Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine

Ho Young Kang  
*Washington University School of Medicine in St. Louis*

Jay Srinivasan  
*Washington University School of Medicine in St. Louis*

Roy Curtiss III  
*Washington University School of Medicine in St. Louis*

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Immune Responses to Recombinant Pneumococcal PspA Antigen Delivered by Live Attenuated Salmonella enterica Serovar Typhimurium Vaccine

Ho Young Kang,† Jay Srinivasan, and Roy Curtiss, III*

Department of Biology, Washington University, St. Louis, Missouri 63130

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Attenuated Salmonella enterica serovar Typhimurium expressing recombinant antigens from other pathogens elicits primarily a Th1-type dominant immune response to both recombinant and Salmonella antigens. The immunogenicity and appropriate subcellular location of the recombinant antigen in the Salmonella vaccine strain may contribute to augmenting immune responses by facilitating adequate exposure of recombinant antigen to antigen-presenting cells for processing. To allow for secretion from gram-negative bacteria and overexpression of antigen, a DNA fragment encoding a highly antigenic α-helical region of PspA (pneumococcal surface protein A) was subcloned downstream from the β-lactamase signal sequence in the multicopy Asd− pYA3493 vector to create pYA3494. pYA3493 was derived from a class of Asd+ vectors with reduced expression of Asd to minimize selective disadvantage and enhance immunization of expressed recombinant antigens. The S. enterica serovar Typhimurium vaccine strain was constructed by the introduction of deletion mutations Δcrp-28 and ΔasdA16. Approximately 50% of the recombinant PspA (rPspA) expressed in a Salmonella strain harboring pYA3494 was detected in the combined supernatant and periplasmic fractions of broth-grown recombinant Salmonella. After a single oral immunization in BALB/c mice with 10⁷ CFU of the recombinant Salmonella vaccine strain carrying pYA3494, immunoglobulin G (IgG) antibody responses were stimulated to both the heterologous antigen rPspA and Salmonella lipopolysaccharide (LPS) and outer membrane proteins (OMPs). About half, and even more at later times after immunization, of the antibodies induced to rPspA were IgG1 (indicating a Th2-type response), whereas 60 to 70% of the antibodies to LPS and 80 to 90% of those to OMPs were IgG2a (indicating a Th1-type response). A sublethal infection with Streptococcus pneumoniae WU2 boosted PspA antibody levels and maintained IgG2a/IgG1 ratios similar to those seen before the challenge. Oral immunization with Salmonella-PspA vaccine protected 60% of immunized mice from death after intraperitoneal challenge with 50 times the 50% lethal dose of virulent S. pneumoniae WU2.

Orally administered Salmonella enterica serovar Typhimurium colonizes the gut-associated lymphoid tissue (Peyer’s patches) and the secondary lymphatic tissues, including the liver and spleen, to elicit anti-Salmonella immune responses during infection of the mouse. (17). The immune responsiveness to orally administered Salmonella has been applied to develop live attenuated oral Salmonella vaccines (13). Attenuated Salmonella vaccines have been constructed by introduction of mutations in the genes required for virulence, including the cyclic AMP receptor protein gene (crp) (12). Crp is a global regulator involved in a variety of biological functions, including carbohydrate utilization (4). Attenuated Salmonella vaccine strains have been genetically modified to express another pathogen’s antigen (s) specified by multicity plasmids. These recombinant vaccines induce immunity to the pathogen whose antigen gene is expressed as well as to Salmonella. It is essential that the antigen-specifying plasmids in Salmonella vaccines are stably maintained during the in vivo colonization process. A balanced-lethal host-vector system based on the essential bacterial gene for aspartate β-semialdehyde dehydrogenase (asd) has been used to specify recombinant antigens from Asd− plasmids that are retained in vivo in Salmonella vaccine strains with the asd gene deleted (16, 36).

Streptococcus pneumoniae is a human pathogen that causes life-threatening diseases, including community-acquired pneumonia, otitis media, meningitis, and bacteremia, in persons of all ages (35). S. pneumoniae is the leading cause of childhood pneumonia worldwide, resulting in about 3 million deaths per year (21). The recent emergence of antibiotic-resistant strains has the potential to threaten the treatment of pneumococcal disease in the near future (5). Thus, the development of a safe, effective, and lower-cost antipneumococcal vaccine is urgently needed. Capsular polysaccharide-based pneumococcal vaccines are currently available and are moderately effective. A 23-valent pneumococcal polysaccharide vaccine is recommended for the prevention of infection in adults (48), and a 7-valent conjugated polysaccharide vaccine is licensed for use in children (49). However, vaccination with the pneumococcal polysaccharide vaccine does not reduce the frequency of hospitalization, costs, and mortality caused by pneumococcal pneumonia (23), which reinforces the need for effective new vaccines.

Studies on the protective efficacy of subunit vaccines may further the development of a more protective pneumococcal vaccine. The pneumococcal PspA (pneumococcal surface protein A) protein has been evaluated and considered to be a
Expression of recombinant PspA (rPspA) in this recombinant vaccine strain was somewhat toxic, such that the stability and protection of mice against challenge with virulent S. pneumoniae (6, 8, 9, 25). Native PspA Rx1 (PspA originating from S. pneumoniae strain Rx1) contains several functional domains: an N-terminal signal sequence, an α-helical region, a proline-rich domain, 10 tandem-repeat choline-binding regions, and a 17-amino-acid residue carboxy terminus. Pneumococcal protection assays with mice immunized with various recombinant PspARx1 oligopeptides showed that the synthetic PspA reached the supernatant about 25% of the synthesized PspA reached the supernatant after 24 h of growth as a standing culture in the presence of diaminopimelic acid (DAP). This phenomenon forced us to design an improved plasmid vector to enable stable expression of rPspA in attenuated Salmonella. An additional goal of our research is to construct recombinant attenuated Salmonella vaccines that induce higher immune responses to the foreign expressed antigen than to Salmonella antigens.

In this work, we constructed a stable multicopy Asd+ anti- gen expression vector encoding the β-lactamase signal sequence-based periplasmic secretion plasmid for the expression of His-tagged PspA. This plasmid was designed to translocate PspA into the periplasmic space of the Salmonella vaccine strain, although about 25% of the synthesized PspA reached the supernatant fluid without cell lysis. We report the immunogenicity, type of immune responses, and protection against both Salmonella and S. pneumoniae in mice immunized with a Salmonella vaccine expressing rPspA by an improved antigen expression system.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriophage P22HTa (46) was used for generalized transduction. Escherichia coli and S. enterica serovar Typhimurium vaccine cultures were grown at 37°C in Lennox broth (29) or Luria-Bertani (LB) broth or on LB agar (1). MacConkey agar (Difco, Detroit, Mich.) supplemented with 1% sugar was used for fermentation assays. When required, antibiotics were added to culture media at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 30 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 15 µg/ml. DAP was added (50 µg/ml) for the growth of Asd- strains (36). LB agar containing 5% sucrose was used for sucB gene-based

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Derivation, source, or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>F- λ- φ80 Δ(lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4</td>
<td>36</td>
</tr>
<tr>
<td>MGN-617</td>
<td>thi-1 thr-1 leuB6 hisX422 lacY1 glmV44 ΔasdA4 recA1 RP4 Tc4-21::Mu</td>
<td>44</td>
</tr>
</tbody>
</table>

*S. enterica* serovar Typhimurium

χ3339 SL1344 hisG
χ4550 SR-11 gyrA1816 Δcrp-1 ΔasdA1 Δzif-4::Tn10 Δcyr-1
χ4746 χ3339 nadA540::Tn10 Δ(galE-chl-uvrB)-1005; Tet<sup>+</sup>
χ8499 hisG Δcrp-28
χ8501 hisG Δcrp-28 ΔasdA16
χ8554 hisG ΔasdA16
χ8599 hisG ΔasdA16 atrB13::MudJ
JF2430 LT2 atrB13::MudJ
S. pneumoniae WU2 Wild-type virulent, encapsulated type 3

Plasmids

| pYA3193 | Asd<sup>+</sup> vector harboring 1.5-kb C-terminally truncated *pspA* gene; pUCori | 37 |
| pYA3333 | Asd<sup>+</sup>; pBRori | Lab collection |
| pYA3334 | Asd<sup>+</sup>; pUCori | Lab collection |
| pYA3341 | Asd<sup>+</sup>; pUCori | This study |
| pYA3342 | Asd<sup>+</sup>; pBRori | This study |
| pYA3493 | pYA3342 derivative β-lactamase signal sequence-based periplasmic secretion plasmid | This study |
| pYA3494 | 0.7-kb DNA encoding the α-helical region of PspA in pYA3493 | This study |
| pYA3496 | 0.7-kb DNA encoding the α-helical region of PspA in pYA3496 | This study |
| pBR322 | Cloning vector; Ap<sup>+</sup> Tc<sup>+</sup> | 3 |
| pMEG-443 | Recombinant suicide plasmid to generate *Salmonella* ΔasdA16 mutant; Ap<sup>+</sup> Cm<sup>+</sup> | 27 |
| pMEG-493 | Recombinant suicide plasmid to generate *Salmonella* Δcrp-28 mutant; Ap<sup>+</sup> Cm<sup>+</sup> | Megan Health Inc. |

<sup>a</sup> Ap<sup>+</sup>, ampicillin resistance; Cm<sup>+</sup>, chloramphenicol resistance; Km<sup>+</sup>, kanamycin resistance; Tc<sup>+</sup>, tetracycline resistance.

**pneumococcal vaccine candidate** because of its immunogenicity and protection of mice against challenge with virulent S. pneumoniae (6, 8, 9, 25). Native PspARx1 (PspA originating from S. pneumoniae strain Rx1) contains several functional domains: an N-terminal signal sequence, an α-helical region, a proline-rich domain, 10 tandem-repeat choline-binding regions, and a 17-amino-acid residue carboxy terminus. Pneumococcal protection assays with mice immunized with various recombinant PspARx1 oligopeptides showed that the α-helical domain contains the protective epitopes (7). In a previous study, mice orally immunized with an S. enterica serovar Typhimurium vaccine strain expressing a recombinant PspARx1 (from the ATG start codon specifying the native signal sequence, the entire α-helical domain, and up to the fifth tandem repeat) showed PspA-specific immune responses and were protected against challenge with virulent S. pneumoniae (37). Expression of recombinant PspA (rPspA) in this recombinant Salmonella vaccine strain was somewhat toxic, such that the high-copy-number plasmid pYA3193 (pUC ori) was relatively unstable. Thus, approximately 50% of cells lost the plasmid after 24 h of growth as a standing culture in the presence of diaminopimelic acid (DAP). This phenomenon forced us to design an improved plasmid vector to enable stable expression of rPspA in attenuated Salmonella. An additional goal of our research is to construct recombinant attenuated Salmonella vaccines that induce higher immune responses to the foreign expressed antigen than to Salmonella antigens. In this work, we constructed a stable multicopy Asd<sup>+</sup> antigen expression vector encoding the β-lactamase signal sequence-fused in frame to the immunogenic α-helical region of PspA. This plasmid was designed to translocate PspA into the periplasmic space of the Salmonella vaccine strain, although about 25% of the synthesized PspA reached the supernatant fluid without cell lysis. We report the immunogenicity, type of immune responses, and protection against both Salmonella and S. pneumoniae in mice immunized with a Salmonella vaccine expressing rPspA by an improved antigen expression system.
counterselection in allelic exchange experiments (18). *S. pneumoniae* WU2 was cultured in brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth grown to 0.5% optical density at 600 nm (OD600).

**General DNA procedures.** DNA manipulations were carried out as described by Sambrook et al. (45). Transformation of *E. coli* and *Salmonella* was done by electroporation (Bio-Rad, Hercules, Calif.). Transformants containing Asd− plasmids were selected on L agar plates without DAP. Only clones containing the recombinant plasmids were able to grow under these conditions. Transfer of recombinant suicide plasmids to *Salmonella* was accomplished by conjugation using *E. coli* MG1615 (Asd−) (44) as the plasmid donor. Bacteriophage P22HIT int-mediated general transduction was performed by standard methods (52). PCR amplification was employed to obtain DNA fragments for cloning and for verification of chromosomal deletions. The PCR conditions were as follows: denaturation at 95°C for 20 s, primer annealing at 55°C for 30 s, and extension at 72°C for 2 min. The PCR products were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie brilliant blue R250 (Sigma, St. Louis, Mo.) staining. For immunoblotting, proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin in 10 mM Tris-HCl buffer (pH 7.4) and incubated with mouse monoclonal antibodies to *Salmonella* outer membrane proteins. The cell pellets were resuspended in 800 μl of 100 mM Tris-HCl buffer (pH 9.8) containing 0.9% NaCl (pH 7.4) and incubated with 1:100 diluted protein. The cell lysates were centrifuged at 132,000 × g for 1 h to separate the soluble fraction and intact cell envelopes. The soluble fraction contained the cytoplasmic proteins. To isolate the outer membrane fraction, total envelope pellets were suspended in 4 ml of 20 mM Tris-HCl (pH 8.6) containing 1% Sarkosyl and incubated for 30 min on ice. The outer membrane fraction was obtained as a pellet after centrifugation at 132,000 × g for 1 h. The pellet was resuspended in 4 ml of 20 mM Tris-HCl buffer (pH 8.6). The original culture supernatant was filtered (0.22 μm-pore filter), and secreted proteins were precipitated with 10% trichloroacetic acid (1 h, 4°C). An equal volume of each fraction sample was separated by SDS-PAGE for Western blot analysis. By using the outer membrane protein preparation procedure described above, *Salmonella* outer membrane proteins (SOMPs) were prepared from *S. enterica* serovar Typhimurium X4746 cells grown in LB broth without galactose for analysis by enzyme-linked immunosorbent assay (ELISA). The use of SOMPs obtained from X4746 precludes LPS-O-antigen contamination.

**Construction of an *S. enterica* serovar Typhimurium vaccine strain.** The Δcrp mutation was introduced into *S. enterica* serovar Typhimurium χ3339 by allelic exchange using the suicide vector pMEG-493 to yield χ2499. The presence of the 680-bp deletion was confirmed by PCR with a primer set flanking crp (5′-AAA GTGCAAAGTGAAAGC-3′ and 5′-CGTAGACGACGATGTGCTG-3′) and a strain phenotype of Mal− and nonmotility. The ΔasdA16 mutation was then introduced into χ2499 by allelic exchange using the suicide vector pMEG-443 integrated into a strain with the ΔasdA16 mutation, followed by sucrose selection to eliminate the suicide vector to yield χ5801 (27). The presence of the 1,242-bp asd deletion in χ5801 was confirmed by PCR using flanking a primer set (5′-CGGAAATGTTCCCTCTCTAACG-3′ and 5′-TATCTGCTGCTCGCTCCTAC-3′).

**Immunization of mice.** Two groups of five inbred 7-week-old female BALB/c mice were deprived of food and water for 4 h before infection. The recombinant *S. enterica* serovar Typhimurium χ5801(pYA3943) vaccine (1.9 × 109 CFU) grown in LB broth to an OD600 of 0.8 was orally administered to BALB/c mice. The recombinant *S. enterica* serovar Typhimurium χ5801(pYA3943) vaccine (2 × 109 CFU in 20 μl of BSG) was used as a vector control. Food and water were returned to the immunized mice 30 min after immunization. Blood was obtained by retro-orbital puncture with heparinized capillary tubes at biweekly intervals. Following centrifugation at 4,000 × g for 5 min, the serum was removed from the whole blood and stored at −20°C. Vaginal secretion specimens were collected in a 50-μl BSG wash and stored at −20°C (59).

**Pneumococcal challenge.** To observe the immune response caused by pneumococcal infection after vaccination, a sublethal dose of 3.8 × 106 *S. pneumoniae* W2U CFU in 200 μl of BSG was administered by intravenous (i.v.) injection to *S. enterica* serovar Typhimurium vaccine-immunized BALB/c mice at 16 weeks after primary immunization. The secondary immunization was performed by intraperitoneal injection of 50 μl BSG. The 50% lethal dose (LD50) of *S. pneumoniae* W2U in BALB/c mice was >106 CFU by i.v. administration (67). ELISA. ELISA was used to assay antibodies in vaginal secretions and serum to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPsPa. Polystyrene 96-well flat-bottom microtiter plates (DYNatech Laboratories Inc., Chantilly, Va.) were coated with *S. enterica* serovar Typhimurium LPS and SOMPs (100 ng/well; Sigma), SOMPs (100 ng/well), or purified rPsPa (100 ng/well). Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied with 100-μl volumes in each well. The coated plates were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. Free binding sites were blocked with a blocking buffer (phosphate-buffered saline [pH 7.4], 0.1% Tween 20, and 1% bovine serum albumin). Vaginal secretions and sera obtained from the same experimental group (five mice per group) were pooled and diluted 1:10 and 1:600, respectively. A 100-μl volume of diluted sample was added to individual wells in duplicate and incubated for 2 h at 37°C. Plates were treated with blocking buffer, goat anti-mouse IgG, or IgA (Sigma Chemical Co., Milwaukee, Ala.) for sera and IgA for vaginal secretions. Wells were developed with streptavidin-alkaline phosphatase conjugate (Southern Biotechnology Inc., Birmingham, Ala.) for sera and IgA for vaginal secretions. Absorbance was measured by an automated ELISA plate (model EL311SX; Biotek, Winooski, Vt.). Absorbance

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RESULTS

Construction of Asd+ vectors to use for antigen expression. Vaccine strains harboring multicopy Asd+ vectors (pBR ori or pUC ori) containing the entire asd gene with its promoter synthesized the Asd protein at a much higher level than necessary to complement the chromosomal asd mutation in a balanced-letal host-vector system. In fact, the 200- to 300-fold excess production of Asd in a strain such as χ8554 (Δasd16) with the pYA3334 Asd+ vector (pUC ori) increases the generation time slightly and the LD50 10-fold compared to the same strain with an Asd+ vector with the pSC101 ori or p15A ori. In an attempt to reduce the level of Asd, the asd promoter region was deleted to determine whether there would be sufficient transcription to permit a promoterless asd gene to complement the chromosomal Δasd16 mutation. The asd gene sequence was amplified by PCR starting at bp 286 and ending at bp 1421 of the S. enterica serovar Typhimurium asd sequence (accession number AF015781) with an N-terminal BgII site and a C-terminal XhoI site. This sequence contains the Shine-Dalgarno (SD) sequence for ribosome recognition but lacks the −35 and −10 promoter sequence and ends just after the asd gene TAG stop codon. The BgII-XhoI DNA fragment was used to construct Asd+ vectors pYA3342 (pBR ori) and pYA3341 (pUC ori) (Table 1). It was possible to clone this fragment onto pSC101 ori or p15A ori vectors, but this did not result in sufficient Asd to permit construction of balanced-lethal systems with strains such as χ8554 which could grow in the absence of DAP. Both pYA3342 and pYA3341 complemented the asd mutations of E. coli χ6212 and S. enterica serovar Typhimurium χ4550. Salmonella strains possessing pYA3342 and pYA3341 produced significantly reduced amounts of Asd protein (39 kDa) compared to strains containing plasmids that had asd genes with the asd native promoter (Fig. 1). pYA3342 and pYA3341 in a Δasd S. enterica serovar Typhimurium strain such as χ8554 yielded recombinants that had wild-type LD50s following oral inoculation of BALB/c mice. Plasmid pYA3342 (Fig. 2A) was used for further construction.

Since export of PsP into the periplasmic space of Salmonella was inefficient and caused toxicity when the export depended on the signal sequence for PsP (37), we constructed a recombinant plasmid by cloning a DNA fragment specifying the signal sequence of β-lactamase which is efficiently translated into the periplasmic space in Salmonella. A 105-bp DNA fragment (nucleotides 4049 to 4153 of the sequence under accession number J01749) of the β-lactamase gene was amplified from the pYA3193 DNA template with a pair of primers (N-terminal, 5′-GCATTCATGAGTATTCAA CATTCC3′-BspHI [underlined]; C-terminal, 5′-CCGGAAATT CCTCAGCATTTTACGCT3′-EcoRI [underlined]). The PCR-amplified fragment included the N terminus of β-lactamase from the ATG start codon through the signal sequence (23 amino acids) plus 12 amino acids of the N terminus of the mature β-lactamase. These additional 12 amino acid residues were included to increase the efficiency of secretion of the recombinant protein (53). The 105-bp PCR product was digested with BspHI and EcoRI enzymes and cloned into the NcoI site (compatible with the BspHI site) and EcoRI site of the Asd+ vector pYA3342, resulting in plasmid pYA3493 (Fig. 2B). The in-frame position of the β-lactamase signal sequence was confirmed by nucleotide sequencing. Transcription promoted by Prrec can be stopped by the 5′ST172 transcriptional terminator located following the multicloning sites. pYA3493 was stably maintained for 50 or more generations in E. coli χ6212 and S. enterica serovar Typhimurium (Δasd) hosts grown in the presence or absence of DAP.

Construction of the rPspA-expressing plasmid. A highly immunogenic α-helical region of PsP from amino acid residue 3 to 257 (765 bp; 255 amino acids) of the mature PsP6212 protein (588 amino acids) was selected to use as a test antigen in antigen delivery by a Salmonella carrier. The 765-bp DNA fragment of the pspA gene of S. pneumoniae Rx1 was PCR amplified from the pYA3193 DNA template with a pair of primers (N-terminal, 5′-CCGGAAATT CCTCAGCATTTTACGCT3′-EcoRI [underlined]; C-terminal, 5′-CCGGAAATT CCTCAGCATTTTACGCT3′-EcoRI [underlined]). The PCR-amplified fragment included the N terminus of β-lactamase from the ATG start codon through the signal sequence (23 amino acids) plus 12 amino acids of the N terminus of the mature β-lactamase. These additional 12 amino acid residues were included to increase the efficiency of secretion of the recombinant protein (53). The 105-bp PCR product was digested with BspHI and EcoRI enzymes and cloned into the NcoI site (compatible with the BspHI site) and EcoRI site of the Asd+ vector pYA3342, resulting in plasmid pYA3493 (Fig. 2B). The in-frame position of the β-lactamase signal sequence was confirmed by nucleotide sequencing. Transcription promoted by Prrec can be stopped by the 5′ST172 transcriptional terminator located following the multicloning sites. pYA3493 was stably maintained for 50 or more generations in E. coli χ6212 and S. enterica serovar Typhimurium (Δasd) hosts grown in the presence or absence of DAP.

Expression and subcellular localization of rPsP in Salmonella. An S. enterica serovar Typhimurium strain was constructed to examine expression and subcellular localization of rPsP. The attB13::MudJ allele (15), causing constitutive expression of β-galactosidase, in S. enterica serovar Typhimurium...
JF2430 was transduced into *S. enterica* serovar Typhimurium \( \text{H9273} \) by P22HT int-mediated generalized transduction (52), resulting in \( \text{H9273} \text{8599} \) (hisG \( \text{H9004} \) asdA16 atrB13::MudJ). \( \text{H9273} \text{8599} \) was Lac\( ^+ \) on MacConkey agar plus lactose and DAP. \( \text{H9252} \)-Galactosidase production from the atrB13::MudJ allele in \( \text{H9273} \text{8599} \) was used as a cytoplasmic protein marker and as an indicator of membrane leaking in the examination of subcellular fractionations. No periplasmic protein marker was used, since the use of the \( \text{H9252} \)-lactamase signal sequence in the pYA3494 construct precluded use of \( \text{H9252} \)-lactamase and the commercially available monoclonal antibody to the *E. coli* maltose binding protein (Sigma) did not react with this protein from *S. enterica* serovar Typhimurium. To observe rPspA expression, plasmid pYA3494 was introduced into *S. enterica* serovar Typhimurium \( \text{H9273} \text{8599} \). \( \text{H9273} \text{8599} \) harboring pYA3493 (vector alone) was used as the control (data not shown).

With the expectation of the periplasmic secretion of the rPspA, various subcellular fractions, including cytoplasm, periplasm, outer membrane, and culture supernatant of \( \text{H9273} \text{8599} \) (pYA3494), were prepared to examine the location of rPspA. Although the calculated size of rPspA was approximately 30 kDa, PspA-specific monoclonal antibody XI126 reacted with an approximately 35-kDa protein (Fig. 4). Aberrant migration of a PspA protein has been seen in previous studies (37, 54). Although a large amount of the rPspA resided in the cytoplasmic fraction, half of the rPspA was detected in the periplasmic fraction and the culture supernatant fluid. Little or no rPspA was detected in the outer membrane fraction. Denaturing analyses of immunoreactive bands showed that approximately 50% of the rPspA was located in the combined periplasm (25%) and culture supernatants (25%). In the immunoblot analyses of subcellular fractions with anti-\( \text{H9252} \)-galactosidase and -OmpC monoclonal antibodies, the \( \text{H9252} \)-galactosidase and OmpC proteins were detected in the cytoplasm and outer membrane fractions, respectively, suggesting that the rPspA detected in the periplasmic fraction and culture supernatant fluid was actively secreted instead of resulting from nonspecific membrane leaking or cell death by lysis.

Recombinant *S. enterica* serovar Typhimurium \( \Delta \text{crp-28} \) vaccine expressing rPspA antigen. pYA3493 (vector control) and pYA3494 (encoding rPspA) were electroporated into the \( \text{H9004} \text{crp-28} \text{H9004} \text{asdA16} \) strain \( \text{H9273} \text{8501} \). The *S. enterica* serovar Typhimurium \( \chi8501 \) (\( \Delta \text{crp-28} \Delta \text{asdA16} \)) vaccine strain containing pYA3494 expressed the rPspA protein at an approximate molecular mass of 35 kDa. In the analyses of Coomassie blue-stained SDS-polyacrylamide gels, the amount of rPspA protein was as much as approximately 1 to 2% of the total \( \text{H9273} \text{8501} \) proteins (Fig. 5). With results consistent with those seen in the rPspA localization analysis (75% of rPspA cell associated [50% in cytoplasm and 25% in periplasm] and 25% of rPspA secreted), the rPspA expressed in the \( \chi8501 \) vaccine strain was secreted into the culture supernatant along with other secreted proteins. To examine the stability of plasmids pYA3493 and pYA3494 in *Salmonella* \( \chi8501 \) in vitro,
χ8501 cells containing pYA3493 and pYA3494 were cultured with daily passage of 1:1,000 dilutions for five consecutive days in LB broth containing DAP. All χ8501 clones examined (300 clones/day) kept the Asd<sup>+</sup> plasmid pYA3493 and pYA3494, indicating that pYA3493 and pYA3494 were very stable in the χ8501 vaccine strain. Cells obtained from the last-day culture of the stability test expressed amounts of the 35-kDa rPspA equivalent to 30-μl volumes of the culture at an OD<sub>600</sub> of 0.8, except for supernatant fluids, were analyzed by SDS-PAGE, and the rPspA was detected by immunoblotting with PspA-specific monoclonal antibody Xi126 (33). β-Galactosidase and OmpC were used as fractionation controls for cytoplasmic and outer membrane fractions, respectively. Standards are indicated to the left. Lanes: 1, total cell lysate; 2, cytoplasm; 3, periplasm; 4, outer membrane; 5, concentrated supernatant (750 μl); 6, supernatant (10 μl).

Immune responses in mice after oral immunization with the recombinant S. enterica serovar Typhimurium vaccines. All mice orally administered a single dose of 1.3 × 10<sup>8</sup> CFU S. enterica serovar Typhimurium χ8501(pYA3493) (vector control) survived for the 30-day monitoring period. Those mice were protected for a 30-day observation period against wild-type χ3339 (LD<sub>50</sub>, <10<sup>6</sup> CFU) challenge (1.7 × 10<sup>9</sup> CFU) 30 days after the initial immunization. There were no survivors in a group of unimmunized mice challenged with 1.7 × 10<sup>9</sup> CFU of χ3339. These results indicate that χ8501 with an Asd<sup>+</sup> vector is avirulent for mice and elicits a protective immune response against challenge with S. enterica serovar Typhimurium.

A single dose of S. enterica serovar Typhimurium χ8501(pYA3494) (1.9 × 10<sup>9</sup> CFU) or χ8501(pYA3493) (control, 2 × 10<sup>9</sup> CFU) was orally administered to 7-week-old female BALB/c mice. All immunized mice survived, and we did not observe any signs of disease in the immunized mice during the entire experimental period. The antibody responses to Salmonella LPS and SOMPs and to the foreign antigen rPspA in the sera and the vaginal secretions of the immunized mice were measured. The serum IgG responses to LPS, SOMPs, and rPspA are presented in Fig. 6. At 2 weeks after administration, little IgG response to the antigens was observed. Maximal anti-LPS, -SOMP, and -rPspA IgG levels without boost immunization were detected at 6 weeks postimmunization.

At 17 weeks postimmunization, we infected mice i.v. with a sublethal dose (3.8 × 10<sup>5</sup> CFU) of the virulent S. pneumoniae WU2 strain to monitor the changes of anti-rPspA antibody titers. Sublethal i.v. infection with S. pneumoniae did not kill mice immunized with the χ8501(pYA3493) or χ8501 (pYA3494) vaccine. Because native PspA is a highly immunogenic pneumococcal surface protein, the pneumococcal challenge boosted rPspA-specific immune responses in χ8501 (pYA3494)-immunized mice (Fig. 6). In comparison to the anti-rPspA IgG level (A<sub>405</sub>, 0.81) at 12 weeks after S. enterica...
serovar Typhimurium χ8501(pYA3494) immunization, the pneumococcal challenge boosted 53% more anti-rPspA IgG (A405, 1.24) 1 week later. This suggests that the S. enterica serovar Typhimurium χ8501-rPspA vaccine induces immunological memory for a rapid responsiveness to subsequently administered PspA antigen. Anti-rPspA IgG was not detected in sera obtained from mice immunized with χ8501(pYA3493), the vector control vaccine. The χ8501(pYA3493) vaccine elicited anti-LPS and -SOMP IgG responses with kinetics and levels similar to those induced by χ8501(pYA3494). These results suggest that Salmonella-delivered rPspA antigen had minimal influence on the immune response to Salmonella itself.

IgA levels, mostly secretory IgA, for LPS, SOMPs, and rPspA in the vaginal fluids of immunized mice were measured. The χ8501(pYA3493) and χ8501(pYA3494) vaccines elicited anti-LPS and anti-SOMP IgA. rPspA-specific IgA was detected in the vaginal fluids from mice immunized with χ8501 (pYA3494) but not in those from mice immunized with the χ8501(pYA3493) vector-only control (Fig. 7).

IgG isotype analyses. The types of immune responses to Salmonella LPS and SOMPs and to the rPspA were further examined by measuring the levels of IgG isotype subclasses IgG2a and IgG1. The Th1-helper cells direct cell-mediated immunity and promote class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote class switching to IgG1 (39, 51). IgG2a isotype dominant responses were observed for the Salmonella LPS and SOMP antigens (Fig. 8). The IgG2a/IgG1 ratios for anti-LPS and -SOMPs in sera obtained from mice immunized with χ8501(pYA3493) were not significantly different from those from mice immunized with χ8501(pYA3494) (LPS, P = 0.07; SOMPs, P = 0.054). The IgG2a/IgG1 ratios for anti-SOMPs (ranging from 6.4 to 11.5) are higher than those for LPS (ranging from 1.1 to 2.5), with statistical significance [χ8501(pYA3493), P = 0.0004; χ8501(pYA3494), P = 0.0003]. Th1-type dominant immune responses are frequently observed after immunization with attenuated Salmonella (30, 40). In contrast to the type of immune responses to LPS and SOMPs, a Th1- and Th2-type mixed response was observed for the rPspA antigen. Although the IgG2a levels were higher than IgG1 levels in the early

FIG. 5. Expression of rPspA in the S. enterica serovar Typhimurium vaccine strain. χ8501 harboring pYA3494 (specifying rPspA) or pYA3493 (vector control) was cultured in LB broth at 37°C. Total cells (equivalent to 7.5 × 10⁸ cells) and concentrated culture supernatants (Sup.) (equivalent to 750 μl of supernatant of cultures at an OD₆₀₀ of 0.8) were subjected to SDS-PAGE analysis. Left panel, Coomassie brilliant blue-stained gel. Right panel, immunoblot of the duplicated gel with PspA-specific monoclonal antibody Xi126. Molecular markers are indicated to the left. PspA proteins are indicated by arrows. Lanes 1 and 2, protein profiles of χ8501(pYA3493) and χ8501(pYA3494), respectively.

FIG. 6. Serum IgG responses to S. enterica serovar Typhimurium LPS and SOMPs and to rPspA. The data represent IgG antibody levels induced in mice orally immunized with χ8501(pYA3493) (vector control) and χ8501(pYA3494) (expressing rPspA) at the indicated weeks after immunization. ELISA and data analysis are described in Materials and Methods. Arrows indicate sublethal i.v. infection with S. pneumoniae WU2.
phase (up to 8 weeks postimmunization), the level of anti-rPspA IgG1 isotype antibodies gradually increased. After 10 weeks postimmunization, a 1:1 ratio of IgG2a to IgG1 or IgG1 dominant responses was detected (Fig. 8). The pneumococcal i.v. challenge stimulated more IgG1 responses (mean IgG2a/IgG1 ratio, 0.83) to PspA than seen before challenge (mean IgG2a/IgG1 ratio, 1.49), with statistical significance ($P = 0.023$).

FIG. 7. Secretory IgA responses to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPspA. The data represent anti-LPS, -SOMP, and -rPspA IgA antibody levels in vaginal secretions of BALB/c mice orally immunized with $\chi 8501(p$YA3493) (vector control) and $\chi 8501(p$YA3494) (expressing rPspA) at weeks 4, 6, 8, and 10 after immunization.

FIG. 8. Serum IgG2a and IgG1 responses to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPspA. The data represent IgG2a and IgG1 subclass antibody levels to *Salmonella* LPS and SOMPs and to rPspA in sera of BALB/c mice orally immunized with $\chi 8501(p$YA3493) (vector control) and $\chi 8501(p$YA3494) (expressing rPspA) at the indicated weeks after immunization. Arrows indicate sublethal i.v. infection with *S. pneumoniae* WU2. Anti-rPspA IgG2a and IgG1 responses of $\chi 8501(p$YA3493) (negative control) are not shown. The overall IgG2a/IgG1 ratios (means \pm standard deviations) for each antigen are shown above the bars. Statistical analyses were performed with a paired Student t test to compare the IgG2a/IgG1 ratios for antigens. $P$ values of <0.05 were considered significant. The results were as follows: (i) LPS for $\chi 8501(p$YA3494) versus $\chi 8501(p$YA3493), $P > 0.05$; (ii) SOMPs for $\chi 8501(p$YA3493) versus $\chi 8501(p$YA3494), $P > 0.05$; (iii) LPS for $\chi 8501(p$YA3493) versus SOMPs for $\chi 8501(p$YA3493), $P < 0.05$; (iv) LPS for $\chi 8501(p$YA3494) versus SOMPs for $\chi 8501(p$YA3493), $P < 0.05$; (v) PspA before versus after sublethall challenge of *S. pneumoniae*, $P < 0.05$; (vi) LPS for $\chi 8501(p$YA3494) versus PspA for $\chi 8501(p$YA3494), $P > 0.05$ (before challenge) and $P < 0.05$ (after challenge); (vii) SOMPs for $\chi 8501(p$YA3494) versus PspA for $\chi 8501(p$YA3494), $P < 0.05$ (before and after challenge).
the strength of the immune response elicited by vaccine strains expressing foreign antigens. In the development of attenuated *Salmonella*-based multivalent vaccines, a preferable system would have a recombinant antigen secreted from the cytoplasm of *Salmonella* vaccines (19, 20). β-Lactamase, encoded by the ampicillin resistance gene, is a well-characterized periplasmic secreted protein in gram-negative bacteria (43). The β-lactamase gene contained in plasmid pBR322 was originally obtained from an *S. enterica* serovar Paratyphi B isolate (3, 14). It is well known that β-lactamase produced from pBR322 is secreted to the periplasmic space of gram-negative bacteria, and its translocation depends upon the presence of a signal sequence consisting of 23 amino acid residues at the N terminus (26, 43). Evidence obtained from previous studies confirms that the signal sequence plus an additional 12 amino acids of the mature β-lactamase are required to translocate β-lactamase through the cytoplasmic membrane of gram-negative bacteria (28, 53). Fusion of a protein to the β-lactamase signal peptide promotes the secretion of the fusion protein into the periplasm of *E. coli* (42, 53). We reasoned that a protein antigen attached to the β-lactamase signal peptide should be secreted into the periplasm of *Salmonella* vaccine strains. The pYA3493 plasmid (Fig. 2B) constructed in this study was designed to use for the periplasmic secretion of recombinant antigens for antigen delivery by *Salmonella* vaccines.

The recombinant plasmid pYA3494 (pBR ori) (Fig. 3) was constructed for the periplasmic secretion of the α-helical region of the PspA protein. In contrast to the previously described PspA-specifying pYA3193 (37), pYA3494 was maintained stably (100%) over 50 generations in the *S. enterica* serovar Typhimurium vaccine strain grown in the presence of DAP. Both *E. coli* and *Salmonella* containing pYA3494 expressed the rPspA protein with an apparent molecular mass of 35 kDa, and the rPspA proteins were detected in the periplasm (25%) as well as in the culture supernatant (25%) (Fig. 4). The N-terminal His<sub>n</sub>-tagged rPspA protein (no apparent signal peptide) expressed in *S. enterica* serovar Typhimurium χ8599 containing pYA3496 was detected only in the cytoplasm and not in the periplasm of the *Salmonella* host (data not shown). These results suggest that the signal peptide and 12 residues of the N terminus of β-lactamase (present in pYA3494) promote the periplasmic secretion of rPspA. The mechanism to explain how rPspA was translocated from the periplasm outside cells (Fig. 4 and 5) remains unknown. The secondary and tertiary structures of rPspA may permit it to traverse through the *Salmonella* outer membrane. Alternatively, accumulated rPspA in the periplasm may be encapsulated in membrane vesicles which are discharged from the cells. Membrane vesicles have been identified in many gram-negative bacteria (2), and Ciofu et al. (10) reported that β-lactamase was packaged into membrane vesicles and secreted from *Pseudomonas aeruginosa*. Approximately 50% of the rPspA remained in the cytoplasm, perhaps because the amount of endogenous signal peptidase necessary to process all of the overexpressed rPspA is limiting. Alternatively, it has been reported that the C-terminal sequence of mature β-lactamase is important for the efficient periplasmic secretion of β-lactamase, along with the signal sequence (28).

The immunogenicity and appropriate subcellular location of rPspA in the *Salmonella* vaccine strain may contribute to the

### DISCUSSION

Attenuated *Salmonella* strains constructed by recombinant DNA technology have been developed as live vaccines for humans and animals to prevent disease caused by *Salmonella* infections. The ability of live recombinant *Salmonella* vaccines to colonize the gut-associated lymphoid tissue (Peyer’s patches) and visceral lymphoid tissues following oral administration is beneficial in that it stimulates all arms of the immune response, including mucosal, humoral, and cellular immunities (13, 17, 34). Recombinant *Salmonella* vaccines have also been developed as multivalent vaccines to deliver recombinant antigens originating from viruses, bacteria and parasites (13, 34).

Analysis of convalescent-phase sera from patients or animals infected with bacterial pathogens reveals that the proteins located in the envelopes of or secreted by the bacterial pathogens act as dominant immunogens for the immune responses (31, 38, 58). These observations indicate that envelope and secreted proteins are highly immunogenic and/or more readily interact with antigen-presenting cells due to their subcellular location. Translocation of such highly immunogenic antigens into the cell envelope or secretion from the cell should increase

<table>
<thead>
<tr>
<th>Vaccine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>rPspA expression&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Protection&lt;sup&gt;c&lt;/sup&gt; (% alive)</th>
<th>Days to death</th>
</tr>
</thead>
<tbody>
<tr>
<td>χ8501(pYA3494)</td>
<td>+</td>
<td>60&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5, 5</td>
</tr>
<tr>
<td>χ8501(pYA3493)</td>
<td>−</td>
<td>0</td>
<td>&gt;21, &gt;21, &gt;21</td>
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<tr>
<td>None (unimmunized)</td>
<td>NA</td>
<td>0</td>
<td>1, 2, 2, 3, 3</td>
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<sup>a</sup> Mice were orally immunized a total of two times at 10-week intervals with 1.0×10<sup>9</sup> CFU of indicated vaccine strain per dose.

<sup>b</sup> rPspA expressed; −, rPspA not expressed; NA, not applicable.

<sup>c</sup> Five weeks after the last immunization, mice (five mice per group) were challenged intraperitoneally with approximately 4.8×10<sup>3</sup> CFU of virulent *S. pneumoniae* WU2. The LD<sub>50</sub> of WU2 by intraperitoneal infection in unimmunized BALB/c mice was <10<sup>2</sup> (data not shown). Mortality was monitored for 3 weeks after pneumococcal challenge. Significance was determined by chi-square analysis. *, P < 0.05.

### Evaluation of protective immunity

To examine the ability of *Salmonella*-rPspA vaccines to protect against pneumococcal infection, BALB/c mice were immunized with either *S. enterica* serovar Typhimurium χ8501(pYA3493) (dose of 1.3×10<sup>9</sup> CFU) or χ8501(pYA3494) (dose of 1.7×10<sup>9</sup> CFU). At 10 weeks after the initial immunization, a second dose of 10<sup>9</sup> CFU of each vaccine was administered. We did not detect weakness or disease signs in vaccinated mice during the immunization periods. At 5 weeks after the second immunization, mice were challenged intraperitoneally with 4.8×10<sup>3</sup> CFU (50 times the LD<sub>50</sub>) of *S. pneumoniae* WU2. The LD<sub>50</sub> of WU2 by intraperitoneal infection in unimmunized BALB/c mice was <10<sup>2</sup> (data not shown). Mortality was monitored for 3 weeks after pneumococcal challenge. Significance was determined by chi-square analysis. *, P < 0.05.

### TABLE 2. Oral immunization with rPspA-expressing *S. enterica* serovar Typhimurium χ8501(pYA3494) vaccine protects BALB/c mice against challenge with virulent *S. pneumoniae* strain WU2

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<td>&gt;21, &gt;21, &gt;21</td>
</tr>
<tr>
<td>None (unimmunized)</td>
<td>NA</td>
<td>0</td>
<td>1, 2, 2, 3, 3</td>
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<sup>a</sup> Mice were orally immunized a total of two times at 10-week intervals with 1.0×10<sup>9</sup> CFU of indicated vaccine strain per dose.

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augmented immune responses by facilitating adequate exposure of rPspA antigen to antigen-presenting cells for processing. BALB/c mice orally immunized with a single dose of S. enterica serovar Typhimurium χ8501 expressing rPspA showed immune responses to both Salmonella antigens and rPspA. Although there was variation in antibody levels between samples due to mucus materials in vaginal washings, mucosal anti-rPspA IgA was detected in vaginal washings only of mice immunized with Salmonella-rPspA vaccine (Fig. 7). It is likely that mucosal immunity will act as a primary immune defense system against natural infection by S. pneumoniae. Detection of anti-rPspA IgG with a typical antibody response in sera from Salmonella-rPspA vaccine-immunized mice (Fig. 6) indicates that the χ8501(pYA3494) vaccine likely reached appropriate lymphoid tissues to stimulate a systemic immune response. Similar levels of anti-LPS and -SOMP IgG were induced by strains χ8501(pYA3493) (vector) and χ8501(pYA3494), suggesting that rPspA-specific immunity did not interfere with immunity against Salmonella itself.

In T-cell-dependent, antigen-specific immune responses, Th1 cells direct cell-mediated immunity and promote class switching to IgG2a. Th2 cells direct humoral immunity and promote class switching to IgG1 and IgA (39, 51). To date, the switching to IgG2a. Th2 cells direct humoral immunity and suggesting that rPspA-specific immunity did not interfere with immunity against Salmonella itself.

However, there are some issues that need to be evaluated in future studies. The factors causing augmented Th2-type immune responses will be investigated. It remains to be examined whether rPspA secretion could be associated with the formation of membrane vesicles. Further modifications of the rPspA antigen expression construct may be required to enhance the rPspA secretion and to induce antibodies which can opsonize diverse pneumococcal strains with high avidity. Answers to these questions will subsequently improve the recombinant attenuated Salmonella-based pneumococcal vaccine.

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