Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment

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We have devised and constructed a biological containment system designed to cause programmed bacterial cell lysis with no survivors. We have validated this system, using *Salmonella enterica* serovar Typhimurium vaccines for antigen delivery after colonization of host lymphoid tissues. The system is composed of two parts. The first component is *Salmonella typhimurium* strain χ8937, with deletions of *asdA* and arabinose-regulated expression of *murA*, two genes required for peptidoglycan synthesis and additional mutations to enhance complete lysis and antigen delivery. The second component is plasmid pYA3681, which encodes arabinose-regulated *murA* and *asdA* expression and C2-regulated synthesis of antisense *asdA* and *murA* mRNA transcribed from the P22 P_R promoter. An arabinose-regulated *c2* gene is present in the chromosome. χ8937(pYA3681) exhibits arabinose-dependent growth. Upon invasion of host tissues, an arabinose-free environment, transcription of *asdA*, *murA*, and *c2* ceases, and concentrations of their gene products decrease because of cell division. The drop in C2 concentration results in activation of P_R, driving synthesis of antisense mRNA to block translation of any residual *asdA* and *murA* mRNA. A highly antigenic α-helical domain of *Streptococcus pneumoniae* Rx1 PspA was cloned into pYA3681, resulting in pYA3685 to test antigen delivery. Mice orally immunized with χ8937(pYA3685) developed antibody responses to PspA and *Salmonella* outer membrane proteins. No viable vaccine strain cells were detected in host tissues after 21 days. This system has potential applications with other Gram-negative bacteria in which biological containment would be desirable.

programmed cell lysis | rPspA | Rx1

Live attenuated pathogens, such as *Salmonella enterica*, have been developed as homologous vaccines and as carriers of heterologous antigens because of their capacity for efficient mucosal antigen delivery (1, 2). A variety of attenuating mutations (reviewed in refs. 1 and 2) and antibiotic-free balanced-lethal plasmid stabilization systems has been developed for this purpose (1, 3–5). However, biological containment systems are required to address the potential risk posed by the unintentional release of these genetically modified organisms into the environment, a subject of considerable concern (6, 7). Such release can lead to unintentional immunizations and the possible transfer of cloned genes that might represent virulence attributes in some cases. A number of mutations have been identified in *Salmonella typhimurium*, including *shdA*, *misL*, and *ratB*, that reduce environmental shedding in mice without negatively influencing immunogenicity (8). Although these mutations lead to a reduction in fecal shedding, it is unclear how long these strains will persist in the environment. Therefore, more effective systems need to be developed. Our approach has been to develop a biological containment system that will allow the vaccine strain time to colonize the host lymphoid tissues, a requirement for inducing a robust immune response (9, 10) and eventually lead to cell death by lysis, thus preventing spread of the vaccine strain into the environment.

The intracellular location of antigens in a recombinant attenuated *Salmonella* vaccine (RASV) can significantly influence the level of induced immune response upon immunization (11). Thus, if the antigen is retained in the cytoplasm and must be released by the actions of the immunized host, the immune response to the antigen is not as strong as when the antigen is secreted (11, 12). We hypothesize that the release of an expressed antigen by a RASV delivery strain within the lymphoid tissues of an immunized animal by programmed lysis would further enhance the immune response to the expressed antigen.

Streptococcus pneumoniae is the leading cause of childhood pneumonia worldwide (13). The recent emergence of drug-resistant strains has provided a strong incentive for preventing pneumococcal infections by vaccination. However, the capsular polysaccharide pneumococcal vaccines currently used to immunize adults are neither immunogenic nor protective in young children due to poor antibody responses (14). Recently, a conjugate pneumococcal vaccine was developed for children that contains the seven most common pneumococcal capsular polysaccharides conjugated individually to a genetic toxoid of diphtheria toxin. This vaccine protects children from invasive disease with strains of the seven capsular types present in the vaccine (15). Unfortunately, at more than $200 per child, the conjugate vaccine is too expensive for generalized use in the developing world. Moreover, since licensure of the seven-valent conjugate vaccine in 2000, pneumococci have evolved to seven common capsular types, some of which are not included in the vaccine, and more frequent infections with many of the other known 83 capsular types have been reported (16, 17). As a result, the efficacy of the vaccine is becoming seriously compromised.

The pneumococcal surface protein A (PspA) is highly immunogenic in mice and humans (18, 19). In previous studies, mice orally immunized with an *S. typhimurium* vaccine strain expressing the α-helical domain of *S. pneumoniae* strain Rx1 PspA generated PspA-specific immune responses and were protected against challenge with virulent *S. pneumoniae* (20). Further, mice immunized with an improved RASV antigen expression system in which the β-lactamase signal sequence, which directs proteins to the periplasm, was fused in frame to the immuno-

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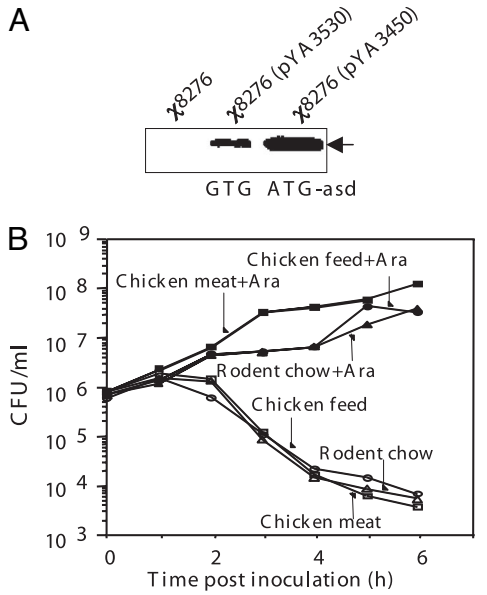

Fig. 1. The expression of Asd protein from the *asd* gene with ATG or GTG start codon and muramic acid-less death assay. (*A*) Western blot was performed with cell lysates of *S. typhimurium* strain χ8276 (Δ*asdA16*) and its derivatives cultured in LB broth with 0.2% arabinose. DAP was included in the medium for strain χ8276. Asd protein was detected by using rabbit anti-Asd serum. The 39-kDa Asd protein is indicated by an arrow. (*B*) Growth of *Salmonella* strain χ8645 (ΔP_{murA7}::*araC* P_{BAD} *murA*) with and without arabinose in the indicated media.

genic α-helical region of PspA developed strong anti-PspA immune responses and were protected against both *Salmonella* and *S. pneumoniae* challenge (21).

In this work, we constructed a RASV programmed bacterial lysis system vectoring the β-lactamase-PspA fusion protein. It was shown that this fusion protein is directed to the periplasm, but only ≈10–20% is released into the extracellular environment (21). This new system combines the features of a previously described antigen expression plasmid with a programmed bacterial lysis system designed to release antigen into the host tissues to induce an efficacious immune response and to provide biological containment of the RASV.

Results

Construction of a Regulated Programmed Lysis System. Diaminopimelic acid (DAP) and muramic acid are essential components of the peptidoglycan layer of the bacterial cell wall (22). The *asdA* gene encodes an enzyme essential for DAP synthesis and the *murA* gene encodes the first enzyme in muramic acid synthesis (23, 24).

To test the feasibility of an arabinose-regulated *asdA*-based lysis system, we introduced the Δ*asdA16* deletion mutation into *S. typhimurium* UK-1 resulting in strain χ8276 [supporting information (SI) Table S1]. We then introduced plasmid pYA3450, which carries the *asdA* gene with ATG start codon transcribed from the P_{BAD} promoter, which is activated by the AraC protein in the presence of arabinose (25), into χ8276 to yield χ8276(pYA3450). However, we found that growth of this strain was not arabinose-dependent, indicating that the level of residual transcription from the P_{BAD} promoter in the absence of arabinose was sufficient to produce enough Asd for growth. In addition, χ8276(pYA3450) also retained wild-type virulence in mice (data not shown). To reduce translational efficiency, we changed the *asdA* start codon from ATG to GTG, generating plasmid pYA3530. The GTG start codon significantly decreased the level of Asd expression (Fig. 1*A*). However, strain χ8276(pYA3530) still exhibited arabinose-independent growth and did not lyse in media devoid of arabinose. This problem was overcome by additional modifications described below, including addition of the *murA* gene.

Unlike lethal *asdA* deletions, which can be overcome by the addition of DAP to the growth medium, *murA* deletions, also lethal, cannot be overcome by nutritional supplements. Therefore, a conditional-lethal *murA* mutation was created by replacing the chromosomal *murA* promoter with the *araC* P_{BAD} activator-promoter. We introduced the ΔP_{murA7}::*araC* P_{BAD} *murA* mutation into wild-type *S. typhimurium* resulting in strain χ8645. To evaluate the predicted arabinose-dependent *murA* transcription, the strain was inoculated with and without arabinose into several media containing nutritional components that are likely to be encountered by a vaccine strain, including 1% rodent chow, 1% chicken feed, or 1% chicken breast meat in minimal medium (26). As expected, growth was not only dependent on the presence of arabinose, but bacterial titers dropped in the absence of arabinose, indicative of cell lysis (Fig. 1*B*).

We combined the *asdA* and *murA* systems, providing redundant mechanisms to ensure cell death. However, as described above, we first needed to reduce the amount of Asd produced from our plasmid. The *araC* P_{BAD} promoter-activator we used for all of the previously described constructs was derived from an *Escherichia coli* B/r strain (27). We discovered that when we substituted the *araC* P_{BAD} promoter-activator from *E. coli* K-12 strain χ289, transcription from the plasmid was more tightly regulated and arabinose-dependent growth was achieved (data not shown). We then inserted a *murA* gene in between the P_{BAD} promoter and the *asdA* gene to further decrease the transcription level of *asdA*. Finally, we introduced P22 P_R, a C2-regulated promoter, with opposite polarity at the 3′ end of the *asd* gene to interfere with transcription of the plasmid *asdA* and *murA* genes and to direct synthesis of antisense mRNA to block translation of mRNA transcribed from these genes during programmed lysis when arabinose is absent. Transcription terminators flank all plasmid domains so that expression in one domain does not affect the transcriptional activities of any other domain. The resulting plasmid was designated pYA3681 (Fig. 2*A*).

The host strain for this system was constructed by introducing a Δ*asdA* mutation into the ΔP_{murA7}::*araC* P_{BAD} *murA* mutant strain χ8645. To facilitate regulation of P22 P_R in plasmid pYA3681 (see above), we introduced the Δ*asdA19* deletion/insertion mutation, in which the P22 phage C2 repressor gene under transcriptional control of the P_{BAD} promoter was inserted into the Δ*asdA16* deletion (Fig. S1*A*). Three additional mutations designed to enhance lysis and facilitate antigen delivery were also included in this strain (Fig. S1*A*). The Δ(*gmd-fcl*)*-26* mutation deletes genes encoding enzymes for GDP-fucose synthesis, thereby precluding the formation of colanic acid, a polysaccharide made in response to stress associated with cell wall damage (28). This mutation was included because we have observed that under some conditions, *asdA* mutants can survive if they produce copious amounts of colanic acid (29). Therefore, by deleting the genes required for colanic acid synthesis, we circumvent this possibility. The Δ*relA1123* mutation uncouples cell wall-less death from dependence on protein synthesis to further ensure that the bacteria do not survive *in vivo* or after excretion and to allow for maximum antigen production in the face of amino acid starvation resulting from a lack of aspartate semialdehyde synthesis due to the *asdA* mutation (30, 31). This regulated lysis system *S. typhimurium* strain also has potential for use as a DNA vaccine delivery vector. Therefore, we included a Δ*endA* mutation, which eliminates the periplasmic endonuclease I enzyme (32), to increase plasmid survival upon its release into the host cell. The resulting strain, χ8937 (Δ*asdA19*::*araC* P_{BAD} *c2* ΔP_{murA7}::*araC* P_{BAD} *murA* Δ*(gmd-fcl)-26* Δ*relA1123* Δ*endA2311*), requires both arabinose and DAP for growth (Fig. S1*B*).

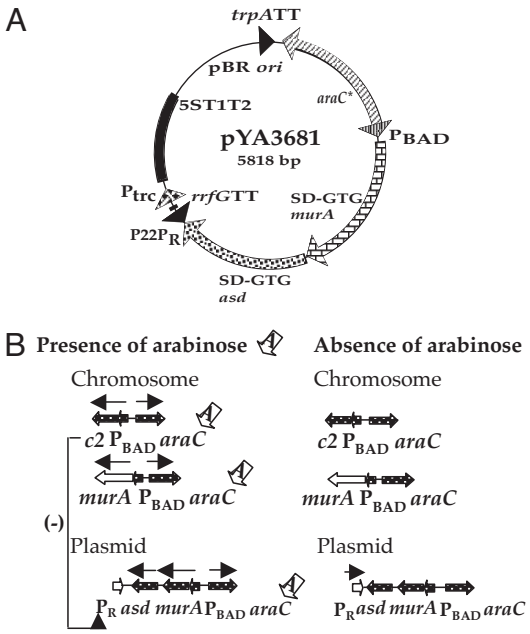

Fig. 2. The regulated programmed lysis system. (*A*) Map of plasmid pYA3681. Plasmid sequences include the *trpA*, *rrfG*, and 5S ribosomal RNA transcriptional terminators; the P_{BAD}, P_{trc} and P22 P_R promoters; the *araC* gene; and the start codon-modified *murA* and *asdA* genes. (*B*) Diagram of model illustrating the regulatory interactions in the programmed lysis system. Details of this system are outlined in *Results*.

pYA3681 was introduced into *S. typhimurium* χ8937. Growth of the resulting strain χ8937(pYA3681) required arabinose (Fig. S1*B*). The plasmid was stably maintained for 50 or more generations when grown in the presence of arabinose and DAP (data not shown). In the presence of arabinose, the plasmid-encoded copies of *asdA* and *murA* and the chromosomally encoded copies of *murA* and *c2* are transcribed from their respective P_{BAD} promoters, allowing for bacterial growth and repression of the P22 P_R promoter by C2 (Fig. 2*B*). In the absence of arabinose, the P_{BAD} promoters cease to be active, with no further synthesis of Asd and MurA or C2. The concentrations of Asd, MurA, and C2 decrease because of cell division. The decreased concentration of Asd and MurA leads to reduced synthesis of DAP and muramic acid and imbalanced synthesis of the peptidoglycan layer of the cell wall. As the C2 concentration drops, P22 P_R is derepressed, resulting in P_R-directed synthesis of antisense mRNA, which blocks translation of residual *asdA* and *murA* mRNA. These concerted activities lead to cell lysis.

Regulated Programmed Lysis and Biological Containment Properties after Colonization of Lymphoid Tissues. The regulated lysis vaccine strain χ8937(pYA3681) grew well in LB broth supplemented with 0.02% arabinose, but began to die after 1 h of incubation in LB broth without arabinose (Fig. 3*A*). To evaluate cell lysis, release of the cytoplasmic enzyme β-galactosidase into culture supernatants was used as an indicator. The *atrB13*::Mud*J* allele, which directs constitutive expression of β-galactosidase (21) was transduced into *S. typhimurium* wild-type as a nonlysis control and into vaccine strain χ8937, resulting in strains χ9379 and χ9380, respectively. We then introduced plasmid pYA3681 into χ9380 to yield χ9380(pYA3681). The ratio of β-galactosidase activity in the supernatant (released β-galactosidase) or cell pellet (retained cell-associated β-galactosidase) versus total β-galactosidase activity (supernatant plus cell pellet) indicated the extent of cell lysis. Release of β-galactosidase by strain χ9380(pYA3681) occurred only in medium lacking arabinose

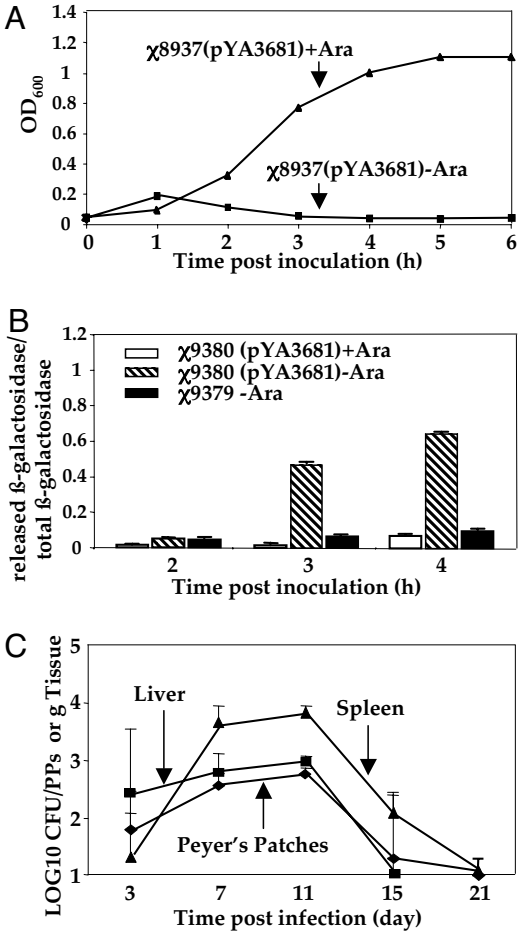

Fig. 3. *In vitro* and *in vivo* lysis of programmed lysis system in the absence of arabinose. (*A*) The growth curves of strain χ8937(pYA3681) with arabinose-regulated *asdA* and *murA* expression in LB broth with or without the addition of 0.02% arabinose. (*B*) The ratio of released β-galactosidase versus total β-galactosidase when strain χ9380(pYA3681) with arabinose-regulated *asdA* and *murA* expression and constitutive *lacZ* expression was grown in LB broth with or without 0.02% arabinose; the wild-type strain χ9379 modified to express *lacZ* acts as a nonlysis system control. (*C*) Colonization of mice with *S. typhimurium* χ8937(pYA3681) after P.O. inoculation with 10^9 CFU bacteria. The limits of detection for this assay were 10 CFU per Peyer's patch or gram of tissue.

(Fig. 3*B*). Conversely, the amount of cell-associated β-galactosidase decreased over time when χ9380(pYA3681) was grown in medium without arabinose, but no decrease was seen in media containing arabinose (data not shown). β-galactosidase release was not observed when the wild-type control strain χ9379 was grown without arabinose. These results are consistent with our expectations for the arabinose-regulated cell lysis phenotype.

To evaluate virulence, BALB/c mice were orally inoculated with doses in excess of 10^9 CFU of the host-vector strain χ8937(pYA3681), a dose 50,000 times the LD_{50} of the wild-type parent strain, χ3761. During the 30-day observation period after dosing, we observed no deaths or signs of illness in any of the mice. Colonization by strain χ8937(pYA3681) was evaluated in 8-week old female mice orally inoculated with 10^9 CFU. The strain transiently colonized lymphoid tissues (Fig. 3*C*), and no bacteria were recovered by 4 weeks after inoculation. No arabinose-independent *Salmonella* mutants were recovered at any time during this experiment. These results indicate that a wild-type *Salmonella* strain engineered with this programmed

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lysis system is attenuated and is efficiently cleared from the host after colonization of lymphoid tissues.

Construction of the rPspA Rx1-expressing Plasmid. It was shown that a recombinant protein fusing the first 35 aa of β-lactamase to the α-helical region of PspA (rPspA Rx1) is highly immunogenic when delivered by a recombinant avirulent *S. typhimurium* (21). We used a similar fusion to evaluate the ability of our regulated lysis strain to deliver an antigen to host tissues. A DNA fragment encoding the in-frame fusion of the β-lactamase leader sequence from plasmid pBR322 to rPspA Rx1 (α-helical region of PspA from amino acid residue 3 to 257of mature $PspA_{Rx1}$) was inserted into pYA3681 to yield pYA3685 (Fig. S2). The antigen is constitutively expressed from the P_{trc} promoter. Expression of rPspA Rx1 in *S. typhimurium* χ8937(pYA3685) grown in media with arabinose was detected by Western blot analysis with the anti-PspA monoclonal antibody (Fig. S3*A*), and we confirmed that the fraction of antigen secreted to the periplasm was similar to that reported (data not shown) (21). The strain did not grow on LB agar without arabinose and expression of the recombinant antigen did not interfere with programmed lysis when χ8937(pYA3685) was grown in LB broth without arabinose (Fig. S3*B*).

Immune Responses in Mice after Oral Immunization with the Regulated Programmed Lysis Host-vector System. The antibody responses to *Salmonella* outer membrane proteins (SOMPs) and to the foreign antigen rPspA Rx1 in sera and vaginal secretions of the immunized mice were measured (Fig. 4). The maximum serum IgG response to PspA was observed at 6 weeks, and responses at all time points were significantly greater than in the control group, where no response was detected ($P < 0.05$) (Fig. 4*A*). The anti-SOMP IgG response was slower to develop in mice vaccinated with χ8937(pYA3685) than in the control group, with significant differences between groups at weeks 2 and 6 ($P < 0.05$). This could be a result of differences in the ability of the two strains to survive systemically brought about by the antigen load in χ8937(pYA3685). However, both χ8937(pYA3681) and χ8937(pYA3685) elicited equivalent anti-SOMP IgA responses in vaginal secretions, with no significant differences between groups after 2 weeks, whereas rPspA Rx1-specific IgA was detected only in samples from mice immunized with vaccine strain χ8937(pYA3685) ($P < 0.05$) (Fig. 4*B*).

The type of immune responses to SOMPs and the rPspA Rx1 were further examined by measuring the levels of IgG isotype subclasses IgG2a and IgG1 (Fig. 4*C*). 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 (33). IgG2a isotype dominant responses were observed for the SOMP antigens indicating that the vaccine induced a strong cellular immune response against *Salmonella*. In contrast to the strong Th1 responses to SOMPs, a Th1- and Th2-type mixed response was observed for the rPspA Rx1 antigen (Fig. 4*C*).

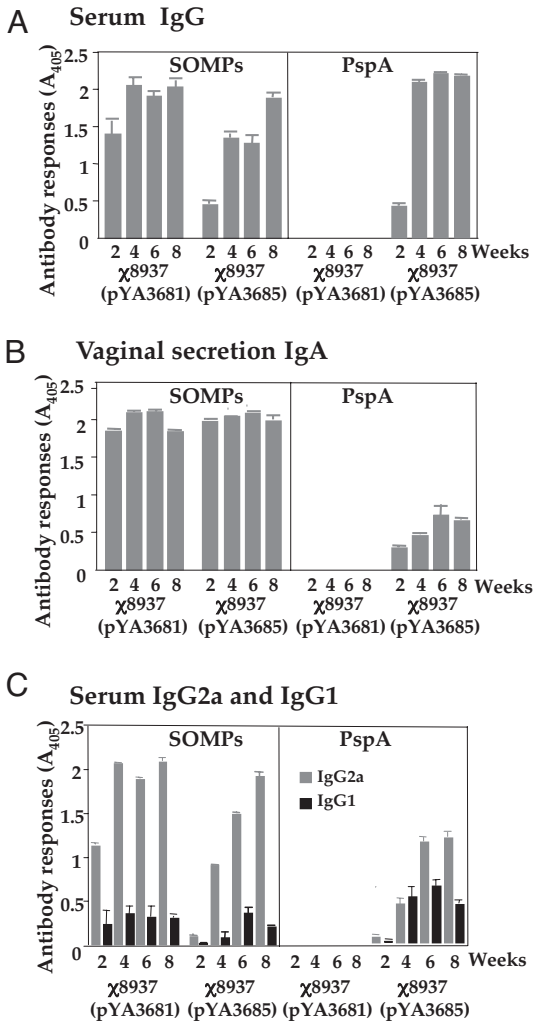

Fig. 4. Immune responses in mice after oral immunization with χ8937(pYA3685) (rPspA Rx1) and χ8937(pYA3681) (vector control) as determined by ELISA. (*A*) IgG antibody against *S. typhimurium* SOMPs and rPspA Rx1 in a 1:1,280 dilution of serum. (*B*) Anti-SOMP and -rPspA Rx1 IgA antibody levels in a 1:10 dilution of vaginal secretions. (*C*) Serum IgG2a and IgG1 responses to SOMPs and rPspA. Gray bars, IgG2a; black bars, IgG1. Serum was diluted 1:400.

Discussion

Our long-term goals are to develop RASVs for oral administration, protecting humans and animals against a variety of mucosal pathogens. Immunization with live *Salmonella* vaccines introduces the potential for release of the bacteria into the environment, possibly leading to unintended immunizations. The objective of this study was to construct and evaluate a biological containment system that would be consistent with the requirements for efficacious vaccination, in particular, colonization of host lymphoid tissues for an amount of time sufficient for optimal antigen delivery. RASV are capable of delivering a variety of bacterial, viral, fungal, and parasitic antigens, thereby eliciting humoral and cellular immunity in the immunized host (1, 34–36). Immune responses, especially antibody responses, are enhanced when the antigen is released into the extracellular environment as opposed to being sequestered in the bacterial cytoplasm (11, 12).

These considerations led us to develop a RASV containment/delivery system capable of releasing antigen by cell lysis within the immunized animal. We used the tightly regulated *araC* P_{BAD} activator-promoter system to construct a strain/plasmid system that directs regulated arabinose-dependent, programmed lysis. An arabinose-regulated cell lysis system should not be undermined by release into the environment, where stream and groundwater levels of arabinose are in the submicromolar range (37). Studies in our laboratory have shown that in our *Salmonella* strains, P_{BAD} is not activated by 13 μM (0.0002%) arabinose (S. Wang, personal communication).

We chose the *asdA* gene as the primary driver of cell lysis, because it is known that, unlike some lethal mutations, a lack of Asd results in both cell death and cell lysis (38). To further facilitate containment, we also included the *murA* gene in our scheme (Fig. S1*A*). The plasmid copy of *murA* was derived from

E. coli to reduce the potential for recombination with the *S. typhimurium* chromosomal copy, a possible escape strategy for the cell. Finally, we included the P22 P_R promoter, driving transcription of antisense mRNA to silence any residual mRNA transcripts that may arise from the plasmid copies of *asdA* or *murA* in the absence of arabinose. In our system, the C2 repressor, which inhibits P22 P_R transcription, is only synthesized in the presence of arabinose. Thus, in the arabinose-limiting environment in host tissues, C2 is not made and antisense mRNA is transcribed.

The data show that the system we have devised results in cell lysis in the absence of arabinose and clearance of the strain from host tissues (Fig. 3). More importantly, our strain was fully capable of delivering a test antigen and inducing a robust immune response comparable to that of a vaccine strain without this containment system (Fig. 4 and data not shown), thereby demonstrating that this system has all of the features required for biological containment of a RASV.

This system can be modified to suit a number of different needs for antigen delivery. We can add mutations that will delay lysis to allow additional time for the RASV to colonize host tissues. For example, we have deleted additional genes from the arabinose operon to prevent arabinose metabolism, thereby maintaining an effective arabinose concentration in the cytoplasm for a longer period. Strains with these arabinose gene deletions are currently being evaluated for use as antigen or DNA delivery vectors.

The regulated lysis system also has potential as a DNA vaccine vector delivery system. An *asdA* deletion mutant of *Shigella flexneri* has been used to deliver DNA in animals (39), but the immune responses were weak, presumably because the cells did not persist long enough to efficiently invade host tissues. A Δ*asdA* mutant of *E. coli* has also been used to deliver DNA in tissue culture (40). However, our system, whether used for *Shigella*, *E. coli*, or *Salmonella* (41), provides the vaccine with adequate time to establish itself in host tissues before lysis occurs, thereby enhancing the probability of efficient DNA delivery. Last, this system could be modified to provide effective biological containment for genetically engineered bacteria used for a diversity of purposes in addition to vaccines.

Materials and Methods

Bacterial Strains and Plasmids. Bacterial strains and plasmids used are listed in Table S1. *S. typhimurium* strains with *asdA* gene deletions were grown at 37°C in LB broth or on LB agar (42) supplemented with 50 μg/ml DAP (3). Transformants containing *araC* P_{BAD} *asdA murA* plasmids were selected on LB agar plates containing 0.2% arabinose. We used 0.02% arabinose in LB broth cultures to prevent pH changes in the medium caused by metabolism of arabinose that may affect the physiology of the bacterial cells. LB agar, containing 5% sucrose and no sodium chloride, was used for *sacB* gene-based counterselection in allelic exchange experiments (43). For mouse inoculation, *Salmonella* strains were grown with aeration after inoculation with a 1/20 dilution of a nonaerated static overnight culture. When required, antibiotics were added to culture media at the following concentrations: 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 50 μg/ml kanamycin.

General DNA Procedures. See *SI Materials and Methods*.

Strain Characterization. Vaccine strains were compared with vector controls for stability of plasmid maintenance, arabinose-dependent growth and antigen synthesis. Molecular genetic attributes were confirmed by PCR with appropriate primers. Lipopolysaccharide profiles of *Salmonella* strains were examined by methods described in ref. 44. For detection of PspA in RASV, 12 or 2 μl of cultures at an OD_{600} of 0.8 were subjected to SDS/PAGE or immunoblot analysis, respectively.

Construction of Regulated Programmed Lysis *S. typhimurium* Vaccine Host-Vector System. See *SI Materials and Methods* and Table S2.

Examination of Cell Lysis *in Vitro*. Overnight cultures of strains were grown in LB broth supplemented with 0.002% arabinose. We used 0.002% arabinose to prevent the accumulation of arabinose within bacterial cells to allow us to detect cell lysis during the short time frame used for this experiment. Cultures were diluted 1: 400 into fresh prewarmed LB broth with or without 0.02% arabinose. β-galactosidase activity in supernatant and cell-pellet fractions were assayed at indicated time points as described in ref. 45.

Colonization of Mice with the Regulated Programmed Lysis *Salmonella* Vaccine Strain. Seven-week-old female BALB/c mice (three mice for each time point) were deprived of food and water for 4 h before oral administration of *Salmonella* vaccine strains. These strains were grown with aeration in LB broth supplemented with 0.02% arabinose to an optical density at 600 nm (OD_{600}) of 0.85 from a nonaerated static overnight culture. CFU (1.3×10^9) of χ8937(pYA3681) in 20 μl of buffered saline containing 0.01% gelatin (BSG) was orally administered to the mice at the back of the mouth with a pipette tip. Food and water were returned to the animals 30 to 45 min later. Mice were killed at indicated times, and their Peyer's patches, spleens, and livers were collected aseptically. Tissues were homogenized and plated on LB agar with 0.2% arabinose to evaluate colonization and persistence and onto LB agar plates without arabinose to screen for arabinose-independent mutants.

Immunization of Mice. Groups of five 7-week-old female BALB/c mice were orally vaccinated with either 1.3×10^9 CFU *S. typhimurium* vaccine strain χ8937(pYA3685) (expressing rPspA Rx1) or 1.1×10^9 CFU host-vector control χ8937(pYA3681) as described above. A second oral dose of 1.2×10^9 CFU χ8937(pYA3685) or 1.1×10^9 CFU χ8937(pYA3681) was given 1 week later. The immunized mice were monitored for 60 days for evidence of illness by observing them daily for evidence of diarrhea, ruffled (ungroomed) fur, or irritability. None of these symptoms of infection were observed in any of the mice.

Blood was collected at weeks 2, 4, 6, and 8 after immunization. Serum fractions were stored at −20°C. Vaginal secretion specimens were collected by wash with 50 μl of BSG, solid material was removed by centrifugation and secretion samples were stored at −20°C (46).

Antigen Preparation. rPspA Rx1 protein and *S. typhimurium* SOMPs were purified as described in ref. 21.

ELISA. The procedures used for detection of antibody have been described in refs. 20 and 21. Briefly, polystyrene 96-well flat-bottom microtiter plates (Nunc) were coated with *S. typhimurium* SOMPs (100 ng per well) or purified rPspA Rx1 (100 ng per well). Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied with 100-μl volumes in each well. Vaginal secretions obtained from the same experimental group were pooled and diluted 1:10, and sera were diluted 1:1,280 for detection of IgG and 1: 400 for IgG1 and IgG2a, respectively. A 100-μl volume of diluted sample was added to individual wells in duplicate. Plates were treated with biotinylated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology) for sera and IgA for vaginal secretions.

Statistical Analysis. Most data were expressed as means ± standard error. The means were evaluated with one-way ANOVA and least significant difference test for multiple comparisons among groups. $P < 0.05$ was considered statistically significant.

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