVs. In addition, while it is known that mice have a common mucosal system and intranasal immunization with nonreplicating immunogens can lead to robust immune responses at distal sites, including in vaginal secretions (36), it is well established that there is some compartmentalization of the mucosal system (discussed in reference 37). Such compartmentalization can lead to a reduction in the amount of antigen-specific antibody at distal sites. This, too, may have played a role in our results.

Future studies will determine whether changing the localization of PspA to the surfaces of OMVs instead of in the lumen will elicit superior mucosal antibody responses. Taken together, these results indicate that mucosal immunization with OMVs loaded with a heterologous antigen is a viable method for inducing protective immunity.

Further studies will be required to see whether the anti-PspA immune responses can be enhanced by changing the location of antigen within the OMVs or whether immune responses against heterologous proteins can be improved by increasing the dose of OMVs administered. It will also be of interest to investigate whether the mucosal immunity provided by OMVs loaded with PspA or other pneumococcal proteins will provide protection against colonization by *S. pneumoniae*.

Vesicles produced by bacterial pathogens can facilitate delivery of toxins directly into host cells. ClyA-containing vesicles from *Salmonella* enhances toxin delivery 8-fold in tissue culture compared to purified protein (35). Heat-labile toxin from enterotoxigenic *E. coli* is most likely delivered exclusively by OMVs (12, 16). Thus, OMV production by pathogenic bacteria has evolved into an efficient protein delivery system (11, 16) that may be exploited for other purposes, including vaccine development.

Therefore, the results reported here may provide useful information regarding the design of live attenuated *Salmonella* vaccines. *Salmonella* is known to produce OMVs inside host cells (9), and *Salmonella*-derived OMVs have been shown to stimulate dendritic cells and prime both B and T cell responses (1). Previous work in our laboratory showed that a live *Salmonella* vaccine designed to secrete PspA was more immunogenic than an isogenic strain that produced PspA only in the cytoplasm (13). It is tantalizing to speculate that one reason for the enhanced immunogenicity of the former strain is due to the production of PspA-containing vesicles *in vivo*. Production of these proteoliposome structures *in vivo* could provide a second route of antigen delivery, in addition to delivery into host lymphoid tissues directly by *Salmonella*. If this were true, then it is possible that identification and inclusion of mutations that enhance vesicle production by live *Salmonella* vaccines without reducing the overall fitness of the cell, in conjunction with a design that facilitates secretion of the antigen to the periplasm, will increase the overall immunogenicity of the vectored antigen.

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