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A Recombinant Attenuated *Salmonella enterica* Serovar Typhimurium Vaccine Encoding *Eimeria acervulina* Antigen Offers Protection against *E. acervulina* Challenge▽

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Coccidiosis is a ubiquitous disease caused by intestinal protozoan parasites belonging to several distinct species of the genus *Eimeria*. Cell-mediated immunity (CMI) is critically important for protection against *Eimeria*; thus, our approach utilizes the bacterial type III secretion system (TTSS) to deliver an antigen directly into the cell cytoplasm of the immunized host and into the major histocompatibility complex class I antigen-processing pathway for induction of CMI and antigen-specific cytotoxic T-lymphocyte responses in particular. To accomplish this goal, *Eimeria* genes encoding the sporozoite antigen EASZ240 and the merozoite antigen EAMZ250 were fused to the *Salmonella enterica* serovar Typhimurium effector protein gene *sptP* in the parental pYA3653 vector, yielding pYA3657 and pYA3658, respectively. SptP protein is secreted by the TTSS of *Salmonella* and translocated into the cytoplasm of immunized host cells. The host strain chromosomal copy of the *sptP* gene was deleted and replaced by a reporter gene, *xylE*. The newly constructed vectors pYA3657 and pYA3658 were introduced into host strain χ8879 (Δ*phoP233* Δ*sptP1033*::*xylE*Δ *asdA16*). This strain is an attenuated derivative of the highly virulent strain UK-1. When strain χ8879(pYA3653) as the vector control and strain χ8879 harboring pYA3657 or pYA3658 were used to orally immunize day-of-hatch chicks, colonization of the bursa, spleen, and liver was observed, with peak titers 6 to 9 days postimmunization. In vitro experiments show that the EASZ240 antigen is secreted into the culture supernatant via the TTSS and that it is delivered into the cytoplasm of Int-407 cells by the TTSS. In vivo experiments indicate that both humoral and cell-mediated immune responses are induced in chickens vaccinated with a recombinant attenuated *Salmonella* serovar Typhimurium vaccine, which leads to significant protection against *Eimeria* challenge.

Coccidiosis is a prevalent disease caused by intestinal protozoan parasites belonging to one of several species of *Eimeria* (8). This disease is of great economic importance, costing poultry producers worldwide more than 800 million dollars annually (54). The conventional approach to controlling coccidiosis has been the use of prophylactic chemotherapy. However, the emergence of drug-resistant *Eimeria* strains, coupled with the high cost of new drug development, has focused interest on the development of efficacious vaccines (5).

The complex life cycle of *Eimeria* results in complex innate and adaptive immune responses to this pathogen generated by the infected host, ultimately conferring resistance to reinfection. However, the immunity generated by one species of *Eimeria* does not render chickens immune to reinfection by one of the other six species of *Eimeria*. In addition, some *Eimeria* spp. have been shown to exhibit a high degree of immunovariability, leading to an observed lack of cross-protective immunity among geographically isolated strains (2, 37). Thus, designing a vaccine that induces cross-protective immunity to all seven species of *Eimeria* known to infect chickens is a great challenge, since there are no known cross-species-protective antigens. The primary target tissue for the invasion and development of *Eimeria* is the intestinal epithelium, and the first line of defense against this parasite is the development of immunity in the underlying mucosa-associated lymphoid tissues (MALT). In chickens, MALT consists of a variety of specialized lymphoid compartments such as Peyer patches, cecal tonsils, and the bursa of Fabricius. Within these compartments reside various cell types such as epithelial cells, T and B lymphocytes, macrophages, dendritic cells, mast cells, and natural killer (NK) cells, all of which act in concert to generate an immune response and defend against pathogens (36).

It is generally accepted that cell-mediated immunity (CMI), mediated mainly by antigen-specific and nonspecific activation of macrophages and T lymphocytes, plays a major role in protection against this parasite, while the role of humoral immunity is less defined and appears to be less important (32). Several authors have shown that NK cells and cytotoxic $CD8^+$ and helper $CD4^+$ T lymphocytes (including cytokines secreted by these cells) at the mucosal site of infection are very important in protection against this parasite (4, 34, 39, 56). Changes in subpopulations of intestinal T cells were found to correlate with disease in chickens following primary and secondary infections with *Eimeria acervulina*. Chickens that were less susceptible to *Eimeria* infection had significantly higher numbers of $CD8^+$ intraepithelial lymphocytes and manifested lower levels of oocyst production than chickens that were more susceptible to *Eimeria* (33). Development of Th1-type gamma

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interferon (IFN-γ)-mediated immunity appears to be dominant during *Eimeria* infections. Administration of exogenous recombinant IFN-γ to chickens significantly hindered the intracellular development of *Eimeria* parasites and reduced body weight loss (35).

Attenuated strains of *Eimeria*, so-called precocious lines, induce protective immunity in chickens and have been used successfully to control coccidiosis. Yet these strains are very costly to produce, have a limited shelf life, and have the potential to revert to the pathogenic wild type (42). Thus, construction of recombinant vaccines has become an attractive and promising approach to control this disease.

Recombinant attenuated *Salmonella* vaccine (RASV) strains have been developed for use as vectors for delivery of heterologous antigens to the gastrointestinal mucosa and other lymphoid tissues (50). The type III secretion system (TTSS), a complex *Salmonella* virulence organelle encoded within *Salmonella* pathogenicity island 1 (SPI-1), has been used successfully for antigen delivery (9, 44). The SPI-1 TTSS consists of more than 20 proteins and is critically important for virulence during the intestinal phase of infection (15). This needle-shaped organelle spans the inner and outer *Salmonella* membranes and, upon contact with eukaryotic cells, injects *Salmonella* effector proteins into the cytoplasm of host cells. Translocated *Salmonella* effector proteins then modulate cellular functions and signal transduction pathways of the host cells (14). Some of these *Salmonella* effector proteins have been used as vehicles for delivery of various antigens into the cytoplasm of host cells (44). A protective major histocompatibility complex class I epitope of lymphocytic choriomeningitis virus, when delivered fused to the *Salmonella* effector protein SptP, generated strong and persistent virus-specific cytotoxic T-lymphocyte (CTL) responses in mice (51). Virus-specific CTLs were present 135 days after the last immunization and were quantitatively sufficient to provide protective immunity against challenge with lymphocytic choriomeningitis virus (51). Similarly, Evans and coworkers (9) delivered fragments of the simian immunodeficiency virus (SIV) Gag protein fused to the effector protein SopE and were able to prime virus-specific $CD4^+$ and $CD8^+$ T-cell responses in rhesus macaques. However, the T-cell responses stimulated in this study were insufficient to protect against an intrarectal challenge with SIVmac239.

Therefore, the objective of the present study was to investigate the potential of RASV strains as delivery vectors of sporozoite and merozoite antigens of *E. acervulina* and the ability of RASV to induce in chickens antigen-specific CMI responses that would confer immunity and protection against *E. acervulina* challenge. *E. acervulina* is one of the most prevalent and highly pathogenic species of *Eimeria* and is considered to be one of the most important species for the poultry industry (49). We chose to use EASZ240 and EAMZ250 antigens because past research has shown partial efficacy with both clones in protecting against *E. acervulina* challenge (23, 24, 29), and the two clones (EASZ240 and EAMZ250) were obtained from cDNA libraries from this species (22). For expression of the recombinant antigens, we used Asd^+ plasmids that are retained in vivo in *Salmonella* vaccine strains harboring a deletion of the *asd* gene (13, 41), which is essential for synthesis of the bacterial cell wall component diaminopimelic acid (DAP). *Eimeria* protein antigens were fused to the 180-amino-acid translocation domain of the effector protein SptP (11) to be delivered by the *Salmonella* TTSS directly into the cytoplasm of intestinal cells of the immunized chickens.

In this work, we report RASVs that express and deliver *E. acervulina* antigens into the culture supernatant and into the cytosol of intestinal epithelial cells. In addition, these vaccines induce protective immunity against *E. acervulina* challenge in immunized chickens.

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 P22HT*int* (48) was used for generalized transduction. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium cultures were grown at 37°C in Lennox broth (31), RM medium (Invitrogen), Luria-Bertani (LB) broth, or Luria-Bertani agar (3). MacConkey agar (Difco, Detroit, Mich.) supplemented with 1% lactose 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; tetracycline, 15 μg/ml. DAP was added (50 μg/ml) for the growth of Asd^- strains (41). LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in allelic exchange experiments (16). *E. coli* strain LMG194 (Invitrogen), used for expression of EASZ240 and EAMZ250 antigens (Table 1), was grown in RM medium (Invitrogen).

General DNA procedures. DNA manipulations were conducted using standard procedures (45, 46). *E. coli* and *Salmonella* serovar Typhimurium were transformed by electroporation (Bio-Rad, Hercules, Calif.). 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. Recombinant suicide plasmids were transferred to *Salmonella* by conjugation using *E. coli* χ7213 (43) as the plasmid donor. Bacteriophage P22HT*int*-mediated general transduction was performed by standard methods (52). The constitutive expression of β-galactosidase in *Salmonella* serovar Typhimurium χ8879 and χ8916 was made possible by introducing the *atrB13*::*mudJ* allele (10) by P22-mediated transduction from χ4574, resulting in strains χ9085 and χ9086, respectively. PCR amplification was employed to obtain DNA fragments for cloning and for verification of chromosomal deletion mutations. Nucleotide sequencing reactions were performed using ABI Prism fluorescent Big Dye terminators according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems, Norwalk, Conn.).

Characterization of phenotype. The phenotype of the vaccine strain χ8879 was confirmed by spraying 0.25% catechol (Sigma, St. Louis, Mo.) onto the colonies plated on Luria-Bertani agar. The *xylE* gene codes for the enzyme catechol 2,3-dioxygenase, which converts the colorless substance catechol to 2-hydroxymuconic semialdehyde, a yellow product. Colonies of cells that express the *xylE* gene turn yellow shortly after being exposed to catechol (20). MacConkey agar supplemented with 1% lactose was used to detect *Salmonella* serovar Typhimurium in tissue samples. The presence of the *asdA16* mutation in *Salmonella* serovar Typhimurium was confirmed by PCR and by the inability of the strain to grow on media without DAP (41). Lipopolysaccharide (LPS) profiles of *Salmonella* serovar Typhimurium strains were examined by previously described methods (19).

SDS-PAGE and immunoblot analyses. Protein samples were boiled for 5 min and separated by discontinuous 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie brilliant blue R250 (Sigma) staining. For immunoblotting, proteins separated by SDS-PAGE were transferred eletrophoretically to nitrocellulose membranes. The membranes were blocked with 5% skim milk in 100 mM Tris containing 0.9% NaCl and 0.1% Tween 20 (pH 7.4) and were incubated first with either mouse monoclonal antibodies specific for SptP, anti-EASZ240 rabbit polyclonal antibodies, or mouse anti–β-galactosidase antibodies (Sigma) and then with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin G (Southern Biotechnology, Birmingham, Ala.). Immunoreactive bands were detected by the addition of nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma). The reaction was stopped after 2 to 5 min by washing the membranes several times with large volumes of deionized water.

Plasmid construction. *E. coli* χ6212 was used as an intermediate host strain for cloning. Plasmid pYA3536 was generated by partially digesting and deleting the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Derivation or source
Strains		
E. coli		
χ6212	F⁻ λ⁻ ϕ80 Δ(*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*	Lab collection
χ7213	*thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44* Δ*asdA4 recA1* RP4 2-Tc::Mu [λ*pir*]; Km^r	Lab collection
LMG194	F⁻ Δ*lacX74 galE thi rpsL* Δ*phoA* (PvuII) Δ*ara714 leu*::Tn*10*	Invitrogen
Salmonella serovar Typhimurium		
χ3761	UK-1 wild type	Lab collection
χ8879	Δ*asdA16* Δ*phoP233* Δ*sptP1033*::*xylE*	This study
χ9085	Δ*asdA16* Δ*phoP233* Δ*sptP1033*::*xylE hisG atrB13*::*mudJ*	χ8879
χ8916	Δ*asdA16* Δ*phoP233*	Lab collection
χ9086	Δ*asdA16* Δ*phoP233 hisG atrB13*::*mudJ*	χ8916
Plasmids		
pYA3653	Plasmid Asd+; p15Aori	This study
pYA3657	566-bp DNA encoding EASZ240 in pYA3653	This study
pYA3658	792-bp DNA encoding EAMZ250 in pYA3653	This study
pYA3620	β-Lactamase signal sequence-based periplasmic secretion plasmid	Lab collection
pYA3731	566-bp DNA encoding EASZ240 in pYA3620	This study
pYA3696	566-bp DNA encoding EASZ240 in pBAD/HisC; Ap^r	This study
pYA3697	792-bp DNA encoding EAMZ250 in pBAD/HisC, Ap^r	This study

P_{trc} region of pYA3332 (with BglII and BamHI). A 2,172-bp sequence harboring the promoter region of the *sicP* gene (12) to downstream of the entire *sptP* gene (Fig. 1B) was PCR amplified from *Salmonella* serovar Typhimurium UK-1 chromosomal DNA (forward primer, 5′-TAA**CCCGGG**ATATGTGTTCCGATGCG-3′; reverse primer, 5′-CCC**AAGCTT**CAGCTTGCCGTCGTCATA-3′; restriction enzyme sequences are shown in bold) and cloned into SmaI-HindIII-digested pYA3536 to yield pYA3539. To delete 1,119 bp encoding the *sptP* C-terminal region, a fragment of 2.7 kb containing the C-terminally truncated *sptP* gene, the *sicP* promoter region, and the *asd* gene was amplified by PCR (forward primer, NcoI 5′-CGATG**CCATGG**AGTAAAGGTTGCTTAC-3′; reverse primer, XbaI 5′-CGCCTC**TAGA**TTTCAGTGCAATT-3′). A fragment of pYA3332 (1,469 bp, harboring the 5S T1T2 transcriptional terminator and p15A replicon) was generated by digesting pYA3332 with NcoI and XbaI and was then ligated to a 2.7-kb fragment generated by PCR to generate plasmid pYA3653 (Fig. 1A). The *EASZ240* gene of *E. acervulina* (21) was amplified by PCR (forward primer, EcoRI 5′-CG**GAATTC**GCGTTTCTTTGTATTTCCTTAC-3′; reverse primer, BamHI 5′-GTA**GGATCC**CATCAAGTGGTTGTGCACTGG-3′) and cloned into pYA3653, fused to the truncated *sptP* gene (encoding the 180-amino-acid translocation domain) (30), to generate plasmid pYA3657. Similarly, the *EAMZ250* gene of *E. acervulina* (21) was amplified by PCR (forward primer, EcoRI 5′-CG**GAATTC**GCCTTTGCCCTTTTCTCCTCCT-3′; reverse primer, BamHI 5′-GTA**GGATCC**CGCACAATCCGCTCTGGCAGT-3′) and cloned into pYA3653, fused to the truncated *sptP* gene, to generate plasmid pYA3658 (Fig. 1A).

For antigen delivery by the β-lactamase secretion system, vector pYA3620 (generously provided by In-Soo Lee) was used. The *EASZ240* gene was PCR amplified (forward primer, EcoRI 5′-CCG**GAATTC**CGTTTCTTTGTATTTCCTTACTC-3′; reverse primer, SalI 5′-ACGC**GTCGAC**AGCGTAATCTGGAACATCGTATGGGTAGAAGCCGCCCTGGTACAGGT-3′) and cloned into pYA3620 to yield vector pYA3731 (Fig. 2A). In-frame cloning was confirmed by nucleotide sequencing.

Determination of plasmid stability. Three milliliters of LB broth supplemented with 50 μg/ml DAP was inoculated with 3 μl (1:1,000) of the overnight culture of vaccine strain χ8879 (harboring pYA3653, pYA3657, or pYA3658) or χ8916 (harboring pYA3620 or pYA3731). Inoculated cultures were grown standing for 14 h (approximately 10 generations), after which time 3 μl was taken and inoculated into 3 ml of LB broth supplemented with 50 μg/ml DAP (1:1,000). Newly inoculated cultures were grown standing for 14 h. This process was repeated for 5 consecutive days (approximately 50 generations). To determine the proportions of cells retaining the Asd$^+$ plasmids, from each culture (for 5 consecutive days) after 14 h of growth, dilutions of 10^{-5} and 10^{-6} were plated onto LB agar plates supplemented with 50 μg/ml DAP and grown overnight. The following morning, 100 colonies from each vaccine construct were picked and patched onto LB agar plates that were either left unsupplemented or supplemented with 50 μg/ml DAP. Colonies that grew on LB agar only or on LB agar supplemented with 50 μg/ml DAP were counted, and the percentage of clones retaining the plasmids was determined.

Cloning, expression, and purification of the EASZ240 and EAMZ250 antigens. The *E. acervulina* sporozoite and merozoite genes *EASZ240* and *EAMZ250*, respectively, were cloned into a pBAD/HisC plasmid (Invitrogen) downstream from the metal-binding domain encoded by the polyhistidine and Xpress epitope tags to yield plasmids pYA3696 and pYA3697. In-frame cloning was confirmed by nucleotide sequencing. Expression of the EASZ240 and EAMZ250 proteins by LMG194 cells harboring pYA3696 and pYA3697, respectively, was detected by Western blot analysis (Fig. 3) using primary anti-Xpress monoclonal antibodies (Invitrogen) and alkaline phosphatase-conjugated rabbit anti-mouse secondary antibodies (Sigma). Optimal protein expression was observed after induction with 0.002% arabinose in RM medium. EASZ240 protein was purified using a Ni^{2+} affinity column (Sigma). 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. Western blot analysis using anti-Xpress monoclonal antibodies (Invitrogen) was performed to identify the purified protein.

Secretion of SptP-EASZ240 into the culture supernatant. RASV strains were grown in 250 ml of LB broth containing 300 mM NaCl with gentle aeration (100 rpm in a 500-ml flask) to an optical density at 600 nm (OD_{600}) of 0.6 (28). Cells were separated from the culture supernatant by centrifugation at 7,000 × *g* for 15 min. The culture supernatant was then filtered through a 0.22-μm-pore-size filter to remove any remaining bacteria. Culture supernatant proteins were precipitated with 10% trichloroacetic acid (TCA) (Sigma) for 2 h at 4°C and pelleted by centrifugation at 10,000 × *g* for 20 min. The pellets were resuspended in 5 ml of ice-cold phosphate-buffered saline (PBS), and proteins were precipitated for 2 h in 20 ml of ice-cold acetone. After centrifugation at 10,000 × *g* for 20 min, the pellets were washed with 1.5 ml of ice-cold acetone, centrifuged, dried, and resuspended in 250 μl of ice-cold PBS. Samples (15 μl) were run on SDS-PAGE gels and transferred to a nitrocellulose membrane to be analyzed by Western blotting. The secreted SptP-EASZ240 fusion protein was detected with either primary polyclonal antibodies (rabbit anti-EASZ240) or mouse anti-SptP antibodies (generously provided by Jorge Galan). To determine that the antigen is secreted actively via the TTSS rather than being released in the culture supernatant due to cell lysis, supernatant and pellet samples were analyzed by immunoblotting for the presence of β-galactosidase by using anti-β-galactosidase antibodies (Sigma). β-Galactosidase production by χ9085 was used as a cytoplasmic

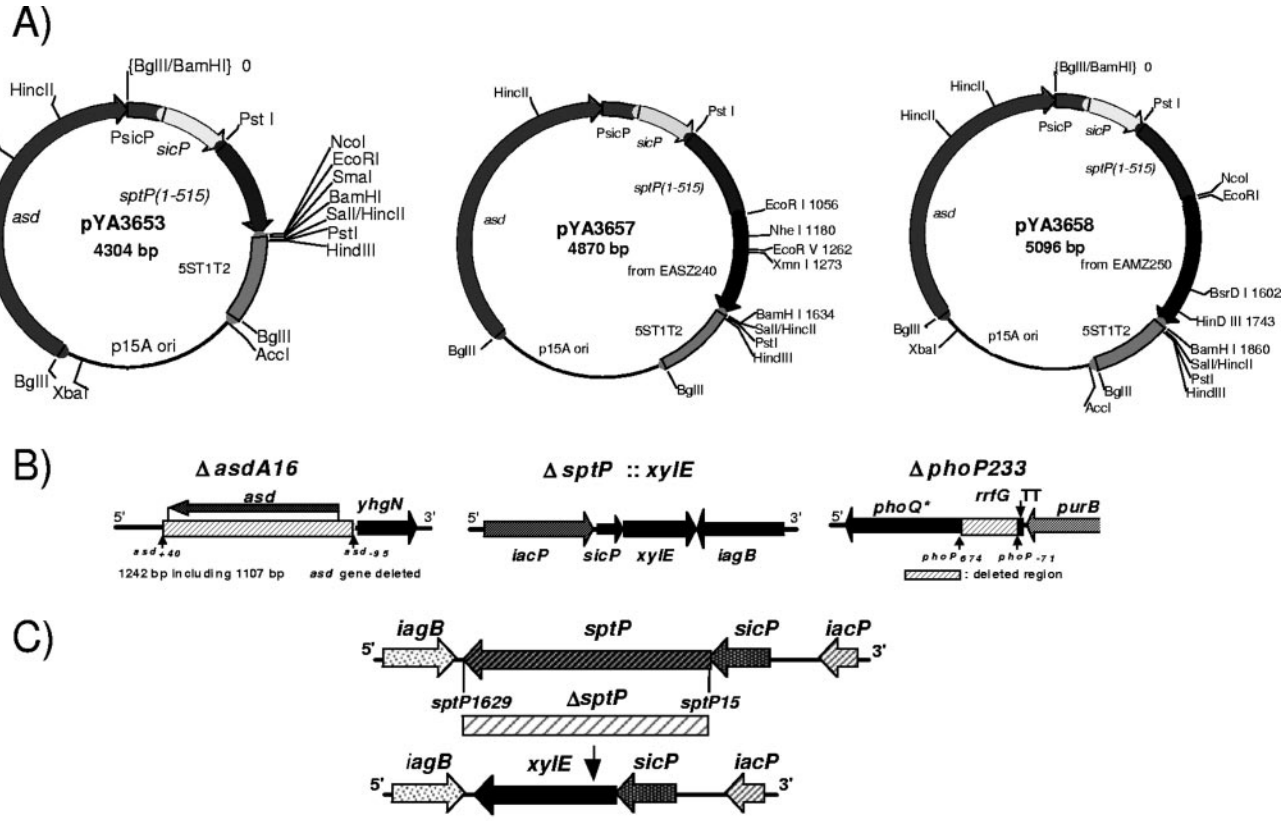

FIG. 1. (A) Vectors constructed for delivery of *Eimeria* antigens EASZ240 and EAMZ250 via the TTSS. The *EASZ240* and *EAMZ250* genes of *E. acervulina* were cloned into the Asd$^+$ vector pYA3653, and the products were fused to a truncated *Salmonella sptP* gene to generate vectors pYA3657 and pYA3658, respectively (B) Vaccine strain *Salmonella* serovar Typhimurium χ8879 (Δ*sptP1033*::*xylE* Δ*asdA16* Δ*phoP233*) chromosomal deletion map. (C) The deleted *sptP* gene was replaced with a reporter gene, *xylE*.

protein marker and as an indicator of membrane leaking in the examination of SptP-EASZ240 secretion into the culture supernatant.

***Salmonella* subcellular fractionation.** The periplasmic fraction was prepared by a modification of the lysozyme-osmotic shock method (27, 55). Fifty-milliliter cultures, grown in LB broth to an OD_{600} of 0.8, were then centrifuged at 7,000 × *g* for 15 min. The supernatant fluid was collected and filtered with 0.22-μm-pore-size filters, and supernatant proteins were precipitated with 10% TCA for 2 h at 4°C and pelleted by centrifugation at 10,000 × *g* for 20 min. The protein pellets were resuspended in ice-cold PBS (secreted protein fraction). 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 $MgCl_2$. After gentle agitation, the suspension was incubated for 15 min in an ice bath. Cells were removed by centrifugation at 7,000 × *g* for 15 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 10 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 in 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). An equal volume of each fraction sample was separated by SDS-PAGE for Western blot analysis. For analysis by enzyme-linked immunosorbent assay (ELISA) of anti-outer membrane protein (anti-OMP) serum antibodies generated in immunized chickens, *Salmonella* OMPs were prepared from *Salmonella* serovar Typhimurium χ4746 by using the OMP preparation procedure described above. The use of this strain for OMP preparation precludes contamination by LPS O antigen. Commercially available LPS (Sigma) was used for determination of titers against this antigen in the sera of immunized and nonimmunized chicks.

Detection of the SptP-EASZ240 fusion protein translocated into intestinal epithelial cell cytosol. The translocation assays described below were conducted according to the procedures described previously (6). Int-407 cells (ATCC, Manassas, Va.) were grown to 70 to 80% confluence in 100-mm tissue culture plates in Dulbecco's modified Eagle medium (GIBCO, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Invitrogen). *Salmonella* serovar Typhimurium vaccine strains were grown in LB broth to an OD_{600} of 0.9 and pelleted by centrifugation (7,000 × *g* for 15 min), and cell pellets were resuspended in Hanks balanced salt solution (HBSS; Sigma). Int-407 monolayers were infected with the appropriate vaccine strain at a multiplicity of infection of 50 for 90 min. The infection medium was removed, and cells were washed three times with HBSS. The infection medium and cell washes were pooled and centrifuged at 7,000 × *g* for 15 min to pellet the bacteria (fraction consisting of nonadherent bacteria). The bacterial cell pellet was resuspended in 200 μl of ice-cold PBS. The supernatant was filtered through 0.22-μm-pore-size syringe filters, and proteins were recovered by precipitation with 10% TCA (bacterium-free infection medium) for 1 h at 4°C. Int-407 cells were further incubated for 1 h in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 100 μg/ml gentamicin to kill any extracellular adhering bacteria. Cells were washed three times with HBSS and then treated with proteinase K (30 μg/ml in HBSS) for 15 min to remove cell surface-associated proteins. Proteinase K (Sigma) treatment was terminated by addition of 3 ml HBSS containing 2 mM phenylmethylsulfonyl fluoride. Int-407 cells were collected by slow-speed cen-

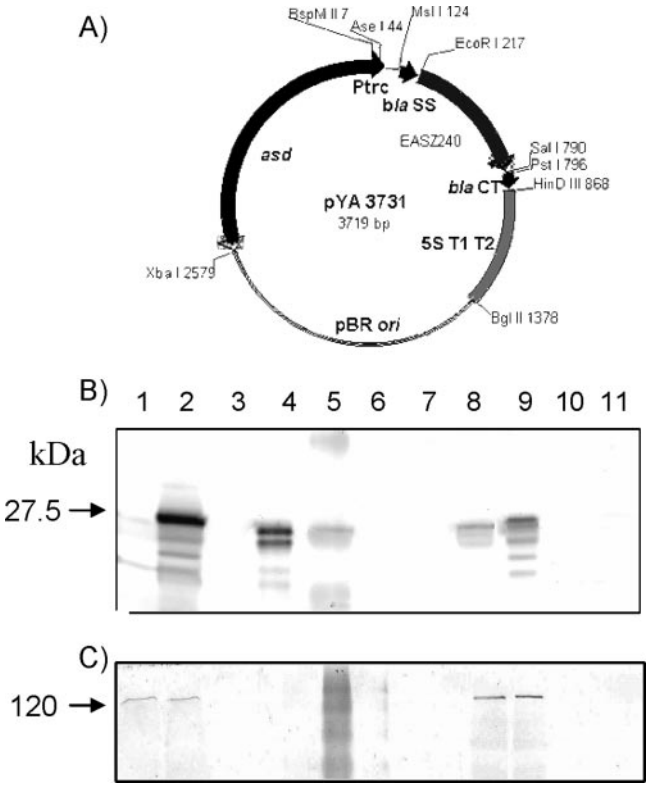

FIG. 2. (A) Map of the a*sd*$^+$ antigen expression vector pYA3731. A 0.74-kb EcoRI-SalI fragment of PCR-amplified *EASZ240* was cloned into the EcoRI and SalI sites of pYA3620. (B) Subcellular location of expressed EASZ240 protein in *Salmonella* serovar Typhimurium χ9086(pYA3731). (C) β-Galactosidase was used as a fractionation control for the cytoplasmic proteins and was detected with anti-β-galactosidase antibodies (Sigma). Lanes for panels B and C: 1 and 2, total-cell lysate; 3 and 4, concentrated supernatant; 5, protein standard (Invitrogen); 6 and 7, outer membrane fraction; 8 and 9, cytoplasmic fraction; 10 and 11, periplasmic fraction. Lanes 1, 3, 6, 8, and 10, RASV strain χ9086 harboring vector control pYA3620; lanes 2, 4, 7, 9, and 11, RASV strain χ9086 harboring vector pYA3731.

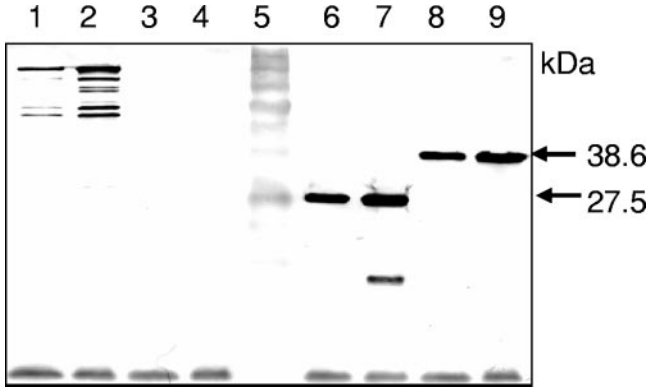

FIG. 3. Expression of EASZ240 and EAMZ250 antigens by pYA3696 and pYA3697, respectively. The *EASZ240* and *EAMZ250* genes of *E. acervulina* were cloned into plasmid pBAD/HisC (Invitrogen). Protein expression was induced by addition of 0.002% arabinose to RM medium. Expression was detected by Western blotting using anti-Xpress antibodies (Invitrogen). For each sample, either 1 or 5 μl of sample was loaded. Lanes: 1 and 2, pBAD/LacZ (positive control); 3 and 4, pBAD/HisC (negative control); 5, protein standard (Invitrogen); 6 and 7, pYA3696 (expressing EASZ240); 8 and 9, pYA3697 (expressing EAMZ250).

trifugation (500 × *g* for 12 min) and then lysed with 1 ml of HBSS containing 0.1% Triton X-100 (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The cell lysate was treated with DNase (10 μg/ml; Sigma) and RNase (10 μg/ml; Sigma) for 15 min at room temperature, followed by centrifugation at 10,000 × *g* for 15 min. The supernatant was filtered through a 0.220-μm-pore-size filter and proteins precipitated with 10% TCA (Triton X-100-soluble fraction) for 2 h at 4°C. The Int-407 cell pellet was suspended in 200 μl of ice-cold PBS (Triton X-100-insoluble fraction). The presence of the SptP-EASZ 240 protein was detected by a polyclonal primary antibody (rabbit anti-EASZ240), which was then recognized by an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit immunoglobulin G).

Chickens. Specific-pathogen-free fertile white leghorn eggs were obtained from SPAFAS Inc. (Roanoke, Ill.) and hatched in Humidaire (New Madison, Ohio) incubator-hatchers in Washington University's animal facilities. At hatching, chicks were randomly assigned to Horsfall isolators equipped with HEPA filters according to vaccine treatment group. Chicks were raised on a schedule of 23 h of light and 1 h of darkness, with feed and water provided for ad libitum consumption. The diet consisted of antibiotic-free chick starter (Purina Mills, St. Louis, Mo.). All experiments were conducted in accordance with protocols approved by the Washington University Animal Studies Committee.

Animal infectivity. RASV strains were grown overnight in LB broth at 37°C. The following day, 100 ml of LB broth was inoculated with an overnight culture (1:100) and grown with aeration (180 rpm) at 37°C to an OD_{600} of 0.8 to 0.9. Cells were pelleted by centrifugation at room temperature (7,000 × *g* for 15 min), and the pellet was resuspended in 1 ml of buffered saline with gelatin (BSG). To determine the titer of RASV strains used to inoculate chickens, dilutions of the RASV strains were plated onto MacConkey agar supplemented with 1% lactose. Newly hatched chicks were orally inoculated with 50 μl of BSG containing 1×10^9 CFU of an RASV strain. This dose of RASV was shown to be appropriate to ensure the colonization and persistence of RASVs in the lymphoid tissues and has no deleterious effects on the health of the chickens. At days 3, 6, 9, 12, and 15 after oral immunization, spleen, cecum, bursa, and liver samples were collected and homogenized to a total volume of 1 ml in BSG, and dilutions of 10^{-1} to 10^{-9} (depending on the tissue) were plated onto MacConkey agar to determine the numbers of viable bacteria (Table 2). Samples that were positive by enrichment in selenite cysteine broth (18) were recorded as <10 CFU/g, and negative samples as were recorded as 0 CFU.

Delayed-type hypersensitivity (DTH) response. Two-week-old chicks were orally immunized with 1×10^9 CFU of the respective vaccine strain in 50 μl of BSG. The same dose of vaccine was administered 7 days later. One week after secondary immunization, 100 μg (in 100 μl PBS) of EASZ240 protein was injected into the toe web of the left foot between the third and fourth digits. The right foot was injected with 100 μl of sterile PBS and served as a negative control to eliminate the background swelling induced at the site of injection by nonspecific inflammatory reactions. The thickness of the toe web was measured with a digital micrometric caliper before antigen injection and at 24 and 48 h after antigen or PBS injection. Data are expressed as the difference in toe web swelling between the left foot and the right (control) foot.

Lymphocyte proliferation assays. In the first experiment, single-cell suspensions from spleens of nonimmunized chicks (negative control), chicks immunized with χ8879 harboring pYA3653 (vector control), and chicks immunized with χ8879 harboring pYA3657 (expressing the EASZ240 antigen) were prepared by finely mincing spleen tissue and pushing it through a 60-μm nylon mesh with ice-cold Dulbecco's PBS. We were also interested in testing whether boosting immunization via β-lactamase secretion is a more potent inducer of CMI than boosting immunization via the TTSS. Thus, we repeated the experiment and added a vaccination treatment group in which chicks received a primary immunization via the TTSS (χ8879 harboring pYA3657) at the age of 2 weeks and then were immunized via the β-lactamase secretion system a week later (χ8916 harboring pYA3731). In both experiments, splenic or peripheral blood lymphocytes (PBLs) were obtained by Ficoll density gradient separation (Histopaque 1077; Sigma), washed twice, and subsequently resuspended in RPMI 1640 (supplemented with 2% heat-inactivated chicken serum) to a final concentration of 5×10^6 lymphocytes/ml. One hundred microliters of lymphocyte cell suspension was plated in each well of 96-well plates in triplicate. Lymphocytes were stimulated with 100 μl of either concanavalin A (ConA) (20 μg/ml), EASZ240 antigen (100 μg/ml), or RPMI 1640. After 24 h of culture at 41°C under 5% CO_2, 20 μl of Alamar Blue dye (Alamar, Sacramento, Calif.) was added to each well (17). At 72 h after the dye addition, the absorbance was measured at 570 and 600 nm with

TABLE 2. Isolation of RASV strain from chicken tissues at various times following inoculation

Plasmid[a] and no. of days after inoculation	Log_{10} colony count of RASV strain isolated per g of tissue (geometric mean ± SE)[b]			
	Liver	Spleen	Bursa	Cecum
pYA3653				
3	2.13 ± 2.17[c]	2.26 ± 2.24[c]	6.81 ± 0.63	8.42 ± 0.09
6	4.03 ± 1.77	5.57 ± 1.01	6.74 ± 0.63	8.58 ± 0.85
9	3.68 ± 2.04	6.24 ± 0.56	6.30 ± 0.88	8.89 ± 0.36
12	2.67[d]	4.43[d]	6.71 ± 0.36	9.94 ± 1.44
15	2.1[d]	2.37[d]	5.06 ± 0.27	7.54 ± 0.49
pYA3657				
3	NA	NA	6.73 ± 1.1	8.56 ± 0.32
6	2.71 ± 0.17	4.81 ± 0.66	5.99 ± 0.21	8.48 ± 0.47
9	1.72 ± 1.58[c]	3.34 ± 2.90[c]	5.39 ± 1.04	9.20 ± 0.38
12	1.82[d]	2.05 ± 1.97[c]	5.75 ± 0.55	8.10 ± 0.84
15	3.82[d]	2.43 ± 2.30[c]	6.13 ± 0.30	8.68 ± 0.74
pYA3658				
3	2.08 ± 1.80[c]	2.08 ± 2.93[c]	6.48 ± 0.52	8.03 ± 0.37
6	2.69 ± 2.69[c]	4.92 ± 1.41	5.75 ± 0.77	7.70 ± 0.11
9	3.54 ± 1.65	4.24 ± 3.70[c]	6.12 ± 1.28	9.55 ± 0.75
12	0.00 ± 0.00	3.72 ± 3.22[c]	5.77 ± 0.42	9.11 ± 0.14
15	4.33[d]	3.43 ± 3.22[c]	5.31 ± 0.69	9.19 ± 0.82

[a] Groups of 15 chicks were inoculated on the day of hatching with *Salmonella* serovar Typhimurium χ8879 (Δ*sptP1033*::*xylE* Δ*asdA16* Δ*phoP233*) harboring plasmid pYA3653, pYA3657, or pYA3658.
[b] Obtained from three chickens. NA, data not available.
[c] One chick had <10 CFU/g of tissue.
[d] Two chicks had <10 CFU/g of tissue.

a microplate reader. The specific absorbance was obtained by subtracting the absorbance at 600 nm from the absorbance at 570 nm. The change in specific absorbance was determined by subtracting the absorbance of unstimulated cells from the absorbance of EASZ240- or ConA-stimulated cells (1).

***Eimeria* challenge.** Outbred male chickens (Sexsal; Moyers Hatchery, Quakertown, Pa.) were assigned to six groups with15 chickens per group in Petersime starter cages (Petersime Incubator Co., Gettysburg, Ohio). The chickens were inoculated orally with 10^9 CFU of RASV strain χ8879 harboring pYA3653 (group 3), pYA3657 (group 4) (EASZ240), pYA3658 (group 5) (EAMZ250), or a combination of pYA3657 and pYA3658 (group 6) in 50 μl BSG. Control groups included chickens that were inoculated with 50 μl BSG only (groups 1 and 2). All chickens were immunized orally 1 week after the primary immunization. Chickens in groups 1 and 2 (controls), 3, and 5 (EAMZ250) received the same inoculum as in the primary immunization. For boosting immunization, the EASZ240 antigen was delivered via the β-lactamase secretion system (RASV strain χ8916 harboring pYA3731) to groups 4 and 6. *Salmonella* serovar Typhimurium harboring the same EAMZ250-expressing plasmid, pYA3658, was used in inoculation of group 6. The body weight of each chicken in groups 1 to 6 was determined 3 weeks after the booster immunization. After weighing, chickens in groups 2 to 6 were inoculated by crop gavage with 10^5 *E. acervulina* oocysts. The challenge dose of 10^5 oocysts was based on titration studies done 1 week earlier on Sexsal chickens of a similar age to provide a 20 to 25% weight loss. On day 6 post-challenge infection, chickens in groups 1 to 6 were terminated by cervical dislocation. Body weight and intestinal lesion scores were obtained using standard procedures (26).

Statistical analysis. Data were analyzed with a one-way or a three-way design by analysis of variance using the GLM (general linear models) procedure of SAS (47). Group means were separated by Tukey's or Duncan's multiple-comparison procedures and declared significantly different at a P value of <0.05. Data are expressed as means ± standard errors of the means.

RESULTS

Plasmid stability. The expression of *Eimeria* antigens by our RASVs was accomplished by introducing recombinant plasmids harboring either the *EASZ240* or the *EAMZ250* gene.

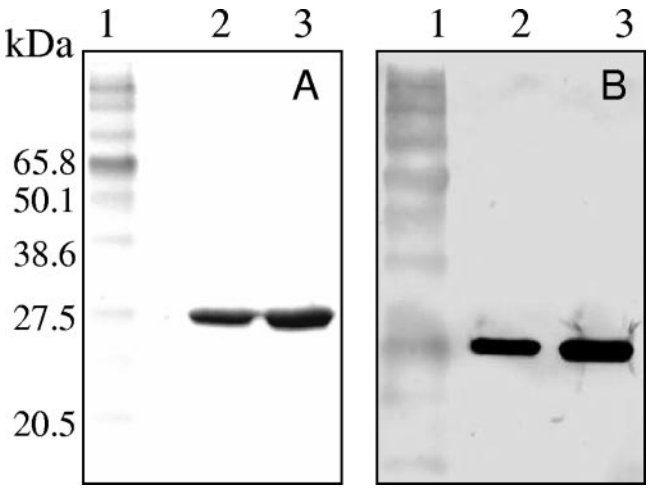

FIG. 4. EASZ240 protein purified by a aNi^{2+} affinity column. Either 5 or 10 μg of purified protein was loaded per well and analyzed by SDS-PAGE (A) or by Western blotting using anti-Xpress antibodies (Invitrogen) (B). Lanes: 1, protein standard (Invitrogen); 2, 2.5 μg protein; 3, 5 μg protein.

Therefore, plasmid stability is very important for antigen delivery and for the overall success of the RASVs. The *asd* gene of *Salmonella* serovar Typhimurium was used as a selective determinant for plasmid maintenance and complements the chromosomal Δ*asd* mutation (41). This balanced-lethal system was designed in order to avoid the use of antibiotic resistance as a selective marker, which is prohibited by the Food and Drug Administration. All four vectors used in our studies—pYA3653, pYA3657, pYA3658, and pYA3731—complemented the *asd* mutations of *Salmonella* serovar Typhimurium host strains χ8879 and χ8916. All these plasmids were stably maintained for 50 or more generations in *Salmonella* serovar Typhimurium (Δ*asd*) hosts grown in the presence or absence of DAP (data not shown). There were no differences between the LPS profiles of χ8879, χ8916 (with or without Asd^+ plasmids), and the wild-type strain, *Salmonella* serovar Typhimurium χ3761 (data not shown).

Expression and purification of the EASZ240 and EAMZ250 proteins. Expression of the EASZ240 and EAMZ250 antigens of *E. acervulina* by strain LMG194 harboring plasmid pYA3696 or pYA3697 was induced by addition of 0.002% arabinose to the culture medium (Fig. 3). After Ni^{2+} affinity purification, a single band of EASZ240 protein was observed by SDS-PAGE (Fig. 4A) and Western blot analysis (Fig. 4B). This protein was used to generate polyclonal antibodies in rabbits and to measure DTH responses and antigen-specific lymphocyte proliferation. For reasons not clear to us, we were unable to purify the EAMZ250 protein in spite of our attempts, and we were unable to make the necessary reagents to further characterize the RASV harboring this antigen. Thus, we chose to focus our efforts on using EASZ240 as a model antigen to demonstrate antigen secretion and translocation by our vaccine constructs and to characterize the immune responses elicited by the RASV harboring this antigen.

Secretion of the SptP-EASZ240 fusion protein into the culture supernatant. Many environmental factors modulate TTSS secretion of effectors into the culture medium. To determine

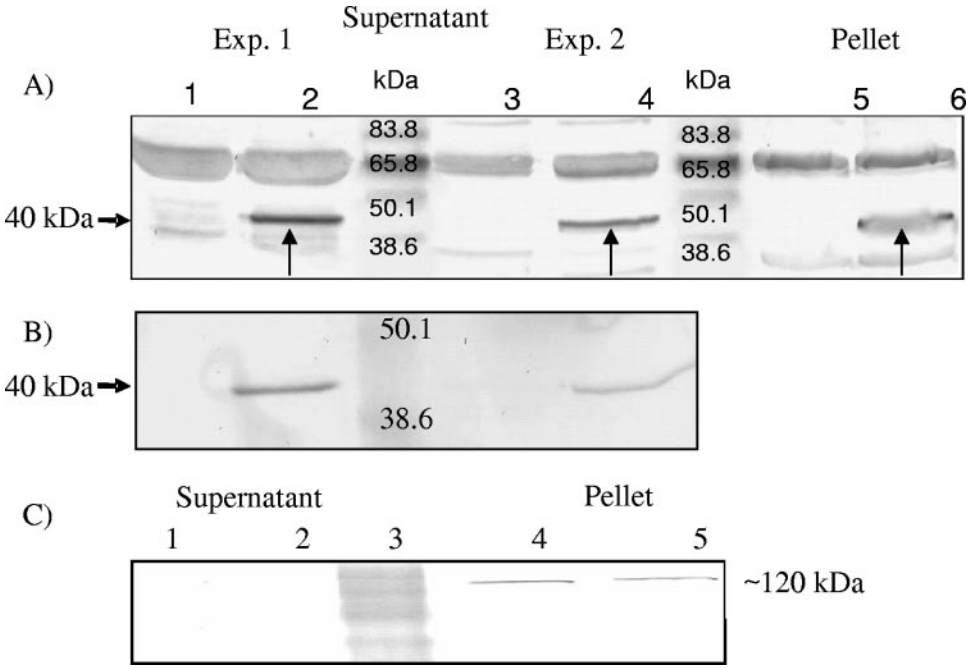

FIG. 5. Secretion of EASZ240 into the culture supernatant. The RASVs were grown under conditions of high osmolarity and low aeration to simulate the conditions of the intestinal environment. The secreted SptP-EASZ240 fusion protein was detected by immunoblotting using either rabbit anti-EASZ240 polyclonal antibodies (A) or anti-SptP antibodies (B). Lanes: 1, 3, and 5, RASV strain χ9085 harboring vector control pYA3653; 2, 4, and 6, RASV strain χ9085 harboring pYA3657. (C) β-Galactosidase was used as a control to determine whether the release of EASZ240 by χ9085 into the culture supernatant was due to cell lysis. Lanes: 1 and 4, χ9085 harboring vector pYA3653; 2 and 5, χ9085 harboring vector pYA3657; 3, protein standard (Invitrogen).

whether the SptP-EASZ240 fusion protein was being secreted into the culture supernatant via the TTSS, RASVs were grown under conditions that induce protein secretion by the TTSS, such as high osmolarity (300 mM NaCl), high pH (pH 8.2), and low aeration. In two similar experiments, the fusion protein was secreted into the supernatant via the TTSS (Fig. 5). No protein of similar size was detected in the cultures of χ8879 or χ9085 harboring pYA3653 (control group). β-Galactosidase production by χ9085 was used as a cytoplasmic protein marker and as an indicator of membrane leaking in the examination of SptP-EASZ240 secretion into the culture supernatant. By immunoblot analyses of the culture supernatant and the cell pellet fractions, β-galactosidase was detected only in the pellet fraction (Fig. 5C), suggesting that the EASZ240 protein detected in the culture supernatant was actively secreted by the TTSS rather than being released by nonspecific membrane leaking or due to cell death and lysis.

Subcellular localization of the EASZ240 antigen specified by pYA3731 in *Salmonella* serovar Typhimurium. To determine the location of the EASZ240 antigen, subcellular fractionations were performed as described previously (27). In the examination of EASZ240 antigen secretion into the culture supernatant, as with RASV delivering the antigen by the TTSS, β-galactosidase produced from χ9086 was used as a cytoplasmic protein marker and as an indicator of membrane leaking and cell lysis. A large amount of EASZ240 antigen was found either in the cytoplasmic fraction or secreted into the culture supernatant (Fig. 2). Little or no antigen was detected in the outer membrane and periplasmic fractions. This finding was surprising to us and is in contrast to the results of studies (27) showing that approximately 50% of the pneumococcal antigen PspA (pneumococcal surface protein A), secreted via the β-lactamase system, was located in the combined periplasm (25%) and culture supernatants (25%). β-Galactosidase was detected only in the pellet fraction, not in the supernatant, indicating that there was active antigen secretion into the culture medium rather than antigen release due to cell lysis.

Translocation of the SptP-EASZ240 fusion protein into the cytosol of Int-407 cells. We determined whether infection of Int-407 cells with an RASV expressing the SptP-EASZ240 fusion protein would result in translocation of SptP-EASZ240 protein into the cytosol of Int-407 cells. Thus, at 90 min after

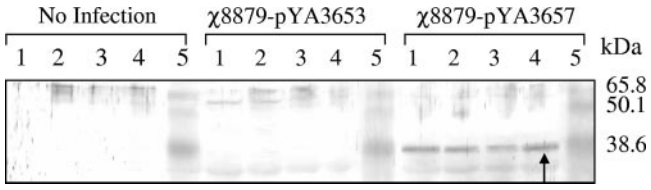

FIG. 6. Translocation of SptP-EASZ240 fusion protein across the Int-407 membrane. The fractionation of Int-407 cells infected with RASV expressing plasmid-encoded SptP-EASZ240 protein is shown. Lanes: 1, whole-cell lysates of nonadherent bacteria (infection medium); 2, bacteria-free, filtered infection medium; 3, Triton X-100-insoluble Int-407 cell lysate containing internalized bacteria; 4, Triton X-100-soluble fraction (cytoplasmic) containing translocated SptP-EASZ240 protein; 5, protein standard (Invitrogen). Equal amounts of sample were loaded for fractions 1 to 3 (in lanes 1 to 3). In lane 4, three times more sample from the cytoplasmic fraction was loaded; the arrow points to the band representing the translocated antigen.

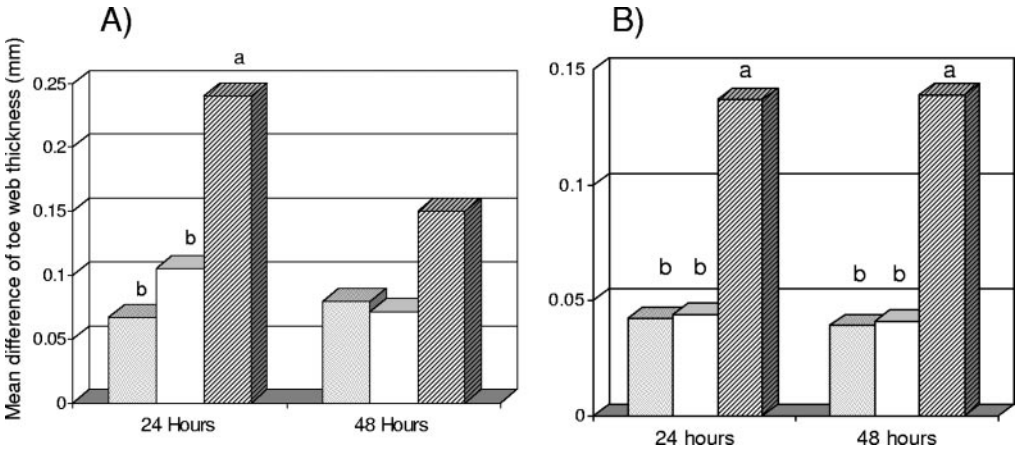

FIG. 7. Toe web swelling responses of chickens immunized with χ8879 harboring pYA3657 (dark patterned bars) or pYA3653 (open bars) and of nonimmunized chickens (light patterned bars). (A) Experiment 1; (B) experiment 2. Swelling was measured at 24 and 48 h after antigen administration. Bars represent the mean difference in swelling between a saline-injected toe web and an antigen-injected toe web. Means that do not share superscripts (a and b) are significantly different from each other ($P < 0.05$).

infection, biochemical fractionations of Int-407 cells were carried out as described previously (6). Fractions that were analyzed by Western blotting for the presence of the SptP-EASZ240 fusion protein were (i) bacterium-free infection medium, (ii) nonadherent bacteria separated from the infection medium, (iii) Triton X-100-insoluble fraction containing internalized bacteria, and (iv) Triton X-100-soluble fraction containing cytoplasmic proteins. To ensure that there was no nonspecific binding of the SptP-EASZ240 protein to the surfaces of Int-407 cells before Triton X-100 lysis, cells were treated with proteinase K. After 60 min of infection with χ8879 harboring pYA3657 at a multiplicity of infection of 50, SptP-EASZ240 protein was observed in all fractions analyzed, including the cytoplasmic fraction of Int-407 cells. No bands corresponding to the size of the SptP-EASZ240 fusion protein were detected in Int-407 cells infected with χ8879 harboring pYA3653 (vector control) or in uninfected cells (Fig. 6).

Evaluation of cell-mediated immunity. DTH assays were conducted in two separate experiments as a simple means of detecting antigen-specific CMI induced by RASV (Fig. 7 A and B). In both experiments, there was significant toe web swelling in chickens vaccinated with the RASV strain expressing the EASZ240 antigen. By comparison, the swelling of the toe webs of chickens immunized with a vaccine that did not express the EASZ240 antigen and of nonimmunized control chickens was significantly lower, indicating that the response observed for RASV-EASZ240-immunized chicks was antigen specific rather than a response to *Salmonella* antigens. The magnitude of the response and the vaccine treatment differences differed in the two experiments, which can be attributed to the inherent experimental variability.

Lymphocyte proliferation assays. Lymphocyte proliferation assays were conducted in order to better assess cell-mediated immune responses for immunized and control chicks. Spleno-

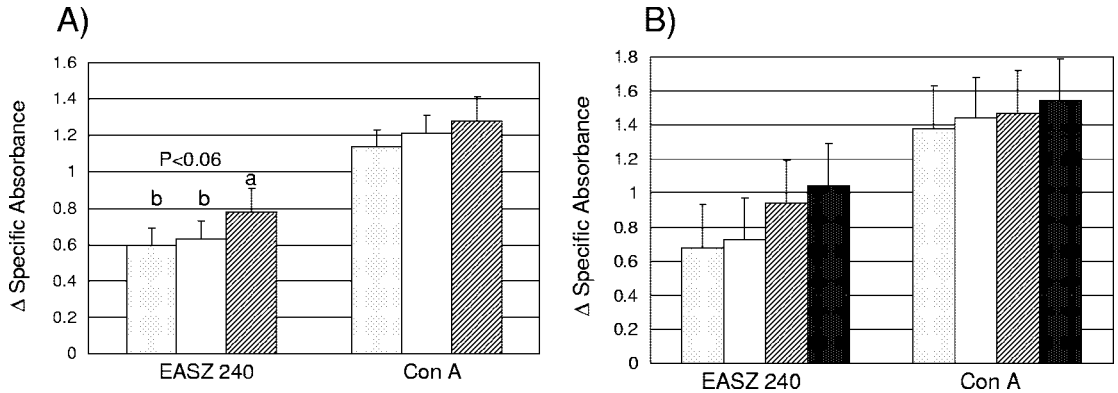

FIG. 8. In vitro proliferation of splenocytes or PBLs isolated from either nonimmunized chickens (light patterned bars), chickens immunized with χ8879 harboring pYA3657 (dark patterned bars) or pYA3653 (open bars), or chickens immunized with χ8916 harboring pYA3731 (solid bars). Splenocytes (A) or PBLs (B) isolated from immunized chicks were stimulated with either EASZ240 or ConA in triplicate. After 24 h of culture, 20 μl/well of Alamar Blue was added. Plates were read at 72 h after the addition of Alamar Blue on an ELISA reader using 570- and 600-nm filters. The change in specific absorbance was derived by subtracting the mean absorbance of unstimulated cells from the mean absorbance of EASZ240- or ConA-stimulated cells. In experiment 1 (A), TTSS vectors were used for primary and secondary immunizations, while in experiment 2 (B), one additional group of chicks was immunized with TTSS vectors and boosted with a β-lactamase secretion vector (pYA3731).

TABLE 3. Body weight gain and lesion scores of chickens immunized with RASV strains[a]

Group	Primary immunization (wk 1)	Secondary immunization (wk 2)	Oocyst challenge[b] (wk 5)	Weight gain (g)[c,d] (wk 6)	Lesion score[d,e] (wk 6)
1	BSG	BSG	No	157 A	0 D
2	BSG	BSG	Yes	129 C	2.1 A
3	pYA3653	pYA3653	Yes	130 C	1.7 ABC
4	pYA3657 (EASZ240)	pYA3731 (EASZ240)	Yes	135 BC	1.4 C
5	pYA3658 (EAMZ250)	pYA3658 (EAMZ250)	Yes	147 AB	1.5 C
6	pYA3657 (EASZ240) pYA3658 (EAMZ250)	pYA3731 (EASZ240) pYA3658 (EAMZ250)	Yes	142 BC	2.0 AB

[a] Chickens were immunized with RASV strain χ8879 (Δ*sptP1033*::*xylE* Δ*asdA16* Δ*phoP233*) harboring plasmid pYA3653, pYA3657, or pYA3658 or with RASV strain χ8916 (Δ*phoP233* Δ*asdA16*) harboring plasmid pYA3731.
[b] Chickens in groups 2 to 6 were inoculated with 10^5 *E. acervulina* oocysts.
[c] Right before the oocyst challenge and on day 6 after challenge infection, body weights were recorded and weight gain was calculated.
[d] Values followed by the same capital letter are not significantly different ($P < 0.05$).
[e] Intestinal lesion scores were obtained using standard procedures.

cytes (experiment 1) (Fig. 8A) or PBLs (experiment 2) (Fig. 8B) were isolated from 10 chicks per group and induced to proliferate with ConA (a T-cell mitogen) or purified EASZ240 antigen. Spontaneous background proliferation measured for RPMI-stimulated splenocytes or PBLs was subtracted from proliferation measured for ConA- or EASZ240-stimulated cultures. No significant differences in lymphocyte proliferation were observed among the three groups of chicks in response to stimulation with ConA (Fig. 8A and B). However, chicks immunized with χ8879 harboring pYA3653 or with χ8879 harboring pYA3657 exhibited only a numerically higher degree of proliferation than the nonimmunized control group. Upon stimulation with the EASZ240 antigen, lymphocytes isolated from chicks immunized with χ8879 harboring pYA3657 exhibited enhanced proliferation ($P < 0.06$) compared to the control groups. There were no differences in the proliferation of lymphocytes isolated from nonimmunized chicks and chicks immunized with χ8879 harboring pYA3653 (vector control) upon stimulation with the EASZ240 antigen (Fig. 8A). In the second experiment, we tested whether immunizing chicks via β-lactamase secretion after priming immunization via the TTSS would further enhance CMI over that of chicks immunized via the TTSS only. When stimulated with the EASZ240 antigen, lymphocytes isolated from chicks immunized via the TTSS and β-lactamase secretion exhibited enhanced proliferation compared to all the other groups, although the difference was not statistically significant ($P < 0.07$) (Fig. 8B).

Challenge of chickens and protection by RASV. For the challenge experiments, in addition to RASV expressing SptP-EASZ240, we also used RASV harboring pYA3658, which expressed a merozoite antigen, EAMZ250, fused to the 180-amino-acid translocation domain of the SptP effector protein. As expected, the lowest body weight gains and the highest lesion scores were observed for the *E. acervulina*-challenged control group (group 2). Weight gain for the challenged control group was nearly 80% of weight gain for the nonchallenged group (group 1) (Table 3). Chickens immunized with RASV expressing the EASZ240 antigen exhibited lower lesion scores (group 4); however, body weight gain was depressed as in the challenged control group (Table 3). Chickens immunized with RASV expressing EAMZ250 merozoite antigen (group 5) had higher weight gain and lower lesion scores than the nonimmunized and challenged control group ($P < 0.05$). In addition, the body weight gain was not significantly different from that of the nonimmunized and unchallenged group, group 1 ($P < 0.05$). Coadministration of RASV expressing both antigens did not significantly improve lesion scores; however, body weight gain was significantly higher for this group than for the nonimmunized challenged birds (group 2) ($P < 0.05$) (Table 3) or the vector control (group 3). These data suggest that immune responses elicited to the EASZ240 antigen lead to little or no protection against challenge by *E. acervulina*. By comparison, the EAMZ250 antigen is shown to be more protective against a virulent challenge.

DISCUSSION

Vaccination remains one of the most effective methods for managing the infectious diseases of agriculturally important animals and diminishing their negative economic impact. Ideally a vaccine should elicit innate and adaptive immune responses that result in protection of vaccinated animals against a challenge by the targeted pathogen. In addition, a vaccine should be easily administered and its use economically feasible. Due to a lack of affordable and convenient vaccines and the emergence of drug-resistant strains of *Eimeria*, there is an ongoing effort to develop new approaches to antigen delivery (5).

Attenuated *Salmonella* strains have been used in chickens as means of inducing immunity against *Salmonella* infection (7). Salmonellae are able to colonize gut-associated lymphoid tissue and invade via the intestinal mucosa; thus, they induce both mucosal and systemic immune responses (38, 40, 53). The immune responses induced by *Salmonella* are diverse, including both humoral and cell-mediated responses (38, 40). In addition, recombinant *Salmonella* strains have been developed for the delivery of heterologous viral, bacterial, and parasite antigens (9, 27, 44).

We have successfully cloned and expressed *E. acervulina* antigens in RASV strains and demonstrated that our RASVs do secrete the EASZ240 model antigen into the culture supernatant and are able to inject this antigen into the cytoplasm of Int-407 cells. When the antigen is delivered via the TTSS of RASVs, it induces antigen-specific mucosal and systemic immune responses (9, 44). All live stages of *Eimeria* from invasion to the formation of oocysts occur within intestinal tissues; thus,

inducing mucosal immunity to this parasite is of paramount importance. One important feature of RASVs is their ability to colonize and persist in mucosal and deep tissues and induce immune responses without causing disease. The attenuating mutations that were introduced into RASV strains for delivery of *Eimeria* antigens did not impair their ability to colonize and persist in deep tissues (Table 2).

In vivo stimulation of lymphocyte proliferation by our antigen points to the induction of antigen-specific lymphocytes that proliferate upon contact with the antigen. Other authors have shown induction of antigen-specific CTLs as well as increased PBL proliferation in response to antigen stimulation in vitro for animals vaccinated with RASV using TTSS antigen delivery (9). In addition, splenocytes isolated from *E. acervulina*-immune chicks showed antigen-specific proliferation and IFN-γ production when stimulated in vitro with the recombinant 3-1E merozoite antigen of *E. acervulina* (35), indicating that *E. acervulina* merozoite and antigens do induce cell-mediated immune responses. Moreover, immunization of chicks with this antigen induced protective immunity against live *E. acervulina* challenge (36).

Delayed-type hypersensitivity has been used extensively as a simple measure of CMI in general. DTH responses to *Salmonella* antigens have been demonstrated repeatedly following infections of chickens (18) and rodents (53) with various *Salmonella* serotypes. In our studies, we used this method to assess the induction of cell-mediated immunity in chicks immunized with RASV. To detect antigen-specific T-cell responses, DTH measurements were taken, and lymphocyte proliferation assays were conducted, at 10 days after the last immunization. Even though we did not examine the histology of the toe swelling, it is unlikely that the swelling of the toe web observed in our experiments could be attributed to an antibody-mediated inflammatory reaction, since delivery of an antigen by the TTSS is not designed to induce, and does not induce, any substantial amount of antigen-specific antibodies, while it does induce appreciable titers of antibody to *Salmonella* LPS and OMP antigens (unpublished observations). Considering that antigen delivery by RASVs occurs for as long as RASVs invade and colonize host tissues, it is difficult to predict the optimal time frame for detecting the antigen-specific peak CMI responses elicited. Another important factor that will affect the degree of antigen-specific immune responses detected is the immunogenicity of a chosen antigen. Thus, we feel that the best measure of RASV efficacy is the protection against a target pathogen that is determined following a challenge. We show that chicks that are immunized at the age of 1 week with an RASV expressing a single *Eimeria* antigen do develop some protective immunity to *E. acervulina*. This finding is very promising, since maximal natural challenge by this parasite does not occur until chickens reach the age of 3 to 5 weeks (5), by which age they would have had enough time to develop protective immunity to challenge. Future studies will explore the possibility of inducing protective immunity to *Eimeria* in birds less than 1 week old, by administering a single immunization dose.

Although a number of recombinant *Eimeria* proteins have been produced and used for immunizations, none have been found to be 100% effective against coccidiosis infection (25). Therefore, immunity to *Eimeria* that is induced by RASVs can be diversified either by including multiple *Eimeria* antigens in one RASV or by administering a mixture of RASVs harboring multiple antigens. In our studies, coadministration of the EASZ240 and EAMZ250 antigens did not have an additive effect in inducing protective immunity. This could be explained by the fact that the EASZ240 antigen alone did not induce significant protection (although there was some improvement in body weight gain and lesion scores), pointing to the observation that immune responses induced to this antigen are marginally relevant for protection against challenge. In addition, the dose of the EAMZ250-expressing RASV was cut in half when it was coadministered with the EASZ240-expressing RASV, resulting in diminished protection against challenge (Table 3, group 6). Thus, the choice of an *Eimeria* antigen, as demonstrated by challenge and protection experiments, is very critical for the efficacy of live RASVs.

Since delivery of antigens by the TTSS has been shown to be a very good way of priming the mucosal cytotoxic T-cell responses to heterologous antigens (9), we hypothesized that using another delivery mechanism such as β-lactamase, which is shown to induce both Th1- and Th2-mediated immune responses (27), would be a more efficient way of boosting the immune response initiated by TTSS delivery. To test this hypothesis, we immunized chicks with an RASV delivering the EASZ240 antigen via the TTSS, followed by a boosting immunization a week later with either an RASV delivering the EASZ240 antigen via the TTSS or an RASV delivering the antigen via β-lactamase secretion. We observed enhanced (although not statistically significant [$P < 0.07$]) proliferation of lymphocytes isolated from chicks that received primary immunization via TTSS delivery, followed by a secondary immunization via β-lactamase delivery, compared to that of chicks that were immunized via TTSS delivery twice (Fig. 8B). Moreover, chicks that received boosting immunization via β-lactamase delivery had significantly higher DTH responses ($P < 0.05$) than chicks that were immunized via TTSS delivery twice (data not shown). Based on these observations, we decided to use β-lactamase secretion for boosting immunization in challenge experiments and to test whether this immunization strategy would translate into better protection against *Eimeria* challenge. We did not observe such a response, and the only conclusion we could draw is that immune responses induced to this antigen are not relevant for protection against *Eimeria* challenge. This conclusion is further supported by other scientists who have observed a lack of protection against *Eimeria acervulina* challenge for chicks immunized with the EASZ240 antigen (Mark C. Jankins, personal communications). Studies employing novel antigens are warranted in order to better address the question of optimizing immunization strategies and to explore the effect that the dose of RASV has on its immunogenicity. Such studies are ongoing.

In conclusion, we demonstrate the expression and secretion of large protozoan antigens by RASV strains and the induction of antigen-specific immune responses. Immunity induced to the EAMZ250 antigen of *Eimeria* appears to be more protective than that induced to the EASZ240 antigen. Our current work is focused on investigating the immunogenicity of additional *Eimeria* antigens and employing different strategies of antigen delivery by RASV. In addition, we are optimizing vaccination parameters such as the dose of RASV adminis-

tered, the timing of the primary and boosting immunization, and novel attenuation strategies for more efficient antigen delivery and enhanced immune responses in vaccinated chickens.

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