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

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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 $\Delta crp-28$ and $\Delta asdA16$. 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 10^9 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 multicopy 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

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Derivation, source, or reference
Strains		
<i>E. coli</i>		
χ6212	F ⁻ λ ⁻ φ80 Δ(<i>lacZYA-argF</i>) <i>endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4</i>	36
MGN-617	<i>thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44 ΔasdA4 recA1</i> RP4 2-Tc::Mu [λ <i>pir</i>]; Km ^r	44
<i>S. enterica</i> serovar Typhimurium		
χ3339	SL1344 <i>hisG</i>	22
χ4550	SR-11 <i>gyrA1816 Δcrp-1 ΔasdA1 Δ(zhf-4::Tn10) Δcya-1</i>	47
χ4746	χ3339 <i>nadA540::Tn10</i> Δ(<i>galE-chl-uvrB</i>)-1005; Tet ^r	Lab collection
χ8499	<i>hisG Δcrp-28</i>	χ3339
χ8501	<i>hisG Δcrp-28 ΔasdA16</i>	χ8499
χ8554	<i>hisG ΔasdA16</i>	χ3339
χ8599	<i>hisG ΔasdA16 atrB13::MudJ</i>	χ8554
JF2430	LT2 <i>atrB13::MudJ</i>	15
<i>S. pneumoniae</i> WU2	Wild-type virulent, encapsulated type 3	6
Plasmids		
pYA3193	Asd ⁺ vector harboring 1.5-kb C-terminally truncated <i>pspA</i> gene; pUCori	37
pYA3333	Asd ⁺ ; pBRori	Lab collection
pYA3334	Asd ⁺ ; pUCori	Lab collection
pYA3341	Asd ⁺ ; pUCori	This study
pYA3342	Asd ⁺ ; 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 pYA3342 for the expression of His-tagged PspA	This study
pBR322	Cloning vector; Ap ^r Tc ^r	3
pMEG-443	Recombinant suicide plasmid to generate <i>Salmonella ΔasdA16</i> mutant; Ap ^r Cm ^r	27
pMEG-493	Recombinant suicide plasmid to generate <i>Salmonella Δcrp-28</i> mutant; Ap ^r Cm ^r	Megan Health Inc.

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

pneumococcal vaccine candidate because of its immunogenicity 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 PspA_{Rx1} 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 PspA_{Rx1} (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 diamino pimelic acid (DAP). This phenomenon forced us to construct 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⁺ 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.

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 P22HTint (46) was used for generalized transduction. *Escherichia coli* and *S. enterica* serovar Typhimurium 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 *sacB* gene-based

counterselection in allelic exchange experiments (18). *S. pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract (6).

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* MGN-617 (*Asd*⁻) (44) as the plasmid donor. Bacteriophage P22HT *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 deletion mutations. The PCR conditions were as follows: denaturation at 95°C for 20 s, primer annealing at 55°C for 20 s, polymerization at 72°C for 2 min, and a final extension at 72°C for 10 min. Nucleotide sequencing reactions were performed using ABI Prism fluorescent Big Dye terminators according to the instructions of the manufacturer (PE Biosystems, Norwalk, Conn.).

Characterization of phenotype. MacConkey agar supplemented with 1% maltose was used to detect the phenotypes of *Salmonella* *crp* mutants. Motility was evaluated by observing *Salmonella* spread on a semisolid medium composed of 1% casein enzyme hydrolysate, 0.5% NaCl, and 0.5% agar. Triphenyltetrazolium chloride (50 µg/ml) was added to motility medium to observe *Salmonella* as red cells. The presence of the *ΔasdA16* mutation in *Salmonella* was confirmed by inability of the strain to grow on media without DAP (36). Lipopolysaccharide (LPS) profiles of *Salmonella* strains were examined by previously described methods (24).

SDS-PAGE and immunoblot analyses. Protein samples were boiled for 5 min and then 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-0.9% NaCl (pH 7.4) and incubated with mouse monoclonal antibodies specific for PspA (Xi126) (32), OmpC (50), or β-galactosidase (Sigma) and then with a peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Bio-Rad). Immunoreactive bands were detected by the addition of 4-chloro-1-naphthol (Sigma) in the presence of H₂O₂. The reaction was stopped after 2 min by washing with several large volumes of deionized water.

Purification of rPspA. For overexpression of His₆-tagged PspA, a fragment of the *pspA* gene specifying the α-helical region from amino acid residue 3 to 257 of the mature PspA protein of *S. pneumoniae* Rx1 was PCR amplified from pYA3193 (37) template DNA using a pair of primers (N terminal, 5'CCGGAA TCCATCACCATCACCATCACTCTCCGCTAGCCAGTCAGT3'; C terminal, 5'GGGAAGCTTCTATTATTCTACATTATTGTT3'). The 0.8-kb amplified fragment was then cloned into the pYA3342 vector, resulting in pYA3496 (Table 1). The N-terminal primer contains an *Eco*RI site (underlined) and six consecutive histidine codons (alternate use of CAT and CAC; boldface) for His₆ tagging at the N terminus. The C-terminal primer specifies two consecutive stop codons (TAA TAG; boldface) followed by a *Hind*III site (underlined). In-frame cloning was confirmed by nucleotide sequencing. *E. coli* χ6212 harboring pYA3496 expressed a large amount of soluble His₆-tagged rPspA in its cytoplasm. According to the protocol of the manufacturer (Qiagen, Valencia, Calif.), rPspA protein was purified by an affinity purification process with Ni²⁺-nitrilotriacetic acid-agarose support. The protein purity was verified by Coomassie blue staining of SDS-polyacrylamide gels, and the total amount of purified protein was determined by using the Pierce (Rockford, Ill.) protein assay kit with bovine serum albumin as a standard. Immunoblotting with the Xi126 PspA monoclonal antibody (32) was performed to confirm the purified protein.

Salmonella subcellular fractionation. The periplasmic fraction was prepared by a modification of the lysozyme-osmotic shock method (56). Cultures grown in LB broth to an optical density at 600 nm (OD₆₀₀) of 0.8 were centrifuged at 7,000 × g for 10 min, and the supernatant fluid was saved for analysis of secreted proteins. The cell pellets were resuspended in 800 µl of 100 mM Tris-HCl buffer (pH 8.6) containing 500 mM sucrose and 0.5 mM EDTA. Hen egg white lysozyme (40 µl of a 4-mg/ml stock solution) was added, followed immediately by the addition of 3.2 ml of 50 mM Tris-HCl buffer (pH 8.6) containing 250 mM sucrose, 0.25 mM EDTA, and 2.5 mM MgCl₂. After gentle agitation, the suspension was incubated for 15 min in an ice bath. Cells were removed by centrifugation at 7,000 × g for 6 min followed by filtration of the supernatant through a 0.45 µm-pore-size filter. The filtered supernatant fluid served as the periplasmic fraction. Cells resuspended in 4 ml of 20 mM Tris-HCl (pH 8.6) were disrupted by two passages through a French pressure cell (American Instrument

Company, Silver Spring, Md.). Cell lysates were centrifuged at 7,000 × g at 4°C for 6 min to remove unbroken cells. The supernatant fluid was then centrifuged at 132,000 × g at 4°C for 1 h to separate the soluble fraction and insoluble 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 at 4°C 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-size 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 χ4746 cells grown in LB broth without galactose for analysis by enzyme-linked immunosorbent assay (ELISA). The use of SOMPs obtained from χ4746 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 χ8499. The presence of the 680-bp deletion was confirmed by PCR with a primer set flanking *crp* (5'-AAA GTCGCAATGGAAGGC-3' and 5'-CGTAGACGACGATGGTCTTG-3') and a strain phenotype of Mal⁻ and nonmotility. The *ΔasdA16* mutation was then introduced into χ8499 by P22HT *int* transduction from χ8554 with the suicide vector pMEG-443 integrated into a strain with the *ΔasdA16* mutation, followed by sucrose selection to eliminate the suicide vector to yield χ8501 (27). The presence of the 1,242-bp *asd* deletion in χ8501 was confirmed by PCR using flanking a *asd* primer set (5'-CGGAAATGATTCCCTTCTAACG-3' and 5'-TATCTGCGTCTCCTACCTCAG-3') (27).

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 χ8501 (pYA3494) vaccine (1.9 × 10⁹ CFU in 20 µl of phosphate-buffered saline containing 0.01% gelatin [BSG]) grown in LB broth to an OD₆₀₀ of 0.8 was orally administered to BALB/c mice. The recombinant *S. enterica* serovar Typhimurium χ8501 (pYA3493) 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 × 10⁵ *S. pneumoniae* WU2 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 ability of the *Salmonella*-PspA vaccine to protect the immunized mice against *S. pneumoniae* was assessed by intraperitoneal challenge with 4.8 × 10³ CFU of *S. pneumoniae* in 100 µl of BSG. The 50% lethal dose (LD₅₀) of *S. pneumoniae* WU2 in BALB/c mice was >10⁶ CFU by i.v. administration (Larry McDaniel, personal communication) and <10² CFU by intraperitoneal administration (37). Significant differences in mortality were determined by chi-square analysis at day 21 after challenge. A difference was considered significant at a *P* value of <0.05.

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 (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 biotinylated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Inc., Birmingham, Ala.) for sera and IgA for vaginal secretions. Wells were developed with streptavidin-alkaline phosphatase conjugate (Southern Biotechnology) followed by *p*-nitrophenylphosphate substrate (Sigma) in diethanolamine buffer (pH 9.8). Color development (absorbance) was recorded at 405 nm using an automated ELISA plate (model EL311SX; Biotek, Winooski, Vt.). Absorbance

readings two times higher than preimmune serum baseline values were considered positive reactions.

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-lethal host-vector system. In fact, the 200- to 300-fold excess production of Asd in a strain such as χ 8554 (Δ *asd16*) 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 Δ *asdA16* 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 *Bgl*II site and a C-terminal *Xba*I 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 *Bgl*II-*Xba*I 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 χ 8554 which could grow in the absence of DAP. Both pYA3342 and pYA3341 complemented the *asd* mutations of *E. coli* χ 6212 and *S. enterica* serovar Typhimurium χ 4550. *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 Δ *asd* *S. enterica* serovar Typhimurium strain such as χ 8554 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 PspA into the periplasmic space of *Salmonella* was inefficient and caused toxicity when the export depended on the signal sequence for PspA (37), we constructed a recombinant plasmid by cloning a DNA fragment specifying the signal sequence of β -lactamase which is efficiently transported 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 PCR amplified from the pBR322 DNA template by using a pair of primers (N-terminal, 5'GCATTTCATGAGTATTCAA CATTTC3'-*Bsp*HI [underlined]; C-terminal, 5'CCGGAATTCTTCAGCATCTTTACT3'-*Eco*RI [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 di-

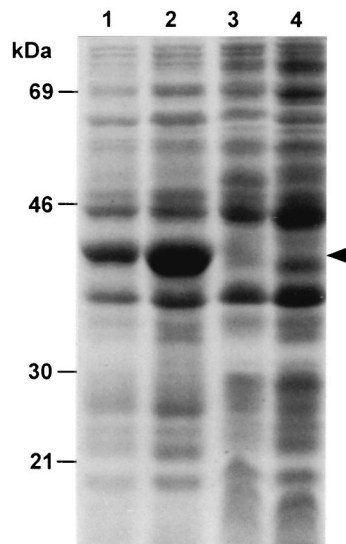


FIG. 1. Reduced expression of Asd protein by deletion of the *asd* gene promoter region. SDS-PAGE was performed with cell lysates of *S. enterica* serovar Typhimurium χ 4550 with pYA3333 (entire *asd* gene with P_{asd}, pBR *ori*), pYA3334 (entire *asd* gene with P_{asd}, pUC *ori*), pYA3342 (promoterless SD-*asd* gene, pBR *ori*), and pYA3341 (promoterless SD-*asd* gene, pUC *ori*). Standards are indicated to the left, and Asd protein (39 kDa) is indicated by an arrow. Lanes: 1, pYA3333; 2, pYA3334; 3, pYA3342; 4, pYA3341.

gested with *Bsp*HI and *Eco*RI enzymes and cloned into the *Nco*I site (compatible with the *Bsp*HI site) and *Eco*RI 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 P_{trc} can be stopped by the 5ST1T2 transcriptional terminator located following the multicloning sites. pYA3493 was stably maintained for 50 or more generations in *E. coli* χ 6212 and *S. enterica* serovar Typhimurium (Δ *asd*) hosts grown in the presence or absence of DAP.

Construction of the rPspA-expressing plasmid. A highly immunogenic α -helical region of PspA from amino acid residue 3 to 257 (765 bp; 255 amino acids) of the mature PspA_{Rx1} 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'CCGGAATTCTCTCCCGTAGCCA GTCAGTCT3'; C-terminal, same as used in the construction of His₆-tagged PspA, which introduces the TAA TAG stop codons after the *pspA* coding sequences). The PCR product, digested with *Eco*RI and *Hind*III enzymes, was cloned into the *Eco*RI and *Hind*III sites of pYA3493, resulting in pYA3494 (Fig. 3). The in-frame fusion of the rPspA with the β -lactamase signal sequence was confirmed by nucleotide sequencing. *E. coli* χ 6212 harboring pYA3494 expressed rPspA as approximately 1% of the total cell protein (data not shown).

Expression and subcellular localization of rPspA in *Salmonella*. An *S. enterica* serovar Typhimurium strain was constructed to examine expression and subcellular localization of rPspA. The *atrB13::MudJ* allele (15), causing constitutive expression of β -galactosidase, in *S. enterica* serovar Typhimurium

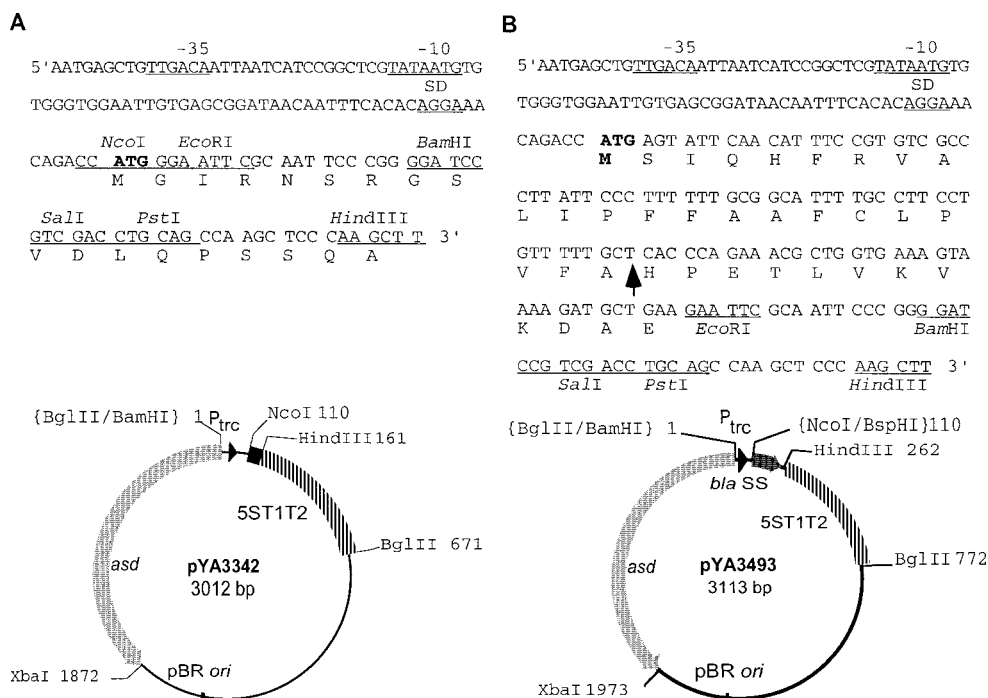


FIG. 2. Asd⁺ antigen expression vectors. (A) Asd⁺ vector pYA3342. The map of pYA3342 and the nucleotide sequences of the P_{trc} promoter region and multicloning sites are shown. (B) Periplasmic secretion Asd⁺ vector pYA3493. A DNA fragment encoding the β -lactamase signal sequence and 12 amino acid residues of the N terminus of mature β -lactamase of plasmid pBR322 was positioned under the control of the P_{trc} promoter of the Asd⁺ vector pYA3342 (pBR ori). The map of pYA3493 and the nucleotide sequences of the P_{trc} promoter region, β -lactamase signal sequence (*bla* SS), and multicloning sites are shown. The P_{trc} sequences for -35, -10, and SD are indicated, and the translation start codon is in boldface. An arrow within the sequence indicates the signal peptidase cleavage site. Unique restriction enzyme sites in the multicloning site are indicated. 5ST1T2 is a transcriptional terminator.

JF2430 was transduced into *S. enterica* serovar Typhimurium χ 8554 by P22HTint-mediated generalized transduction (52), resulting in χ 8599 (*hisG* Δ *asdA16* *atrB13::MudJ*). χ 8599 was Lac⁺ on MacConkey agar plus lactose and DAP. β -Galactosidase production from the *atrB13::MudJ* allele in χ 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 β -lactamase signal sequence in the pYA3494 construct precluded use of β -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 χ 8599. χ 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 χ 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. Densitometry 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- β -galactosidase and -OmpC monoclonal antibodies, the β -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 Δ crp-28 vaccine expressing rPspA antigen. pYA3493 (vector control) and pYA3494 (encoding rPspA) were electroporated into the Δ crp-28 Δ asdA16 strain χ 8501. The *S. enterica* serovar Typhimurium χ 8501 (Δ crp-28 Δ 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 χ 8501(pYA3494) 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 χ 8501 vaccine strain was secreted into the culture supernatant along with other secreted proteins. To examine the stability of plasmids pYA3493 and pYA3494 in *Salmonella* χ 8501 in vitro,

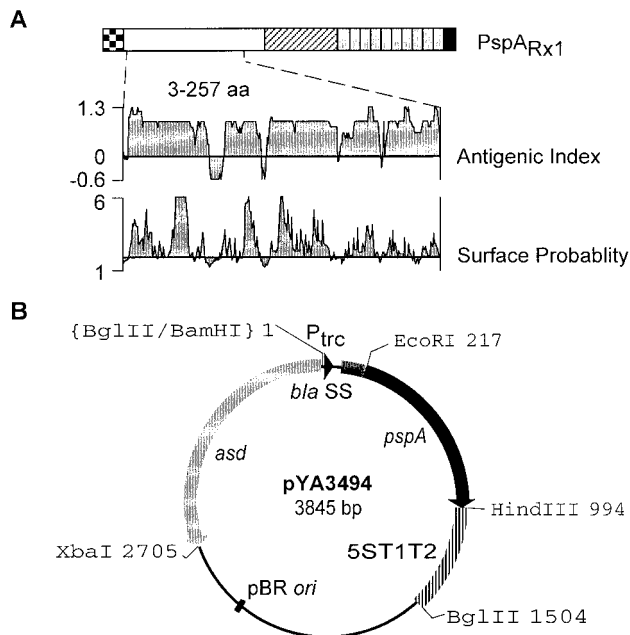


FIG. 3. Recombinant plasmid pYA3494 for PspA expression. (A) The PspA region used in this study. Functional domains of native PspA from *S. pneumoniae* (PspA_{Rx1}) are diagrammed. Dotted box, leader sequence (31 amino acids [aa]); open box, immunodominant α -helical region (aa 1 to 288); hatched box, proline-rich region (aa 289 to 370); 10 gray boxes, choline-binding repeats (aa 371 to 571); black box, C terminus (aa 572 to 588). Dotted lines represent the limit of the rPspA region used in this study. Bioinformatic analyses of the rPspA for antigenic index and surface probability are presented. Analyses were performed with the Protean module of the Lasergene sequence analysis software. (B) Map of recombinant plasmid pYA3494. A 0.7-kb *EcoRI*-*HindIII* fragment of PCR-amplified DNA of *pspA*_{Rx1} was cloned into the *EcoRI* and *HindIII* sites of pYA3493 (Fig. 2B). The cloned fragment included the immunogenic α -helical region of PspA including amino acids 3 through 257 of mature PspA (255 amino acids).

χ 8501 cells containing pYA3493 and pYA3494 were cultured with daily passage of 1:1,000 dilutions for five consecutive days in L broth containing DAP. All χ 8501 clones examined (300 clones/day) kept the Asd⁺ plasmid pYA3493 and pYA3494, indicating that pYA3493 and pYA3494 were very stable in the χ 8501 vaccine strain. Cells obtained from the last-day culture of the stability test expressed amounts of the 35-kDa rPspA similar to those from the first day (data not shown), suggesting stable expression of rPspA without rearrangements.

Immune responses in mice after oral immunization with the recombinant *S. enterica* serovar Typhimurium vaccines. All mice orally administered a single dose of 1.3×10^9 CFU *S. enterica* serovar Typhimurium χ 8501(pYA3493) (vector control) survived for the 30-day monitoring period. Those mice were protected for a 30-day observation period against wild-type χ 3339 (LD₅₀, $<10^6$ CFU) challenge (1.7×10^9 CFU) 30 days after the initial immunization. There were no survivors in a group of unimmunized mice challenged with 1.7×10^7 CFU of χ 3339. These results indicate that χ 8501 with an Asd⁺ vector is avirulent for mice and elicits a protective immune response against challenge with *S. enterica* serovar Typhimurium.

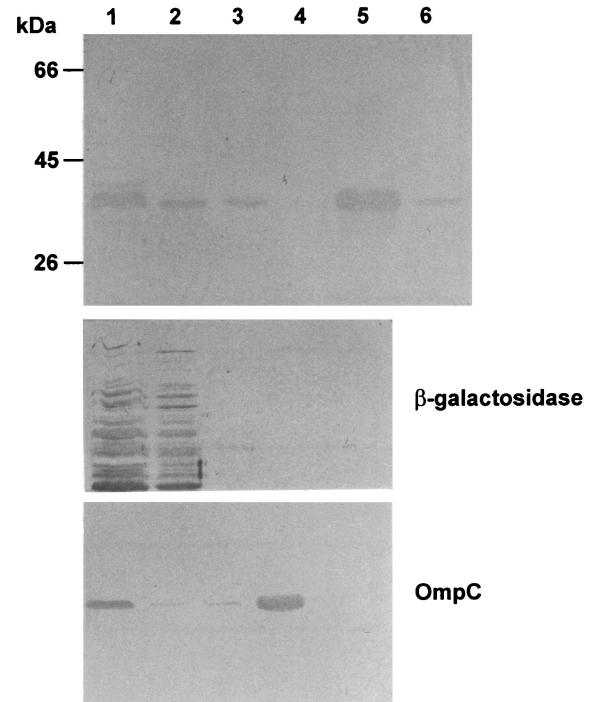


FIG. 4. Subcellular location of expressed rPspA in *S. enterica* serovar Typhimurium. Subcellular fractions were prepared from *S. enterica* serovar Typhimurium χ 8599(pYA3494) cells grown in LB broth at 37°C by the procedures described in Materials and Methods. Fractions equivalent to 30- μ l volumes of the culture at an OD₆₀₀ 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 μ l); 6, supernatant (10 μ l).

A single dose of *S. enterica* serovar Typhimurium χ 8501(pYA3494) (1.9×10^9 CFU) or χ 8501(pYA3493) (control, 2×10^9 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 (3.8×10^5 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 χ 8501(pYA3493) or χ 8501(pYA3494) vaccine. Because native PspA is a highly immunogenic pneumococcal surface protein, the pneumococcal challenge boosted rPspA-specific immune responses in χ 8501(pYA3494)-immunized mice (Fig. 6). In comparison to the anti-rPspA IgG level (A_{405} , 0.81) at 12 weeks after *S. enterica*

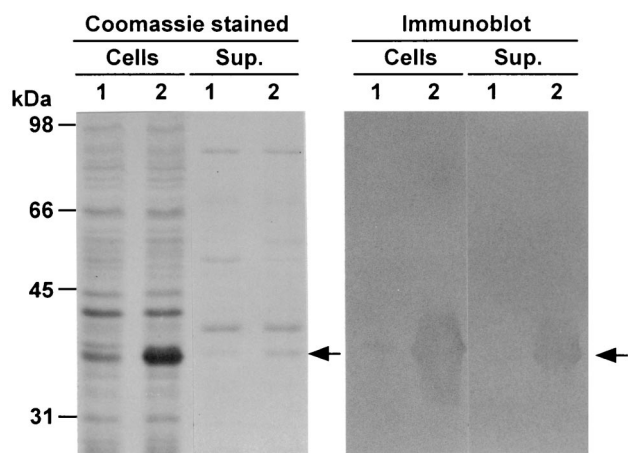


FIG. 5. Expression of rPspA in the *S. enterica* serovar Typhimurium vaccine strain. χ 8501 harboring pYA3494 (specifying rPspA) or pYA3493 (vector control) was cultured in LB broth at 37°C. Total cells (equivalent to 7.5×10^8 cells) and concentrated culture supernatants (Sup.) (equivalent to 750 μ l 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 χ 8501(pYA3493) and χ 8501(pYA3494), respectively.

serovar Typhimurium χ 8501(pYA3494) immunization, the pneumococcal challenge boosted 53% more anti-rPspA IgG (A_{405} , 1.24) 1 week later. This suggests that the *S. enterica* serovar Typhimurium χ 8501-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 χ 8501(pYA3493),

the vector control vaccine. The χ 8501(pYA3493) vaccine elicited anti-LPS and -SOMP IgG responses with kinetics and levels similar to those induced by χ 8501(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 χ 8501(pYA3493) and χ 8501(pYA3494) vaccines elicited anti-LPS and anti-SOMP IgA. rPspA-specific IgA was detected in the vaginal fluids from mice immunized with χ 8501(pYA3494) but not in those from mice immunized with the χ 8501(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 χ 8501(pYA3493) were not significantly different from those from mice immunized with χ 8501(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 [χ 8501(pYA3493), $P = 0.0004$; χ 8501(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

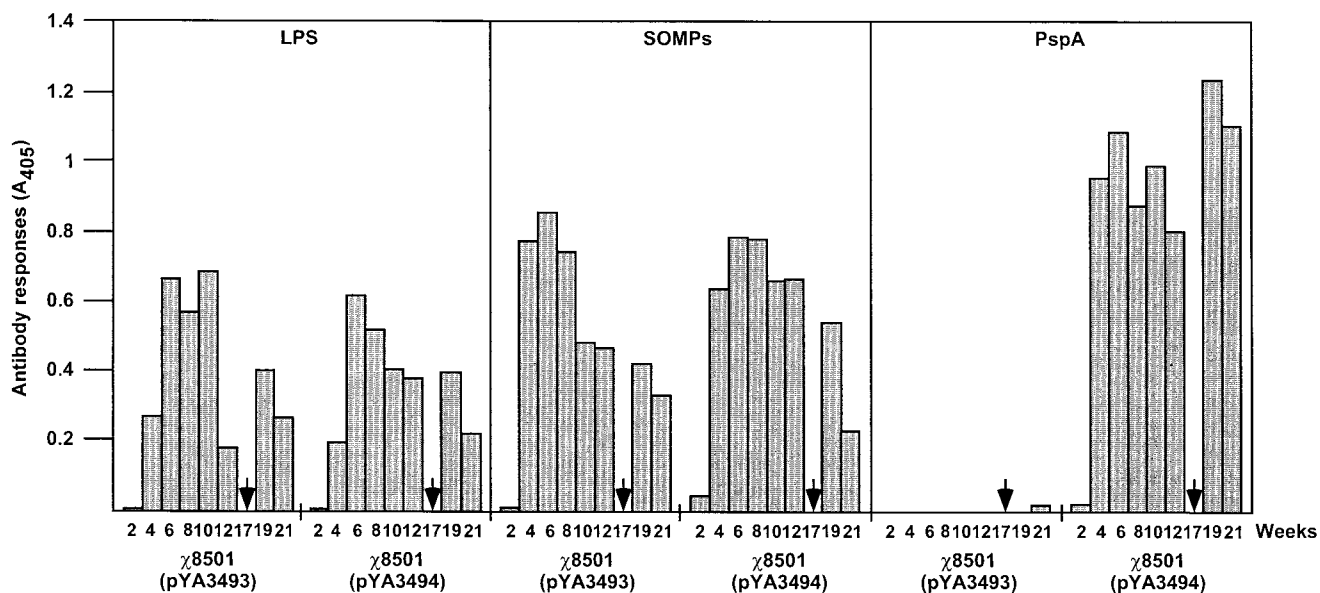


FIG. 6. Serum IgG responses to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPspA. The data represent IgG antibody levels induced in mice orally immunized with χ 8501(pYA3493) (vector control) and χ 8501(pYA3494) (expressing rPspA) at the indicated weeks after immunization. ELISA and data analysis are described in Materials and Methods. Arrows indicate sublethal i.v. infection with *S. pneumoniae* WU2.

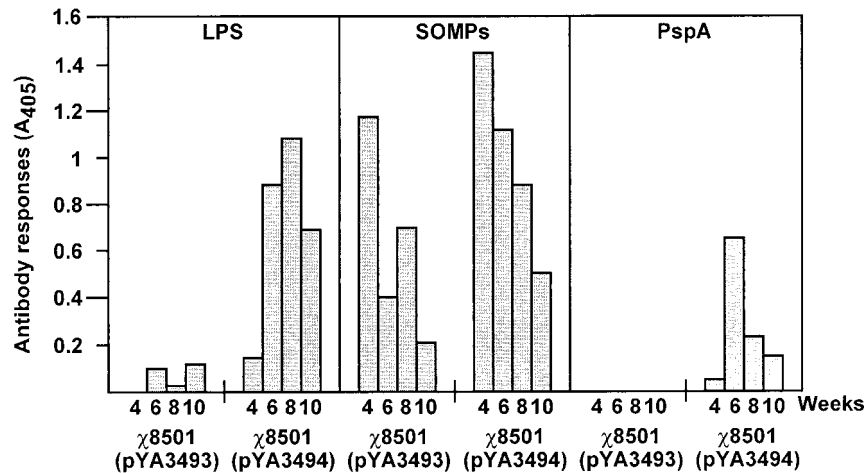


FIG. 7. Secretory IgA responses to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPspA. The data represent anti-LPS, -SOMP, and -rPspA IgA antibody levels in vaginal secretions of BALB/c mice orally immunized with χ 8501(pYA3493) (vector control) and χ 8501(pYA3494) (expressing rPspA) at weeks 4, 6, 8, and 10 after immunization.

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 ($P = 0.023$).

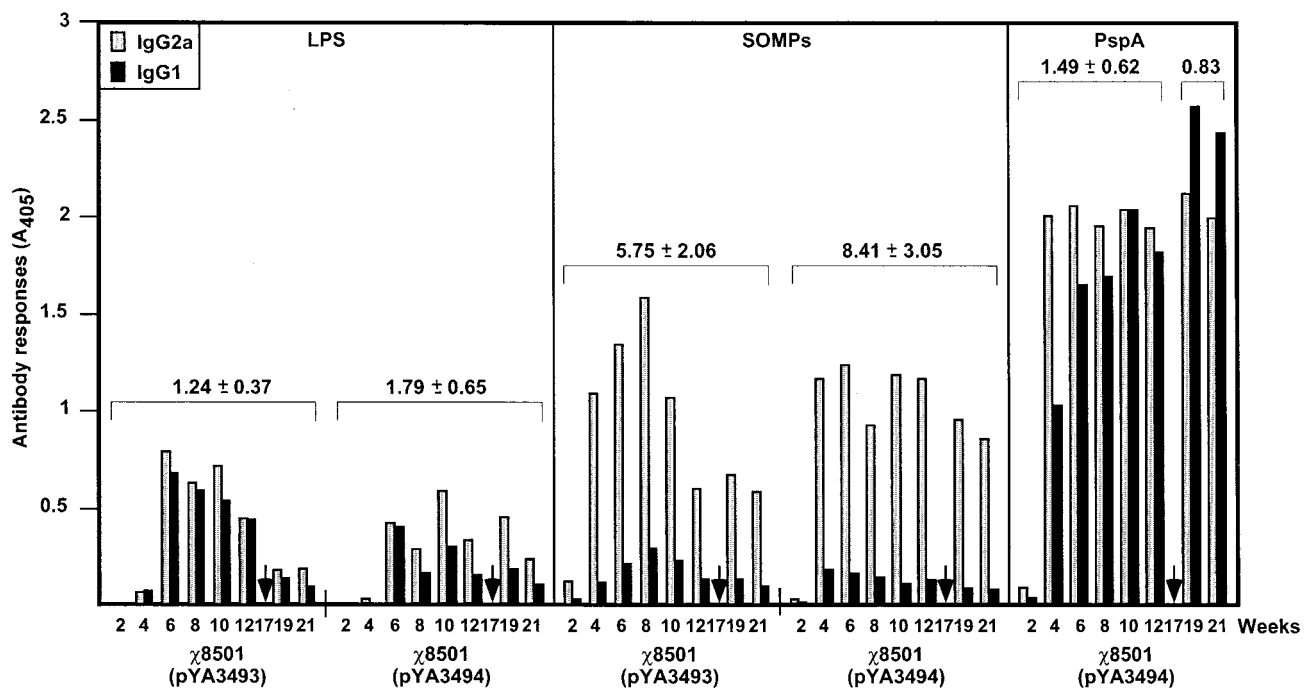


FIG. 8. Serum IgG2a and IgG1 responses to *S. enterica* serovar Typhimurium LPS and SOMPs and to rPspA. The data represent IgG2a and IgG1 subclass antibody levels to *Salmonella* LPS and SOMPs and to rPspA in sera of BALB/c mice orally immunized with χ 8501(pYA3493) (vector control) and χ 8501(pYA3494) (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 χ 8501(pYA3493) (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 χ 8501(pYA3493) versus χ 8501(pYA3494), $P > 0.05$; (ii) SOMPs for χ 8501(pYA3493) versus χ 8501(pYA3494), $P > 0.05$; (iii) LPS for χ 8501(pYA3493) versus SOMPs for χ 8501(pYA3493), $P < 0.05$; (iv) LPS for χ 8501(pYA3494) versus SOMPs for χ 8501(pYA3494), $P < 0.05$; (v) PspA before versus after sublethal challenge of *S. pneumoniae*, $P < 0.05$; (vi) LPS for χ 8501(pYA3494) versus PspA for χ 8501(pYA3494), $P > 0.05$ (before challenge) and $P < 0.05$ (after challenge); (vii) SOMPs for χ 8501(pYA3494) versus PspA for χ 8501(pYA3494), $P < 0.05$ (before and after challenge).

TABLE 2. Oral immunization with rPspA-expressing *S. enterica* serovar Typhimurium χ 8501(pYA3494) vaccine protects BALB/c mice against challenge with virulent *S. pneumoniae* strain WU2

Vaccine ^a	rPspA expression ^b	Protection ^c (% alive)	Days to death
χ 8501(pYA3494)	+	60*	5, 5 >21, >21, >21
χ 8501(pYA3493)	-	0	1, 2, 2, 3, 3
None (unimmunized)	NA	0	1, 2, 2, 2, 3

^a Mice were orally immunized a total of two times at 10-week intervals with $\sim 10^9$ CFU of indicated vaccine strain per dose.

^b +, rPspA expressed; -, rPspA not expressed; NA, not applicable.

^c Five weeks after the last immunization, mice (five mice per group) were challenged intraperitoneally with approximately 4.8×10^3 CFU of virulent *S. pneumoniae* WU2. The LD₅₀ of WU2 by intraperitoneal infection in unimmunized BALB/c mice was $< 10^2$ (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 χ 8501(pYA3493) (dose of 1.3×10^9 CFU) or χ 8501(pYA3494) (dose of 1.7×10^9 CFU). At 10 weeks after the initial immunization, a second dose of 10^9 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 4.8×10^3 CFU (50 times the LD₅₀) of *S. pneumoniae* WU2. Sixty percent of the mice immunized with χ 8501(pYA3494) were protected from pneumococcal challenge, with statistical significance ($P < 0.05$). This challenge dose killed 100% of unimmunized and χ 8501(pYA3493)-immunized mice (Table 2). Following challenge, mice unimmunized or immunized with χ 8501(pYA3493) died much faster, with death at a mean of day 2, than mice immunized with χ 8501(pYA3494), which died at a mean of day 5.

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

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_{RX1}. 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 His₆-tagged rPspA protein (no apparent signal peptide) expressed in *S. enterica* serovar Typhimurium χ 8599 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

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 χ 8501 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 χ 8501(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 χ 8501(pYA3493) (vector) and χ 8501(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 mechanism determining Th1- or Th2-type immunity to a given antigen is not well understood. Th2-type immune responses are rare in the immune response elicited by attenuated *Salmonella* vaccines (11, 41). While Th1-type dominant responses were observed for the LPS and SOMP in this study, mixed Th1- and Th2-type immune responses were observed for rPspA (Fig. 8). After 10 weeks postimmunization, the Th2-type response became dominant. The mechanisms stimulating these types of immune responses by the *Salmonella*-rPspA vaccine remain unknown. It has been reported that oral immunization of purified PspA with the mucosal adjuvant cholera toxin in mice elicited Th2-type immune responses (57). In the development of pneumococcal vaccines, the anti-rPspA IgG isotype switching to a mixed or IgG1-dominant type along with a mucosal IgA response, indicating induction of a strong Th2-type humoral immune response, is preferred to prevent colonization of *S. pneumoniae* in the respiratory tract (pneumonia) or ear mucosa (otitis media) (33, 55). A sublethal challenge of *S. pneumoniae* boosted the rPspA immune responses with maintenance of the IgG1 isotype dominant response observed before infection.

Mice orally vaccinated with *Salmonella*-rPspA vaccine were significantly protected (60%) ($P < 0.05$) against virulent *S. pneumoniae* WU2 intraperitoneal challenge at 10 weeks postimmunization. These results indicate that the *Salmonella* χ 8501 vaccine, which expresses the rPspA by an improved antigen expression system, induces protective immunity against pneumococcal infection, and they are consistent with the results demonstrating that the α -helical portion of PspA represents a protective domain (7).

In conclusion, the results of this study demonstrate that a recombinant attenuated *Salmonella* vaccine expressing rPspA antigen elicited mixed Th1- and Th2-type immune responses to rPspA and protected mice against pneumococcal challenge. Recombinant attenuated *Salmonella*-rPspA vaccines may have great potential for use for inducing effective protection against pneumococci which establish infection via a mucosal surface.

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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