Supporting Information

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SI Materials and Methods

DNA and Genetic Procedures. DNA manipulations were carried out as described in Sambrook *et al.* (1). Transformation of bacterial strains was routinely done by electroporation (2), by using Gene Pulser Xcell System (Bio-Rad). Transformants containing Asd^+ plasmids were selected on LB agar plates without DAP. Only clones containing the recombinant plasmids were able to grow under these conditions. PCR amplification was used to obtain DNA fragments for verification of chromosomal deletion mutations.

SDS/PAGE and immunoblot analyses protein samples were boiled for 5 min and subsequently separated by SDS/PAGE. For immunoblotting, proteins separated by SDS/PAGE were transferred to nitrocellulose membranes. After blocking membranes with 3% skim milk in 10 mM Tris-0.9% NaCl (pH 7.4), pneumococcal surface protein A (PspA) was detected with rabbit polyclonal antibody specific for PspA (University of Alabama at Birmingham), followed by the addition of an AP-conjugated goat anti-rabbit IgG (Sigma). Immunoreactive bands were visualized by the addition of BCIP/NBT solution (Sigma). The reaction was stopped after 2 min by washing with large volumes of deionized water several times.

ELISA. ELISAs were performed as previously described (3). Briefly, polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories) were coated with 100 ng/well of either LPS, *Salmonella* outer membrane protein (SOMP), or purified rPspA. Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied in 100-μL volumes to each well. Plates were incubated overnight at 4 °C. Free binding sites were blocked with PBS (pH 7.4), containing 0.1% Tween 20 (PBS-T) and 1% BSA. A 100-μL volume of series diluted sample was added to individual wells in triplicate and incubated for 1h at 37 °C. Plates were treated with biotinylated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology). 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 by using an automated ELISA plate reader (model EL311SX; Biotek). Absorbance readings 0.1 higher than PBS control values were considered positive reactions.

IL-4 and IFN-γ ELISPOT. ELISPOTs were performed as previously described (4). Briefly, PVDF membrane plates (Millipore) were prewetted with EtOH, washed with sterile H_2O, and coated with 100 μL of mAbs IL-4 or IFN-γ (BD PharMingen) at 2 μg/mL, in PBS overnight at 4 °C. The wells were washed with PBS and blocked with RPMI with 10% FCS. After that, 50 μL cell medium (RPMI-1640 supplemented with 10% FCS, 2 mM l-glutamine, 100 IU/mL penicillin, and streptomycin and 1% Hepes) and 50 μL of cells (100,000 per well) in cell medium with or without stimulation with rPspA at 5 μg/ml were added per well and incubated in the plates overnight in 5% CO_2 at 37 °C. The next day, the cell suspensions were discarded and the plates washed with PBS-T. Biotinylated mAb IL-4 or IFN-γ (BD PharMingen) at 0.5 μg/mL in PBS-T with 1% FCS was added and incubated at room temperature for 2 h. After washing with PBS-T, 100 μL/well of avidin peroxidase diluted 1:1,000 (vol/vol) in PBS-T containing 1% FCS were added followed by incubation for 1 h at room temperature. AEC (3-amina-9-ethycarbazole) substrate was prepared according to manufacturer's specifications (Vector Laboratories), and after washing with PBS-T, 100 μL of substrate was added per well. Spots were developed for 15 min at room temperature. Plates were dried and analyzed by using an automated CTL ELISPOT Reader System (Cellular Technology).

Measurement of Cytokine Concentrations. Cytokine concentrations were determined by using the Bio-Plex Protein Array System (Bio-Rad) (5). Cytokine-specific antibody-coated beads (Bio-Rad) were used for these experiments. Serum samples were incubated with antibody-coupled beads for 1 h with continuous shaking. The beads were washed 3 times with wash buffer to remove unbound protein and then incubated with biotinylated detection cytokine-specific antibody for 1 h with continuous shaking. The beads were washed once more and were then incubated with streptavidin–phycoerythrin for 10 min. After incubation, the beads were washed and resuspended in assay buffer, and the constituents of each well were drawn up into the flow-based Bio-Plex Suspension Array System, which identifies each different color bead as a population of protein and quantifies each protein target based on secondary antibody fluorescence. Cytokine concentrations were automatically calculated by Bio-Plex Manager software by using a standard curve derived from a recombinant cytokine standard. Multiple readings were made on each bead set.

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3. Kang HY, Srinivasan J, Curtiss R, III (2002) Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine. *Infect Immun* 70:1739–1749.
4. Sedgwick JD, Holt PG (1983) A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J Immunol Methods* 57:301–309.
5. Kerr JR, *et al.* (2004) Circulating cytokines and chemokines in acute symptomatic parvovirus B19 infection: Negative association between levels of pro-inflammatory cytokines and development of B19-associated arthritis. *J Med Virol* 74:147–155.
6. Curtiss R, III, *et al.* (2007) in *Virulence Mechanisms of Bacterial Pathogens*, ed Brogden K, *et al.* (American Society for Microbiology, Washington, DC), pp 297–313.
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8. Briles DE, *et al.* (1996) PspA, a protection-eliciting pneumococcal protein: Immunogenicity of isolated native PspA in mice. *Vaccine* 14:858–867.

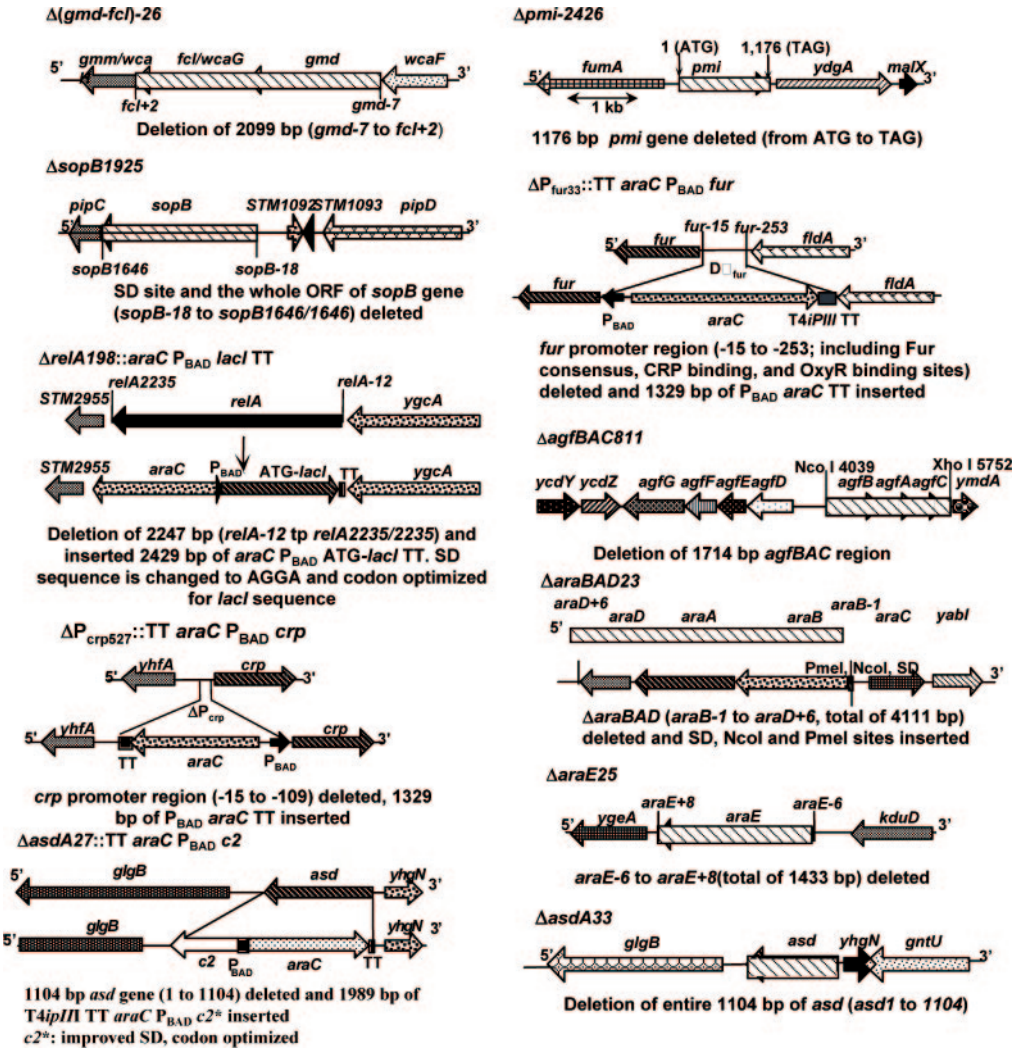

Fig. S1. Schematic representations of the mutations in *Salmonella enterica* strains χ9088 and χ9558.

Fig. S2. Western blotting showing the synthesis of PspA (Rx1) in different *Salmonella typhimurium* mutants. Strains were grown in LB broth overnight at 37 °C. Plasmid-containing χ9088 and χ9558 cultures were supplemented with 0.2% mannose or 0.2% mannose and 0.05% arabinose, respectively. Equal numbers of cells from each culture were pelleted, suspended in loading buffer, and boiled. Equal volumes of cell lysates were subjected to SDS/PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody specific for PspA lanes: molecular mass markers (positions are indicated in kilodaltons) (lane 1); χ8133(pYA3493) (lane 2); χ8133(pYA3634) (lane 3); χ9088(pYA3493) (lane 4); χ9088(pYA3634) (lane 5); χ9558(pYA3493) (lane 6); χ9558(pYA3634) (lane 7). Due to the presence of arabinose in LB broth, the PspA expression of χ9558(pYA3634) has been partially repressed.

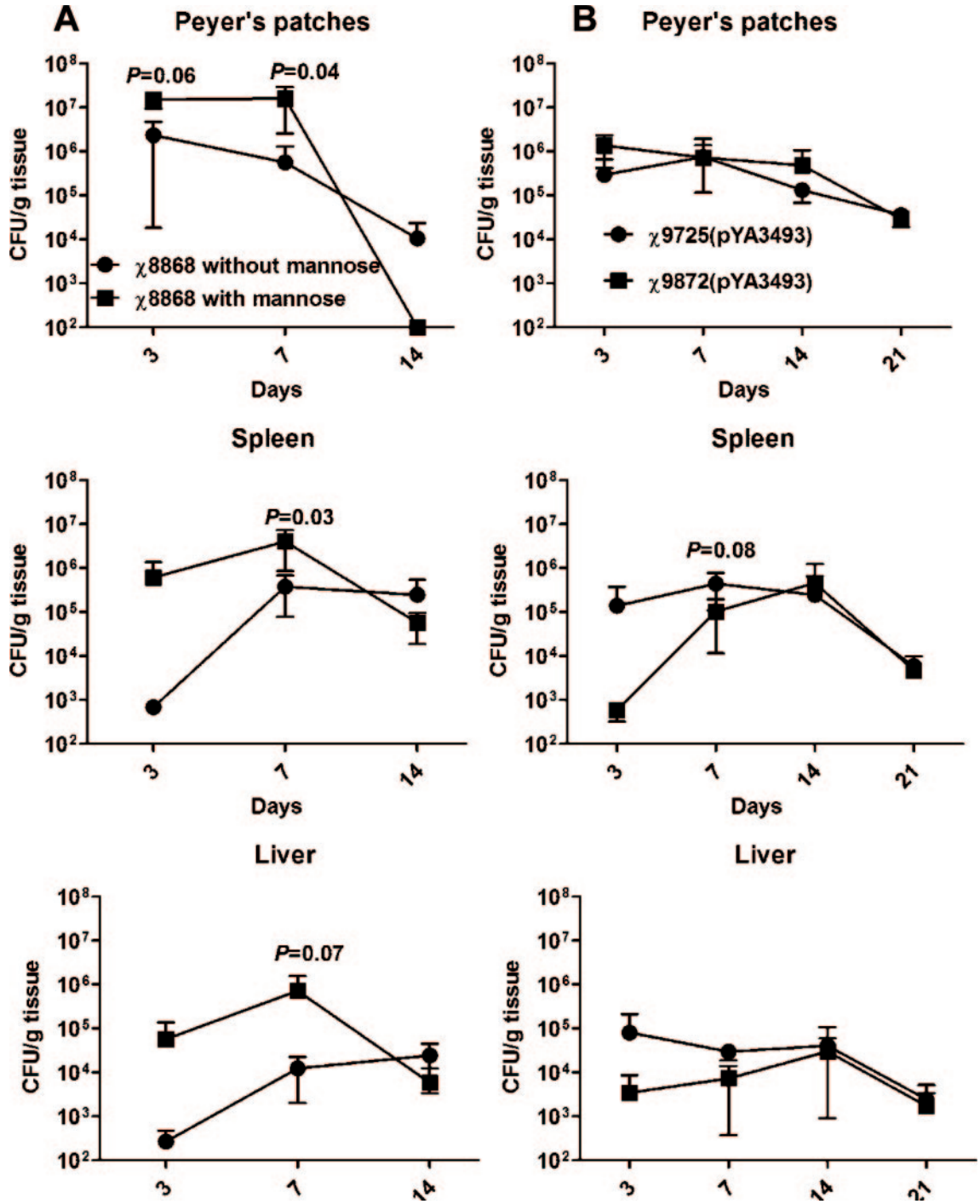

Fig. S3. Comparison of colonization kinetics for the Δ*pmi* strain χ8868 with 0.2% mannose and χ8868 without mannose (*A*), and the *fur* mutants χ9725(pYA3493) (ΔP_{fur81}::TT *araC* P_{BAD} *fur*) and χ9872(pYA3493) (*fur-1*) (*B*). At each time point, 3 mice per group were euthanized, and Peyer's patches, spleen, and liver were collected, homogenized, and plated on the plates. *Salmonella* colonies were counted and expressed in cfu per gram of tissue. *P* values shown in the representation are compared between the 2 groups in the graphs.

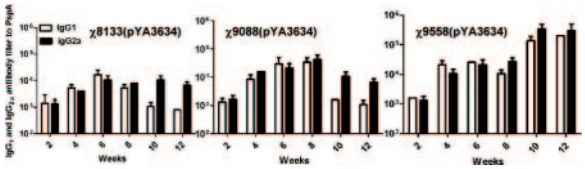

Fig. S4. Serum IgG2a and IgG1 responses to rPspA measured by ELISA. The data represent IgG2a and IgG1 subclass antibody levels to rPspA in pooled sera from BALB/c mice orally immunized with the indicated strains at various times after immunization. Error bars represent variation between triplicate wells. The ratios of $I\gamma\Gamma1$: $I\gamma\Gamma2\alpha$ at 12 weeks are 1:8.3 for χ8133(pYA3634) immunized mice; 1:9.4 for χ9088(pYA3634) immunized mice and 1:1.5 for χ9558(pYA3634) immunized mice.

Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or ref.
Strains		
S. typhimurium		
χ8133	Δcya-27 Δcrp-27 ΔasdA16	Lab collection
χ9088	ΔP_{fur33}::TT araC P_{BAD} fur Δpmi-2426 Δ(gmd-fcl)-26 ΔasdA33	6
χ9558	Δpmi-2426 Δ(gmd-fcl)-26 ΔP_{fur81}::TT araC P_{BAD} fur $\Delta Pcrp_{527}$::TT araC P_{BAD} crp ΔasdA27::TT araC P_{BAD}c2 ΔaraE25 ΔaraBAD23 ΔrelA198::araC P_{BAD} lacI TT ΔsopB1925 ΔagfBAC811	7
χ8868	Δpmi-2426 Δ(gmd-fcl)-26	Lab collection
χ9725	ΔasdA33 ΔP_{fur81}::TT araC P_{BAD} fur ΔaraBAD23	Lab collection
χ9872	ΔasdA33 Δfur-1 zbf-5123::Tn10	Lab collection
S. pneumoniae WU2	Wild-type virulent, encapsulated type 3	8
Plasmids		
pYA3493	Plasmid Asd^+; pBRori β-lactamase signal sequence-based periplasmic secretion plasmid	3
pYA3634	0.7-kb DNA encoding the α-helical region of PspA from amino acid 3 to amino acid 286 in pYA3493	6

Table S2. Immunization with regulated delayed attenuation *S. typhimurium* vaccine strains stimulates systemic cytokine production

Mouse groups	Cytokine concentration, pg/mL						
	IL-2	IL-4	IL-5	IL-10	IL-12	GM-CSF	TNF-α
BSG	6.5 ± 0.71	13.4 ± 1.27	8.5 ± 1.41	7.8 ± 1.06	15.0 ± 0.71	12.5 ± 1.41	9.9 ± 0.14
χ8133 (pYA3493)	7.3 ± 0.35	18.3 ± 0.35	10.2 ± 1.20	8.5 ± 0.00	17.7 ± 0.92	13.8 ± 1.06	11.3 ± 0.35
χ8133* (pYA3634)	12.0 ± 1.27	27.7 ± 5.44	21.8 ± 3.89	21.7 ± 3.04	42.3 ± 9.55	28.0 ± 2.12	32.3 ± 3.18
χ9088 (pYA3493)	9.5 ± 0.00	27.4 ± 1.56	17.0 ± 0.00	14.0 ± 1.41	29.9 ± 3.39	20.9 ± 0.57	18.8 ± 1.77
χ9088* (pYA3634)	11.5 ± 0.71	37.3 ± 1.41	24.5 ± 2.12	22.5 ± 0.71	49.7 ± 1.20	31.7 ± 3.75	31.0 ± 2.12
χ9558 (pYA3493)	11.8 ± 0.35	34.8 ± 1.06	30.5 ± 2.12	25.8 ± 1.06	53.3 ± 1.41	39.2 ± 1.02	37.8 ± 1.06
χ9558*,** (pYA3634)	15.0 ± 1.41	46.3 ± 1.06	32.0 ± 1.41	26.5 ± 1.48	59.0 ± 8.13	39.8 ± 7.78	39.7 ± 3.32

*, compared with BSG group, significantly higher overall systemic cytokine production, $P < 0.01$. **, compared with χ8133(pYA3634) group, significantly higher overall systemic cytokine production, $P < 0.05$.