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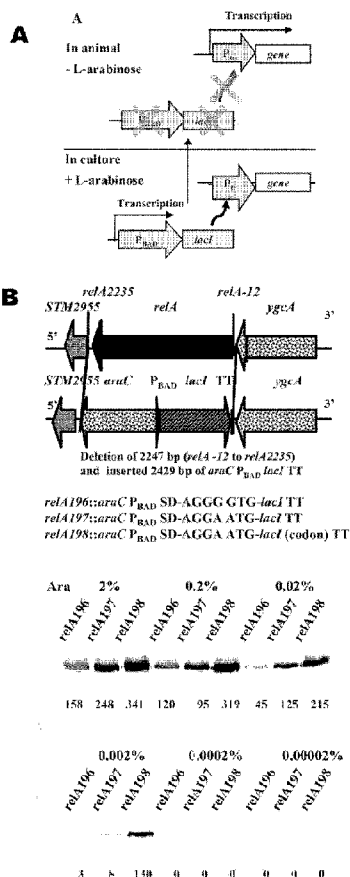
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FIG. 19



(57) Abstract: The invention relates to compositions and methods for making and using recombinant bacteria that are capable of regulated attenuation and/or regulated expression of one or more antigens of interest.



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REGULATED SYNTHESIS OF ANTIGEN AND/OR REGULATED ATTENUATION TO ENHANCE VACCINE IMMUNOGENICITY AND/OR SAFETY

GOVERNMENTAL RIGHTS

[0001] This invention was made with government support under Grant Nos. 5RO1DE006669, 5RO1AI056289, RO1AI24533, and RO1AI057885 awarded by the National Institutes of Health, and Grant Nos. 99-35204-8572, 2001-02994, and 2003-35204-13748, awarded by the United States Department of Agriculture. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0002] The invention relates to compositions and methods for making and using recombinant bacteria that are capable of regulated attenuation and/or regulated expression of at least one nucleic acid encoding an antigen of interest.

BACKGROUND OF THE INVENTION

[0003] Recombinant microorganisms have widespread utility and importance. One use of these microorganisms is as live vaccines to produce an immune response. Live vaccines are most effective when they produce high levels of antigen. However, the synthesis of a recombinant antigen encoded by a highly expressed nucleic acid sequence may be deleterious to the microorganism. Because of this, regulated (as opposed to constitutive) expression systems have been identified and utilized where the recombinant nucleic acid sequence of interest is operably linked to control elements that allow expression of significant amounts of the recombinant nucleic acid sequence only when it is induced, derepressed or activated. Examples include the *cspA* nucleic acid sequence promoter, the *phoA* nucleic acid sequence promoter, P_{BAD} (in an *araC*-P_{BAD} system), the *trp* promoter, the *tac* promoter, the *trc* promoter, λ P_L, P22 P_R, *mal* promoters, *rha* promoter, *xyl* promoter, and the *lac* promoter. These promoters may mediate transcription at low temperature, at low phosphate levels, in the presence of arabinose, in the presence of at low tryptophan levels, in the presence of rhamnose, in the presence of xylose, and in the presence of lactose (or other *lac* inducers).

[0004] When the recombinant microorganism is used as a vertebrate live vaccine, certain considerations must be taken into account. To provide a benefit beyond that of a nonliving vaccine, the live vaccine microorganism must attach to, invade, and survive in lymphoid tissues of the vertebrate and expose these immune effector sites to antigen for an extended period of time. Through this continual stimulation, the vertebrate's immune system becomes more highly reactive to the antigen than with a nonliving vaccine. Therefore, preferred live vaccines are attenuated pathogens of the vertebrate, particularly pathogens that colonize the gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT) or bronchial-associated lymphoid tissue (BALT). An additional advantage of these attenuated pathogens over nonliving vaccines is that these pathogens have elaborate mechanisms to gain access to lymphoid tissues, and thus efficient exposure to the vertebrate's immune system can be expected. In contrast, nonliving vaccines will only provide an immune stimulus if the vaccine is passively exposed to the immune system, or if host mechanisms bring the vaccine to the immune system.

[0005] Pathogenic bacteria may be attenuated by mutation so that upon infection, host disease symptomology is not elicited. Most means of attenuation, however, make live vaccine strains more susceptible than wild-type strains to environmental stresses encountered after inoculation into the animal or human host. Consequently, fewer bacteria survive to colonize the GALT, NALT and/or BALT with a reduction in effective immunogenicity of the vaccine. Thus these attenuation mechanisms hyperattenuate the vaccine, precluding the candidate vaccine from either reaching or persisting in lymphoid tissues to a sufficient extent or duration to permit induction of a protective immune response against the wild-type pathogen of interest. Thus, there is a need in the art for methods of regulating the expression of the attenuated phenotype. This allows the live vaccine strain to display abilities similar to a wild-type virulent parental pathogen in order to successfully colonize effector lymphoid tissues prior to the display and imposition of the full attenuated phenotype to preclude induction of disease symptoms.

[0006] Since immune responses induced against foreign antigens are proportional to the levels of antigen synthesized by the recombinant attenuated bacterial vaccine (1,3), the placement of the nucleic acid sequence for the foreign

antigen on a multi-copy plasmid vector is much preferable to the insertion of the nucleic acid sequence for the foreign antigen into the chromosome of the attenuated bacterial vaccine vector. This is because the level of foreign antigen synthesis is generally proportional to the number of copies of the nucleic acid sequence for the foreign antigen expressed within the attenuated bacterial host.

[0007] Since plasmid-containing recombinant attenuated bacterial vaccines overproduce large amounts of antigen that provide no advantage to the vaccine, the plasmid vectors are often lost over time after immunization (4). In many cases, ten percent or less of the recombinant attenuated bacterial vaccine isolated from the immunized vertebrate retains the plasmid after three or four days. When this plasmid loss occurs, the immune response is directed more against the attenuated bacterial host vaccine itself rather than against the expressed foreign antigen.

[0008] As stated above, the level of immune response to a foreign antigen is generally proportional to the level of expression of the nucleic acid sequence encoding the antigen. Encoding the protective antigen on a plasmid vector is important in maximizing the production of that protective antigen, which is very much correlated with the ability to induce high level protective immune responses by production of mucosal and systemic antibodies against the protective antigen. Unfortunately, overexpression of nucleic acid encoding a foreign antigen is often toxic such that it reduces the rate of growth and therefore the ability of the attenuated bacterial vaccine to colonize lymphoid tissues. As a consequence, the ultimate immunogenicity is sharply diminished. For this reason, it is necessary to balance the ability of the vaccine to colonize and grow in lymphoid tissues with the ability to synthesize the foreign antigen.

SUMMARY OF THE INVENTION

[0009] One aspect of the present invention encompasses a recombinant bacterium capable of the regulated expression of at least one nucleic acid sequence encoding an antigen of interest, and capable of regulated attenuation. The bacterium comprises at least one chromosomally integrated nucleic acid sequence encoding a repressor operably linked to a regulatable promoter and a vector comprising a nucleic acid sequence encoding at least one antigen of interest operably linked to a promoter regulated by the repressor, such that the expression of the nucleic acid sequence

encoding the antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level expression of the nucleic acid sequence encoding the antigen in a host. The bacterium further comprises a regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably-linked to, at least one nucleic acid sequence of an attenuation protein.

[0010] Another aspect of the invention encompasses a recombinant bacterium capable of regulated expression of at least one nucleic acid sequence encoding an antigen of interest. The bacterium comprises at least one chromosomally integrated nucleic acid sequence encoding a repressor operably linked to a regulatable promoter, wherein the nucleic acid sequence encoding the repressor and/or promoter have been modified from the wild-type nucleic acid sequence so as to optimize the expression level of the nucleic acid sequence encoding the repressor, and a vector comprising at least one nucleic acid sequence encoding an antigen of interest operably linked to a promoter regulated by the repressor, such that the expression of the nucleic acid sequence encoding the antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level expression of the nucleic acid sequence encoding the antigen in a host.

[0011] Yet another aspect of the invention encompasses a recombinant bacterium capable of regulated attenuation. The bacterium comprises a modified regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence encoding an attenuation protein.

[0012] Other aspects and iterations of the invention are described more thoroughly below.

BRIEF DESCRIPTION OF THE FIGURES

[0013] **Fig. 1** depicts an illustration of various deletion-insertion mutations of *relA* and various embodiments of *lacI* optimization. The schematic shows the deletion of 2247 bp (*relA* -12 to *relA* 2235/2235) and insertion of 2429 bp of *araC* P_{BAD} *lacI* TT. The optimized sequences of $\Delta relA196::araC$ P_{BAD} *lacI* TT (SEQ ID NO:1), $\Delta relA197::araC$ P_{BAD} *lacI* TT (SEQ ID NO:2), and $\Delta relA198::araC$ P_{BAD} (SEQ ID NO:3) *lacI** TT show variations of SD sequences and start codons. *lacI**:

codon optimized.

[0014] **Fig. 2** depicts an illustration of a chromosomal map of the deletion-insertion mutation of *endA* and various embodiments of *lacI* optimization. The schematic shows the deletion of 719 bp (*endA* -11 to *endA* 708/708) and insertion of 2429 bp of *araC* P_{BAD} *lacI* TT. The optimized sequences of $\Delta endA19::araC$ P_{BAD} *lacI* TT (SEQ ID NO:4), $\Delta endA20::araC$ P_{BAD} *lacI* TT (SEQ ID NO:5), and $\Delta endA21::araC$ P_{BAD} *lacI** TT (SEQ ID NO:6) show variations of SD sequences and start codons.

[0015] **Fig. 3** depicts an illustration of nucleic acid sequence modification analyses of SD-*lacI* in $\Delta endA19$ and $\Delta relA196$ (SEQ ID NO:7), SD-*lacI* in $\Delta endA20$ and $\Delta relA197$ (SEQ ID NO:8), and SD-*lacI* in $\Delta endA21$ and $\Delta relA198$ (SEQ ID NO:9). The amino acid sequence of LacI is included (SEQ ID NO:10).

[0016] **Fig. 4** depicts an illustration of a chromosomal map of various deletion-insertion mutations of *asdA* and various embodiments of *c2* optimization. The schematic shows the deletion of 1104 bp from the *asd* nucleic acid sequence (1 to 1104 not including TAG stop codon) and the insertion of 1989 bp of *c2* P_{BAD} *araC* TT. The optimized sequences of $\Delta asdA18::TT$ *araC* P_{BAD} *c2* (SEQ ID NO:11), $\Delta asdA20::TT$ *araC* P_{BAD} *c2** (SEQ ID NO:12), $\Delta asdA21::TT$ *araC* P_{BAD} *c2** (SEQ ID NO:13), and $\Delta asdA27::TT$ *araC* P_{BAD}* *c2*** (SEQ ID NO:14) show variations of SD sequences and start codons. P_{BAD}*: the -10 sequence is improved from TACTGT to TATAAT; *c2**: SD-codon optimized; *c2***: SD-codon optimized and second codon is modified to AAA from AAT.

[0017] **Fig. 5** depicts an illustration of *c2* original sequence (SEQ ID NO:15) aligned with the optimized sequence (SEQ ID NO:16). The amino acid sequence of the optimized sequence is shown (SEQ ID NO:17). According to the actual DNA sequence data, the amino acid at position 49 has been altered to serine (S) from aspartic acid (N) (AAC to AGC).

[0018] **Fig. 6** depicts an illustration of the original (SEQ ID NO:18) and the modified (SEQ ID NO:19) P_{BAD} region and the original (SEQ ID NO:20) and modified (SEQ ID NO:21) *c2* sequence. The original C2 amino acid sequence (SEQ ID NO:22) is compared to the optimized sequence (SEQ ID NO:23).

[0019] **Fig. 7** depicts an illustration of a chromosomal map of the deletion-insertion mutation of the *crp* promoter region. The schematic shows the deletion of the *crp* promoter region (-15 to -109) and the insertion of 1335bp of TT *araC* P_{BAD}. TT: T4 *ipIII* Transcription Terminator

[0020] **Fig. 8** depicts an illustration of a chromosomal map of various deletion-insertion mutations of the *fur* promoter region. The schematic shows the deletion of the *fur* promoter region (-15 to -253; including Fur consensus, Crp binding, and OxyR binding sites) and the insertion of 1335 bp of P_{BAD} *araC* TT. The optimized sequences of $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ (SEQ ID NO:24), $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$ (SEQ ID NO:25), and $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ (SEQ ID NO:26) show variations of SD sequences and start codons.

[0021] **Fig. 9** depicts an illustration of a chromosomal map of various deletion-insertion mutations of the *phoPQ* promoter region. The schematic shows the deletion of the *phoPQ* promoter region (-12 to -109) and the insertion of 1335 bp of P_{BAD} *araC* TT. The optimized sequences of $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ (SEQ ID NO:27), $\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$ (SEQ ID NO:28), and $\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$ (SEQ ID NO:29), $\Delta P_{phoPQ174}::TT\ araC\ P_{BAD}\ phoPQ$ (SEQ ID NO:30), $\Delta P_{phoPQ175}::TT\ araC\ P_{BAD}\ \Delta GAG-phoPQ$ (SEQ ID NO:31), and $\Delta P_{phoPQ176}::TT\ araC\ P_{BAD}\ \Omega CTC-phoPQ$ (SEQ ID NO:32) show variations of SD sequences and start codons.

[0022] **Fig. 10** depicts an illustration of a chromosomal map of the deletion-insertion mutation of the *rpoS* promoter region. The schematic shows the deletion of 36 bp of the *rpoS* promoter region (*rpoS*-13 to -48) and the insertion of 1335 bp of P_{BAD} *araC* TT.

[0023] **Fig. 11** depicts an illustration of a chromosomal map of the deletion-insertion mutation of the *murA* promoter region. The schematic shows the deletion of 42 bp between *murA* and *yrbA* and the insertion of 1335 bp of P_{BAD} *araC* TT.

[0024] **Fig. 12** depicts an illustration of the pYA3493 plasmid.

[0025] **Fig. 13** depicts an illustration of the pYA3634 plasmid.

[0026] **Fig. 14** depicts an illustration of the pYA3681 plasmid.

[0027] **Fig. 15** depicts a photograph and a graph showing results from a western blot analysis on *Salmonella* UK-1 $\Delta relA::araC\ P_{BAD}\ lacI$ (GTG vs ATG vs ATG codon) TT mutations using rabbit LacI antiserum.

[0028] **Fig. 16** depicts a photograph showing results from a western blot analysis on *Salmonella* UK-1 $\Delta asdA::TT araC P_{BAD} c2$ mutants using polyclonal C2 antiserum.

[0029] **Fig. 17** depicts an illustration of a chromosomal map of the deletion-insertion mutation of the *araC* P_{BAD} region and the DNA sequence of P22 P_R *araB* region (SEQ ID NO: 33). The schematic shows the deletion of 1169 bp including 846 bp of *araC* and 323 bp of the P_{BAD} region and the insertion of 91 bp of P22 P_R sequence.

[0030] **Fig. 18** depicts a graph showing survival after *S. pneumoniae* challenge.

[0031] **Fig. 19** depicts principle of regulated delayed expression and constructions of strains with $\Delta relA::araC P_{BAD} lacI$ TT cassette. (A) Principle of regulated delayed expression. This system includes a chromosomal repressor gene, *lacI*, expressed from the arabinose-regulated *araC* P_{BAD} promoter. LacI regulates expression from a plasmid promoter, P_{trc} that directs antigen synthesis. In the presence of arabinose, LacI is produced which binds to P_{trc} , blocking antigen encoding sequence expression. In host tissues, an arabinose poor environment, the concentration of LacI will decrease with each cell division allowing increased antigen synthesis, thus inducing an immune response. (B) Strains with different levels of LacI encoding sequence expression due to altered SD-sequence, start codon and codon usage in the *lacI* gene were inserted into the *relA* gene site of the *Salmonella* genome. The deletion-insertion deleted 2247 bp in *relA* (*relA* -12 to *relA* 2235) and inserted 2429 bp of *araC* $P_{BAD} lacI$ TT cassette. These mutations were also introduced into strains with the $\Delta pabA \Delta pabB \Delta asdA \Delta araBAD$ genotype to provide attenuation, selection for a balanced-lethal vector (Asd^+) and to block arabinose metabolism. (C) Western blot analysis of $\Delta relA::araC P_{BAD} lacI$ (GTG vs. ATG vs. ATG codon optimized) mutations using rabbit anti-LacI antiserum. The strains were grown in 3XD media with different concentrations of arabinose. The samples were normalized by cell number. Densitometry was measured by Quantityone software. The number shows the relative densitometry.

[0032] **Fig. 20** depicts two graphs showing that higher expression of LacI encoding sequence does not change growth. The strains were grown in LB media with 0% (dashed line) and 0.2% arabinose (solid line). The OD_{600} was measured at 40 min

intervals.*, χ 9097, $\blacktriangle$, χ 9095, $\bullet$, χ 9101, $\blacksquare$, χ 9241 (a) Strains without antigen encoding sequence expression plasmid. DAP was included in the growth medium for these strains. (b) Strains with antigen encoding sequence expression plasmid pYA4088.

[0033] **Fig. 21** depicts a series of graphs illustrating the effect of arabinose on *gfp* expression and the kinetics of LacI decrease and PspA antigen increase. (A) Different repression levels of GFP synthesis achieved by varied concentrations of arabinose. These strains have plasmid pYA4090 expressing GFP with P_{trc} promoter. Overnight nutrient broth cultures grown without arabinose were diluted 1:100 into pre-warmed nutrient broth with 2%, 0.2%, 0.02%, 0.002% and 0% arabinose. When OD₆₀₀ reached 0.4, samples were diluted 1:100 into PBS and subjected to FACS analysis. (B) Kinetics of GFP synthesis. Overnight cultures with different concentrations of arabinose were diluted 1:100 into pre-warmed nutrient broth with arabinose; grown to OD₆₀₀ of 0.6, and diluted 1:100 into the same pre-warmed media without arabinose. The process was repeated two more times. At each time point of OD₆₀₀ about 0.6, samples were diluted 1:200 into PBS and subjected to FACS analysis. (C) Kinetics of LacI decrease and PspA antigen increase. Overnight cultures with 0.2% arabinose were diluted 1:100 into pre-warmed LB media with 0.2% arabinose, grown to an OD₆₀₀ of 0.6, and then diluted 1:10 into pre-warmed LB media without arabinose. The process was repeated four times. At each time point of OD₆₀₀ about 0.6, equal numbers of samples were taken for western blot analysis using anti-LacI and anti-PspA antisera. The densitometry was measured by Quantityone software.

[0034] **Fig. 22** depicts a graph showing the stability of LacI protein in different strains. XL1-Blue (*lacI^q* *E. coli*) was grown in LB media. Strains χ 8990, χ 9080 and χ 9226 were grown in 3XD media with 0.2% arabinose to OD₆₀₀ of 0.6 and washed 2 times with 3XD media without arabinose. Chloramphenicol were added to 50 μ g/ml. Samples were taken before washing (pre 0), just after adding chloramphenicol (0), and at 1, 2, 4, 6, 8, 24 h and subjected to western blot analysis. The samples were normalized by cell number. The densitometry was measured by Quantityone software.

[0035] **Fig. 23** depicts a graph showing antibody titers against LPS and PspA. These strains were transformed with plasmid pYA4088 expressing *S. pneumoniae* PspA Rx1 antigen and vector plasmid pYA3493. Strains were grown with

0.2% arabinose in LB medium before inoculating mice. A) anti-LPS antibody titers; B) anti-PspA antibody titers.

[0036] **Fig. 24** depicts a graph showing a survival curve after challenge with virulent *S. pneumonia* WU2 strain. Female BALB/c mice were immunized with a single dose of the indicated strains grown in LB containing 0.2% arabinose. Eight weeks after immunization, mice were challenged with 250 LD₅₀ of virulent *S. pneumoniae* WU2. All mice immunized with PspA-expressing strains were significantly protected ($p < 0.05$). Mice vaccinated with strain $\chi 9101(pYA4088)$ showed significantly higher protection than mice vaccinated with the other strains ($p < 0.05$). The remaining vaccinated groups were not significantly different from each other ($p > 0.05$).

[0037] **Fig. 25** depicts schematics illustrating different deletion-insertion mutations resulting in arabinose-regulated virulence trait. (A) The schematic shows the deletion of the *fur* promoter region (-15 to -253; including Fur consensus, Crp binding, and OxyR binding sites) and the insertion of 1335 bp of P_{BAD} *araC* TT to create the $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ insertion-deletion mutation. (B) The schematic shows the deletion of the *phoPQ* promoter region (-12 to -109) and the insertion of 1335 bp of P_{BAD} *araC* TT to create the $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ deletion-insertion mutation. (C) The schematic shows the deletion of 36 bp of the *rpoS* promoter region (-13 to -48) and the insertion of 1335 bp of P_{BAD} *araC* TT to create the $\Delta P_{rpoS183}::TT\ araC\ P_{BAD}\ rpoS$ deletion-insertion mutation. (D) The schematic shows the deletion of the *crp* promoter region (-15 to -109) and the insertion of 1335bp of TT *araC* P_{BAD} to create the $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ insertion-mutation.

[0038] **Fig. 26** depicts several photographs illustrating the phenotypes of strains with deletion-insertion mutations to enable arabinose-dependent expression of virulence traits. (A) $\chi 9021$ with $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ mutation streaked on MacConkey maltose agar without and with 0.2 percent arabinose. (B) $\chi 8848$ with $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ and $\chi 9107$ with $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ and $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ mutations spotted on CAS agar plates without and with 0.2 percent arabinose to visualize siderophore production. (C) $\chi 8918$ with $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ and $\chi 9108$ with $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ and $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ mutations streaked on X-P plates without and with 0.2 percent arabinose to reveal acid

phosphatase activity. (d) $\chi 8956$ with $\Delta P_{rpoS183}::TT \text{ araC } P_{BAD} rpoS$ and $\chi 9064$ with $\Delta P_{rpoS183}::TT \text{ araC } P_{BAD} rpoS$ and $\Delta P_{crp527}::TT \text{ araC } P_{BAD} crp$ mutations streaked on glycogen-indicator agar without and with 0.2 percent arabinose and sprayed with iodine indicator solution.

[0039] **Fig. 27** depicts an illustration of deletion mutations precluding breakdown of arabinose and enhancing retention of arabinose taken up by bacterial cells. (A) The schematic shows the deletion of a total of 4110 bp (*araB*₂ to *araD*₊₅₂) and the insertion of 22 bp of SD, *NcoI* and *PmeI* at *araB*₂ to create the $\Delta araBAD23$ mutation. (B) The schematic shows the deletion of a total of 1432 bp (*araE*-5 to *araE*+8) to create the $\Delta araE25$ mutation.

[0040] **Fig. 28** depicts several photographs illustrating the stability of Crp, Fur, RpoS and PhoP proteins during incubation of cultures induced for synthesis of these proteins prior to addition of 30 and 200 μ g chloramphenicol/ml of culture. Rabbit antibodies raised against His-tagged Crp, Fur and PhoP were used for western blot analyses. Mouse monoclonal antibodies for RpoS was purchased from Neoclone. $\chi 9021$ (ΔP_{crp527}), $\chi 8848$ (ΔP_{fur33}), $\chi 8956$ ($\Delta P_{rpoS183}$) and $\chi 8918$ ($\Delta P_{phoPQ107}$) were grown in LB broth with 0.2 percent arabinose for these studies.

[0041] **Fig. 29** depicts several photographs illustrating the decrease in amounts of Crp, Fur, RpoS and PhoP proteins as a consequence of growth of $\chi 9021$ (ΔP_{crp527}), $\chi 8848$ (ΔP_{fur33}), $\chi 8956$ ($\Delta P_{rpoS183}$) and $\chi 8918$ ($\Delta P_{phoPQ107}$) in the absence of arabinose. The same bacterial strains as used for the results shown in Figure 28, were grown in nutrient broth with 0.2 percent arabinose and at the commencement of sampling to measure the amounts of proteins, the cultures were diluted 1:4 into prewarmed nutrient broth lacking arabinose. Rabbit antibodies raised against His-tagged Crp, Fur and PhoP were used for western blot analyses. Mouse monoclonal antibody against RpoS was purchased from Neoclone. Synthesis of the Crp, Fur and PhoP proteins continues until after the third 1:4 dilution whereas the amount of the RpoS protein decreases considerably after the first 1:4 dilution.

[0042] **Fig. 30** depicts a photograph of the LPS profiles of vaccine strains in silver-stained SDS -PAGE. Bacteria were lysed with SDS, treated with proteinase K, and analyzed by SDS-PAGE followed by LPS-specific silver staining. Lane 1,

χ 9088(pYA3634) (grown in nutrient broth without mannose); Lane 2, χ 9558(pYA3634) (grown in nutrient broth without mannose); Lane 3, χ 9088(pYA3634) (grown in nutrient broth with 0.5% mannose); Lane 4, χ 9558(pYA3634) (grown in nutrient broth with 0.5% mannose); Lane 5, χ 9088(pYA3634) (grown in LB broth); Lane 6, χ 9558(pYA3634) (grown in LB broth).

[0043] **Fig. 31** depicts an illustration showing different regulated delayed attenuation constructs in vaccine strains. To create the Δpmi -2426 mutation, 1176 bp of the *pmi* nucleic acid sequence was deleted (from ATG to TAG). To create the $\Delta P_{crp527::TT} araC P_{BAD} crp$ mutation, 95 bp of the *crp* promoter region (-15 to -109) was deleted and 1335 bp of $P_{BAD} araC TT$ was inserted. To create the $\Delta P_{fur33::TT} araC P_{BAD} fur$ mutation, 239 bp of the *fur* promoter region (-15 to -253; including Fur consensus, Crp binding, and OxyR binding sites) was deleted and 1335 bp of $P_{BAD} araC TT$ was inserted.

[0044] **Fig. 32** depicts a photograph showing western blot data of the synthesis of PspA Rx1 by different *S. Typhimurium* mutants. Cell lysates of *S. Typhimurium* mutants containing PspA Rx1 were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose, which was subsequently probed with a polyclonal antibody specific for PspA. Lanes: 1, molecular mass markers (positions are indicated in kilodaltons); 2, χ 8133(pYA3493); 3, χ 8133(pYA3634); 4, χ 9088(pYA3493); 5, χ 9088(pYA3634); 6, χ 9558(pYA3493); 7, χ 9558(pYA3634). Due to the presence of arabinose in LB broth, the PspA synthesis of χ 9558(pYA3634) has been suppressed partly.

[0045] **Fig. 33** depicts a series of graphs showing serum IgG responses to rPspA (A) and to *S. Typhimurium* LPS (B) and SOMP (C) measured by ELISA. The data represent IgG antibody levels in mice orally immunized with χ 8133(pYA3493) (vector control), χ 8133(pYA3634) (expressing rPspA), χ 9088(pYA3493) (vector control), χ 9088(pYA3634) (expressing rPspA), χ 9558(pYA3493) (vector control) and χ 9558(pYA3634) (expressing rPspA) at the indicated weeks after immunization. $p < 0.05$ for anti-rPspA serum IgG antibody levels of χ 9558(pYA3634) and χ 9088(pYA3634) immunized mice with that of the χ 8133(pYA3634) immunized mice at week 8. $p < 0.01$ for the anti-rPspA serum IgG antibody levels of χ 9558(pYA3634) immunized mice

compared to χ 8133(pYA3634) immunized mice at week 10 and 12. $p < 0.05$ for the anti-rPspA serum IgG levels of χ 9558(pYA3634) immunized mice compared to χ 9088(pYA3634) immunized mice at week 8, 10 and 12.

[0046] **Fig. 34** depicts a series of graphs showing serum IgG2a and IgG1 responses to rPspA measure by ELISA. The data represent IgG2a and IgG1 subclass antibody levels to rPspA in sera of BALB/c mice orally immunized with the indicated strains at various times after immunization. The ratios of IgG1: IgG2a at 12 weeks are 1:8.3 for χ 8133(pYA3634) immunized mice (A), 1:9.4 for χ 9088(pYA3634) immunized mice (B) and 1:1.5 for χ 9558(pYA3634) immunized mice (C).

[0047] **Fig. 35** depicts a series of graphs showing antigen-specific stimulation of IFN- γ (A) or IL-4 (B) production. Splenectomies were performed on euthanized BALB/c mice at 8 weeks following immunization. BSG controls were also included. Splenocytes were harvested from three mice per group. ELISPOT analyses were performed as described in Materials and Methods. The results are presented as ELISPOTS per million splenocytes minus any background ELISPOTS from unpulsed mock controls. One-way ANOVA and LSD methods were adopted to compare the secretion levels of IL-4 or IFN- γ between different groups. $p < 0.001$ when compare χ 9558(pYA3634) and χ 9088(pYA3634) with χ 8133(pYA3634) for both secretion levels of IL-4 and IFN- γ . $p < 0.01$ when compare χ 9558(pYA3634) with χ 9088(pYA3634) for the secretion levels of IFN- γ .

[0048] **Fig. 36** depicts photomicrographs of conventional light microscopy of H&E-stained lung tissue samples of WU2 challenged mice (10 $\times$ 40). (A) *S. pneumoniae* caused focal consolidation with extensive mononuclear and polymorphonuclear infiltration and loss of alveolar structure in mice that succumbed to the infection. (B) Lungs from survivors appeared normal without extensive cellular infiltration.

[0049] **Fig. 37** depicts the synthesis of Asd protein from the *asd* gene with ATG or GTG start codon and muramic acid-less death assay. (A) The western blot was performed with cell lysates of *S. Typhimurium* strain χ 8276 (Δ *asdA16*) and its derivatives cultured in LB broth with 0.2% arabinose. DAP was included in the medium for strain χ 8276. Asd protein was detected using rabbit anti-Asd serum. The 39 kDa

Asd protein is indicated by an arrow. (B) Growth of *Salmonella* strain χ 8645 ($\Delta P_{murA7}::araC P_{BAD} murA$) with and without arabinose in the indicated media.

[0050] **Fig. 38** depicts an illustration and a series of photographs showing the defined deletion mutations of strain χ 8937 and nutritional requirements. (A) The defined deletion chromosomal mutations in wild-type *Salmonella* UK-1 and in strain χ 8937. P: promoter, TT: transcriptional terminator. (B) The growth of host strain χ 8937 alone or strain χ 8937 harboring pYA3681 on LB agar plates with or without supplementations.

[0051] **Fig. 39** depicts a diagram illustrating the regulatory interactions in the programmed lysis system.

[0052] **Fig. 40** depicts a series of graphs illustrating the in vitro and in vivo lysis of the programmed lysis system in the absence of arabinose. (A) The growth curves of strain χ 8937(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 χ 9380(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 χ 9379 modified to express *lacZ* acts as a non-lysis system control. (C) Colonization of mice with *S. Typhimurium* χ 8937(pYA3681) following P.O. inoculation with 10^9 CFU bacteria. The limits of detection for this assay were 10 CFU/PP or g of tissue.

[0053] **Fig. 41** depicts the synthesis of PspA Rx1 and arabinose-dependent growth of χ 8937(pYA3685). (A) Map of plasmid pYA3685. 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 start codon-modified *murA* and *asdA* genes, and the *bla-pspA* fusion protein. (B) The synthesis of rPspA Rx1 in the programmed lysis *S. Typhimurium* strain χ 8937(pYA3685) grown in LB broth with 0.02% arabinose at 37°C. Aliquots of mid-log phase cultures were subjected to SDS-PAGE or immunoblot analysis. The immunoblot was probed with anti-PspA antibody. PspA proteins are indicated by arrows. Lanes 1 and 2, protein from χ 8937(pYA3685) and χ 8937(pYA3681), respectively. (C) Growth curves of PspA-producing strain χ 8937(pYA3685) in LB broth with or without the addition of 0.02% arabinose.

[0054] **Fig. 42** depicts a series of graphs showing Immune responses in mice after oral immunization with χ 8937(pYA3685) (rPspA Rx1) and χ 8937(pYA3681) (vector control) as determined by ELISA. (A) IgG antibody against *S. Typhimurium* SOMP and rPspA Rx1 in a 1:1280 dilution of serum. (B) Anti-SOMP and –rPspA Rx1 IgA antibody levels in a 1:10 dilution of vaginal secretions.

[0055] **Fig. 43** depicts a graph showing IgG isotype analyses. Serum IgG2a and IgG1 responses to SOMP and rPspA. IgG2a (gray bars) and IgG1 (dark bars). Serum was diluted 1:400.

DETAILED DESCRIPTION OF THE INVENTION

[0056] The present invention provides, in some embodiments, a recombinant bacterium capable of regulated expression of at least one nucleic acid sequence encoding an antigen of interest. In other embodiments, the invention provides a recombinant bacterium capable of regulated attenuation. In exemplary embodiments, the invention provides a recombinant bacterium capable of both regulated expression of at least one nucleic acid sequence encoding an antigen of interest and regulated attenuation.

[0057] In each of the embodiments herein, the recombinant bacterium typically belongs to the Enterobacteriaceae. The Enterobacteria family comprises species from the following genera: *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophonus*, *Brenneria*, *Budvicia*, *Buttiauxella*, *Candidatus Phlomobacter*, *Cedeceae*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Photorhabdus*, *Plesiomonas*, *Pragia*, *Proteus*, *Providencia*, *Rahnella*, *Raoultella*, *Salmonella*, *Samsonia*, *Serratia*, *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia*, *Yokenella*. In certain embodiments, the recombinant bacterium is typically a pathogenic species of the Enterobacteriaceae. Due to their clinical significance, *Escherichia coli*, *Shigella*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella*, *Providencia* and *Yersinia* are considered to be particularly useful. In other embodiments, the recombinant bacterium may be a species or strain commonly used for a vaccine.

[0058] Some embodiments of the instant invention comprise a species or subspecies of the *Salmonella* genera. For instance, the recombinant bacterium may be a *Salmonella enterica* serovar. In an exemplary embodiment, a bacterium of the invention may be derived from *S. typhimurium*, *S. typhi*, *S. paratyphi*, *S. gallinarum*, *S. enteritidis*, *S. choleraesuis*, *S. arizona*, or *S. dublin*.

[0059] A recombinant bacterium of the invention derived from *Salmonella* may be particularly suited to use as a vaccine. Infection of a host with a *Salmonella* strain typically leads to colonization of the gut-associated lymphoid tissue (GALT) or Peyer's patches, which leads to the induction of a generalized mucosal immune response to the recombinant bacterium. Further penetration of the bacterium into the mesenteric lymph nodes, liver and spleen may augment the induction of systemic and cellular immune responses directed against the bacterium. Thus the use of recombinant *Salmonella* for oral immunization stimulates all three branches of the immune system, which is particularly important for immunizing against infectious disease agents that colonize on and/or invade through mucosal surfaces.

[0060] In an alternative embodiment, a bacterium of the invention may be a bacterium included in Table 1 below.

Table 1

Strain	Genotype or relevant characteristics
<i>Escherichia coli</i>	
χ289	F ⁻ λ ⁻ glnV42 T3 ^r
χ6097	F ⁻ araD139 Δ(proAB-lac) λ ⁻ φ80dlacZΔM15 rpsL ΔasdA4 Δ(zhf-2::Tn10) thi-1
χ6212	F ⁻ Δ(argF-lacZYA)-U169 glnV44 λ ⁻ deoR φ80dlacZΔM15 gyrA96 recA1 relA1 endA1 ΔasdA4 Δ(zhf-2::Tn10) thi-1 hsdR17
χ7213	thr-1 leuB6 fhuA21 lacY1 glnV44 recA1 ΔasdA4 Δ(zhf-2::Tn10) thi-1 RP4-2-Tc::Mu [λpir]; Km ^r
χ7232	endA1 thr-1 hsdR17 (r _K ⁻ , m _K ⁺) supE44 gyrA recA1 ΔrelA1 Δ(argF-lacZYA)-U169 [λpir] deoR Φ80dlacZΔM15
χ7370	F ⁻ araD139 Δ(ara-leu)-7697 ΔlacX74 Δlon-4 galK deoR ΔcsgA4 mcrA galU φ80dlacZΔM15 ΔfliC38 Δ(wcaL-wza)-19 recA1 endA1 nupG rpsL Δ(fimA-H) Δ(mcrBC-hsdRMS-mrr)
χ7385	F ⁻ araD139 Δ(ara-leu)-7697 Δ(lacAYZOPI)-X74 Δlon-4 ΔompT0523::TT araC P _{BAD} T7 pol TT galK deoR ΔcsgA4 mcrA galU φ80dlacZΔM15 ΔfliC38 Δ(wcaL-wza)-19 recA1 endA1 nupG rpsL Δ(fimA-H) Δ(mcrBC-hsdRMS-mrr) ΔasdA99
BL21 (DE3)	F ⁻ ompT hsdS _B (r _B ⁻ , m _B ⁻) gal dcm (DE3)
Top 10	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1

Strain	Genotype or relevant characteristics
XL1-blue	<i>araD139 Δ(ara-leu)7697 galU galK rpsL (Str^r) endA1 nupG recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^qZΔM15 Tn10 (Tet^r)].</i>
<i>Salmonella enterica</i> Typhimurium UK-1	
χ3761	UK-1 wild type
χ8060	Δ <i>pabA1516</i>
χ8133	Δ <i>cya-27</i> Δ <i>crp-27</i> Δ <i>asdA16</i>
χ8276	Δ <i>asdA16</i>
χ8289	Δ <i>asdA19::TTaraC</i> P _{BAD} <i>c2</i>
χ8442	Δ <i>pabA1516</i> Δ <i>pabB232</i>
χ8477	Δ <i>araE25</i>
χ8645	ΔP _{murA7::TT} <i>araC</i> P _{BAD} <i>murA</i>
χ8767	Δ <i>araBAD23</i>
χ8831	Δ(<i>gmd-fcl</i>)-26
χ8844	Δ <i>endA2311</i>
χ8848	ΔP _{fur33::TT} <i>araC</i> P _{BAD} <i>fur</i>
χ8854	Δ <i>endA2311</i> Δ <i>asdA19::TT</i> <i>araC</i> P _{BAD} <i>c2</i> ΔP _{murA7::TT} <i>araC</i> P _{BAD} <i>murA</i> Δ <i>araE25</i> Δ <i>araBAD1923</i>
χ8882	Δ <i>relA1123</i>
χ8914	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i>
χ8918	ΔP _{phoPQ107::TT} <i>araC</i> P _{BAD} <i>phoPQ</i>
χ8937	Δ <i>asdA19::araC</i> P _{BAD} <i>c2</i> ΔP _{murA7::araC} P _{BAD} <i>murA</i> Δ(<i>gmd-fcl</i>)-26 Δ <i>relA1123</i> Δ <i>endA2311</i>
χ8956	ΔP _{rpoS183::TT} <i>araC</i> P _{BAD} <i>rpoS</i>
χ8960	Δ <i>asdA18::TT</i> <i>araC</i> P _{BAD} <i>c2</i>
χ8989	Δ <i>endA19::araC</i> P _{BAD} <i>lacI</i> TT
χ8990	Δ <i>relA196::araC</i> P _{BAD} <i>lacI</i> TT
χ9000	ΔP _{fur33::TT} <i>araC</i> P _{BAD} <i>fur</i> ΔP _{phoPQ107::TT} <i>araC</i> P _{BAD} <i>phoPQ</i>
χ9021	ΔP _{crp527::TT} <i>araC</i> P _{BAD} <i>crp</i>
χ9064	ΔP _{rpoS183::TT} <i>araC</i> P _{BAD} <i>rpoS</i> ΔP _{crp527::TT} <i>araC</i> P _{BAD} <i>crp</i>
χ9080	Δ <i>relA197::araC</i> P _{BAD} <i>lacI</i> TT
χ9088	Δ <i>pmi-2426</i> Δ(<i>gmd-fcl</i>)-26 ΔP _{fur33::TT} <i>araC</i> P _{BAD} <i>fur</i> Δ <i>asdA33</i>
χ9095	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>relA196::araC</i> P _{BAD} <i>lacI</i> TT Δ <i>araBAD23</i>
χ9097	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i>
χ9101	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>relA197::araC</i> P _{BAD} <i>lacI</i> TT Δ <i>araBAD23</i>
χ9107	ΔP _{fur33::TT} <i>araC</i> P _{BAD} <i>fur</i> ΔP _{crp527::TT} <i>araC</i> P _{BAD} <i>crp</i>
χ9108	ΔP _{phoPQ107::TT} <i>araC</i> P _{BAD} <i>phoPQ</i> ΔP _{crp527::TT} <i>araC</i> P _{BAD} <i>crp</i>
χ9109	ΔP _{fur33::TT} <i>araC</i> P _{BAD} <i>fur</i> ΔP _{phoPQ107::TT} <i>araC</i> P _{BAD} <i>phoPQ</i> ΔP _{crp527::TT} <i>araC</i> P _{BAD} <i>crp</i>
χ9225	Δ <i>endA21::araC</i> P _{BAD} <i>lacI</i> TT
χ9226	Δ <i>relA198::araC</i> P _{BAD} <i>lacI</i> TT
χ9241	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198::araC</i> P _{BAD} <i>lacI</i> TT
χ9269	ΔP _{fur81::TT} <i>araC</i> P _{BAD} <i>fur</i>

Strain	Genotype or relevant characteristics
χ 9273	$\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$
χ 9275	$\Delta asdA21::TT\ araC\ P_{BAD}\ c2$
χ 9302	$\Delta asdA20::TT\ araC\ P_{BAD}\ c2$
χ 9339	$\Delta sifA26\ \Delta asdA18::TT\ araC\ P_{BAD}\ c2$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta araBAD23$
χ 9340	$\Delta alr-3\ \Delta dadB4\ \Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ\ \Delta recJ1516$ $\Delta recF126\ \Delta asdA18::TT\ araC\ P_{BAD}\ c2$
χ 9362	$\Delta pmi-2426\ \Delta (gmd-fcl)-26\ \Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta asdA18::TT\ araC\ P_{BAD}\ c2$ $\Delta araE25\ \Delta araBAD23\ \Delta relA198::araC\ P_{BAD}\ lacI\ TT$
χ 9371	$\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9372	$\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9373	$\Delta pmi-2426\ \Delta (gmd-fcl)-26\ \Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta asdA21::TT\ araC\ P_{BAD}\ c2\ \Delta araE25$ $\Delta araBAD23\ \Delta relA198::araC\ P_{BAD}\ lacI\ TT$
χ 9379	χ 3761 $\Delta atrB13::MudJ$
χ 9380	χ 9379 $\Delta atrB13::MudJ$
χ 9382	$\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ\ \Delta araBAD23$
χ 9383	$\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ\ \Delta araBAD23$
χ 9402	$\Delta pmi-2426\ \Delta (gmd-fcl)-26\ \Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta asdA21::TT\ araC\ P_{BAD}\ c2\ \Delta araE25$ $\Delta araBAD23\ \Delta relA198::araC\ P_{BAD}\ lacI\ TT\ \Delta sopB1925$
χ 9412	$\Delta pmi-2426\ \Delta (gmd-fcl)-26\ \Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta asdA21::TT\ araC\ P_{BAD}\ c2$ $\Delta araE25\ \Delta araBAD23\ \Delta relA198::araC\ P_{BAD}\ lacI\ TT$ $\Delta P_{murA7}::TT\ araC\ P_{BAD}\ murA$
χ 9413	$\Delta pmi-2426\ \Delta (gmd-fcl)-26\ \Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta asdA18::TT\ araC\ P_{BAD}\ c2$ $\Delta araE25\ \Delta araBAD23\ \Delta relA198::araC\ P_{BAD}\ lacI\ TT$ $\Delta P_{murA7}::TT\ araC\ P_{BAD}\ murA$
χ 9442	$\Delta P_{murA12}::TT\ araC\ P_{BAD}\ murA$
χ 9443	$\Delta (araC\ P_{BAD})-5::P22\ P_R\ araBAD$
χ 9444	$\Delta asdA34::TT$
χ 9477	$\Delta asdA27::TT\ araC\ P_{BAD}\ c2$
χ 9509	$\Delta relA198::araC\ P_{BAD}\ lacI\ TT\ \Delta araBAD23$
χ 9521	$\Delta P_{murA12}::TT\ araC\ P_{BAD}\ murA\ \Delta (araC\ P_{BAD})-5::P22\ P_R\ araBAD$
χ 9527	$\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur\ \Delta (araC\ P_{BAD})-5::P22\ P_R\ araBAD$
χ 9533	$\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp\ \Delta (araC\ P_{BAD})-5::P22\ P_R\ araBAD$
χ 9541	$\Delta P_{phoPQ176}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9542	$\Delta P_{phoPQ175}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9543	$\Delta P_{phoPQ174}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9548	$\Delta P_{phoPQ175}::TT\ araC\ P_{BAD}\ phoPQ\ \Delta araBAD23$
χ 9549	$\Delta P_{phoPQ174}::TT\ araC\ P_{BAD}\ phoPQ\ \Delta araBAD23$
χ 9550	$\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur\ \Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$
χ 9551	$\Delta asdA34::TT\ \Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9552	$\Delta asdA34::TT\ \Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$
χ 9553	$\Delta asdA34::TT\ \Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$

Strain	Genotype or relevant characteristics
χ 9558	$\Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{crp527}::TT araC P_{BAD} crp \Delta asdA27::TT araC P_{BAD} c2 \Delta araE25 \Delta araBAD23 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta agfBAC811$
χ 9569	$\Delta endA20::araC P_{BAD} lacI TT$
Salmonella enterica Typhi ISP1820	
χ 9421	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::araC P_{BAD} lacI TT \Delta araE25 \Delta araBAD23 \Delta tviABCDE10 \Delta agfBAC811$
χ 9633	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::araC P_{BAD} lacI TT \Delta araE25 \Delta araBAD23 \Delta tviABCDE10 \Delta agfBAC811 PhoP^+ \Delta asdA33$
Salmonella enterica Typhi Ty2	
χ 9205	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur33}::TT araC P_{BAD} fur RpoS^-$
χ 9114	$\Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta P_{crp527}::TT araC P_{BAD} crp RpoS^-$
χ 9213	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur33}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ RpoS^-$
χ 9369	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur33}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta relA198::araC P_{BAD} lacI TT RpoS^-$
χ 9639	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::araC P_{BAD} lacI TT \Delta araE25 \Delta tviABCDE10 \Delta agfBAC811 PhoP^+ \Delta asdA33 RpoS^-$
χ 9640	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::araC P_{BAD} lacI TT \Delta araE25 \Delta tviABCDE10 \Delta agfBAC811 PhoP^+ RpoS^+ \Delta asdA33$
Salmonella enterica Paratyphi A	
χ 9515	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta agfBAC811 \Delta relA198::araC P_{BAD} lacI TT$
χ 9608	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta agfBAC811 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta araE25 \Delta araBAD23 \Delta asdA33$
χ 9651	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{phoPQ107}::TT araC P_{BAD} phoPQ \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta agfBAC811 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta araE25 \Delta(araC P_{BAD})-5::P22 P_R araBAD$
χ 9763	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta agfBAC811 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta araE25 \Delta araBAD23 PhoP^+$
χ 9857	$\Delta P_{crp527}::TT araC P_{BAD} crp \Delta P_{fur81}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta agfBAC811 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta araE25 \Delta araBAD23 PhoP^+ \Delta asdA33$

Strain	Genotype or relevant characteristics
<i>Streptococcus pneumoniae</i>	
Rx1	PspA Clade 2, Capsule Type Rough
WU2	PspA Clade 2, Capsule Type 3
D39	PspA Clade 2, Capsule Type 2
Δ = deletion P = promoter p = plasmid TT = transcription terminator	

I. Regulated expression

[0061] The present invention encompasses a recombinant bacterium capable of regulated expression of at least one nucleic acid sequence encoding an antigen of interest. Generally speaking, the bacterium comprises a chromosomally integrated nucleic acid sequence encoding a repressor and a vector. Each is discussed in more detail below.

(a) *chromosomally integrated nucleic acid sequence encoding a repressor*

[0062] A recombinant bacterium of the invention that is capable of the regulated expression of at least one nucleic acid sequence encoding an antigen comprises, in part, at least one chromosomally integrated nucleic acid sequence encoding a repressor. Typically, the nucleic acid sequence encoding a repressor is operably linked to a regulatable promoter. The nucleic acid sequence encoding a repressor and/or the promoter may be modified from the wild-type nucleic acid sequence so as to optimize the expression level of the nucleic acid sequence encoding the repressor.

[0063] Methods of chromosomally integrating a nucleic acid sequence encoding a repressor operably-linked to a regulatable promoter are known in the art and detailed in the examples. Generally speaking, the nucleic acid sequence encoding a repressor should not be integrated into a locus that disrupts colonization of the host by the recombinant bacterium, or attenuates the bacterium. In one embodiment, the nucleic acid sequence encoding a repressor may be integrated into the *relA* nucleic acid sequence. In another embodiment, the nucleic acid sequence encoding a repressor may be integrated into the *endA* nucleic acid sequence.

[0064] In some embodiments, at least one nucleic acid sequence encoding a repressor is chromosomally integrated. In other embodiments, at least two,

or at least three nucleic acid sequences encoding repressors may be chromosomally integrated into the recombinant bacterium. If there is more than one nucleic acid sequence encoding a repressor, each nucleic acid sequence encoding a repressor may be operably linked to a regulatable promoter, such that each promoter is regulated by the same compound or condition. Alternatively, each nucleic acid sequence encoding a repressor may be operably linked to a regulatable promoter, each of which is regulated by a different compound or condition.

i. repressor

[0065] As used herein, “repressor” refers to a biomolecule that represses transcription from one or more promoters. Generally speaking, a suitable repressor of the invention is synthesized in high enough quantities during the in vitro growth of the bacterial strain to repress the transcription of the nucleic acid encoding an antigen of interest on the vector, as detailed below, and not impede the in vitro growth of the strain. Additionally, a suitable repressor will generally be substantially stable, i.e. not subject to proteolytic breakdown. Furthermore, a suitable repressor will be diluted by about half at every cell division after expression of the repressor ceases, such as in a non-permissive environment (e.g. an animal or human host).

[0066] The choice of a repressor depends, in part, on the species of the recombinant bacterium used. For instance, the repressor is usually not derived from the same species of bacteria as the recombinant bacterium. For instance, the repressor may be derived from *E. coli* if the recombinant bacterium is from the genus *Salmonella*. Alternatively, the repressor may be from a bacteriophage.

[0067] Suitable repressors are known in the art, and may include, for instance, LacI of *E. coli*, C2 encoded by bacteriophage P22, or C1 encoded by bacteriophage λ . Other suitable repressors may be repressors known to regulate the expression of a regulatable nucleic acid sequence, such as nucleic acid sequences involved in the uptake and utilization of sugars. In one embodiment, the repressor is LacI. In another embodiment, the repressor is C2. In yet another embodiment, the repressor is C1.

ii. regulatable promoter

[0068] The chromosomally integrated nucleic acid sequence encoding a repressor is operably linked to a regulatable promoter. The term “promoter”, as used herein, may mean a synthetic or naturally-derived molecule that is capable of conferring, activating or enhancing expression of a nucleic acid. A promoter may comprise one or more specific transcriptional regulatory sequences to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid. The term “operably linked,” as used herein, means that expression of a nucleic acid is under the control of a promoter with which it is spatially connected. A promoter may be positioned 5' (upstream) of the nucleic acid under its control. The distance between the promoter and a nucleic acid to be expressed may be approximately the same as the distance between that promoter and the native nucleic acid sequence it controls. As is known in the art, variation in this distance may be accommodated without loss of promoter function.

[0069] The regulated promoter used herein generally allows transcription of the nucleic acid sequence encoding a repressor while in a permissive environment (i.e. in vitro growth), but ceases transcription of the nucleic acid sequence encoding a repressor while in a non-permissive environment (i.e. during growth of the bacterium in an animal or human host). For instance, the promoter may be sensitive to a physical or chemical difference between the permissive and non-permissive environment. Suitable examples of such regulatable promoters are known in the art.

[0070] In some embodiments, the promoter may be responsive to the level of arabinose in the environment. Generally speaking, arabinose may be present during the in vitro growth of a bacterium, while typically absent from host tissue. In one embodiment, the promoter is derived from an *araC*-P_{BAD} system. The *araC*-P_{BAD} system is a tightly regulated expression system which has been shown to work as a strong promoter induced by the addition of low levels of arabinose (5). The *araC-araBAD* promoter is a bidirectional promoter controlling expression of the *araBAD* nucleic acid sequences in one direction, and the *araC* nucleic acid sequence in the other direction. For convenience, the portion of the *araC-araBAD* promoter that mediates expression of the *araBAD* nucleic acid sequences, and which is controlled by the *araC* nucleic acid sequence product, is referred to herein as P_{BAD}. For use as described herein, a cassette with the *araC* nucleic acid sequence and the *araC-araBAD* promoter may be used. This

cassette is referred to herein as *araC*-P_{BAD}. The AraC protein is both a positive and negative regulator of P_{BAD}. In the presence of arabinose, the AraC protein is a positive regulatory element that allows expression from P_{BAD}. In the absence of arabinose, the AraC protein represses expression from P_{BAD}. This can lead to a 1,200-fold difference in the level of expression from P_{BAD}.

[0071] Other enteric bacteria contain arabinose regulatory systems homologous to the *araC araBAD* system from *E. coli*. For example, there is homology at the amino acid sequence level between the *E. coli* and the *S. Typhimurium* AraC proteins, and less homology at the DNA level. However, there is high specificity in the activity of the AraC proteins. For example, the *E. coli* AraC protein activates only *E. coli* P_{BAD} (in the presence of arabinose) and not *S. Typhimurium* P_{BAD}. Thus, an arabinose regulated promoter may be used in a recombinant bacterium that possesses a similar arabinose operon, without substantial interference between the two, if the promoter and the operon are derived from two different species of bacteria.

[0072] Generally speaking, the concentration of arabinose necessary to induce expression is typically less than about 2%. In some embodiments, the concentration is less than about 1.5%, 1%, 0.5%, 0.2%, 0.1%, or 0.05%. In other embodiments, the concentration is 0.05% or below, e.g. about 0.04%, 0.03%, 0.02%, or 0.01%. In an exemplary embodiment, the concentration is about 0.05%.

[0073] In other embodiments, the promoter may be responsive to the level of maltose in the environment. Generally speaking, maltose may be present during the in vitro growth of a bacterium, while typically absent from host tissue. The *malT* nucleic acid encodes MalT, a positive regulator of four maltose-responsive promoters (P_{PQ}, P_{EFG}, P_{KBM}, and P_S). The combination of *malT* and a *mal* promoter creates a tightly regulated expression system that has been shown to work as a strong promoter induced by the addition of maltose (6). Unlike the *araC*-P_{BAD} system, *malT* is expressed from a promoter (P_T) functionally unconnected to the other *mal* promoters. P_T is not regulated by MalT. The *malEFG-malKBM* promoter is a bidirectional promoter controlling expression of the *malKBM* nucleic acid sequences in one direction, and the *malEFG* nucleic acid sequences in the other direction. For convenience, the portion of the *malEFG-malKBM* promoter that mediates expression of the *malKBM* nucleic acid sequence, and which is controlled by the *malT* nucleic acid sequence product, is

referred to herein as P_{KBM} , and the portion of the *malEFG-malKBM* promoter that mediates expression of the *malEFG* nucleic acid sequence, and that is controlled by the *malT* nucleic acid sequence product, is referred to herein as P_{EFG} . Full induction of P_{KBM} requires the presence of the MalT binding sites of P_{EFG} . For use in the vectors and systems described herein, a cassette with the *malT* nucleic acid sequence and one of the *mal* promoters may be used. This cassette is referred to herein as *malT*- P_{mal} . In the presence of maltose, the MalT protein is a positive regulatory element that allows expression from P_{mal} .

[0074] In still other embodiments, the promoter may be sensitive to the level of rhamnose in the environment. Analogous to the *araC*- P_{BAD} system described above, the *rhaRS*- P_{rhaB} activator-promoter system is tightly regulated by rhamnose. Expression from the rhamnose promoter (P_{rha}) is induced to high levels by the addition of rhamnose, which is common in bacteria but rarely found in host tissues. The nucleic acid sequences *rhaBAD* are organized in one operon that is controlled by the P_{rhaBAD} promoter. This promoter is regulated by two activators, RhaS and RhaR, and the corresponding nucleic acid sequences belong to one transcription unit that is located in the opposite direction of the *rhaBAD* nucleic acid sequences. If L-rhamnose is available, RhaR binds to the P_{rhaRS} promoter and activates the production of RhaR and RhaS. RhaS together with L-rhamnose in turn binds to the P_{rhaBAD} and the P_{rhaT} promoter and activates the transcription of the structural nucleic acid sequences (7). Full induction of *rhaBAD* transcription also requires binding of the Crp-cAMP complex, which is a key regulator of catabolite repression (7).

[0075] Although both L-arabinose and L-rhamnose act directly as inducers for expression of regulons for their catabolism, important differences exist in regard to the regulatory mechanisms. L-Arabinose acts as an inducer with the activator AraC in the positive control of the arabinose regulon. However, the L-rhamnose regulon is subject to a regulatory cascade; it is therefore subject to even tighter control than the *araC* P_{BAD} system. L-Rhamnose acts as an inducer with the activator RhaR for synthesis of RhaS, which in turn acts as an activator in the positive control of the rhamnose regulon. In the present invention, rhamnose may be used to interact with the RhaR protein and then the RhaS protein may activate transcription of a nucleic acid sequence operably-linked to the P_{rhaBAD} promoter.

[0076] In still other embodiments, the promoter may be sensitive to the level of xylose in the environment. The *xylR*-P_{xyIA} system is another well-established inducible activator-promoter system. Xylose induces xylose-specific operons (*xyIE*, *xyIFGHR*, and *xyIAB*) regulated by XylR and the cyclic AMP-Crp system. The XylR protein serves as a positive regulator by binding to two distinct regions of the *xyI* nucleic acid sequence promoters. As with the *araC*-P_{BAD} system described above, the *xylR*-P_{xyIAB} and/or *xylR*-P_{xyIFGH} regulatory systems may be used in the present invention. In these embodiments, *xylR* P_{xyIAB} *xylose* interacting with the XylR protein activates transcription of nucleic acid sequences operably-linked to either of the two P_{xyI} promoters.

[0077] The nucleic acid sequences of the promoters detailed herein are known in the art, and methods of operably-linking them to a chromosomally integrated nucleic acid sequence encoding a repressor are known in the art and detailed in the examples.

iii. modification to optimize expression

[0078] A nucleic acid sequence encoding a repressor and regulatable promoter detailed above, for use in the present invention, may be modified so as to optimize the expression level of the nucleic acid sequence encoding the repressor. The optimal level of expression of the nucleic acid sequence encoding the repressor may be estimated, or may be determined by experimentation (see the Examples). Such a determination should take into consideration whether the repressor acts as a monomer, dimer, trimer, tetramer, or higher multiple, and should also take into consideration the copy number of the vector encoding the antigen of interest, as detailed below. In an exemplary embodiment, the level of expression is optimized so that the repressor is synthesized while in the permissive environment (i.e. in vitro growth) at a level that substantially inhibits the expression of the nucleic acid encoding an antigen of interest, and is substantially not synthesized in a non-permissive environment, thereby allowing expression of the nucleic acid encoding an antigen of interest.

[0079] As stated above, the level of expression may be optimized by modifying the nucleic acid sequence encoding the repressor and/or promoter. As used herein, "modify" refers to an alteration of the nucleic acid sequence of the repressor

and/or promoter that results in a change in the level of transcription of the nucleic acid sequence encoding the repressor, or that results in a change in the level of synthesis of the repressor. For instance, in one embodiment, modify may refer to altering the start codon of the nucleic acid sequence encoding the repressor. Generally speaking, a GTG or TTG start codon, as opposed to an ATG start codon, may decrease translation efficiency ten-fold. In another embodiment, modify may refer to altering the Shine-Dalgarno (SD) sequence of the nucleic acid sequence encoding the repressor. The SD sequence is a ribosomal binding site generally located 6-7 nucleotides upstream of the start codon. The SD consensus sequence is AGGAGG, and variations of the consensus sequence may alter translation efficiency. In yet another embodiment, modify may refer to altering the distance between the SD sequence and the start codon. In still another embodiment, modify may refer to altering the -35 sequence for RNA polymerase recognition. In a similar embodiment, modify may refer to altering the -10 sequence for RNA polymerase binding. In an additional embodiment, modify may refer to altering the number of nucleotides between the -35 and -10 sequences. In an alternative embodiment, modify may refer to optimizing the codons of the nucleic acid sequence encoding the repressor to alter the level of translation of the mRNA encoding the repressor. For instance, non-A rich codons initially after the start codon of the nucleic acid sequence encoding the repressor may not maximize translation of the mRNA encoding the repressor. Similarly, the codons of the nucleic acid sequence encoding the repressor may be altered so as to mimic the codons from highly synthesized proteins of a particular organism. In a further embodiment, modify may refer to altering the GC content of the nucleic acid sequence encoding the repressor to change the level of translation of the mRNA encoding the repressor.

[0080] In some embodiments, more than one modification or type of modification may be performed to optimize the expression level of the nucleic acid sequence encoding the repressor. For instance, at least one, two, three, four, five, six, seven, eight, or nine modifications, or types of modifications, may be performed to optimize the expression level of the nucleic acid sequence encoding the repressor.

[0081] By way of non-limiting example, when the repressor is LacI, then the nucleic acid sequence of LacI and the promoter may be altered so as to increase the level of LacI synthesis. In one embodiment, the start codon of the LacI repressor

may be altered from GTG to ATG. In another embodiment, the SD sequence may be altered from AGGG to AGGA. In yet another embodiment, the codons of *lacI* may be optimized according to the codon usage for highly synthesized proteins of *Salmonella*. In a further embodiment, the start codon of *lacI* may be altered, the SD sequence may be altered, and the codons of *lacI* may be optimized.

[0082] Methods of modifying the nucleic acid sequence encoding the repressor and/or the regulatable promoter are known in the art and detailed in the examples.

iv. transcription termination sequence

[0083] In some embodiments, the chromosomally integrated nucleic acid sequence encoding the repressor further comprises a transcription termination sequence. A transcription termination sequence may be included to prevent inappropriate expression of nucleic acid sequences adjacent to the chromosomally integrated nucleic acid sequence encoding the repressor and regulatable promoter.

(b) vector

[0084] A recombinant bacterium of the invention that is capable of the regulated expression of at least one nucleic acid sequence encoding an antigen comprises, in part, a vector. The vector comprises a nucleic acid sequence encoding at least one antigen of interest operably linked to a promoter. The promoter is regulated by the chromosomally encoded repressor, such that the expression of the nucleic acid sequence encoding an antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level synthesis of the antigen in an animal or human host.

[0085] As used herein, "vector" refers to an autonomously replicating nucleic acid unit. The present invention can be practiced with any known type of vector, including viral, cosmid, phasmid, and plasmid vectors. The most preferred type of vector is a plasmid vector.

[0086] As is well known in the art, plasmids and other vectors may possess a wide array of promoters, multiple cloning sequences, transcription terminators, etc., and vectors may be selected so as to control the level of expression of

the nucleic acid sequence encoding an antigen by controlling the relative copy number of the vector. In some instances in which the vector might encode a surface localized adhesin as the antigen, or an antigen capable of stimulating T-cell immunity, it may be preferable to use a vector with a low copy number such as at least two, three, four, five, six, seven, eight, nine, or ten copies per bacterial cell. A non-limiting example of a low copy number vector may be a vector comprising the pSC101 ori.

[0087] In other cases, an intermediate copy number vector might be optimal for inducing desired immune responses. For instance, an intermediate copy number vector may have at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 copies per bacterial cell. A non-limiting example of an intermediate copy number vector may be a vector comprising the p15A ori.

[0088] In still other cases, a high copy number vector might be optimal for the induction of maximal antibody responses. A high copy number vector may have at least 31, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 copies per bacterial cell. In some embodiments, a high copy number vector may have at least 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, or 400 copies per bacterial cell. Non-limiting examples of high copy number vectors may include a vector comprising the pBR ori or the pUC ori.

[0089] Additionally, vector copy number may be increased by selecting for mutations that increase plasmid copy number. These mutations may occur in the bacterial chromosome but are more likely to occur in the plasmid vector.

[0090] Preferably, vectors used herein do not comprise antibiotic resistance markers to select for maintenance of the vector.

i. antigen

[0091] As used herein, "antigen" refers to a biomolecule capable of eliciting an immune response in a host. In some embodiments, an antigen may be a protein, or fragment of a protein, or a nucleic acid. In an exemplary embodiment, the antigen elicits a protective immune response. As used herein, "protective" means that the immune response contributes to the lessening of any symptoms associated with infection of a host with the pathogen the antigen was derived from or designed to elicit a response against. For example, a protective antigen from a pathogen, such as

Mycobacterium, may induce an immune response that helps to ameliorate symptoms associated with *Mycobacterium* infection or reduce the morbidity and mortality associated with infection with the pathogen. The use of the term “protective” in this invention does not necessarily require that the host is completely protected from the effects of the pathogen.

[0092] Antigens may be from bacterial, viral, mycotic and parasitic pathogens, and may be designed to protect against bacterial, viral, mycotic, and parasitic infections, respectively. Alternatively, antigens may be derived from gametes, provided they are gamete specific, and may be designed to block fertilization. In another alternative, antigens may be tumor antigens, and may be designed to decrease tumor growth. It is specifically contemplated that antigens from organisms newly identified or newly associated with a disease or pathogenic condition, or new or emerging pathogens of animals or humans, including those now known or identified in the future, may be expressed by a bacterium detailed herein. Furthermore, antigens for use in the invention are not limited to those from pathogenic organisms. The selection and recombinant synthesis of antigens has been previously described by Schodel (9) and Curtiss (10). Immunogenicity of the bacterium may be augmented and/or modulated by constructing strains that also express sequences for cytokines, adjuvants, and other immunomodulators.

[0093] Some examples of microorganisms useful as a source for antigen are listed below. These may include microorganisms for the control of plague caused by *Yersinia pestis* and other *Yersinia* species such as *Y. pseudotuberculosis* and *Y. enterocolitica*, for the control of gonorrhea caused by *Neisseria gonorrhoea*, for the control of syphilis caused by *Treponema pallidum*, and for the control of venereal diseases as well as eye infections caused by *Chlamydia trachomatis*. Species of *Streptococcus* from both group A and group B, such as those species that cause sore throat or heart diseases, *Erysipelothrix rhusiopathiae*, *Neisseria meningitidis*, *Mycoplasma pneumoniae* and other *Mycoplasma*-species, *Hemophilus influenza*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, other *Bordetella* species, *Escherichia coli*, *Streptococcus equi*, *Streptococcus pneumoniae*, *Brucella abortus*, *Pasteurella hemolytica* and *P. multocida*, *Vibrio cholera*, *Shigella* species, *Borrellia* species, *Bartonella* species, *Helicobacter pylori*, *Campylobacter*

species, *Pseudomonas* species, *Moraxella* species, *Brucella* species, *Francisella* species, *Aeromonas* species, *Actinobacillus* species, *Clostridium* species, *Rickettsia* species, *Bacillus* species, *Coxiella* species, *Ehrlichia* species, *Listeria* species, and *Legionella pneumophila* are additional examples of bacteria within the scope of this invention from which antigen nucleic acid sequences could be obtained. Viral antigens may also be used. Viral antigens may be used in antigen delivery microorganisms directed against viruses, either DNA or RNA viruses, for example from the classes Papovavirus, Adenovirus, Herpesvirus, Poxvirus, Parvovirus, Reovirus, Picornavirus, Myxovirus, Paramyxovirus, Flavivirus or Retrovirus. Antigens may also be derived from pathogenic fungi, protozoa and parasites.

[0094] Certain embodiments encompass an allergen as an antigen. Allergens are substances that cause allergic reactions in a host that is exposed to them. Allergic reactions, also known as Type I hypersensitivity or immediate hypersensitivity, are vertebrate immune responses characterized by IgE production in conjunction with certain cellular immune reactions. Many different materials may be allergens, such as animal dander and pollen, and the allergic reaction of individual hosts will vary for any particular allergen. It is possible to induce tolerance to an allergen in a host that normally shows an allergic response. The methods of inducing tolerance are well-known and generally comprise administering the allergen to the host in increasing dosages.

[0095] It is not necessary that the vector comprise the complete nucleic acid sequence of the antigen. It is only necessary that the antigen sequence used be capable of eliciting an immune response. The antigen may be one that was not found in that exact form in the parent organism. For example, a sequence coding for an antigen comprising 100 amino acid residues may be transferred in part into a recombinant bacterium so that a peptide comprising only 75, 65, 55, 45, 35, 25, 15, or even 10, amino acid residues is produced by the recombinant bacterium. Alternatively, if the amino acid sequence of a particular antigen or fragment thereof is known, it may be possible to chemically synthesize the nucleic acid fragment or analog thereof by means of automated nucleic acid sequence synthesizers, PCR, or the like and introduce said nucleic acid sequence into the appropriate copy number vector.

[0096] In another alternative, a vector may comprise a long sequence of nucleic acid encoding several nucleic acid sequence products, one or all of which may

be antigenic. In some embodiments, a vector of the invention may comprise a nucleic acid sequence encoding at least one antigen, at least two antigens, at least three antigens, or more than three antigens. These antigens may be encoded by two or more open reading frames operably linked to be expressed coordinately as an operon, wherein each antigen is synthesized independently. Alternatively, the two or more antigens may be encoded by a single open reading frame such that the antigens are synthesized as a fusion protein.

[0097] In certain embodiments, an antigen of the invention may comprise a B cell epitope or a T cell epitope. Alternatively, an antigen to which an immune response is desired may be expressed as a fusion to a carrier protein that contains a strong promiscuous T cell epitope and/or serves as an adjuvant and/or facilitates presentation of the antigen to enhance, in all cases, the immune response to the antigen or its component part. This can be accomplished by methods known in the art. Fusion to tetanus toxin fragment C, CT-B, LT-B and hepatitis virus B core are particularly useful for these purposes, although other epitope presentation systems are well known in the art.

[0098] In further embodiments, a nucleic acid sequence encoding an antigen of the invention may comprise a secretion signal. In other embodiments, an antigen of the invention may be toxic to the recombinant bacterium.

ii. promoter regulated by repressor

[0099] The vector comprises a nucleic acid sequence encoding at least one antigen operably-linked to a promoter regulated by the repressor, encoded by a chromosomally integrated nucleic acid sequence. One of skill in the art would recognize, therefore, that the selection of a repressor dictates, in part, the selection of the promoter operably-linked to a nucleic acid sequence encoding an antigen of interest. For instance, if the repressor is LacI, then the promoter may be selected from the group consisting of LacI responsive promoters, such as P_{trc} , P_{lac} , P_{T7lac} and P_{tac} . If the repressor is C2, then the promoter may be selected from the group consisting of C2 responsive promoters, such as P22 promoters P_L and P_R . If the repressor is C1, then the promoter may be selected from the group consisting of C1 responsive promoters, such as λ promoters P_L and P_R .

[0100] In each embodiment herein, the promoter regulates expression of a nucleic acid sequence encoding the antigen, such that expression of the nucleic acid sequence encoding an antigen is repressed when the repressor is synthesized (i.e. during in vitro growth of the bacterium), but expression of the nucleic acid sequence encoding an antigen is high when the repressor is not synthesized (i.e. in an animal or human host). Generally speaking, the concentration of the repressor will decrease with every cell division after expression of the nucleic acid sequence encoding the repressor ceases. In some embodiments, the concentration of the repressor decreases enough to allow high level expression of the nucleic acid sequence encoding an antigen after about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 divisions of the bacterium. In an exemplary embodiment, the concentration of the repressor decreases enough to allow high level expression of the nucleic acid sequence encoding an antigen after about 5 divisions of the bacterium in an animal or human host.

[0101] In certain embodiments, the promoter may comprise other regulatory elements. For instance, the promoter may comprise *lacO* if the repressor is LacI. This is the case with the lipoprotein promoter P_{lpp} that is regulated by LacI since it possesses the LacI binding domain *lacO*.

[0102] In one embodiment, the repressor is a LacI repressor and the promoter is P_{trc} .

iii. expression of the nucleic acid sequence encoding an antigen

[0103] As detailed above, generally speaking the expression of the nucleic acid sequence encoding the antigen should be repressed when the repressor is synthesized. For instance, if the repressor is synthesized during in vitro growth of the bacterium, expression of the nucleic acid sequence encoding the antigen should be repressed. Expression may be “repressed” or “partially repressed” when it is about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or even less than 1% of the expression under non-repressed conditions. Thus although the level of expression under conditions of “complete repression” might be exceeding low, it is likely to be detectable using very sensitive methods since repression can never be absolute.

[0104] Conversely, the expression of the nucleic acid sequence encoding the antigen should be high when the expression of the nucleic acid sequence encoding

the repressor is repressed. For instance, if the nucleic acid sequence encoding the repressor is not expressed during growth of the recombinant bacterium in the host, the expression of the nucleic acid sequence encoding the antigen should be high. As used herein, "high level" expression refers to expression that is strong enough to elicit an immune response to the antigen. Consequently, the copy number correlating with high level expression can and will vary depending on the antigen and the type of immune response desired. Methods of determining whether an antigen elicits an immune response such as by measuring antibody levels or antigen-dependant T cell populations or antigen-dependant cytokine levels are known in the art, and methods of measuring levels of expression of antigen encoding sequences by measuring levels of mRNA transcribed or by quantitating the level of antigen synthesis are also known in the art. For more details, see the examples.

(c) *crp* cassette

[0105] In some embodiments, a recombinant bacterium of the invention may also comprise a $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$ deletion-insertion mutation. Since the *araC* P_{BAD} cassette is dependent both on the presence of arabinose and the binding of the catabolite repressor protein Crp, a $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$ deletion-insertion mutation may be included as an additional means to reduce expression of any nucleic acid sequence under the control of the P_{BAD} promoter. This means that when the bacterium is grown in a non-permissive environment (i.e. no arabinose) both the repressor itself and the Crp protein cease to be synthesized, consequently eliminating both regulating signals for the *araC* P_{BAD} regulated nucleic acid sequence. This double shut off of *araC* P_{BAD} may constitute an additional safety feature ensuring the genetic stability of the desired phenotypes.

[0106] Generally speaking, the activity of the Crp protein requires interaction with cAMP, but the addition of glucose, which may inhibit synthesis of cAMP, decreases the ability of the Crp protein to regulate transcription from the *araC* P_{BAD} promoter. Consequently, to avoid the effect of glucose on cAMP, glucose may be substantially excluded from the growth media, or variants of *crp* may be isolated that synthesize a Crp protein that is not dependent on cAMP to regulate transcription from

P_{BAD}. This strategy may also be used in other systems responsive to Crp, such as the systems responsive to rhamnose and xylose described above.

(d) attenuation

[0107] In each of the above embodiments, a recombinant bacterium of the invention capable of regulated expression may also be attenuated. "Attenuated" refers to the state of the bacterium wherein the bacterium has been weakened from its wild type fitness by some form of recombinant or physical manipulation. This includes altering the genotype of the bacterium to reduce its ability to cause disease. However, the bacterium's ability to colonize the gut (in the case of *Salmonella*) and induce immune responses is, preferably, not substantially compromised.

[0108] In an exemplary embodiment, a recombinant bacterium may be attenuated as described in section II below. In which case, both regulated attenuation and regulated expression of an antigen encoding sequence may be dependent upon an arabinose regulatable system. Consequently, the concentration of arabinose needed for optimal expression of the regulated antigen encoding sequence may not be the same as the concentration for optimal expression of attenuation. In an exemplary embodiment, the concentration of arabinose for the optimization of both regulated attenuation and regulated expression of sequences encoding antigen will be substantially the same.

[0109] Accordingly, the promoter and/or the nucleic acid sequence encoding an attenuation protein may be modified to optimize the system. Methods of modification are detailed above. Briefly, for example, the SD ribosome binding sequence may be altered, and/or the start codon may be altered from ATG to GTG for the nucleic acid sequences *fur* and *phoPQ*, so that the production levels of Fur and PhoPQ are optimal for both the regulated attenuation phenotype and the regulated expression when growing strains with a given concentration of arabinose. One of skill in the art will appreciate that other nucleic acid sequences, in addition to *fur* and *phoPQ*, may also be altered as described herein in combination with other well-known protocols. In addition, these attenuating nucleic acid sequences may be regulated by other systems using well-established protocols known to one of skill in the art. For example,

they may be regulated using with promoters dependent on addition of maltose, rhamnose, or xylose rather than arabinose.

[0110] Other methods of attenuation are known in the art. For instance, attenuation may be accomplished by altering (e.g., deleting) native nucleic acid sequences found in the wild type bacterium. For instance, if the bacterium is *Salmonella*, non-limiting examples of nucleic acid sequences which may be used for attenuation include: a *pab* nucleic acid sequence, a *pur* nucleic acid sequence, an *aro* nucleic acid sequence, *asd*, a *dap* nucleic acid sequence, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *dam*, *phoP*, *phoQ*, *rfc*, *poxA*, *galU*, *mviA*, *sodC*, *recA*, *ssrA*, *sirA*, *inv*, *hilA*, *rpoE*, *flgM*, *tonB*, *slyA*, and any combination thereof. Exemplary attenuating mutations may be *aroA*, *aroC*, *aroD*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *ompR*, *galE*, and *htrA*.

[0111] In certain embodiments, the above nucleic acid sequences may be placed under the control of a sugar regulated promoter wherein the sugar is present during in vitro growth of the recombinant bacterium, but substantially absent within an animal or human host. The cessation in transcription of the nucleic acid sequences listed above would then result in attenuation and the inability of the recombinant bacterium to induce disease symptoms.

[0112] In another embodiment, the recombinant bacterium may contain one and in some embodiments, more than one, deletion and/or deletion-insertion mutation present in the strains listed in Table 1 above. Furthermore, suicide vectors, as listed in Table 2, and ss described in the Examples below, along with other plasmid vectors, may be used to introduce these deletion and deletion-insertion mutations into strains during their construction.

TABLE 2

Plasmid	Properties
Suicide vector	
pMEG-375	<i>sacRB mobRP4 R6K ori Cm^r Ap^r</i>
pMEG-443	Δ <i>asdA16</i>
pRE112	<i>SacB mobRP4 R6K ori Cm^r</i>
pYA3438	Δ <i>pabB232</i> in pMEG-375
pYA3485	Δ <i>araE25</i> in pMEG-375
pYA3599	Δ <i>araBAD23</i> in pMEG-375
pYA3629	Δ (<i>gmd-fcl</i>)-26 in pMEG-375

pYA3652	Δ endA2311 in pMEG-375
pYA3679	Δ relA1123 in pMEG-375
pYA3722	Δ P _{fur33} ::TT <i>araC</i> P _{BAD} <i>fur</i> in pMEG-375
pYA3723	Δ P _{phoPQ107} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
pYA3735	Δ P _{rpoS183} ::TT <i>araC</i> P _{BAD} <i>rpoS</i> in pRE112
pYA3736	Δ asdA33 in pRE112
pYA3737	Δ asdA18::TT <i>araC</i> P _{BAD} P22 <i>c2</i> in pRE112
pYA3783	pRE112 derived suicide vector to generate GTG- <i>lacI</i> , Δ endA19:: <i>araC</i> P _{BAD} <i>lacI</i> TT mutation, Cm ^r
pYA3784	pRE112 derived suicide vector to generate GTG- <i>lacI</i> , Δ relA196:: <i>araC</i> P _{BAD} <i>lacI</i> TT mutation, Cm ^r
pYA3832	Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> in pRE112
pYA3871	pRE112 derived suicide vector to generate ATG- <i>lacI</i> , Δ endA20:: <i>araC</i> P _{BAD} <i>lacI</i> TT mutation, Cm ^r
pYA3879	pRE112 derived suicide vector to generate ATG- <i>lacI</i> , Δ relA197:: <i>araC</i> P _{BAD} <i>lacI</i> TT mutation, Cm ^r
pYA4062	Δ P _{phoPQ173} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
pYA4063	pRE112 derived suicide vector to generate improved SD ATG- <i>lacI</i> , Δ endA21:: <i>araC</i> P _{BAD} <i>lacI</i> (improved codon) TT mutation, Cm ^r
pYA4064	pRE112 derived suicide vector to generate codon optimized <i>lacI</i> , Δ relA198:: <i>araC</i> P _{BAD} <i>lacI</i> TT mutation, Cm ^r
pYA4109	Δ P _{phoPQ177} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
pYA4138	Δ asdA27::TT <i>araC</i> P _{BAD} P22 <i>c2</i> in pRE112
pYA4177	Δ asdA21::TT <i>araC</i> P _{BAD} P22 <i>c2</i> in pRE112
pYA4180	Δ P _{fur77} ::TT <i>araC</i> P _{BAD} <i>fur</i> in pRE112
pYA4181	Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> in pRE112
pYA4213	Δ asdA20::TT <i>araC</i> P _{BAD} P22 <i>c2</i> in pRE112
pYA4235	Δ P _{murA12} ::TT <i>araC</i> P _{BAD} <i>murA</i> in pRE112
pYA4280	Δ (<i>araC</i> P _{BAD})-5:P22 P _R <i>araBAD</i> in pRE112
pYA4343	Δ P _{phoPQ176} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
pYA4344	Δ P _{phoPQ175} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
pYA4345	Δ P _{phoPQ174} ::TT <i>araC</i> P _{BAD} <i>phoPQ</i> in pRE112
Recombinant vector	
pGEM-3Z	Ap ^r , Cloning vector, pUC <i>ori</i>
pYA3342	pBR <i>ori</i> Asd ⁺ , 3012 bp
Plasmid	Properties
pYA3450	p15A <i>ori</i> <i>araC</i> ^S P _{BAD} SD-ATG <i>asdA</i>
pYA3493	pBR <i>ori</i> <i>bla</i> SS, Asd ⁺ , 3113 bp,
pYA3530	p15A <i>ori</i> <i>araC</i> ^S P _{BAD} SD-GTG <i>asdA</i>
pYA3552	Asd ⁺ vector expression <i>gfp3</i> , pBR <i>ori</i>
pYA3620	pBR <i>ori</i> Asd ⁺ , <i>bla</i> SS <i>bla</i> CT, 3169 bp
pYA3624	Plasmid with tightly regulated <i>araC</i> P _{BAD} cassette, p15A <i>ori</i>
pYA3634	pBR <i>ori</i> <i>bla</i> SS, Asd ⁺ PspA Rx1 aa 3-257
pYA3635	pBR <i>ori</i> <i>bla</i> SS, Asd ⁺ PspA Rx1 aa 3-257 (codon optimized)
pYA3681	pBR <i>ori</i> <i>araC</i> P _{BAD} SD-GTG <i>asdA</i> SD-GTG <i>murA</i> P22 P _R anti-sense mRNA
pYA3685	pBR <i>ori</i> <i>araC</i> P _{BAD} SD-GTG <i>asdA</i> SD-GTG <i>murA</i> P22 P _R anti-sense mRNA, rPspA Rx1
pYA3698	pGEM-3Z with T4 <i>ipIII</i> transcription terminator, Ap ^r

pYA3699	pGEM-3Z with <i>araC</i> P _{BAD} cassette, Ap ^r
pYA3700	<i>AraC</i> P _{BAD} TT cassette plasmid, Ap ^r
pYA3782	P _{BAD} regulated <i>lacI</i> (GTG) in pYA3700-PCR-Topo-Blunt II
pYA3789	Runaway vector harboring <i>araC</i> P _{BAD} P22 c2, GTG-MurA ⁺ , GTG-Asd ⁺ , P _{trc} , pSC101 <i>ori</i> , pUC <i>ori</i>
pYA3856	pBAD-HisA with GTG start codon <i>lacI</i> , His-tagged, Ap ^r
pYA4050	pUC <i>ori</i> <i>araC</i> P _{BAD} SD-GTG <i>murA</i> SD-GTF <i>asd</i> , P22P _R antisense RNA with DNA nuclear Targeting sequence and poly A from SV40, 6941 bp
pYA4088	pBR <i>ori</i> <i>bla</i> SS, Asd ⁺ PspA Rx1 aa 3-285 (codon optimized)
pYA4090	Asd ⁺ vector, P _{trc} <i>gfp3</i> , pBR <i>ori</i>
p = plasmid P = promoter SD = Shine-Dalgarno sequence <i>araC</i> [§] P _{BAD} = <i>E.coli</i> B/r aa = amino acid SS = signal sequence Ap ^r = ampicillin resistance Cm ^r = chloramphenicol resistance	

[0113] The bacterium may also be modified to create a balanced-lethal host-vector system, although other types of systems may also be used (e.g., creating complementation heterozygotes). For the balanced-lethal host-vector system, the bacterium may be modified by manipulating its ability to synthesize various essential constituents needed for synthesis of the rigid peptidoglycan layer of its cell wall. In one example, the constituent is diaminopimelic acid (DAP). Various enzymes are involved in the eventual synthesis of DAP. In one example, the bacterium is modified by using a $\Delta asdA$ mutation to eliminate the bacterium's ability to produce β -aspartate semialdehyde dehydrogenase, an enzyme essential for the synthesis of DAP. One of skill in the art can also use the teachings of U.S. Patent No. 6,872,547 for other types of mutations of nucleic acid sequences that result in the abolition of the synthesis of DAP. These nucleic acid sequences may include, but are not limited to, *dapA*, *dapB*, *dapC*, *dapD*, *dapE*, *dapF*, and *asd*. Other modifications that may be employed include modifications to a bacterium's ability to synthesize D-alanine or to synthesize D-glutamic acid (e.g., $\Delta murl$ mutations), which are both unique constituents of the peptidoglycan layer of the bacterial cell wall.

[0114] Yet another balanced-lethal host-vector system comprises modifying the bacterium such that the synthesis of an essential constituent of the rigid layer of the bacterial cell wall is dependent on a nutrient (e.g., arabinose) that can be supplied during the growth of the microorganism. For example, a bacterium may be comprise the $\Delta P_{murA}::TT araC P_{BAD} murA$ deletion-insertion mutation. This type of mutation makes synthesis of muramic acid (another unique essential constituent of the

peptidoglycan layer of the bacterial cell wall) dependent on the presence of arabinose that can be supplied during growth of the bacterium in vitro.

[0115] However, when arabinose is absent as it is in an animal or human host, the essential constituent of the peptidoglycan layer of the cell wall is not synthesized. This mutation represents an arabinose dependant lethal mutation. In the absence of arabinose, synthesis of muramic acid ceases and lysis of the bacterium occurs because the peptidoglycan layer of the cell wall is not synthesized. It is not possible to generate $\Delta murA$ mutations because they are lethal. The necessary nutrient, a phosphorylated muramic acid, can not be exogenously supplied because enteric bacteria cannot take the nutrient up from the media. Recombinant bacteria with a $\Delta P_{murA}::TT araC P_{BAD} murA$ deletion-insertion mutation grown in the presence of arabinose exhibit effective colonization of effector lymphoid tissues after oral vaccination prior to undergoing lysis due to the inability to synthesize muramic acid.

[0116] Similarly, various embodiments may comprise the $araC P_{BAD} c2$ cassette inserted into the *asd* nucleic acid sequence that encodes aspartate semialdehyde dehydrogenase. Since the *araC* nucleic acid sequence is transcribed in a direction that could lead to interference in the expression of adjacent nucleic acid sequences and adversely affect vaccine strain performance, a transcription termination (TT) sequence is generally inserted 3' to the *araC* nucleic acid sequence. The chromosomal *asd* nucleic acid sequence is typically inactivated to enable use of plasmid vectors encoding the wild-type *asd* nucleic acid sequence in the balanced lethal host-vector system. This allows stable maintenance of plasmids in vivo in the absence of any drug resistance attributes that are not permissible in live bacterial vaccines. In some of these embodiments, the wild-type *asd* nucleic acid sequence may be encoded by the vector described above. The vector enables the regulated expression of an antigen encoding sequence through the repressible promoter. For instance, in one embodiment shown in Figure 13, pYA3634 has the Asd^+ vector specifying the antigen PspA Rx1.

[0117] In one embodiment shown in Figure 4, $\Delta asdA271::TT araC P_{BAD} c2$ has an improved SD sequence and a codon optimized *c2* nucleic acid sequence (Figure 5). The C2 repressor synthesized in the presence of arabinose is used to repress nucleic acid sequence expression from P22 P_R and P_L promoters. In another

embodiment shown in Figure 4, $\Delta asdA27::TT\ araC\ P_{BAD}\ c2$ (the preferred embodiment) has the 1104 base-pair *asd* nucleic acid sequence deleted (1 to 1104, but not including the TAG stop codon) and the 1989 base-pair fragment containing T4 *ipIII* TT *araC* $P_{BAD}\ c2$ inserted. The *c2* nucleic acid sequence in $\Delta asdA27::TT\ araC\ P_{BAD}\ c2$ has a SD sequence that was optimized to TAAGGAGGT. It also has an improved P_{BAD} promoter such that the -10 sequence is improved from TACTGT to TATAAT. Furthermore, it has a codon optimized *c2* nucleic acid sequence, in which the second codon was modified from AAT to AAA (Figure 6).

[0118] In further embodiments, the bacterium may be attenuated by regulating the *murA* nucleic acid sequence encoding the first enzyme in muramic acid synthesis and the *asd* nucleic acid sequence essential for DAP synthesis. These embodiments may comprise the chromosomal deletion-insertion mutations $\Delta asdA19::TT\ araC\ P_{BAD}\ c2$ or $\Delta asdA27::TT\ araC\ P_{BAD}\ c2$ and $\Delta P_{murA7}::TT\ araC\ P_{BAD}\ murA$ or $\Delta P_{murA12}::TT\ araC\ P_{BAD}\ murA$. This host-vector grows in LB broth with 0.1 % L-arabinose, but is unable to grow in or on media devoid of arabinose since it undergoes cell wall-less death by lysis. In some embodiments of the invention, the recombinant bacterium may comprise *araBAD* and *araE* mutations to preclude breakdown and leakage of internalized arabinose such that *asd* and *murA* nucleic acid sequence expression continues for a cell division or two after oral immunization into an environment that is devoid of external arabinose. (For example a strain with the $\Delta P_{murA7}::TT\ araC\ P_{BAD}\ murA$ deletion-insertion mutation undergoes about two cell divisions and then commences to lyse in media made of mouse or chicken feed or chicken breast meat, unless they are supplemented with arabinose.) Either GTG or TTG start codons for the *murA* and *asd* nucleic acid sequences are important to decrease translation efficiency on multi-copy plasmids. This embodiment is illustrated by Figure 14, which shows the plasmid vector pYA3681. This vector contains the *murA* nucleic acid sequence (with altered start codon sequences to decrease translation efficiency) under the control of an *araC* P_{BAD} promoter. Also the second nucleic acid sequence under the direction of this promoter is the *asd* nucleic acid sequence (with altered start codon sequences to decrease translation efficiency). The P22 P_R promoter is in the anti-sense direction of both the *asd* nucleic acid sequence and the *murA* nucleic acid sequence. The P22 P_R is repressed by the C2 repressor made during growth of the strain

in media with arabinose (due to the $\Delta asdA19::TT araC P_{BAD} c2$ deletion-insertion). However C2 concentration decreases due to cell division in vivo to cause P_R directed synthesis of anti-sense mRNA to further block translation of *asd* and *murA* mRNA. The *araC* P_{BAD} sequence is also not from *E. coli* B/r as originally described (5) but represents a sequence derived from *E. coli* K-12 strain $\chi 289$ with tighter control and less leakiness in the absence of arabinose. In the preferred embodiment, transcription terminators (TT) flank all of the domains for controlled lysis, replication, and expression so that expression in one domain does not affect the activities of another domain. As a safety feature, the plasmid *asd* nucleic acid sequence does not replace the chromosomal *asd* mutation since they have a deleted sequence in common, consequently, the *E. coli* *murA* nucleic acid sequence was used in the plasmid instead of using the *Salmonella* *murA* nucleic acid sequence. The recombinant bacterium of this embodiment is avirulent at oral doses in excess of 10^9 CFU to BALB/c mice. In addition to being fully attenuated, this construction exhibits complete biological containment with no in vivo recombinant bacteria survivors detectable after 21 days and no recombinant bacteria survivors during or after excretion. This property enhances vaccine safety and minimizes the potential for vaccination of individuals not intended for vaccination.

II. Regulated attenuation

[0119] The present invention also encompasses a recombinant bacterium capable of regulated attenuation. Generally speaking, the bacterium comprises a chromosomally integrated regulatable promoter. The promoter replaces the native promoter of, and is operably linked to, at least one nucleic acid sequence encoding an attenuation protein, such that the absence of the function of the protein renders the bacterium attenuated. In some embodiments, the promoter is modified to optimize the regulated attenuation.

[0120] In each of the above embodiments described herein, more than one method of attenuation may be used. For instance, a recombinant bacterium of the invention may comprise a regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence encoding an attenuation protein, such that the absence of the function of the

protein renders the bacterium attenuated, and the bacterium may comprise another method of attenuation detailed in section I above.

(a) attenuation protein

[0121] Herein, “attenuation protein” is meant to be used in its broadest sense to encompass any protein the absence of which attenuates a bacterium. For instance, in some embodiments, an attenuation protein may be a protein that helps protect a bacterium from stresses encountered in the gastrointestinal tract or respiratory tract. Non-limiting examples may be the RpoS, PhoPQ, OmpR, Fur, and Crp proteins. In other embodiments, the protein may be a necessary component of the cell wall of the bacterium, such as the protein encoded by *murA*. In still other embodiments, the protein may be listed in Section I(d) above.

[0122] The native promoter of at least one, two, three, four, five, or more than five attenuation proteins may be replaced by a regulatable promoter as described herein. In one embodiment, the promoter of one of the proteins selected from the group comprising RpoS, PhoPQ, OmpR, Fur, and Crp may be replaced. In another embodiment, the promoter of two, three, four or five of the proteins selected from the group comprising RpoS, PhoPQ, OmpR, Fur, and Crp may be replaced.

[0123] If the promoter of more than one attenuation protein is replaced, each promoter may be replaced with a regulatable promoter, such that the expression of each attenuation protein encoding sequence is regulated by the same compound or condition. Alternatively, each promoter may be replaced with a different regulatable promoter, such that the expression of each attenuation protein encoding sequence is regulated by a different compound or condition such as by the sugars arabinose, maltose, rhamnose or xylose.

(b) regulatable promoter

[0124] The native promoter of a nucleic acid encoding an attenuation protein is replaced with a regulatable promoter operably linked to the nucleic acid sequence encoding an attenuation protein. The term “operably linked,” is defined above.

[0125] The regulatable promoter used herein generally allows transcription of the nucleic acid sequence encoding the attenuation protein while in a permissive environment (i.e. in vitro growth), but cease transcription of the nucleic acid sequence

encoding an attenuation protein while in a non-permissive environment (i.e. during growth of the bacterium in an animal or human host). For instance, the promoter may be responsive to a physical or chemical difference between the permissive and non-permissive environment. Suitable examples of such regulatable promoters are known in the art and detailed above.

[0126] In some embodiments, the promoter may be responsive to the level of arabinose in the environment, as described above. In other embodiments, the promoter may be responsive to the level of maltose, rhamnose, or xylose in the environment, as described above. The promoters detailed herein are known in the art, and methods of operably linking them to a nucleic acid sequence encoding an attenuation protein are known in the art.

[0127] In certain embodiments, a recombinant bacterium of the invention may comprise any of the following: $\Delta P_{fur}::TT\ araC\ P_{BAD}\ fur$, $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$, $\Delta P_{phoPQ}::TT\ araC\ P_{BAD}\ phoPQ$, or a combination thereof. (P stands for promoter and TT stands for transcription terminator). Growth of such strains in the presence of arabinose leads to transcription of the *fur*, *phoPQ*, and/or *crp* nucleic acid sequences, but nucleic acid sequence expression ceases in a host because there is no free arabinose. Attenuation develops as the products of the *fur*, *phoPQ*, and/or the *crp* nucleic acid sequences are diluted at each cell division. Strains with the ΔP_{fur} and/or the ΔP_{phoPQ} mutations are attenuated at oral doses of 10^9 CFU, even in three-week old mice at weaning. Generally speaking, the concentration of arabinose necessary to induce expression is typically less than about 2%. In some embodiments, the concentration is less than about 1.5%, 1%, 0.5%, 0.2%, 0.1%, or 0.05%. In certain embodiments, the concentration may be about 0.04%, 0.03%, 0.02%, or 0.01%. In an exemplary embodiment, the concentration is about 0.05%. Higher concentrations of arabinose or other sugars may lead to acid production during growth that may inhibit desirable cell densities. The inclusion of mutations such as $\Delta araBAD$ or mutations that block the uptake and/or breakdown of maltose, rhamnose, or xylose, however, may prevent such acid production and enable use of higher sugar concentrations with no ill effects.

[0128] When the regulatable promoter is responsive to arabinose, the onset of attenuation may be delayed by including additional mutations, such as $\Delta araBAD23$, which prevents use of arabinose retained in the cell cytoplasm at the time

of oral immunization, and/or $\Delta araE25$ that enhances retention of arabinose. Thus, inclusion of these mutations may be beneficial in at least two ways: first, enabling higher culture densities, and second enabling a further delay in the display of the attenuated phenotype that may result in higher densities in effector lymphoid tissues to further enhance immunogenicity.

(c) modifications

[0129] Attenuation of the recombinant bacterium may be optimized by modifying the nucleic acid sequence encoding an attenuation protein and/or promoter. Methods of modifying a promoter and/or a nucleic acid sequence encoding an attenuation protein are the same as those detailed above with respect to repressors in Section I.

[0130] In some embodiments, more than one modification may be performed to optimize the attenuation of the bacterium. For instance, at least one, two, three, four, five, six, seven, eight or nine modifications may be performed to optimize the attenuation of the bacterium.

[0131] In various exemplary embodiments of the invention, the SD sequences and/or the start codons for the *fur* and/or the *phoPQ* virulence nucleic acid sequences may be altered so that the production levels of these nucleic acid products are optimal for regulated attenuation. Figure 8 depicts $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$, whose start codon is changed from ATG to GTG, and $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$, that has a weakened SD sequence as well as the start codon changed from ATG to GTG. Figure 9 depicts $\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$, that has modifications to the start codon as well as the second codon, which was changed from ATG to GTG. Figure 9 also depicts $\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$, wherein the SD sequence has been changed to the weaker AAGG sequence, the start codon was modified, and the second codon was modified from ATG to GTG.

(d) *crp* cassette

[0132] In some embodiments, a recombinant bacterium of the invention may also comprise a $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$ deletion-insertion mutation (Figure 7), as described above. Since the *araC* P_{BAD} cassette is dependent both on the presence of

arabinose and the binding of the catabolite repressor protein Crp, a $\Delta P_{crp}::TT\ araC\ P_{BAD}$ *crp* deletion-insertion mutation may be included as an additional control on the expression of the nucleic acid sequence encoding an attenuation protein.

[0133] Generally speaking, the activity of the Crp protein requires interaction with cAMP, but the addition of glucose, which may inhibit synthesis of cAMP, decreases the ability of the Crp protein to regulate transcription from the *araC* P_{BAD} promoter. Consequently, to avoid the effect of glucose on cAMP, glucose may be substantially excluded from the growth media, or variants of *crp* may be isolated that synthesize a Crp protein that is not dependent on cAMP to regulate transcription from P_{BAD} . This strategy may also be used in other systems responsive to Crp, such as the systems responsive to rhamnose and xylose described above

(e) regulated expression

[0134] In each of the above embodiments, a bacterium capable of regulated attenuation may also be capable of regulated expression of at least one nucleic acid encoding an antigen as detailed in section I above.

[0135] For instance, various embodiments of the present invention may encompass a recombinant pathogenic *Enterobacteriaceae* species comprising deletion-insertion mutations conferring regulated attenuation and regulated expression of a nucleic acid sequence encoding an antigen. In some embodiments, the recombinant bacterium may further comprise at least one chromosomal nucleic acid sequence containing a mutation conferring a lethal phenotype. The mutated chromosomal nucleic acid sequence may be complemented by a plasmid vector containing a functional nucleic acid sequence corresponding to the mutated chromosomal nucleic acid sequence.

III. Vaccine compositions and administration

[0136] A recombinant bacterium of the invention may be administered to a host as a vaccine composition. As used herein, a vaccine composition is a composition designed to elicit an immune response to the recombinant bacterium, including any antigens that may be expressed by the bacterium. In an exemplary embodiment, the immune response is protective, as described above. Immune responses to antigens are

well studied and widely reported. A survey of immunology is given by aul, WE, Stites D *et.al.* and Ogra PL. *et.al.* (11-13). Mucosal immunity is also described by Ogra PL *et.al.* (14).

[0137] Vaccine compositions of the present invention may be administered to any host capable of mounting an immune response. Such hosts may include all vertebrates, for example, mammals, including domestic animals, agricultural animals, laboratory animals, and humans, and various species of birds, including domestic birds and birds of agricultural importance. Preferably, the host is a warm-blooded animal. The vaccine can be administered as a prophylactic or for treatment purposes.

[0138] In exemplary embodiments, the recombinant bacterium is alive when administered to a host in a vaccine composition of the invention. Suitable vaccine composition formulations and methods of administration are detailed below.

(a) vaccine composition

[0139] A vaccine composition comprising a recombinant bacterium of the invention may optionally comprise one or more possible additives, such as carriers, preservatives, stabilizers, adjuvants, and other substances.

[0140] In one embodiment, the vaccine comprises an adjuvant. Adjuvants, such as aluminum hydroxide or aluminum phosphate, are optionally added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. In exemplary embodiments, the use of a live attenuated recombinant bacterium may act as a natural adjuvant. The vaccine compositions may further comprise additional components known in the art to improve the immune response to a vaccine, such as T cell co-stimulatory molecules or antibodies, such as anti-CTLA4. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences naturally found in bacteria, like CpG, are also potential vaccine adjuvants.

[0141] In another embodiment, the vaccine may comprise a pharmaceutical carrier (or excipient). Such a carrier may be any solvent or solid material for encapsulation that is non-toxic to the inoculated host and compatible with the recombinant bacterium. A carrier may give form or consistency, or act as a diluent. Suitable pharmaceutical carriers may include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers not used

for humans, such as talc or sucrose, or animal feed. Carriers may also include stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Carriers and excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington's Pharmaceutical Sciences 19th Ed. Mack Publishing (1995). When used for administering via the bronchial tubes, the vaccine is preferably presented in the form of an aerosol.

[0142] Care should be taken when using additives so that the live recombinant bacterium is not killed, or have its ability to effectively colonize lymphoid tissues such as the GALT, NALT and BALT compromised by the use of additives. Stabilizers, such as lactose or monosodium glutamate (MSG), may be added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process.

[0143] The dosages of a vaccine composition of the invention can and will vary depending on the recombinant bacterium, the regulated antigen, and the intended host, as will be appreciated by one of skill in the art. Generally speaking, the dosage need only be sufficient to elicit a protective immune response in a majority of hosts. Routine experimentation may readily establish the required dosage. Typical initial dosages of vaccine for oral administration could be about 1×10^7 to 1×10^{10} CFU depending upon the age of the host to be immunized. Administering multiple dosages may also be used as needed to provide the desired level of protective immunity.

(b) methods of administration

[0144] In order to stimulate a preferred response of the GALT, NALT or BALT cells, administration of the vaccine composition directly into the gut, nasopharynx, or bronchus is preferred, such as by oral administration, intranasal administration, gastric intubation or in the form of aerosols, although other methods of administering the recombinant bacterium, such as intravenous, intramuscular, subcutaneous injection or intramammary, intrapenial, intrarectal, vaginal administration, or other parenteral routes, are possible.

[0145] In some embodiments, these compositions are formulated for administration by injection (e.g., intraperitoneally, intravenously, subcutaneously,

intramuscularly, etc.). Accordingly, these compositions are preferably combined with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like.

IV. Kits

[0146] The invention also encompasses kits comprising any one of the compositions above in a suitable aliquot for vaccinating a host in need thereof. In one embodiment, the kit further comprises instructions for use. In other embodiments, the composition is lyophilized such that addition of a hydrating agent (e.g., buffered saline) reconstitutes the composition to generate a vaccine composition ready to administer, preferably orally.

[0147] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. Those of skill in the art should, however, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

V. Methods of Use

[0148] A further aspect of the invention encompasses methods of using a recombinant bacterium of the invention. For instance, in one embodiment the invention provides a method for modulating a host's immune system. The method comprises administering to the host an effective amount of a composition comprising a recombinant bacterium of the invention. One of skill in the art will appreciate that an effective amount of a composition is an amount that will generate the desired immune response (e.g., mucosal, humoral or cellular). Methods of monitoring a host's immune response are well-known to physicians and other skilled practitioners. For instance, assays such as ELISA, and ELISPOT may be used. Effectiveness may be determined by monitoring the amount of the antigen of interest remaining in the host, or by

measuring a decrease in disease incidence caused by a given pathogen in a host. For certain pathogens, cultures or swabs taken as biological samples from a host may be used to monitor the existence or amount of pathogen in the individual.

[0149] In another embodiment, the invention provides a method for eliciting an immune response against an antigen in a host. The method comprises administering to the host an effective amount of a composition comprising a recombinant bacterium of the invention

[0150] In still another embodiment, a recombinant bacterium of the invention may be used in a method for eliciting an immune response against a pathogen in an individual in need thereof. The method comprises administering to the host an effective amount of a composition comprising a recombinant bacterium as described herein. In a further embodiment, a recombinant bacterium described herein may be used in a method for ameliorating one or more symptoms of an infectious disease in a host in need thereof. The method comprises administering an effective amount of a composition comprising a recombinant bacterium as described herein.

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EXAMPLES

[0151] The following examples illustrate various iterations of the invention.

Example 1: Regulated Expression of Antigen Encoding Nucleic Acid Sequences

[0152] Antigens, delivered by recombinant attenuated *Salmonella* vaccine strains (RASVs), induce strong systemic and mucosal immune responses that are dependent on several factors including route of immunization [1, 2], expression level [3], cellular location [4], presentation [5], strain background [6, 7] and the inherent immunogenic properties of antigen. Generally, achieving maximal immune responses to

the foreign antigen is directly correlated with the amount of the antigen produced [3, 8], thus it is important that the immunizing bacterial strain produce adequate levels of antigen. However, for RASV, this need must be weighed against the fact that high level antigen production can be a drain on the energy resources of the bacterium, leading to reduced growth rates and a compromised ability to colonize and stimulate effector lymphoid tissues [9]. In addition, some antigens are inherently toxic to vaccine strains for other reasons, leading to a severe inhibition of growth rate and host colonizing potential and, in some cases, death of the RASV. Sometimes, overexpression of foreign proteins can also result in mutations in the promoter or coding sequence of the nucleic acid sequence encoding the antigen, leading to unwanted changes in the level of antigen synthesis or character, thus reducing or compromising the desired immune response. Several approaches have been used to address this problem, including adopting in vivo inducible promoters, including those from the *pagC* [13], *nirB* [14], *spv* and *dps* [15] nucleic acid sequences. In principle, the advantage of using inducible promoters is that only low levels of antigen are produced during in vitro growth and the initial stages of infection. These promoters then upregulate antigen expression once the bacteria reach immunocompetent sites within the host, thus inducing the desired antigen-specific immune response. However, inducible promoters, as they are presently known in the art, are often either too weak in vivo or too strong in vitro, and may be limited by the mode of attenuation [14, 16]. Therefore, there is a need in the art for a system with a promoter that is weakly active in vitro, is capable of strong expression in vivo and whose function is not influenced by the mode of attenuation.

[0153] In this example, the construction of such a system is described utilizing the strong P_{trc} promoter for antigen expression and attenuated *Salmonella enterica* serovar Typhimurium strains expressing different levels of LacI under the control of an arabinose-regulated promoter. Two test antigens were used to evaluate the system. The green fluorescent protein (GFP) was used for in vitro evaluation of the system and the α -helical fragment of the *Streptococcus pneumoniae pspA* nucleic acid sequence [4, 17, 18] was used as the test antigen for immunogenicity studies. *Salmonella* strains were constructed and evaluated for level of LacI synthesis, antigen synthesis and the ability to induce a protective immune response in mice.

Materials and Methods for Example 1

[0154] Bacterial strains, plasmids, media and growth conditions: Bacterial strains and plasmids used are listed in above Table 1 and Table 2, respectively. Bacteria were grown statically overnight at 37°C in LB broth [19], 3XD broth, a buffered Casamino acids medium that includes glycerol as the carbon source [20] or nutrient broth (Difco) as indicated. The second day, the cultures were diluted 1:100 into pre-warmed media with aeration at 37°C. When required, antibiotics and supplements were added at the following concentrations: chloramphenicol, 30 µg/ml; Diaminopimelic acid (DAP), 50 µg/ml [21]; p-aminobenzoic acid (pABA), 10 µg/ml. LB agar without NaCl and containing 5% sucrose was used for *sacB* nucleic acid sequence-based counter selection in allelic exchange experiments [22]. *S. pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract[17].

[0155] General DNA procedures: DNA manipulations were carried out as described by Sambrook et al. [51]. Transformation of bacterial strains was routinely done by electroporation [52] using Nucleic acid sequence Pulser Xcell System (BioRad, 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. Suicide vector and P22-mediated transduction was used to generate defined deletion/ deletion-insertion mutation [53, 54]. Transfer of recombinant suicide plasmids to *Salmonella* was accomplished by conjugation using *E. coli* χ 7213 (Asd⁻) as the plasmid donor [48]. Bacteriophage P22HT *int*-mediated general transduction was performed by standard methods [55]. PCR amplification was employed to obtain DNA fragments for cloning and for verification of chromosomal deletion mutations. Nucleotide sequencing reactions were performed by the DNA lab in Arizona State University.

[0156] Construction of plasmid pYA3700: Plasmid pYA3700 carried a tightly regulated *araC* P_{BAD} TT cassette. To construct this plasmid, two oligonucleotides, 5'-

CCTGGTACCTAGGCCTCTAGATAAATAAAAGCAGTTTACAACCTCCTAGAATTGTGAA
TATATTATCACAATTCTAGGATAGAATAATAAAAGATCTCTGCAGGGC-3' (SEQ ID NO:34) and its complement, corresponding to the T4 *ipIII* transcription terminator [56]

and additional enzyme site (underlined) were annealed, cut with *KpnI*-*PstI*, and cloned into pGEM3Z cut with the same enzymes to create plasmid pYA3698 (Table 2). The *araC* P_{BAD} cassette was amplified using plasmid pYA3624 [57] as template with primer pair 1pBADaraCKpnI (5'-AGAGGTACCCTCGAGGCTAGCCCCAAAAAACGGG-3') (SEQ ID NO:35) and 1pBADaraCXbaI (5'-TGGTCTAGAGTCAAGCCGTCAATTGTCTGATTCTG-3') (SEQ ID NO:36). The PCR fragment was cut with *KpnI*-*XbaI* and cloned into plasmid pGEM3Z to generate plasmid pYA3699 and into pYA3698 to generate the plasmid pYA3700.

[0157] Construction of suicide vector pYA3784: The GTG-*lacI* nucleic acid sequences were amplified from the χ 289 genome using the primer pairs, *lacI* *EcoRI*-3' (5'-GGAATTCTCACTGCCCCGCTTTCCAGTCGGG-3') (SEQ ID NO:37) and GTG *lacI* *XhoI*-5' (5'-CCGCTCGAGAGGGTGGTGAATGTGAAACCAGTAACGTT-3') (SEQ ID NO:38). The resulting 1.1 kb PCR fragment was cloned into pCR-Blunt II-TOPO to create pCR-Blunt II-TOPO-*LacI*(E-X). The *relA* upstream homology region from the χ 3761 genome was amplified using the primer pairs *RelA* N-*HindIII*/*SacI*-5' (5'-CCCAAGCTTGAGCTCGAGGGCGTTCCGGCGCTGGTAGAA-3') (SEQ ID NO:39) and *RelA* N-*BglII*-3' (5'-GAAGATCTAAGGGACCAGGCCTACCGAAG-3') (SEQ ID NO:40). The fragment was cut with *HindIII*-*BglII* and ligated into plasmid pYA3700 at the same restriction sites to generate plasmid pGEM3Z-pBADaraCT4ipIII*relA*-N. Plasmid pYA3700 was cut with *XhoI*-*XbaI* and ligated into pCR-Blunt II-TOPO-*LacI*(E-X) to generate the plasmid pCR-Blunt II-TOPO-*LacI*pBADaraC. This plasmid was cut with *EcoRI*, blunted with Mungbean nuclease and then cut with *HindIII*. Plasmid pGEM3Z-pBADaraCT4ipIII*relA*-N was cut with *XbaI*, blunted with Mungbean nuclease and then cut with *HindIII*. These two fragments were ligated to form the plasmid pCR-Blunt II-TOPO-*LacI*(GTG)pBADaraC-*relA*N. The *relA* downstream homology region was amplified from χ 3761 genome using the primer pairs *RelA* C-*EcoRI*-5' (5'-CGGAATTCACCCCAGACAGTAATCATGTAGCGGCT-3') (SEQ ID NO:41) and *RelA* C-*KpnI*-3' (5'-CGGGTACCCCAGATATTTTCCAGATCTTCAC-3') (SEQ ID NO:42). The fragment was ligated with the pCR-Blunt II-TOPO-*LacI*(GTG)pBADaraC-*relA*N, cut with *XbaI* and blunted with Mungbean nuclease, to generate plasmid pYA3782. The *relA::araC* P_{BAD} *lacI* TT cassette was cut with *KpnI*-*SacI* and cloned into pRE112 to

generate the suicide plasmid pYA3784 harboring the GTG-*lacI*. The ATG-*lacI* was amplified using primers pairs, *lacI* EcoRI-3' (5'-GGAATTCTCACTGCCCGCTTTCCAGTCGGG-3') (SEQ ID NO:43) and *XhoI* SD*-ATG *lacI* -5' (5'-CCGCTCGAGAGGATGGTGAATATGAAACCAGTAACGTT-3') (SEQ ID NO:44) and cloned into pCR-Blunt-II-Topo vector. The codon optimization of ATG-*lacI* was done by the PCR method. Briefly, 22 pairs of overlapping primers covering 15 non-optimized codons used in the *lacI* nucleic acid sequence were PCR amplified. The overlapping PCR products were used as template to be amplified again to get codon optimized ATG-*lacI*. The 15 codons are 35th CGG to CGT, 49th CCC to CCG, 101th CGA to CGT, 155th CCC to CCG, 168th CGA to CGT, 213th ATA to ATC, 216th CGG to CGT, 239th CCC to CCG, 272th GGA to GGT, 320th CCC to CCG, 326th AGA to CGT, 332th CCC to CCG, 339th CCC to CCG, 351th CGA to CGT and 355th CGA to CGT. The codon optimized ATG-*lacI* was also cloned into the pCR-Blunt-II-Topo plasmid. Then the similar strategies were used to generate suicide plasmid pYA3789 with the ATG-*lacI* and suicide plasmid pYA4064 with codon optimized *lacI*.

[0158] Construction of expression plasmid pYA4088: Plasmid pYA3494 carries amino acids 3-257 of native *pspA* Rx1 fused to the first 23 amino acids of *bla* [23]. Nine codons that are rare in *Salmonella* were converted to highly used codons without changing the amino acid sequence to create plasmid pYA3635 using PCR methods [58]. The 9 codons are 2nd CCC to CCG, 57th CTA to CTG, 77th CTA to CTG, 95th ATA to ATC, 113th CGA to CGT, 144th CTA to CTG, 185th AGA to CGT, 186th CTA to CTG, 221st CTA to CTG. Two additional codons, 23rd GCG to GCT and 124th GCT to GCG were also changed to keep the GC content the same. Generally, the overlapping primers covering the 9 non-optimized codons used in the *pspA* Rx1 nucleic acid sequence were PCR amplified. The overlapping PCR products were used as template to be amplified again to get the codon optimized *pspA* Rx1. The final PCR product was cloned into pYA3493 to generate pYA3635. Using pYA3635 as the starting material, the optimized *pspA* sequence was extended an additional 28 amino acids to include a recently identified B cell epitope (S. Hollingshead, personal communication). The extended codon-optimized *pspA* Rx-1 nucleic acid sequence was constructed in 3 steps. First, the optimized *pspA* sequence was amplified using primer set PspA Rx1 forward (5'-TCTCCGGTAGCCAGTCAGTCTAAAGCTGAG-3') (SEQ ID NO:45) and

PspA RX1-a1 (5'-

CTAATTCAGCTTTTTTAGCAGCAATAGTTTTCTCTAAACCTTCTTTAAAGTAGTCTTC
TACATTATTGTTTTCTTC-3') (SEQ ID NO:46). The resulting 820-bp PCR fragment
was used as template in a second PCR reaction using primer set PspA Rx1 forward (5'-
TCTCCGGTAGCCAGTCAGTCTAAAGCTGAG-3') (SEQ ID NO:47) and PspA Rx1-a2
(5'-

TGCTTTCTTAAGGTCAGCTTCAGTTTTTTCTAATTCAGCTTTTTTAGCAGCAATAGTT
TTCTC-3') (SEQ ID NO:48) PspA Rx1-*EcoRI*-s. The resulting 849-bp PCR product was
used as template for a third amplification with the primer set PspA Rx1-*EcoRI*-s (5'-
GGAATTCTCTCCGGTAGCCAGTCAGTCT-3') (SEQ ID NO:49) and PspA Rx1-*HindIII*-
a (5'-TTCAAGCTTATTATGCTTTCTTAAGGTCAGCTTC-3') (SEQ ID NO:50). The 869-
bp PCR product from that reaction was cloned into plasmid pYA3493 [23] using *EcoRI*-
HindIII restriction sites to generate pYA4088. The sequence was verified by sequencing
and enzyme digestion.

[0159] Construction of plasmid pYA4090: Plasmid pYA3552 comprises the
gfp3 nucleic acid sequence, which is a kind gift from Dr. Ho-Young Kang. Plasmid
pYA4090 was constructed by PCR amplification of the 740-bp *gfp3* nucleic acid
sequence using plasmid pYA3552 as template with the primer set GFP-*EcoRI*-s (5'-
GGGAATTCCGATGAGTAAAGGAGAAGAACTTTTC-3') (SEQ ID NO:51) and GFP-
HindIII-a (5'-CGGTGCAAGCTTATTATTTGTATAGTTCATCCATG-3') (SEQ ID NO:52)
and then cloned into pYA3342 using *EcoRI*-*HindIII*.

[0160] Construction of plasmid pYA3438: The plasmid containing the 1.5
kb *pabB* nucleic acid homology was cloned into the *XbaI*-*BamHI* site of pMEG375. The
pabB nucleic acid sequence had 106 bp deleted between the two internal *EcoRV* sites.

[0161] Construction of plasmid pYA3599: The suicide plasmid pYA3599
was used to delete the *araBAD* operon. A 360 bp fragment of the 3' end of *araD* was
generated by PCR using primers *araD*-*BamHI* (5'-CGGGATCCTGGTAGGGAACGAC-
3'; add *BamHI* underlined) (SEQ ID NO:53) and *araD*-*NcoI* (5'-
GATGCCATGGTTTAACTATATTCAGCAAATGCG-3' ; add *NcoI* underlined) (SEQ ID
NO:54), and a 500 bp fragment of 5' end of the *araB* nucleic acid sequence was nucleic
acid generated by PCR using primers *araC*-*NcoI* (5'-
GATGCCATGGTCTGTTTCCTCGTCTTACTCCATCC-3'; add *NcoI* underlined) (SEQ ID

NO:55) and *araC-SphI* (5'-ACATGCGATGCGGACGATCGATAA-3'; add *SphI* underlined) (SEQ ID NO:56). These two fragments were cloned into the *Bam*HI and *SphI* site of pMEG-375 to result in the suicide vector pYA3599.

[0162] Construction of χ 9097: The strain χ 8060 is from Megan Health, Inc. and harbors a *pabA1516* mutation. The χ 8442 strain was constructed by conjugation of χ 7213, harboring plasmid pYA3599, with χ 8060. The χ 8914 strain was constructed by conjugation of χ 7213, harboring plasmid pMEG-443, with χ 8060. The χ 8767 was constructed by conjugation of χ 7213 harboring plasmid pYA3599. The P22 lysate was made on the single cross over by conjugation χ 7213, harboring plasmid pYA3599, with χ 8767 according to Kang's method [59]. The Δ *araBAD*23 mutation was introduced into strain χ 8914 by P22 transduction from (χ 8767:pYA3599) to generate strain χ 9097. The mutation was verified by PCR and formation of a white colony phenotype on MacConkey agar supplemented with 1% arabinose. Minimal agar with/without pABA was used to detect the phenotype associated with the *pabA pabB* mutations. The presence of the *asdA* mutation in *Salmonella* was confirmed by its dependence on DAP for growth [21]. The presence of the 3.3 kb deletion-insertion of *relA* was confirmed by PCR with primer set RelA N-*HindIII*SacI-5' (5'-CCCAAGCTTGAGCTCGAGGGCGTTCCGGCGCTGGTAGAA-3') (SEQ ID NO:57) and RelA C-*KpnI*-3' (5'-CGGGTACCCCAGATATTTTCCAGATCTTCAC-3') (SEQ ID NO:58) and western-blot using anti-LacI antiserum as described below. Lipopolysaccharide (LPS) profiles of *Salmonella* strains were examined as described [60].

[0163] Construction of vectors and strains: Similar strategies to those described above were used to construct pYA3789 and pYA4064 to generate the ATG-*lacI* mutation Δ *relA*197::*araC* P_{BAD} *lacI* TT and the codon optimized ATG-*lacI* mutation Δ *relA*198::*araC* P_{BAD} *lacI* TT, respectively. Plasmid pYA3342 is an Asd⁺ expression vector with promoter P_{trc} [23]. Plasmid pYA4090 is a pYA3342 derivative that codes for *gfp3* expression from the P_{trc} promoter. Details for the construction of plasmid pYA4090 are described herein. Plasmid pYA3493 is a pYA3342 derivative that encodes the first 23 amino acids of β -lactamase [23]. Plasmid pYA4088, derived from pYA3493, carries a cloned fragment of the *S. pneumoniae pspA* nucleic acid sequence, encoding aa 3-285,

that has been codon-optimized for expression in *Salmonella*, and fused to the nucleic acid sequence encoding amino acids 1-23 of β -lactamase.

[0164] Construction and phenotypic characterization of *S. Typhimurium* vaccine strains: The $\Delta relA196::araC$ P_{BAD} *lacI* TT, $\Delta relA197::araC$ P_{BAD} *lacI* TT and $\Delta relA198::araC$ P_{BAD} *lacI* TT mutations were introduced into the *S. Typhimurium* strain $\chi 3761$ by allelic exchange using $\chi 7213$ harboring the suicide vectors pYA3784, pYA3789 and pYA4064 to yield $\chi 8990$, $\chi 9080$ and $\chi 9226$, respectively, and into RASV strain $\chi 9097$ to generate $\chi 9095$, $\chi 9101$ and $\chi 9241$. The presence of the 3.3 kb deletion-insertion was confirmed by PCR and western-blot as described below.

[0165] Western blot analysis: Protein samples were prepared from equal numbers of cells, separated on a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane using Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad). *LacI*, *PspA* and *GroEL* were detected using rabbit polyclonal anti-*LacI*, anti-*PspA* and anti-*GroEL* primary antiserum, respectively, at 1:10,000 dilutions, and a secondary anti-rabbit alkaline phosphatase-conjugated antibody (Sigma, St Louis, MO) at 1:10,000 dilution. Bands were visualized using NBT/BCIP (Sigma,). The bands were scanned and densitometry was measured using Quantity One software (Bio-Rad).

[0166] Growth curves: Standing overnight 37°C cultures of RASV strains $\chi 9095$, $\chi 9097$, $\chi 9101$ and $\chi 9241$, with and without plasmid pYA4088, were grown in LB or LB plus DAP, respectively, containing 0.2% arabinose. The culture was adjusted to the same OD with pre-warmed medium, and then diluted 1:100 into pre-warmed LB or LB-DAP broth with 0.2% arabinose. The optical density at 600 nm (OD₆₀₀) was measured every 40 min. At the final time point, samples of each strain were taken and used for western blot analysis with anti-*LacI* and/or anti-*PspA* antisera.

[0167] Protein stability analysis: *S. Typhimurium* strains $\chi 8990$, $\chi 9080$ and $\chi 9226$ were grown in 3XD medium containing 0.2% arabinose and *E. coli* strain XL1-Blue was grown in LB without arabinose. Standing overnights of each strain were grown at 37°C, diluted 1:100 into fresh media and grown with aeration to an OD₆₀₀ of 0.6. Cells were washed 2 times with fresh medium. Chloramphenicol was added to 50 μ g/ml. Samples taken before adding chloramphenicol (pre 0), just after adding

chloramphenicol (0), and at 1, 2, 4, 6, 8, 24 h were analyzed by western blot. The samples were normalized by cell number before loading onto the gel.

[0168] Flow cytometry analysis: Standing overnight cultures of $\chi 9095$ (pYA4090), $\chi 9097$ (pYA4090), $\chi 9101$ (pYA4090) and $\chi 9241$ (pYA4090) were grown at 37°C in Nutrient Broth without arabinose. Then, 3×10^5 CFU of each strain were added to 3 ml of fresh medium containing 0%, 2%, 0.2%, 0.02% or 0.002% arabinose and grown to an OD₆₀₀ of 0.4. The cultures were diluted 1:10 in PBS and subjected to flow cytometry analysis using Cytomics FC500 (Beckman Coulter, Inc., Fullerton, CA, USA). The data were analyzed by CXP analysis software (Beckman Coulter, Inc.)

[0169] Kinetics of LacI loss and antigen synthesis in pre-induced cultures grown without arabinose: Overnight cultures of strains $\chi 9095$, $\chi 9097$, $\chi 9101$ and $\chi 9241$ carrying either plasmid pYA4088 or plasmid pYA4090 were grown in Nutrient broth with or without 0.2% arabinose. Each culture was adjusted to OD₆₀₀=0.6 and diluted 1:100 into the same pre-warmed medium at 37°C. When cultures reached an OD₆₀₀ of 0.6, the cultures were washed once with nutrient broth without arabinose and diluted 1:100 (for plasmid pYA4090) or 1:10 (for plasmid pYA4088) into pre-warmed nutrient broth without arabinose and grown to an OD₆₀₀ = 0.6. The cultures were diluted into fresh media and the process was repeated twice (for pYA4090 cultures) or three times more (for pYA4088 cultures). Samples were taken at the end of each growth cycle. Samples of strains $\chi 9095$ (pYA4088), $\chi 9097$ (pYA4088), $\chi 9101$ (pYA4088) and $\chi 9241$ (pYA4088) were normalized according to cell number and analyzed by western blot. Bands were scanned and densitometry was measured using Quantity One software (Bio-Rad, Hercules, California). Samples of strains $\chi 9095$ (pYA4090), $\chi 9097$ (pYA4090), $\chi 9101$ (pYA4090) and $\chi 9241$ (pYA4090) were analyzed by flow cytometry.

[0170] Tests of immunogenicity and protection in mice: Strains were grown in LB medium supplemented with 0.2% arabinose to an OD₆₀₀ of 0.8, sedimented by room temperature centrifugation at 6,000 x g for 15 minutes and resuspended in phosphate-buffered saline containing gelatin (BSG) [24]. Groups of female BALB/c mice were orally immunized with 10⁹ CFU of the RASV. Food and water was removed 4 h before inoculation and restored 30 min after inoculation. The inoculum was diluted for titer determination on LB agar plates. Mice were bled at 0, 2, 4, 6, and 8 weeks. Anti-

LPS and anti-PspA Rx1 serum IgG was evaluated by ELISA. Mice were challenged by intraperitoneal injection with 250 LD₅₀ of virulent *S. pneumoniae* WU2 eight weeks after immunization. The mice were observed daily for 21 days after challenge. All animal protocols were approved by ASU IACUC and complied with rules and regulations by American Association for Accreditation of Laboratory Animal Care.

[0171] ELISA: rPspA Rx1 protein was purified as described by Kang et al. [23]. *S. Typhimurium* LPS was obtained from Sigma. The procedure for ELISA has been described [23]. Briefly, polystyrene 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 ng/well of either *S. Typhimurium* LPS or rPspA Rx1 in 100 ml sodium carbonate-bicarbonate coating buffer (pH 9.6). The sera were serially diluted in two-fold steps for detection of IgG. A 100 ml of diluted sample was added to triplicate wells. Plates were treated with biotinylated goat anti-mouse IgG (Southern Biotechnology Inc., Birmingham) and then alkaline phosphatase-labeled streptavidin (Southern Biotechnology Inc., Birmingham). After adding *p*-nitrophenylphosphate substrate solution in diethanolamine buffer (pH 9.8) (Sigma, St. Louis, MO), absorbance was read at 405 nm.

[0172] Statistics: Statistical analyses were performed by using the SPSS software package (SPSS, Chicago, IL). *p* values of ≤ 0.05 were considered significant. Antibody titers were expressed as means $\pm$ standard error. The means were evaluated with One-way Anova and the LSD tests were used for multiple comparisons among groups.

Rationale for the regulated delayed antigen synthesis system

[0173] The P_{trc} promoter is commonly used for constitutive expression of nucleic acid sequences encoding antigens [25, 26]. P_{trc} is a strong promoter in vivo [27], constitutive under most environmental conditions, and is more transcriptionally active both anaerobically and aerobically than the nirB promoter [14]. Although the P_{trc} promoter has been widely used in bacterial expression vectors and in mammalian cell expression systems [28-30], it has been reported that constitutive antigen expression from the similar P_{tac} promoter can affect the colonization capability of RASV [16]. Thus regulating antigen expression from P_{trc} would be beneficial. The P_{trc} promoter can be repressed by LacI. In *Escherichia coli*, the LacI repressor is typically produced at

approximately 8 copies per cell [31, 32] and regulates expression of the lactose metabolic nucleic acid sequences [33] by binding to the *lacO* operator sequence, blocking RNA polymerase from binding to the *lac* promoter. Generally, induction of expression from LacI-repressed promoters is accomplished by the addition of chemical agents, either lactose or IPTG which bind to LacI causing an allosteric change in the protein that leads to its release from *lacO*. However, this is not a practical method of induction for RASVs. Instead, the production of LacI was regulated by placing it under the control of the regulated arabinose-inducible promoter P_{BAD} (Fig. 19A). Transcription from P_{BAD} can be regulated by varying the concentration of arabinose [34, 35]. When the RASV is grown in culture and arabinose is added to the growth medium, LacI is produced which represses transcription from P_{trc} . Once in the host, the transcription from P_{BAD} would cease because free arabinose is very rarely encountered in animal tissues [36], and subsequently, no additional LacI would be produced. The concentration of LacI should then decrease by dilution as the RASV cells divide and antigen production would increase to levels high enough to induce the desired immune response.

Construction of strains with high expression of LacI

[0174] Based on this concept, a tightly-regulated *araC* P_{BAD} *lacI* TT cassette was integrated into the chromosome in the *relA* nucleic acid sequence (Fig. 19B), nucleic acid sequencing defining the *relA196* allele in strain, $\chi 8990$. *relA* was chosen as the integration site because the *relA* mutation is not attenuating nor does it have an effect on colonization [37, 38]. The native *lacI* nucleic acid sequence has a GTG start codon and an AGGG Shine-Dalgarno sequence, leading to expression of only 5-10 molecules each generation [31] and the functional form of LacI repressor is a tetramer [32]. Plasmids with a ColE1 (pBR) replicon are present at 20-30 copies per cell. Thus, it was estimated that at least 80-120 copies of LacI are needed to repress the operator sequences in the expression plasmid to achieve adequate repression. Therefore, in addition to strain $\chi 8990$, which encodes the native *lacI* sequence (GTG-*lacI*), the start codon of *lacI* was modified from GTG to ATG, and the SD sequence was modified from AGGG to the canonical ribosome binding site sequence, AGGA, resulting

in the *relA197* allele in strain $\chi 9080$. This modification increased LacI levels about 2 fold (Fig. 19C). To enhance expression further, the codons of *lacI* were optimized according to the codon usage for highly expressed nucleic acid sequences in *Salmonella*, yielding the *relA198* allele in strain $\chi 9226$. This modification increased LacI levels approximately 4-8 fold over *relA196* (Fig. 19C). The expression levels of LacI for GTG-*lacI*, ATG-*lacI*, and codon optimized-*lacI* were proportional to the arabinose concentration (Fig. 19C). It is anticipated that different antigens may require different levels of repression, so these constructs provide the flexibility needed to produce different amounts of repressor to meet varied requirements.

High expression of LacI does not affect growth.

[0175] The *Salmonella* chromosome does not encode the *lac* operon. Consequently, the effect of LacI production or overproduction on growth was investigated, because this might translate to a reduction in immunogenicity. The growth of all three of the above strains was evaluated in LB broth with or without 0.2% arabinose, and compared to a strain that does not produce LacI. All four strains had similar growth rates, including strain $\chi 9241$ (*relA198*), which produces the most LacI (Fig. 20). Although one of the LacI-producing strains, $\chi 9101$ (*relA197*), grew better than the other strains, there were no large differences in doubling times. Growth at lower concentrations of arabinose gave similar results, but 2% arabinose led to reduced growth of all the LacI-producing strains.

Codon optimized lacI provides the highest repression

[0176] To evaluate the relationship between antigen synthesis and arabinose concentration, plasmid pYA4090, which encodes the *gfp3* nucleic acid sequence under transcriptional control of P_{trc} , was introduced into *S. Typhimurium* strains $\chi 9097$, $\chi 9095$, $\chi 9101$ and $\chi 9241$. Transformants were grown in LB at varying arabinose concentrations and subjected to FACS analysis. The P_{BAD} promoter is subject to autocatalytic regulation, and therefore we were able to evaluate the fraction of cells expressing GFP as a measure of induction [39]. As expected, there was no effect of arabinose on *gfp3* nucleic acid expression in strain $\chi 9097$ (pYA4090), which does not

encode *lacI* (Fig. 21A). When strains χ 9095(pYA4090), χ 9101(pYA4090) and χ 9241(pYA4090) were grown without arabinose, nearly all the cells expressed GFP. No decrease in the number of GFP-positive cells was observed when 0.002% arabinose was included in the growth medium but repression was evident at 0.02% arabinose for all strains. As the arabinose concentration was increased, the number of GFP positive cells dropped substantially for all strains with arabinose-regulated expression of *lacI*. The greatest level of repression was seen in strain χ 9241 (*relA198*), with only 7.6% GFP positive cells in the presence of 2% arabinose. These results are consistent with the expectation that antigen synthesis should be inversely proportional to arabinose concentration and inversely proportional to LacI synthesis (Fig. 21A). Although the *lacI* constructs in χ 9095 (*relA196*) and χ 9101 (*relA197*) produced different amounts of LacI (Fig 19), there was no difference in *gfp* nucleic acid expression between the two strains.

LacI is a stable protein

[0177] Because LacI is not normally synthesized in *S. Typhimurium* and in *E. coli* it is only expressed at low levels and because of the central role LacI plays in the system, the stability of LacI was investigated in these strains, as that could have an impact on the timing of antigen synthesis in vivo. Chloramphenicol was added to mid-exponential phase cultures of *Salmonella* strains χ 8990, χ 9080 and χ 9226 and *E. coli* strain XL1-Blue. The stability of LacI was similar for all strains (Fig. 22). The amount of LacI declined by 50% over the first 2-4 hours in all strains, after which the amount declined very slowly, reaching approximately 20% of the starting levels by 24 hours for the *S. Typhimurium* strains and 40% for the *E. coli* strain. These results indicate that LacI stability is essentially the same in *S. Typhimurium* and *E. coli*, and that the protein is relatively stable. Thus it is expected that the concentration of LacI in these strains, in the absence of arabinose, will decrease primarily due to dilution as a result of cell division.

Time course for the induction of GFP synthesis.

[0178] To evaluate the kinetics of the induction of antigen synthesis after growth in arabinose, the GFP-producing strains were grown in nutrient broth with 0.2%

arabinose. Nutrient broth was chosen as the growth medium because it is derived from animal tissue and should mimic the low arabinose conditions found in host tissues better than LB broth. The arabinose-grown cells were diluted 1:100 into fresh nutrient broth without arabinose and grown to an OD₆₀₀ of 0.6. The cells were diluted and grown twice more in the same way. Each round of growth represented approximately 4.3 cell divisions, for a total of 13.8 nucleic acid sequencerations of growth in the absence of arabinose. Samples were taken at the end of each growth cycle and analyzed by FACS (Fig. 21B). The results indicated that although some strains were not fully repressed by growth in arabinose, the kinetics of induction was similar in all strains leading to nearly full induction for synthesis of GFP by 9.2 nucleic acid sequencerations of growth.

Time course for the induction of PspA antigen synthesis

[0179] As shown above, the system worked as expected using *gfp3* as a model. The system was next evaluated in the context of a vaccine with a clinically relevant antigen, the *S. pneumoniae* PspA Rx1 protein that has been shown to be a potent and protective immunogen [23]. Plasmid pYA4088 was introduced into the strains and their growth rates in LB broth were compared. All of the LacI-producing strains had a growth advantage over strain χ 9097(pYA4088) in the presence of 0.2% arabinose (Fig. 20B), indicating that repressing the expression of the nucleic acid sequence encoding antigen results in faster growth. The LacI-producing strains also grew faster than χ 9097(pYA4088) in the absence of added arabinose. This may be due to trace amounts of arabinose present in the yeast extract used to prepare the LB broth medium, approximately 0.0034% (K. Ameiss, personal communication). Although the amount of LacI produced under these conditions was undetectable by western blot (Fig. 19C), there may have been sufficient LacI present to account for the observed growth advantage.

[0180] Next LacI and PspA synthesis were directly evaluated in cells grown in 0.2% arabinose. This concentration was chosen because there was only a small difference in repression levels between 2% and 0.2% arabinose, (Fig. 21A) and the addition of 2% arabinose resulted in a growth rate reduction. The amount of PspA synthesis was inversely correlated with LacI synthesis, as expected (Fig. 21C). PspA

synthesis levels in strain $\chi 9241(pYA4088)$, which produced the most LacI, were approximately 8-fold less than the $\chi 9097(pYA4088)$ control. The kinetics of induction was evaluated as described above for the GFP-producing strains and it was found that all of the strains produced the same amount of PspA as $\chi 9097(pYA4088)$ after 9.2 nucleic acid sequencerations of growth, indicating full derepression of P_{trc} (Fig. 21C). For most strains, the maximum PspA synthesis was achieved by 6.9 nucleic acid generations.

Regulated delayed expression provides better protection than constitutive expression

[0181] Strains $\chi 9097(pYA4088)$, $\chi 9095(pYA4088)$, $\chi 9101(pYA4088)$ and $\chi 9241(pYA4088)$ were then evaluated for immunogenicity in mice in two separate experiments. The results of both experiments were similar, so the results were pooled. After a single dose, all immunized mice developed high titers against *S. Typhimurium* LPS (Fig. 23A). Compared with vector controls, the strains synthesizing PspA elicited lower LPS titers, although the synthesis of LacI abrogated this effect somewhat. While all the RASV strains carrying pYA4088 induced a strong anti-PspA serum IgG response (Fig. 23B), those strains, $\chi 9095$, $\chi 9101$ and $\chi 9241$ that produced LacI induced significantly higher anti-PspA titers than strain $\chi 9097(pYA4088)$ ($p < 0.05$). Strain $\chi 9241(pYA4088)$ vaccinates produced higher anti-PspA antibodies than the other strains at 6 and 8 weeks ($p < 0.05$). Overall, the serum antibody response was roughly proportional to the amount of LacI produced by each strain. No anti-PspA antibody response was detected in mice vaccinated with the vector only controls.

[0182] When vaccinated mice were challenged with virulent *S. pneumoniae* WU2, all groups that received PspA-producing strains were protected ($p < 0.01$; Fig 24). Among the protected groups, mice vaccinated with strain $\chi 9101(pYA4088)$ showed significantly higher protection than mice vaccinated with the other strains ($p < 0.05$). The remaining vaccinated groups were not significantly different from each other ($p > 0.05$). Interestingly, $\chi 9241(pYA4088)$, the strain that induced the highest anti-PspA antibody titer (Fig. 23B), provided the poorest protection. These results illustrate the need to optimize the amount of LacI and antigen to attain an

optimal immune response and indicate that protection may not be closely correlated with induced antibody titer levels.

Discussion for Example 1

[0183] A regulated delayed expression system has been developed to minimize the negative effects of antigen expression on the host strain and to enhance immunity. An *araC* P_{BAD} *lacI* TT cassette was engineered to synthesize different levels of LacI when grown in the presence of arabinose (Fig. 19C). The amount of LacI produced by the strains was proportional to the amount of arabinose present in the medium, up to 2%, the maximum concentration tested (Fig. 19C). These results differ from what has been observed in *E. coli* where protein synthesis from P_{BAD} reached a maximum at 0.2% arabinose [34]. There are several possible reasons for this difference. First, the source of the *araC* P_{BAD} promoter/activator cassette was *E. coli* K-12, while in the previous studies, the promoter was derived from *E. coli* B/r. Tighter regulation has been observed with a K-12 cassette than with a B/r cassette. Additionally, the previous studies were performed using plasmid copies of P_{BAD}, while herein the P_{BAD} was chromosomally integrated. Moreover, differences in regulation have been observed for a number of promoters when they are present in multiple copies (Kenneth Roland, personal communication). Finally, differences in the arabinose-transport system between *E. coli* and *Salmonella* may have also played a role. *Salmonella* only has one L-arabinose transport system encoded by *araE* [40, 41], which has a low affinity for arabinose, while *E. coli* has both *araE* and the high affinity transport system encoded by *araFGH* [40, 42]. Therefore, in *Salmonella*, higher concentrations of arabinose are likely to be required for full P_{BAD} promoter induction than in *E. coli* [40].

[0184] Although 2% arabinose can induce the maximum LacI synthesis, repression of antigen synthesis was not complete (Fig. 21). One reason for this problem is undoubtedly the fact that the P_{trc} promoter is present on a multicopy plasmid, while LacI is specified by a single nucleic acid sequence on the chromosome. Another reason may be that the binding affinity of the lactose operator sequence in P_{trc} is 10-fold less than the ideal one [43]. An additional consideration is that in the native *E. coli* *lac* operon, there are 3 adjacent operator sequences, facilitating cooperative binding

interactions between three LacI tetramers [44], while there is only one operator in our plasmid [45-47]. Consistent with this hypothesis is the fact that only in strain χ 9241, which produced 3-4 times the amount of LacI as the other strains, was antigen expression nearly shut off completely. Thus it is likely possible to improve the efficiency of antigen repression by modifying lacO for tighter repressor binding. In addition, one can also modify the system by adjusting the amount of arabinose in the growth medium, thereby varying the amount of LacI in the cell.

[0185] In this example, three strains were developed with the idea that different antigens may require more or less LacI to achieve an appropriate balance between the health of the RASV and the optimal antigen expression required for induction of protective immune responses. Antigen expression was reduced in vitro, which led to a faster growth rate by the RASV (Fig. 20). While all the RASV strains described in this example grew faster than the control strain that constitutively expressed PspA, the results from each strain were different with respect to the serum immune response and protective immunity (Fig. 23, Fig. 24). It was anticipated that strain χ 9241 with the *relA198* allele would elicit the highest antibody titers and provide the best protection. This strain did, in fact, yield the highest anti-PspA serum IgG titers (Fig. 23), but it did the poorest job of providing protection against *S. pneumoniae* challenge (Fig. 24). This may be a reflection of differences in the amount of antigen produced by each strain and the timing of the induction of antigen synthesis or that other factors such as cellular immunity might be more important than antibodies for conferring protective immunity.

[0186] In conclusion, a regulated delayed antigen expression system has been developed. This system reduces the negative effects of antigen expression during in vitro growth thereby improving the overall health of the vaccine strain, while allowing for maximum antigen expression in host tissues. This technology should be particularly useful for inducing immune responses to antigens that are toxic to the vaccine strain synthesizing them.

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Example 2: Regulated Attenuation

[0187] Attenuation of *Salmonella* vaccine vectors should decrease, if not eliminate, undesirable disease symptoms, but the nutritional status and health of the population to be vaccinated should be considered. The attenuation should be (i) an inherent property of the vaccine and not depend on fully functional host defenses and immune responses, (ii) not be reversible by diet or by host or microbial modification of diet constituents, and (iii) not permit development of a persistent carrier state. The attenuated vaccine should be sufficiently invasive and persistent to stimulate both strong primary and lasting memory immune responses and should be designed to minimize unnecessary tissue damage. As even attenuated vaccines may cause disease in unlucky individuals, the vaccine should be susceptible to clinically useful antibiotics. Many means to attenuate *Salmonella* vaccines make them less able to tolerate stresses encountered after oral administration including exposure to acid, bile, increasing osmolarity and iron, and decreasing O₂, and/or reduced invasion of the gut associated lymphoid tissue (GALT). The doses of recombinant *Salmonella* vaccines to elicit maximal immune responses are lower for intranasal immunization than they are for oral immunization (1,2). This may be due, in part, to killing of orally administered vaccines by the acid stress of the stomach (3) quickly followed by exposure to bile in the duodenum. We have determined that these two stresses in succession are more effective in causing bacterial cell death than the sum of killing by each stress alone. *Salmonella* possesses a large constellation of genes that confer acid tolerance and resistance to acid stress (4,5) and inactivation of these genes or their inability to be expressed by induction, reduces virulence (6). In this regard, the regulatory proteins RpoS (7), Fur (8), PhoPQ (9) and OmpR (10, 11) are all necessary to confer resistance to acid stress and/or shock in *S. Typhimurium*. Similarly, many genes are turned on in response to exposure to bile and some of these gene products transiently repress invasion while bacteria reside in the intestinal lumen (12 - 14).

[0188] It is important to have mutations contributing to attenuation or other beneficial vaccine attributes that do not impair the abilities of the vaccine to adjust to and/or withstand a diversity of stresses encountered at any location within the gastrointestinal tract if administered orally or in the respiratory tract if administered intranasally. Likewise, the vaccine strain should have wild-type abilities not compromised by attenuating or other mutations to penetrate through mucin, to attach to cells in the mucosal epithelium and be invasive into those cells. To achieve these objectives, means have been developed herein to achieve regulated delayed attenuation in vivo such that the vaccine at the time of immunization exhibits almost the same abilities as a fully virulent wild-type strain to contend with stresses and successfully reach effector lymphoid tissues before display of attenuation to preclude onset of any disease symptoms. The means described herein confer high-level attenuation and superior immunogenicity compared to traditional mutationally attenuated strains.

Materials and Methods for Example 2

[0189] Bacterial strains, media and bacterial growth: All strains for testing in mice are derived from the highly virulent *S. Typhimurium* strain UK-1 (15). All bacterial strains for this example are listed above in Table 1. LB broth and agar (16) are used as complex media for propagation and plating of bacteria. Nutrient broth and agar (Difco), which are devoid of arabinose and mannose, and minimal salts medium and agar (17) were also used. Some studies were done with bacterial strains grown in tissue culture medium to simulate environments to be encountered in vivo. MacConkey agar with 0.5% lactose (Lac), 0.2 or 0.5% arabinose (Ara) or 0.5% maltose (Mal) were used to indicate fermentation of sugars and enumerate bacteria from mice. CAS plates (Schwyn B, Neilands JB, 1987. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem.* 1987 Jan;160(1):47-56), which were used to determine siderophore production, were made by addition of chrome azurol S mixed with Fe^{+3} and hexadecyltrimethyl ammonium bromide (HDTMA) to MOPS basal agar. X-P plates to detect phosphatase activity were made by addition of 50 mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP or XP) to Nutrient agar. Kornberg agar medium plates were prepared as a glycogen indicator agar (18 - 20). Selenite broth, with or without supplements, was used for enrichment of *Salmonella* from tissues, although

later results demonstrated that enrichment with tetrathionate broth gave better results when vaccine strains had multiple mutations. Bacterial growth was monitored spectrophotometrically and by plating for colony counts.

[0190] Molecular and genetic procedures: Methods for DNA isolation, restriction enzyme digestion, DNA cloning and use of PCR for construction and verification of vectors are standard (21). DNA sequence analysis was performed in the DNA Sequence Laboratory in the School of Life Sciences at ASU. All oligonucleotide and/or gene segment syntheses were done commercially. Overlapping PCR amplification with primers designed for specific modifications was used to optimize codons for translational efficiency in *Salmonella* or to alter promoter, ribosome binding/Shine-Dalgarno (SD) and start codon sequences. Conjugational transfer of suicide vectors for generation of unmarked deletion and deletion-insertion mutations was performed by standard methods (22, 23) using the suicide vector donor strain χ 7213 (Table 1). Since live vaccine strains cannot display resistance to antibiotics, means were used to generate defined deletion mutations using suicide vector technologies that did not use drug-resistance markers or leave molecular scars. Subsequently, these unmarked defined deletion mutations with and without specific insertions were introduced into strains using P22HT $\int$ (24, 25) transduction of suicide vectors integrated into the deletion or deletion-insertion mutation followed by selection for sucrose resistance as described (26). Whenever insertion of a regulatory sequence might adversely effect expression of an adjoining gene, a transcription terminator (TT) was included to prevent such consequences. Strong TTs from bacteriophages were generally used. Plasmid constructs were evaluated by DNA sequencing, ability to complement various *S. Typhimurium* mutant strains (Table 1) and for ability to specify synthesis of proteins using gel electrophoresis and western blot analyses. His- or GST-tagged proteins have been produced and used to obtain anti-protein rabbit antisera for western blot analyses.

[0191] Strain characterizations: Exquisite care was taken in strain construction and complete biochemical and genetic characterizations were performed after every step in strain construction. This includes running an LPS gel to make sure rough variants were not selected. Comparative growth analyses were conducted since the objective is to have single and multiple mutant strains grow at similar rates and to

the same density as the wild-type parental strains when grown under permissive conditions. Vaccine strain stability was also evaluated, due to possible recombinational and/or mutational events as described below. Strains are also evaluated for biochemical and metabolic attributes, sensitivity to antibiotics and drugs, serological properties and resistance compared to wild-type parental strains to stresses associated with exposure to acid and bile.

[0192] Cell biology: The ability of various constructed *Salmonella* strains to attach to, invade into and survive in various murine and human epithelial and/or macrophage cell lines are quantitated by well established methods (27, 28) that are used routinely.

[0193] Animal experimentation: BALB/c and C57BL/6 female mice, six to eight weeks of age, were used for most experiments. Mice are held in quarantine one-week before use in experiments. They are deprived of food and water 6 h before oral immunization. No bicarbonate is administered. Food and water are returned 30 min after immunization. Candidate vaccine strains are quantitatively enumerated in various tissues as a function of time after inoculation (29, 30). The inoculation procedures are the same as in the immunization studies. All animals are housed in BL2 containment with filter bonnet covered cages. If high immunogenicity is observed in initial tests after primary immunization, subsequent studies are done to determine the lowest level of vaccine inocula to induce a significant protective immune response to oral or intraperitoneal challenge with the wild-type *S. Typhimurium* UK-1 parental strain χ 3761.

Construction of deletion-insertion mutations to achieve regulated delayed attenuation.

[0194] Four means are described to permit a regulated delayed attenuation phenotype so that vaccine strains at the time of immunization exhibit nearly wild-type attributes for survival and colonization of lymphoid tissues and after five to ten cell divisions become avirulent. These means to achieve regulated delayed attenuation rely on using an *araC* P_{BAD} activator-promoter that is more tightly regulated by arabinose (31) than the original sequence from the *E. coli* B/r strain (32). The promoter was deleted, including all sequences that interact with activator or repressor proteins, for the *fur*, *phoPQ*, *rpoS* and *crp* nucleic acid sequences and substituted by insertion of the improved *araC* P_{BAD} cassette (31) to yield *Salmonella* strains with the $\Delta P_{fur33::TT}$

araC P_{BAD} *fur*, $\Delta P_{\text{phoPQ107}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ phoPQ}$, $\Delta P_{\text{rpoS183}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ rpoS}$ and $\Delta P_{\text{crp527}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ crp}$ deletion-insertion mutations (P stands for promoter and TT stands for transcription terminator). The suicide vectors used to generate these four deletion-insertion mutations are depicted in Figure 25 and are listed in Table 2. A strong phage-derived TT was included at the 3' end of the *araC* nucleic acid sequence in all these constructions since its transcription in the presence of arabinose could often lead to altered over expression of downstream adjacent nucleic acid sequences with the same transcriptional orientation as the *araC* nucleic acid sequence or to diminished expression when the downstream adjacent nucleic acid sequence is in opposite orientation resulting in synthesis of anti-sense mRNA from P_{araC}.

Phenotypic characterization of mutant strains.

[0195] Growth of these strains in the presence of arabinose leads to transcription of the *fur*, *phoPQ*, *rpoS* and/or *crp* nucleic acid sequences but nucleic acid sequence expression ceases in the absence of arabinose. These activities can be readily observed by appropriate tests. Thus $\chi 9021$ with the $\Delta P_{\text{crp527}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ crp}$ deletion-insertion mutation can only ferment maltose when grown in the presence of arabinose and not in the absence of arabinose as revealed by streaking cultures on MacConkey maltose agar without and with 0.2 percent arabinose (Fig. 26a). Similarly, $\chi 8848$ with the $\Delta P_{\text{fur33}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ fur}$ and $\chi 9107$ with $\Delta P_{\text{fur33}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ fur}$ and $\Delta P_{\text{crp527}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ crp}$ mutations reveal siderophore production when streaked on CAS plates without arabinose and no siderophore production when grown in the presence of arabinose (Fig. 26b). $\chi 8918$ with the $\Delta P_{\text{phoPQ107}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ phoPQ}$ and $\chi 9108$ with the $\Delta P_{\text{phoPQ107}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ phoPQ}$ and $\Delta P_{\text{crp527}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ crp}$ mutations when streaked on X-P plates without and with 0.2 percent arabinose reveal acid phosphatase activity due to expression of the PhoP-activated *phoN* nucleic acid sequence only when grown in the presence of arabinose (Fig. 26c). $\chi 8956$ with the $\Delta P_{\text{rpoS183}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ rpoS}$ and $\chi 9064$ with the $\Delta P_{\text{rpoS183}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ rpoS}$ and $\Delta P_{\text{crp527}}::\text{TT } araC \text{ P}_{\text{BAD}} \text{ crp}$ mutations reveal glycogen accumulation when streaked on glycogen-indicator agar with 0.2 percent arabinose and sprayed with iodine indicator solution (Fig. 26d). The presence or absence of RpoS in these strains can also be

revealed by adding hydrogen peroxide to cultures to detect the activity of the RpoS-dependant catalase, KatE, (33-36) when arabinose is present during strain growth. Since Crp positively enhances transcription from P_{BAD} , the inclusion of the $\Delta P_{crp527::TT}$ *araC* P_{BAD} *crp* mutation with other *araC* P_{BAD} regulated nucleic acid sequences causes a tighter cessation of transcription in the absence of arabinose. This is seen by close examination of the photographs in Figure 26. For this reason, the $\Delta P_{crp527::TT}$ *araC* P_{BAD} *crp* mutation is included in all vaccine strains when using *araC* P_{BAD} regulation of virulence nucleic acid sequences.

Stability of Crp, Fur, RpoS and PhoP proteins and their decline during growth in the absence of arabinose.

[0196] Growth of strains with *araC* P_{BAD} regulated nucleic acid sequences in the presence of arabinose results in acid production that can cause cessation of growth. We have therefore included the $\Delta araBAD23$ mutation (Fig. 27) that prevents use of arabinose. Inclusion of this mutation also prevents breakdown of arabinose retained in the cell cytoplasm at the time of oral immunization and inclusion of the $\Delta araE25$ mutation (Fig. 27), which enhances retention of arabinose further delays cessation in expression of *araC* P_{BAD} regulated nucleic acid sequences for an additional cell division or so. The suicide vectors for introducing the $\Delta araBAD23$ and $\Delta araE25$ mutations are listed in Table 2.

[0197] The stability of virulence gene products in strains with each of the *araC* P_{BAD} regulated virulence genes was determined by growing cultures to an OD_{600} of 0.8 in LB broth with 0.2 percent arabinose and then adding 30 μ g chloramphenicol/ml (37) for Crp, Fur and PhoP and 200 μ g chloramphenicol/ml for RpoS (38) to arrest further protein synthesis. As can be seen by the results presented in Figure 28, the Crp, Fur and PhoP proteins are very stable and not significantly degraded, whereas the RpoS protein displays no stability in the log phase (39). However, the RpoS protein seemed to be stable when 50 μ g chloramphenicol/ml was added to saturated overnight stationary phase cultures. When these mutant strains were grown in Nutrient broth with 0.2 percent arabinose to an OD_{600} of 0.8 and then diluted 1 to 4 into Nutrient broth with no added arabinose and these 1 to 4 dilutions continued after each culture again reached an OD_{600} of 0.8, we observed no significant reductions in the amounts of Crp,

Fur and PhoP proteins until a final dilution of 1 to 16 with an arabinose concentration of 0.0125 percent or until a final dilution of 1 to 64 with an arabinose concentration of 0.003125 percent. Thereafter the amount of these proteins decreased by a factor of four for each subsequent 1 to 4 dilution of the culture (Fig. 29). In the case of RpoS protein, we observed a significant amount of reduction within a dilution of 1 to 4 with an arabinose concentration of 0.05 percent (Fig. 29). The decline in the amounts of these proteins in vivo would be expected to be more accelerated since there is no arabinose present in tissues upon invasion of *Salmonella* into the GALT. In other experiments, strains grown in Nutrient broth with 0.2 percent arabinose were sedimented by centrifugation and resuspended at a density one-fourth of the original culture. In this case after growth to the original density, the amounts of each of the four virulence gene proteins was three to four times less than in the culture grown with arabinose. In other experiments, it was determined that the levels of Fur, PhoP, RpoS and Crp synthesis were nearly the same when mutant cultures were grown in LB broth with either 0.05 percent or 0.2 percent arabinose.

Attenuation of mutant strains in orally immunized female BALB/c mice.

[0198] Levels of attenuation were evaluated in *S. Typhimurium* UK-1 strains with different *araC* P_{BAD} regulated virulence nucleic acid sequences by oral inoculation of female BALB/c mice with doses approximating 10⁷, 10⁸ and 10⁹ CFU from cultures grown in LB broth with 0.0, 0.05 and 0.2 percent arabinose. It should be noted, that LB broth contains arabinose in the yeast extract equivalent to a concentration of 0.003 percent based on mass spec analysis. The collective results presented in Table 3 indicate that the strains with the $\Delta P_{\text{phoPQ107::TT } araC \text{ P}_{BAD} \text{ phoPQ}}$, $\Delta P_{\text{rpoS183::TT } araC \text{ P}_{BAD} \text{ rpoS}}$ and $\Delta P_{\text{crp527::TT } araC \text{ P}_{BAD} \text{ crp}}$ deletion-insertion mutations were highly attenuated whereas the strain with the $\Delta P_{\text{fur33::TT } araC \text{ P}_{BAD} \text{ fur}}$ mutation was less attenuated. In this regard, a higher level of attenuation was noted when χ_{8848} was grown in LB broth with no added arabinose and a greater virulence when grown in LB broth with 0.2 percent arabinose. It is evident, however, from the collective results (Table 3) that attenuation develops as the products of the fur, phoPQ, rpoS and/or crp nucleic acid sequences are diluted at each cell division.

Table 3. Attenuation of mutant strains in orally immunized female BALB/c mice ^a

Strain	Genotype	Dose ^b range	Survivors/total	Percent/survivors
χ 8848	ΔP_{fur33}	9.0×10^6 - 2.2×10^9	138/189	73.0
χ 8918	$\Delta P_{phoPQ107}$	9.0×10^6 - 1.2×10^9	182/185	98.4
χ 8956	$\Delta P_{rpoS183}$	9.4×10^6 - 1.5×10^9	179/184	97.3
χ 9021	ΔP_{crp527}	9.5×10^6 - 1.5×10^9	163/164	99.4

^a Mice were seven to eight weeks of age. Bacterial strains were grown in LB broth with 0, 0.05 or 0.2 percent arabinose that did not have a significant effect on levels of attenuation on strains with the $\Delta P_{phoPQ107}::TT \text{ araC } P_{BAD} \text{ phoPQ}$, $\Delta P_{rpoS183}::TT \text{ araC } P_{BAD} \text{ rpoS}$ and $\Delta P_{crp527}::TT \text{ araC } P_{BAD} \text{ crp}$ deletion-insertion mutations but did effect the results for χ 8848 with the $\Delta P_{fur33}::TT \text{ araC } P_{BAD} \text{ fur}$ mutation (see Results).

^b Doses are in CFU.

Abilities of orally administered strains with araC P_{BAD} regulated virulence nucleic acid sequences to induce protective immunity to oral challenge with wild-type S.

Typhimurium UK-1.

[0199] Strains with each of the *araC* P_{BAD} regulated virulence nucleic acid sequences were next evaluated for induction of protective immunity against a challenge with the highly virulent *S. Typhimurium* UK-1 strain χ 3761 (oral LD₅₀ of $1-2 \times 10^4$ CFU). The results in Table 4 reveal that χ 8848 with the $\Delta P_{fur33}::TT \text{ araC } P_{BAD} \text{ fur}$ mutation displayed some virulence even at low doses when grown in LB broth with 0.2 percent arabinose. However, for immunizing doses of 10^7 CFU and higher 100 percent of the survivors developed protective immunity to challenges with 10^8 and 10^9 CFU doses of χ 3761. Thus the $\Delta P_{fur33}::TT \text{ araC } P_{BAD} \text{ fur}$ mutation while displaying moderate attenuation is highly immunogenic. This is a very important attribute of an attenuating mutation to include in a vaccine strain. It was previously reported (40) that χ 8848 with the $\Delta P_{fur33}::TT \text{ araC } P_{BAD} \text{ fur}$ mutation was completely attenuated even at high 10^9 CFU

doses when grown in LB broth with no added arabinose. This observation implies that production of too much Fur protein may diminish attenuation.

Table 4: Oral immunization of mice with χ_{8848} (ΔP_{fur33}) and with survivors challenged orally with wild-type χ_{3761} thirty days later ^a

Immunizing dose ^b		Survivors/ total		Challenge dose ^b		Survivors/ total	
Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
9.0×10^8	1.1×10^9	6/10	4/10	1.0×10^9	1.5×10^9	3/3	2/2
				1.0×10^8	1.5×10^8	3/3	2/2
9.0×10^7	1.1×10^8	7/10	7/10	1.0×10^9	1.5×10^9	2/2	4/4
				1.0×10^8	1.5×10^8	5/5	3/3
9.0×10^6	1.1×10^7	7/10	5/10	1.0×10^9	1.5×10^9	4/4	2/2
				1.0×10^8	1.5×10^8	3/3	3/3
9.0×10^5	1.1×10^6	5/10	8/10	1.0×10^9	1.5×10^9	1/2	1/4
				1.0×10^8	1.5×10^8	0/3	2/4
9.0×10^4	1.1×10^5	10/10	7/10	1.0×10^9	1.5×10^9	0/5	2/4
		—	—	1.0×10^8	1.5×10^8	<u>0/5</u>	<u>3/3</u>
Total (all doses)			66/100				45/66
							68.2%
Total (10^7 - 10^9 doses)			36/60				36/36
							100%

^a Female BALB/c mice were six to eight weeks of age. χ_{8848} was grown in LB broth with 0.2% arabinose.

^b Doses are in CFU.

[0200] The results in Table 5 reveal that χ 8918 with the $\Delta P_{\text{phoPQ107}}::\text{TT}$ *araC* P_{BAD} *phoPQ* deletion-insertion mutation is very attenuated but displays more moderate immunogenicity in regard to inducing protection against challenge with χ 3761. These results suggest that some of the attenuation may be due to a reduced ability of χ 8918 to effectively colonize lymphoid tissues, quite possibly due to the over expression of the *phoPQ* nucleic acid sequences when χ 8918 is grown in LB broth with 0.2 percent arabinose. In accord with this expectation, χ 8918 is better able to colonize Peyer's patches, mesenteric lymph nodes and spleens in orally immunized mice when grown in LB broth without added arabinose than when grown in LB broth with 0.2 percent arabinose. Nevertheless, χ 8918 is still less capable of colonizing these lymphoid tissues than χ 9021 with the $\Delta P_{\text{crp527}}::\text{TT}$ *araC* P_{BAD} *crp* deletion-insertion mutation, which colonizes equally well independent of arabinose concentration in the LB broth.

Table 5. Oral immunization of mice with χ 8918 ($\Delta P_{\text{phoPQ107}}$) and with survivors challenged orally with wild-type χ 3761 thirty days later^a

Immunizing dose ^b		Survivors/ total		Challenge dose ^b		Survivors/ total	
Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
9.0 x 10 ⁸	1.2 x 10 ⁹	10/10	9/10	1.0 x 10 ⁹	1.5 x 10 ⁹	4/5	5/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	4/5	4/4
9.0 x 10 ⁷	1.2 x 10 ⁸	10/10	10/10	1.0 x 10 ⁹	1.5 x 10 ⁹	3/5	4/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	3/5	5/5
9.0 x 10 ⁶	1.2 x 10 ⁷	10/10	9/10	1.0 x 10 ⁹	1.5 x 10 ⁹	2/4	1/4
				1.0 x 10 ⁸	1.5 x 10 ⁸	2/5	2/5
9.0 x 10 ⁵	1.1 x 10 ⁶	10/10	10/10	1.0 x 10 ⁹	1.5 x 10 ⁹	3/5	0/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	0/5	3/5
9.0 x 10 ⁴	1.1 x 10 ⁵	10/10	10/10	1.0 x 10 ⁹	1.5 x 10 ⁹	0/5	0/5

		—	—	1.0×10^8	1.5×10^8	<u>0/5</u>	<u>0/5</u>
Total (all doses)			98/100				45/98
							45.9%
Total (10^7 - 10^9 doses)			58/60				39/58
							67.2%
^a Female BALB/c mice were six to eight weeks of age. χ 8918 was grown in LB broth with 0.2% arabinose.							
^b Doses are CFU.							

[0201] The results in Table 6 confirm the oral avirulence of χ 8956 with the $\Delta P_{rpoS183}::TT \text{ araC } P_{BAD} rpoS$ deletion-insertion mutation. However, the two experiments give very different results on the ability of this strain to induce protective immunity to oral challenge with wild-type *S. Typhimurium*. We therefore repeated the experiment giving oral doses of χ 8956 ($\Delta P_{rpoS183}$) of 1.4×10^7 , 10^8 and 10^9 CFU with 15 survivors at each dose and after challenge with 3.1×10^9 CFU of χ 3761 observed 13, 13 and 14 survivors, respectively, out of 15 mice challenged. It thus appears that the data in the second experiment in Table 6 are more indicative of the correct attenuating and immunogenic phenotype. No differences in results were observed when χ 8956 ($\Delta P_{rpoS183}$) was grown in LB broth with or without arabinose.

Table 6. Oral immunization of mice with χ 8956 ($\Delta P_{rpoS183}$) and with survivors challenged orally with wild-type- χ 3761 thirty days later ^a

Immunizing dose ^b		Survivors/ total		Challenge dose ^b		Survivors/ total	
Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
9.4×10^8	1.5×10^9	9/10	9/10	1.0×10^9	1.5×10^9	0/4	4/4
				1.0×10^8	1.5×10^8	1/5	5/5
9.4×10^7	1.5×10^8	10/10	10/10	1.0×10^9	1.5×10^9	0/5	5/5

				1.0×10^8	1.5×10^8	0/5	3/5
9.4×10^6	1.5×10^7	10/10	9/10	1.0×10^9	1.5×10^9	2/5	5/5
				1.0×10^8	1.5×10^8	0/5	2/4
9.4×10^5	1.5×10^6	10/10	10/10	1.0×10^9	1.5×10^9	0/5	4/5
				1.0×10^8	1.5×10^8	0/5	2/5
9.4×10^4	1.5×10^5	10/10	10/10	1.0×10^9	1.5×10^9	0/5	0/5
		_____	_____	1.0×10^8	1.5×10^8	<u>0/5</u>	<u>0/5</u>
Total (all doses)			97/100				33/97
							34.0%
Total (10^7 - 10^9 doses)			57/60				27/57
							47.4%
^a Female BALB/c mice were six to eight weeks of age. χ 8956 was grown in LB broth with 0.2% arabinose. ^b Doses are in CFU.							

[0202] The results in Table 7 indicate that χ 9021 with the $\Delta P_{crp527}::TT \text{ araC}$ P_{BAD} *crp* deletion-insertion mutation is both highly attenuated and also very immunogenic. Neither of these attributes was altered when the strain was grown in LB broth with or without arabinose.

Table 7. Oral immunization of mice with χ 9021 (ΔP_{crp527}) and with survivors challenged orally with wild-type χ 3761 thirty days later ^a

Immunizing dose ^b		Survivors/ total		Challenge dose ^b		Survivors/ total	
Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
9.5×10^8	1.6×10^9	10/10	10/10	1.0×10^9	1.5×10^9	5/5	5/5

				1.0 x 10 ⁸	1.5 x 10 ⁸	5/5	5/5
9.5 x 10 ⁷	1.6 x 10 ⁸	10/10	10/10	1.0 x 10 ⁹	1.5 x 10 ⁹	5/5	5/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	5/5	5/5
9.5 x 10 ⁶	1.6 x 10 ⁷	10/10	10/10	1.0 x 10 ⁹	1.5 x 10 ⁹	4/5	5/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	5/5	5/5
9.5 x 10 ⁵	1.6 x 10 ⁶	10/10	9/10	1.0 x 10 ⁹	1.5 x 10 ⁹	5/5	3/5
				1.0 x 10 ⁸	1.5 x 10 ⁸	3/5	2/4
9.5 x 10 ⁴	1.6 x 10 ⁵	10/10	10/10	1.0 x 10 ⁹	--	4/5	--
			————	1.0 x 10 ⁸	--	3/5	==
Total (all doses)			99/100				78/89 87.6%
Total (10 ⁷ -10 ⁹ doses)			60/60				59/60 98.3%
^a Female BALB/c mice were six to eight weeks of age. χ 9021 was grown in LB broth with 0.2% arabinose. ^b Doses are in CFU.							

Alterations in strains with the $\Delta P_{fur}::TT\ araC\ P_{BAD}\ fur$ and $\Delta P_{phoPQ}::TT\ araC\ P_{BAD}\ phoPQ$ deletion-insertion mutations to increase the attenuation of the former and increase the immunogenicity of the latter.

[0203] As noted above, $\chi 8848$ with the $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ mutation was more attenuated when grown in LB broth without arabinose and more virulent when grown in LB broth with 0.2 percent arabinose prior to oral inoculation of mice. This implied that overproduction of Fur, which would require more cell divisions in vivo to dilute out, reduced attenuation without adversely altering immunogenicity in mice surviving immunization. Consequently, two derivatives were constructed in which the ATG start codon for the *fur* nucleic acid sequence was changed to GTG, and in one of these, the SD sequence was changed from AGGA to AAGG. The structure of these two mutations, $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$ and $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$, are diagrammed in Figure 8. $\chi 9273$ with the $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$ mutation and $\chi 9269$ with the $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ mutation both synthesize much less Fur as revealed by western blot analysis when grown in LB broth with 0.2 percent arabinose than does $\chi 8848$ with the $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ mutation.

[0204] It was also noted above that the immunogenicity of $\chi 8918$ with the $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ mutation was decreased when the strain was grown in LB broth with 0.2 percent arabinose although its attenuation was independent of the arabinose concentration in LB broth. This implied that over production of PhoP and/or PhoQ decreased induction of immunity to challenge. This inference was also supported by studies that demonstrated that $\chi 8918$ was less able to colonize Peyer's patches, mesenteric lymph nodes and spleen when grown in LB broth with 0.2 percent arabinose than when grown with no added arabinose. Two derivatives were therefore constructed in which the ATG start codon for the *phoP* nucleic acid sequence was changed to GTG and in one of these also changed the SD sequence from AGGA to AAGG. The structure of these two mutations, $\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$ and $\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$, are diagrammed in Figure 9. $\chi 9382$ with the $\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$ mutation and $\chi 9383$ with the $\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$ mutation both synthesize much less PhoP as revealed by western blot analysis when grown in LB broth with 0.2 percent arabinose than does $\chi 8918$ with the $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ mutation.

[0205] Table 8 below contains results that demonstrate the high immunogenicity of χ 9273 with the $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$ mutation and χ 9269 with the $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ mutation with χ 9269 with the $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ mutation demonstrating much better attenuation when grown in LB broth with 0.2 percent arabinose. The data in Table 8 also indicates that both χ 9382 with the $\Delta P_{phoPQ173}::TT\ araC\ P_{BAD}\ phoPQ$ mutation and χ 9383 with the $\Delta P_{phoPQ177}::TT\ araC\ P_{BAD}\ phoPQ$ mutation are completely attenuated when grown in LB broth with 0.2 percent arabinose and display essentially the same immunogenicity that is much improved over that exhibited by χ 8918 with the $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ mutation when it is grown in LB broth with 0.2 percent arabinose.

Table 8. Oral immunization of mice with strains with modified ΔP_{fur} and ΔP_{phoPQ} mutations and with survivors challenged orally with wild-type $\chi 3761$ thirty days later.^a

Strain ^b	Genotype	Immunizing dose ^c	Survivors/total	Challenge dose ^c	Survivors/total
$\chi 9273$	ΔP_{fur77}	1.5×10^9	6/10	1.7×10^9	6/6
		1.7×10^9	6/10	1.6×10^9	6/6
$\chi 9269$	ΔP_{fur81}	1.0×10^9	11/15	8.7×10^8	11/11
		1.0×10^8	15/15	8.7×10^8	15/15
		1.8×10^9	10/10	1.3×10^9	10/10
		1.7×10^9	19/20	1.6×10^9	19/19
$\chi 9382$	$\Delta P_{phoPQ173}$	1.0×10^9	15/15	1.8×10^9	11/15
		1.0×10^8	15/15	1.8×10^9	11/15
		1.0×10^7	15/15	1.8×10^9	12/15
$\chi 9383$	$\Delta P_{phoPQ177}$	1.1×10^9	15/15	1.8×10^9	10/15
		1.1×10^8	15/15	1.8×10^9	11/15
		1.1×10^7	15/15	1.8×10^9	13/15

^aFemale BALB/c mice were six to eight weeks of age. Strains were grown in LB broth with no added arabinose or with 0.05% or 0.2% arabinose with no significant differences noted.

^b $\chi 9382$ and $\chi 9383$ have the $\Delta araBAD23$ deletion (Table 1) in addition to the ΔP_{phoPQ} insertion-deletion mutations the $\Delta araBAD23$ deletion (Table 5).

^cDoses are in CFU.

Abilities of intraperitoneally administered strains with araC P_{BAD} regulated virulence nucleic acid sequences to induce protective immunity to oral challenge with wild-type S. Typhimurium UK-1.

[0206] Although the vaccines were designed for oral administration, it was worthwhile to determine if strains with these mutations, when administered intraperitoneally (i.p.), would also display attenuation and induce immunity to challenge with orally administered wild-type χ 3761. The *S. Typhimurium* UK-1 strain χ 3761 has an LD₅₀ by the i.p. route of less than 10 CFU. Table 9 demonstrates that strains with $\Delta P_{fur}::TT\ araC\ P_{BAD}\ fur$ mutations retain considerable virulence by this route of administration although χ 9269 with the $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ mutation displays the highest attenuation of the three strains evaluated and yet induces complete protective immunity to all survivors when challenged with about 10⁹ CFU of χ 3761. χ 8918 with the $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ mutation displays fairly good attenuation by this route. On the other hand, χ 8956 with the $\Delta P_{rpoS183}::TT\ araC\ P_{BAD}\ rpoS$ mutation and χ 9021 with the $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ mutation are the most attenuated and induce a very high level of protective immunity when delivered at i.p doses in the 10² to 10⁴ CFU range (Table 9).

Table 9. Intraperitoneal immunization of mice with strains with various deletion-insertion mutations conferring regulated delayed oral attenuation and with survivors orally challenged with wild-type χ 3761 thirty days later.

Strain	Genotype	Immunizing dose ^b	Survivors/total	Challenge dose ^b	Survivors/total
χ 8848	ΔP_{fur33}	1.2 x 10 ⁴	0/5	—	
		1.2 x 10 ³	0/5	—	
		1.2 x 10 ²	0/5	—	
		1.2 x 10 ¹	0/5	—	
χ 9273	ΔP_{fur77}	1.6 x 10 ⁴	0/5		

		1.6×10^3	0/5		
		1.6×10^2	0/5		
		1.6×10^1	1/5	1.6×10^9	1/1
χ_{9269}	ΔP_{fur81}	1.7×10^4	1/10	1.6×10^9	1/1
		1.7×10^3	7/10	1.6×10^9	7/7
		1.7×10^2	4/10	1.6×10^9	4/4
		1.7×10^1	6/10	1.6×10^9	6/6
χ_{8918}	$\Delta P_{\text{phoPQ107}}$	1.2×10^5	0/5	—	
		9.6×10^4	6/10	1.5×10^9	4/6
		1.2×10^4	5/5	9.6×10^8	5/5
		9.6×10^3	8/10	1.5×10^9	6/8
		1.3×10^3	5/5	9.6×10^8	2/5
		9.6×10^2	5/5	1.5×10^9	2/5
		1.2×10^2	5/5	9.6×10^8	3/5
χ_{8956}	$\Delta P_{\text{rpoS183}}$	1.5×10^4	5/5	1.0×10^9	5/5
		1.5×10^3	5/5	1.0×10^9	5/5
		1.5×10^2	5/5	1.0×10^9	3/5
		1.5×10^1	5/5	1.0×10^9	3/5
χ_{9021}	ΔP_{crp527}	1.4×10^5	0/5	—	

		1.4×10^4	4/10	1.5×10^9	4/4
		1.4×10^3	10/10	1.5×10^9	10/10
		1.4×10^2	10/10	1.5×10^9	10/10
		1.4×10^1	9/10	1.5×10^9	8/9
^a Female BALB/C mice were six to eight weeks of age. All strains were grown in LB broth with 0.2% arabinose. ^b Doses are in CFU.					

Enhanced control over araC P_{BAD} regulated virulence nucleic acid sequences in vivo by inclusion of the $\Delta P_{crp527::TT}$ araC P_{BAD} crp mutation.

[0207] Maximum levels of transcription of nucleic acid sequences regulated by the araC P_{BAD} system require not only arabinose to interact with the AraC protein but also the Crp protein (41). Thus the $\Delta P_{crp527::TT}$ araC P_{BAD} crp mutation was included in vaccine strains whenever other araC P_{BAD} regulated nucleic acid sequences are included. The benefit of this addition is readily observed by the results previously presented in Figure 7 that demonstrate this tighter regulation in the absence of arabinose in strains that also have the $\Delta P_{crp527::TT}$ araC P_{BAD} crp mutation. This also acts as a backup and should enhance safety and efficacy of vaccine strains.

[0208] Means for delay in the in vivo timing of onset of regulated delayed attenuation. As noted by Guzman et al. (32), the inclusion of mutations that abolish utilization of arabinose can prolong expression of nucleic acid sequences under the control of the araC P_{BAD} system. Onset of attenuation can therefore be delayed by including $\Delta araBAD23$, which prevents use of arabinose retained in the cell cytoplasm at the time of oral immunization, and/or $\Delta araE25$ that enhances retention of arabinose. These mutations are diagrammed in Figure 27.

Discussion for example 2

[0209] Four different means have been described to achieve regulated delayed attenuation of S. Typhimurium vaccine strains such that vaccines at the time of immunization will be better able to withstand the host defense imposed stresses following oral immunization. Some of these constructs have been modified to optimize

attenuation and improve immunogenicity. Although comparative studies with vaccine strains having defined deletion mutations in the *fur*, *phoPQ*, *rpoS* and *crp* nucleic acid sequences might resolve doubt, such comparative studies become difficult to justify based on animal use. These mutations are being included in strains with multiple attenuating mutations.

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Example 3: Improved Immune Responses Induced by RASVs with Regulated Delayed Attenuation and Regulated Delayed Synthesis of Protective Antigen

[0210] Generating a *Salmonella* strain that is safe and also retains its immunogenicity is the biggest challenge in the development of live vaccine candidates

(1). An ideal *Salmonella* vaccine strain should exhibit wild-type abilities to withstand all stresses (enzymatic, acid, osmotic, ionic, etc.) and host defenses (bile, antibacterial peptides, etc.) encountered following oral or intranasal immunization and should exhibit wild-type abilities to colonize and invade host lymphoid tissues while remaining avirulent. A variety of attenuated *Salmonella* strains have been used as live vaccines to induce mucosal and systemic immunity against either the carrier itself or to a vectored antigen (2). More recently developed *Salmonella* vaccine strains carry defined nonreverting mutations that fall into two general categories: metabolic functions and virulence factors (3). Different means for attenuating *Salmonella* have been investigated to develop ideal immune responses (4, 5). Many previously utilized means for *Salmonella* attenuation either reduced vaccine survival due to host-induced stresses and/or reduced colonization of lymphoid effector tissues leading to less than optimal immunogenicity (6, 7). To circumvent these problems, a system for regulated delayed in vivo expression of attenuation has been developed (8). Thus vaccine strains are phenotypically wild-type for host invasion at the time of immunization and become attenuated after colonization of host tissues (8).

[0211] PspA is an important virulence factor found on the surface of all pneumococci (9). It plays a role in colonization of the host and contributes to the ability of pneumococcus to cause invasive disease (10). The N-terminal half of the protein is the α -helical domain which contains protective epitopes based on immunization studies with the full length and truncated PspA fragments (11, 12). Humans naturally infected or colonized with pneumococcus, develop anti-PspA antibodies in both serum and mucosal secretions, with antibody to the α -helical domain of PspA also implicated in preventing pneumococcal carriage.

[0212] Previous work has demonstrated that oral vaccination of mice with a Δ crp *Salmonella* vaccine strain expressing a secreted PspA fusion protein could protect the immunized mice from virulent *S. pneumoniae* WU2 challenge (13). The protection rate was about 60% against a 50 LD₅₀ challenge (13). To increase the effective protective immunity against *S. pneumoniae*, we designed and constructed a new generation of *Salmonella enterica* serovar Typhimurium strains with delayed regulated attenuation (see Example 2) and for one strain with regulated delayed expression of antigen encoding sequences in vivo (Example 1).

[0213] In this example, the immunogenicity is evaluated of two new attenuated *S. Typhimurium* strains transformed with Asd⁺ balanced-lethal plasmids encoding a secreted form of the α -helical region of PspA. Antibody responses, cytokine responses and protective immunity against *S. pneumoniae* WU2 challenge were evaluated. The results attained confirm the hypothesis that vaccine strains with regulated delayed in vivo attenuation including the strain that also exhibited regulated delayed protective antigen synthesis confer a more superior immune response than a vaccine strain with a more traditional means of attenuation.

Materials and methods for Example 3

[0214] Bacterial strains, plasmids, media, and growth conditions: The bacterial strains and plasmids used in this example are listed in Table 1 and 2, respectively. Bacteriophage P22HT_{int} was used for generalized transduction. *S. Typhimurium* cultures were grown at 37°C in LB broth or on LB agar (14). For animal experiments, plasmid-containing χ 9088 and χ 9558 cultures were supplemented with 0.2% mannose or 0.2% mannose and 0.05% arabinose, respectively. No additions were made to the media for growing plasmid-containing χ 8133 cultures. MacConkey agar (Difco, Detroit, Mich.) supplemented with 1% sugar was used for fermentation assays. DAP was added (50 μ g/ml) for the growth of Asd⁻ strains (15). *S. pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract (12).

[0215] Strain construction and characterization: MacConkey agar supplemented with 1% maltose was used to confirm the phenotype of *crp* mutants (13). Chrome Azurol S (CAS) plates were used to confirm the constitutive synthesis of siderophores characteristic of *fur* mutants (16). The presence of the Δ asdA33 and Δ asdA16 mutations in *Salmonella* was confirmed by inability of the strain to grow on media without DAP (15). Lipopolysaccharide (LPS) profiles of *Salmonella* strains were examined as described (17). Plasmid stability was determined as previously described (18). All plasmids were found to be stable for 50 generations of growth in the presence of DAP.

[0216] SDS-PAGE and immunoblot analyses: Protein samples were boiled for 5 min and subsequently separated by SDS-PAGE. For immunoblotting, proteins

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

[0217] Immunization of mice: Female BALB/c mice, 6-7 weeks old, were obtained from Charles River Laboratories. All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Mice were acclimated for 7 days before starting experiments.

[0218] Recombinant attenuated *Salmonella* vaccine (RASV) strains were grown statically overnight in LB broth containing the appropriate supplements at 37°C. The following day, an overnight culture of 1 ml was inoculated into 100 ml of LB broth containing the appropriate supplements and grown with aeration at 37°C to an OD₆₀₀ of 0.8 to 0.9. Cells were pelleted by centrifugation at room temperature (6,000×g for 15 min), and the pellet resuspended in 1 ml of buffered saline with gelatin (BSG). To determine the titer of RASV strains used to inoculate mice, dilutions of the RASV strains were plated onto MacConkey agar supplemented with 1% lactose. Mice were orally inoculated with 20 µl of BSG containing 1×10⁹ CFU of an RASV strain. Blood was obtained by mandibular vein puncture at biweekly intervals. Following centrifugation, the serum was removed from the whole blood and stored at -20°C.

[0219] Antigen preparation: rPspA protein and *S. Typhimurium* outer membrane proteins (SOMPs) were purified as described (13). *S. Typhimurium* LPS was obtained from Sigma. The rPspA clone and purified protein were kind gifts from Dr. Susan Hollingshead at the University of Alabama at Birmingham (19).

[0220] Enzyme linked immunosorbent assay (ELISA): ELISA was used to assay antibodies in serum to *S. Typhimurium* LPS, SOMPs and to rPspA. Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA.) were coated with LPS (100 ng/well; Sigma), SOMP (100 ng/well, our lab), or purified rPspA (100 ng/well). Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied with 100-µl volumes in each well. Plates were incubated overnight at

4°C. Free binding sites were blocked with phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20, and 1% bovine serum albumin. A 100- μ l volume of series diluted sample was added to individual wells in triplicate and incubated for 1h at 37°C. Plates were treated with biotinylated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Inc., Birmingham, Ala.) Wells were developed with streptavidin-alkaline phosphatase conjugate (Southern Biotechnology) followed by *p*-nitrophenylphosphate substrate (Sigma) in diethanolamine buffer (pH 9.8). Color development (absorbance) was recorded at 405 nm using an automated ELISA plate (model EL311SX; Biotek, Winooski, VT.). Absorbance readings 0.1 higher than PBS control values were considered positive reactions.

[0221] Passive transfer of cells and sera: At week 12, sera and spleen cells were harvested from 5 mice per group. The sera were pooled and CD4⁺ T cells were isolated using T-cell enrichment columns (R&D Systems Inc, Minneapolis, MN.), according to the manufacturer's instructions. Spleen cells (1×10^7) or purified CD4⁺ T cells (5×10^6) were suspended in PBS and injected into the lateral tail veins of naive, syngeneic BALB/c mice. Naïve syngeneic BALB/c mice received 100 μ l of serum from a different group of mice through the tail vein. All groups were challenged intraperitoneally after 12h with 5×10^4 CFU of *S. pneumoniae* in 200 μ l of BSG.

[0222] IL-4 and IFN- γ ELISPOTs: At week 8, spleen cells were harvested from 3 mice of each group. ELISPOTs were performed as previously described (20). Briefly, PVDF membrane plates (Millipore, Bedford, MA, USA) were pre-wetted with ethyl alcohol, washed with sterile H₂O and coated with 100 μ l of mAbs IL-4 or IFN- γ (BD PharMingen, San Diego, CA) at 2 μ g/ml, in PBS overnight at 4 °C. The wells were washed with PBS and blocked with RPMI with 10% FCS. After that, 50 μ l cell medium (RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin and 1% HEPES, with or without stimuli and 50 μ l of cells (100,000 per well) in cell medium were added per well and incubated in the plates overnight in 5% CO₂ at 37 °C. The next day, the cell suspensions were discarded and the plates washed with PBS. Biotinylated mAb IL-4 or IFN- γ (BD PharMingen, San Diego, CA) at 0.5 μ g/ml in PBS with 1% FCS was added and incubated at room temperature for 2 h. After washing with PBS, 100 μ l/well of avidin peroxidase diluted 1:1000 (v/v) in PBS-T containing 1% FCS were added followed by incubation for 1 hour at room temperature.

AEC (3-amino-9-ethylcarbazole) substrate was prepared according to manufacturer's (Vector Laboratories, Burlingame, CA) specifications, and 100 μ l of substrate was added per well. Spots were developed for 15 minutes at room temperature. Plates were dried and analyzed by using an automated CTL ELISPOT Reader System (Cellular Technology LTD, Cleveland, OH).

[0223] Measurement of cytokine concentrations: Cytokine concentrations were determined using the Bio-Plex Protein Array System (Bio-Rad, Hercules, CA, USA). Cytokine-specific antibody-coated beads (Bio-Rad) were used for these experiments. The assay quantitates cytokines over a broad range (2–32,000 pg/ml) and eliminates multiple dilutions of high-concentration samples. The samples were prepared and incubated with the antibody-coupled beads for 1 h with continuous shaking. The beads were washed three times with wash buffer to remove unbound protein and then incubated with biotinylated detection cytokine-specific antibody for 1 h with continuous shaking. The beads were washed once more and were then incubated with streptavidin–phycoerythrin for 10 min. After incubation, the beads were washed and resuspended in assay buffer, and the constituents of each well were drawn up into the flow-based Bio-Plex Suspension Array System, which identifies each different color bead as a population of protein and quantifies each protein target based on secondary antibody fluorescence. Cytokine concentrations were automatically calculated by Bio-Plex Manager software using a standard curve derived from a recombinant cytokine standard. Many readings were made on each bead set, further validating the results.

[0224] Pneumococcal challenge: At week 12 the ability of the *Salmonella*-PspA vaccine to protect immunized mice against *S. pneumoniae* was assessed by intraperitoneal challenge with 5×10^4 CFU of *S. pneumoniae* WU2 in 200 μ l of BSG (21). The 50% lethal dose (LD_{50}) of *S. pneumoniae* WU2 in BALB/c mice was 2×10^2 CFU by intraperitoneal administration. Twenty-four hours after intraperitoneal challenge, mice were marked and bled by mandibular vein puncture, and blood samples with 10-fold serial dilutions in saline were plated on brain heart infusion agar containing 5% sheep blood. Bacterial colonies were enumerated after overnight incubation at 37°C.

[0225] Histological examinations: After challenge, mice were euthanized just before dying and survivors were euthanized after 15 days of observation. Fixed lung, spleen and liver specimens were embedded in paraffin wax, sectioned, and

stained with hematoxylin and eosin (H&E). Micrographs were taken with a digital camera.

[0226] Statistical analysis: Most data were expressed as means $\pm$ standard error. The means were evaluated with One-way ANOVA and LSD tests for multiple comparisons among groups. $p < 0.05$ was considered statistically significant.

Regulated attenuation of fur, crp and pmi in χ 9088 ($\Delta P_{fur33}::TT araC P_{BAD} fur \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta asdA33$) and χ 9558 ($\Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta P_{fur81}::TT araC P_{BAD} fur \Delta P_{crp527}::TT araC P_{BAD} crp \Delta asdA27::TT araC P_{BAD} C2 \Delta araE25 \Delta araBAD23 \Delta relA198::araC P_{BAD} lacI TT \Delta sopB1925 \Delta agfBAC811$).

[0227] Regulated delayed attenuation attributes distinguish χ 9088 and χ 9558 from other attenuated Salmonella strains due to a unique combination of arabinose-regulated expression of Fur, Crp and mannose-regulated expression of O-antigen synthesis. Salmonella with *pmi* mutations are attenuated and immunogenic (22). Strains with the $\Delta pmi-2426$ mutation lack phosphomannose isomerase needed to interconvert fructose-6-P and mannose-6-P but synthesize a complete LPS O-antigen when grown in the presence of mannose (Fig. 30). Note that LPS synthesis is dependent on the addition of mannose when cells are grown in nutrient broth, but there is enough mannose in LB broth to enable O-antigen production.

[0228] The other means used to achieve regulated delayed in vivo attenuation was to replace the promoter/operator regions of the *fur* and *crp* nucleic acid sequences with the tightly-regulated, arabinose-dependent *araC* P_{BAD} activator-promoter. (Fig. 31) Growth of these mutant strains in the presence of arabinose leads to transcription of the *fur* and *crp* nucleic acid sequences but nucleic acid sequence expression ceases in the absence of arabinose. Since free arabinose is not found in mammalian tissues, the arabinose-regulated *fur* and *crp* nucleic acid sequences will not be expressed.

Expression of rPspA in Salmonella.

[0229] The recombinant plasmid pYA3634 (pBR *ori*) was constructed for the periplasmic secretion of the α -helical region of the PspARx1 (8)(Table 2). Plasmid

pYA3493 (vector control) and pYA3634 (encoding β -lactamase (*bla*) SS-*pspA*) were electroporated into *S. Typhimurium* strains χ 8133, χ 9088 and χ 9558. The RASV electroporants containing pYA3634 expressed a protein with an approximate molecular mass of 37 kDa, the expected size of the Bla SS-PspA fusion protein encoded by pYA3634, that reacts specifically with an anti-PspA polyclonal antibody (Fig. 32). Plasmid stability was evaluated as described in the Material and Methods. Plasmids were maintained and protein expression was shown to be stable when strains were grown in the presence of DAP for 50 nucleic acid sequencerations.

Antibody responses in mice after oral immunization with the recombinant S. enterica serovar Typhimurium vaccines.

[0230] A dose of RASV χ 8133(pYA3634) (1.9×10^9 CFU), χ 9088(pYA3634) (1.7×10^9 CFU), χ 9558(pYA3634) (1.5×10^9 CFU), χ 8133(pYA3493) (1.8×10^9 CFU), χ 9088(pYA3493) (2.0×10^9 CFU) or χ 9558(pYA3493) (1.5×10^9 CFU/mouse) was orally administered to 7-week-old female BALB/c mice. Mice were boosted with the same dose of the same strain eight weeks later. All immunized mice survived, and no signs of disease were observed in the immunized mice during the entire experimental period. The antibody responses to Salmonella LPS, SOMP and rPspA in the sera of immunized mice were measured (Fig. 33). High serum IgG titers against all antigens, including PspA, were observed by 2 weeks after the primary immunization. The maximum preboost anti-LPS, -SOMP, and -rPspA IgG levels were detected by 6 weeks post immunization. The serum anti- rPspA IgG antibody levels of χ 9558(pYA3634) and χ 9088(pYA3634) immunized mice were significantly higher than the levels in χ 8133(pYA3634) immunized mice ($p < 0.01$, $p < 0.05$).

IgG isotype analyses

[0231] The types of immune responses to rPspA were further examined by measuring the levels of IgG isotype subclasses IgG1 and IgG2a (Fig. 34). 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 (23, 24). Th1-type dominant immune responses are frequently observed after

immunization with attenuated *Salmonella* (25-27). A Th1- and Th2-type mixed response was observed for the rPspA antigen. Although the IgG1 levels were almost the same as IgG2a levels in the early phase, the level of anti-rPspA IgG2a isotype antibodies gradually increased after boosting at 8 weeks. After 12 weeks postimmunization, the ratios of IgG1: IgG2a are 1:8.3 for χ_{8133} (pYA3634) immunized mice, 1:9.4 for χ_{9088} (pYA3634) immunized mice and 1:1.5 for χ_{9558} (pYA3634) immunized mice. The serum anti- rPspA IgG1 and IgG2a antibody levels of χ_{9558} (pYA3634) and χ_{9088} (pYA3634) immunized mice are both significantly higher than those of the χ_{8133} (pYA3634) immunized mice ($p < 0.01$) (Fig. 34).

Antigen-specific stimulation of IL-4 or IFN-g production.

[0232] ELISPOT was used to compare PspA antigen stimulation of IL-4 or IFN-g production by cells from spleens of immunized control mice (Fig. 35). Spleen lymphocytes from χ_{9088} (pYA3634) immunized mice had (114.0 ± 28.8) antigen specific IL-4 secreting cells per 10^6 cells and (315.9 ± 31.5) antigen specific IFN- g secreting cells per 10^6 cells which were both significantly higher than those of the spleen lymphocytes from χ_{8133} (pYA3634) immunized mice ($p < 0.001$) The spleen lymphocytes from χ_{9558} (pYA3634) immunized mice had (108.1 ± 90.25) antigen specific IL-4 secreting cells per 10^6 cells and (280.1 ± 47.10) antigen specific IFN- g secreting cells per 10^6 cells which were also significantly higher than those of the spleen lymphocytes from χ_{8133} (pYA3634) immunized mice ($p < 0.001$).

Status of systemic cytokine environment.

[0233] At 2 weeks after immunization, sera from each group of mice were subjected to Bio-Plex analyses. The secretion profiles were compared (Table 10). The sera from χ_{8133} (pYA3634), χ_{9088} (pYA3634), χ_{9558} (pYA3634) immunized mice have increased levels of cytokine concentrations compared with the BSG control group ($p < 0.01$). χ_{9558} (pYA3634) immunized mice have an increased level of cytokines including both Th1 and Th2 cytokines compared with the χ_{8133} (pYA3634) group ($p < 0.05$), which suggested that the RASV strains caused mixed Th1 and Th2 responses

and our regulated delayed attenuation strain $\chi 9558(pYA3634)$ stimulated stronger cellular immunity and cytokine secretion than $\chi 8133(pYA3634)$, which will facilitate antigen presentation and activation of T and B cells.

Table 10. *S. Typhimurium* vaccine strains with regulated delayed attenuation stimulate higher systemic cytokine production.

Mouse groups	Cytokines concentrations (pg/ml)						
	IL-2	IL-4	IL-5	IL-10	IL-12	GM-CSF	TNF- α
BSG	6.5 $\pm$ 0.71	13.4 $\pm$ 1.27	8.5 $\pm$ 1.41	7.8 $\pm$ 1.06	15.0 $\pm$ 0.71	12.5 $\pm$ 1.41	9.9 $\pm$ 0.14
χ 8133 (pYA3493)	7.3 $\pm$ 0.35	18.3 $\pm$ 0.35	10.2 $\pm$ 1.20	8.5 $\pm$ 0.00	17.7 $\pm$ 0.92	13.8 $\pm$ 1.06	11.3 $\pm$ 0.35
χ 8133 ** (pYA3634)	12.0 $\pm$ 1.27	27.7 $\pm$ 5.44	21.8 $\pm$ 3.89	21.7 $\pm$ 3.04	42.3 $\pm$ 9.55	28.0 $\pm$ 2.12	32.3 $\pm$ 3.18
χ 9088 (pYA3493)	9.5 $\pm$ 0.00	27.4 $\pm$ 1.56	17.0 $\pm$ 0.00	14.0 $\pm$ 1.41	29.9 $\pm$ 3.39	20.9 $\pm$ 0.57	18.8 $\pm$ 1.77
χ 9088 ** (pYA3634)	11.5 $\pm$ 0.71	37.3 $\pm$ 1.41	24.5 $\pm$ 2.12	22.5 $\pm$ 0.71	49.7 $\pm$ 1.20	31.7 $\pm$ 3.75	31.0 $\pm$ 2.12
χ 9558 (pYA3493)	11.8 $\pm$ 0.35	34.8 $\pm$ 1.06	30.5 $\pm$ 2.12	25.8 $\pm$ 1.06	53.3 $\pm$ 1.41	39.2 $\pm$ 1.02	37.8 $\pm$ 1.06
χ 9558 ** # (pYA3634)	15.0 $\pm$ 1.41	46.3 $\pm$ 1.06	32.0 $\pm$ 1.41	26.5 $\pm$ 1.48	59.0 $\pm$ 8.13	39.8 $\pm$ 7.78	39.7 $\pm$ 3.32

** Compared with BSG group of mice all three vaccine strains stimulate significant higher systemic cytokine production $p < 0.01$.

Compared with $\chi 8133(pYA3634)$ group of mice, $\chi 9558(pYA3634)$ group of mice stimulate significantly higher systemic cytokine production $p < 0.05$.

Evaluation of protective immunity.

[0234] To examine the ability of *Salmonella*-rPspA vaccines to protect against pneumococcal infection, mice were challenged intraperitoneally with 5×10^4 CFU (250 times the LD₅₀) of *S. pneumoniae* WU2 four weeks after they were boosted. Eighty-six percent of the mice immunized with $\chi 9088$ (pYA3634), seventy-one percent of the mice immunized with $\chi 9558$ (pYA3634), and twenty-nine percent of the mice immunized with $\chi 8133$ (pYA3634) were protected from pneumococcal challenge, with statistical significance ($p < 0.01$). This challenge dose killed 100% of the non-immunized, and $\chi 8133$ (pYA3493), $\chi 9088$ (pYA3493) and $\chi 9558$ (pYA3493) immunized mice (Table 11). Following challenge, non-immunized mice, and mice immunized with $\chi 8133$ (pYA3493) or $\chi 9088$ (pYA3493) or $\chi 9558$ (pYA3493) died rapidly.

Table 11. Oral immunization with PspA-expressing *Salmonella* strains protects BALB/c mice against i.p. challenge with 5×10^4 CFU of capsular type 3 *S. pneumoniae* WU2

Vaccine strain ^a	PspA expression ^b	Number of challenged mice	Days to death ^c	Protection rate(%)
BSG	NA	10	2,2,2,2,2,2,2,3,3	0
$\chi 8133$ (pYA3493)	-	10	2,2,2,2,2,2,2,2,2	0
$\chi 9088$ (pYA3493)	-	10	2,2,2,2,2,2,2,2,3	0
$\chi 9558$ (pYA3493)	-	10	2,2,2,2,2,2,2,2,3	0
$\chi 8133$ (pYA3634)	+	14	2,2,2,2,2,2,2,3,3,3, >15, >15, >15	21
$\chi 9088$ (pYA3634)	+	14	2, 3, >15, >15, >15, >15, >15, >15, >15, >15, >15, >15	86*
$\chi 9558$ (pYA3634)	+	14	2, 2, 3, 3, >15, >15, >15, >15, >15, >15, >15, >15	71 [#]

^a Mice were orally immunized twice at 8-weeks intervals with the indicated vaccine strains.

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

^c Four weeks after the second oral immunization, mice were challenged in two experiments with approximately 5×10^4 CFU of *S. pneumoniae* WU2. Both experiments gave similar results, and the data have been pooled for presentation and analysis. The LD₅₀ of WU2 in nonimmunized BALB/c mice is 2×10^2 (data not shown).

* $p < 0.001$ versus survival of mice immunized with the $\chi 8133$ (pYA3634);

[#] $p = 0.001$ versus survival of mice immunized with the $\chi 8133$ (pYA3634).

Passive-immunization studies.

[0235] A passive-immunization study was conducted to evaluate the roles of antibody and T-cell mediated immunity afforded by immunization of mice with the recombinant attenuated *Salmonella* vaccines. One hundred microliters of pooled sera, spleen lymphocytes (1×10^7) or purified CD4⁺ T cells (5×10^6) taken from immunized mice or controls were administered by tail vein injection into groups of 5 naïve mice. This was followed 12 hours later by intraperitoneal challenge with 5×10^4 CFU WU2. Mice receiving sera or cells transferred from $\chi 8133$ (pYA3493), $\chi 9088$ (pYA3493), $\chi 9558$ (pYA3493) or BSG immunized mice died with a mean time of 2 days (Table 12). The sera transferred from both $\chi 9088$ (pYA3634) immunized mice and $\chi 9558$ (pYA3634) immunized mice protected all 5 naïve mice from challenge; while the sera transferred from $\chi 8133$ (pYA3634) immunized mice protected 4 mice from challenge, the other mouse died 4 days after challenge. Passive transfer of spleen lymphocytes from $\chi 9088$ (pYA3634) immunized mice protected all 5 naïve mice from challenge; spleen lymphocytes from $\chi 9558$ (pYA3634) immunized mice protected 3 out of 5 mice from challenge; while the spleen cell transfer from $\chi 8133$ (pYA3634) immunized mice showed no protection. All the mice receiving CD4⁺ T cells from any group of immunized mice all died in 2 or 3 days (Table 12).

Table 12. Passive transfer of pneumococcal immunity by serum or lymphocytes from donors immunized with PspA *Salmonella* vaccines

Vaccine strain used to immunize donors ^a	% survival of recipients of ^b		
	Pooled serum ^c	Spleen cells ^d	Purified CD4 ⁺ T cells ^e
BSG	0	0	0
$\chi 8133$ (pYA3493)	0	0	0
$\chi 9088$ (pYA3493)	0	0	0
$\chi 9558$ (pYA3493)	0	0	0

χ8133(pYA3634)	80	0	0
χ9088(pYA3634)	100	100 [*]	0
χ9558(pYA3634)	100	60 [#]	0

^a Mice were orally immunized at day 0 and boosted 8 weeks later with the indicated vaccine strains. Serum and cells were collected 4 weeks after boosting and transferred to groups of 5 naïve mice.

^b All recipient mice were challenged by i.p with 5×10^4 CFU WU2 at 12h after transfer. Survival was calculated 15 days postchallenge.

^c 0.1 ml of serum intravenously.

^d 1×10^7 viable spleen cells intravenously.

^e 5×10^6 viable CD₄⁺ T cells intravenously.

^{*} $p < 0.001$, compared to groups of mice receiving passive transfer from χ8133(pYA3634) immunized or control donors.

[#] $p = 0.001$, compared to groups of mice receiving passive transfer from χ8133(pYA3634) immunized or control donors.

Isolation of S. pneumoniae from blood and histological examinations of challenged mice.

[0236] Twenty-four hours after intraperitoneal challenge, each mouse was marked and bled. *S. pneumoniae* was recovered from the blood of mice which showed significant signs of weakness and listlessness and died within 7 days (6833.3 ± 321.5 CFU/ml), but not from mice that appeared to be healthy and survived past 15 days ($p < 0.001$). Histological analyses showed severe tissue damage to the lung tissue in mice that died after challenge (Fig. 36). Gross visual examination of the lungs of the dead mice from challenge but not the survivors' lungs showed lobar regions of consolidation and hemorrhage. Conventional light microscopy of H&E-stained lung tissue samples of dead mice from challenge demonstrated that *S. pneumoniae* caused focal consolidation with extensive mononuclear and polymorphonuclear infiltration and loss of alveolar structure, which are characteristic of pneumonia.

Discussion of example 3

[0237] Few infectious pathogens can match the global impact of *Streptococcus pneumoniae* (*S. pneumoniae*) on illness, complications, sequelae, health care costs, and death (28). *S. pneumoniae* is the most common cause of community-acquired pneumonia, bacterial meningitis, and acute otitis media (29, 30). The current 23-valent polysaccharide vaccine has been shown to prevent pneumococcal pneumonia

in immunocompetent young adults, but not in elderly persons (31). A 7-valent conjugated polysaccharide vaccine is licensed for use in children. However, the low serotype coverage, need for repeated doses, and high price, may decrease the usefulness of the conjugate vaccine, especially in the developing world (32). Developing an inexpensive, safe and effective vaccine against this pathogen is still an urgent demand.

[0238] The new regulated delayed attenuation strains χ 9088(pYA3634) and χ 9558(pYA3634) induced stronger immune responses to PspA as judged by PspA-specific serum antibody levels, PspA specific lymphocytes cytokine secretion levels, systemic cytokine secretion levels and protection from virulent *S. pneumoniae* challenge. In this example a 10-fold higher challenge dose of *S. pneumoniae* WU2 was used compared to earlier studies (13), while the protection rate increased more than twenty percent.

[0239] Mixed Th1- and Th2-type immune responses were observed for rPspA. The mechanisms stimulating these types of immune responses by the *Salmonella*-rPspA vaccine remain unknown. Host defense against encapsulated bacteria, such as *S. pneumoniae*, depends on the presence of opsonic antibodies specific for capsular polysaccharide (33-35). Therefore, antibody levels measured by ELISA may not adequately reflect the presence of protective antibodies that are capable of triggering leukocyte effector functions (36). Other investigators have suggested that IgG2a is protective during infection with encapsulated bacteria, including pneumococcal infection (36-38). It is possible that under conditions of limiting expression of antibody, in the mouse, IgG2a is highly effective at fixing complement and promoting opsonophagocytosis (39). It is consistent with our result that after boosting, IgG2a becomes the dominant antibody type. Some investigators reported that CD4⁺ T cells are very important for the protection against virulent *S. pneumoniae* challenge (40-41). Our results showed that CD4⁺ T cells from both groups of immunized mice did not confer protection. It might be that for our *Salmonella*-rPspA vaccines, PspA specific antibodies are the most important factors in protection. T cells are important in helping the production of effective antibodies (23, 42, 43), but T cells themselves did not have the ability to protect, at least in this experiment.

[0240] All the control mice challenged intraperitoneally with *S. pneumoniae* WU2 died of septicemia. In surviving mice from the immunized group no bacteria were recovered from blood and also no signs of lung damage were detected, indicating that these vaccines can protect mice from both fatal bacteremia and the pneumonia caused by *S. pneumoniae*.

[0241] Although χ_{9558} (pYA3436) possesses the $\Delta relA198::araC P_{BAD} lacI$ TT deletion-insertion mutation to provide regulated delayed synthesis of the PspA antigen, this feature did not show any benefit to the level of immunogenicity over that induced by χ_{9088} that does not have this attribute that was shown to be highly beneficial in Example 1. There are two probable reasons for this. First, the segment of the Rx1 PspA specified by pYA3634 is shorter and less stressful on recombinant *Salmonella* than the Rx1 PspA specified by the codon optimized sequence in pYA4088 used in the studies reported in Example 1. Second, χ_{9558} possesses some 10 genetic alterations and has a genotype almost identical to *S. Typhi* candidate vaccine strains that will soon be evaluated in human volunteers. This is in contrast to the presence of only four genetic alterations in χ_{9088} . The additional mutations in χ_{9558} are present to render it safe for newborn mice and thus probably result in some degree of overattenuation to reduce immunogenicity. Nevertheless, these constructions are important to evaluate since the historical fact is that almost all single mutations that render *S. Typhimurium* totally avirulent and highly immunogenic in mice when introduced into *S. Typhi* strains and tested in humans are still partially virulent and cause disease. It will thus be important to evaluate isogenic strains that only differ by the presence or absence of the $\Delta relA198::araC P_{BAD} lacI$ TT deletion-insertion mutation. Nevertheless, χ_{9558} (pYA3436) was still far superior to χ_{8133} (pYA3634) in regard to immunogenicity and in inducing protective immunity to pneumococcal challenge.

[0242] In conclusion, the results of this example demonstrated that the *Salmonella* vaccine strains χ_{9088} (pYA3436) and χ_{9558} (pYA3436) featuring the novel regulated delayed in vivo attenuation system are superior not only in inducing PspA specific antibody responses but also in eliciting cellular immunity and cytokine secretion resulting in significant protection of mice against pneumococcal challenge.

References for example 3

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Example 4: Design and Construction of *S. Typhi* and *S. Paratyphi A* Vaccine Strains with Regulated Delayed Attenuation and Regulated Delayed Synthesis of Plasmid Encoded Protective Antigens

[0243] Attenuated mutants of *Salmonella enterica* serovar Typhi (*S. Typhi*) and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) have been extensively studied as multivalent vectors expressing more than 50 different bacterial, viral and protozoan antigens in preclinical and clinical trials (1-5). Recombinant *S. Typhimurium* vaccines (RASVs) administered orally can colonize the gut-associated lymphoid tissue (GALT) and the secondary lymphatic tissues, including the liver and spleen, and elicit mucosal, humoral and cellular immune responses against *Salmonella* and heterologous antigens during infection of the mouse (1, 2). There is also good reason to consider use of attenuated derivatives of *S. Paratyphi A* as vaccine vectors since infections with this pathogen causing enteric fever have been increasing in recent years due to the

cessation in use of the killed TAB vaccine. This has been contributed to by immunization against *S. Typhi* by use of the live Ty21A or conjugate Vi antigen vaccines.

[0244] A number of factors may affect the immune response to protective antigens including the ability of vaccine strains to invade and colonize the host GALT, the stability of the plasmid expression system and the antigen sub-cellular location (1, 2, 6). High-level expression of protective antigens by RASV strains often impose an energy demand that decreases the growth, fitness and ability to colonize lymphoid tissues resulting in further attenuation and a reduction in immunogenicity (2, 7, 8). Means have been developed, such as regulated delayed in vivo antigen synthesis (Example 1), to enhance the ability of vaccine strains to efficiently invade and colonize GALT after oral immunization (7 - 10). Another strategy to improve the immune response is to construct strains with regulated delayed in vivo attenuation such that vaccine strains are better able to withstand the stresses and host defence means that generally reduce survival especially of attenuated vaccine strains. We have addressed this problem by developing means for regulated delayed in vivo attenuations such that vaccine strains are more able to survive these host induced stresses and behave more like wild-type infectious *Salmonella* pathogens at the time of immunization. Thus, the combined use of regulated delayed in vivo attenuation and regulated delayed in vivo synthesis of protective antigens both afford means for the RASV to more efficiently colonize to a higher vaccine cell density effector lymphoid tissues to enhance induction of effective protective immunity.

[0245] We have thus constructed *S. Typhimurium* strains to evaluate in mice with a constellation of mutations to not only provide regulated delayed in vivo attenuation and regulated delayed in vivo synthesis of plasmid-specified protective antigens but to also provide inability to establish persistent carrier states, inability to induce fluid secretion in the intestine, and engender safety for newborns. *S. Typhimurium* UK-1 strains with each of the mutations present in some of these strains and not presented in detail in Examples 1 and 2 are listed in Table 13 along with data from infection of female BALB/c mice to indicate the contributions of these mutations to the level of virulence/attenuation. The suicide vectors used to introduce these mutations

into *Salmonella* strains are listed in Table 2 and the methods for these constructions are given in Examples 1 and 2.

Table 13. Virulence of *S. Typhimurium* UK-1 mutants with single deletion or deletion-insertion mutations

Strain	Genotype	CFU/dose	Survival/total
χ 3761	Wild-type UK-1	4.7×10^8 4.7×10^7 4.7×10^5 4.7×10^4 4.7×10^3	0/5 0/5 0/5 0/5 3/5
χ 3761	Wild-type UK-1	1.0×10^6 1.0×10^5 1.0×10^4 1.0×10^3	0/5 0/5 4/5 5/5
χ 8767	$\Delta araBAD23$ UK-1	1.9×10^5 1.9×10^4 1.9×10^3	1/5 1/5 5/5
χ 8477	$\Delta araE25$ UK-1	1.1×10^8 1.1×10^7 1.1×10^6 1.1×10^5	0/4 1/4 1/4 2/4
χ 8276	$\Delta asdA16$ UK-1	6.0×10^8 9.8×10^8	5/5 4/4
χ 8960	$\Delta asdA18::TT araC P_{BAD} c2$ UK-1	5.5×10^8	5/5
χ 8289	$\Delta asdA19::TT araC P_{BAD} c2$ UK-1	1.0×10^9	4/4
χ 8958	$\Delta asdA33$ UK-1	6.4×10^8	5/5
χ 8844	$\Delta endA2311$ UK-1	8.6×10^6 8.6×10^5 8.6×10^4	0/4 2/4 2/2
χ 8844	$\Delta endA2311$ UK-1	3.0×10^5 3.0×10^4	0/2 1/2
χ 8989	$\Delta endA19::araC P_{BAD} lacI TT$ UK-1	1.0×10^6 1.0×10^5 1.0×10^4	0/5 2/5 2/5
χ 8831	$\Delta(gmd-fcl)-26$ UK-1	5.9×10^5 5.9×10^4	1/4 4/4

		5.9 x 10 ³ 5.9 x 10 ²	4/4 4/4
χ 8831	$\Delta(gmd-fcl)$ -26 UK-1	8.6 x 10 ⁶ 8.6 x 10 ⁵ 8.6 x 10 ⁴ 8.6 x 10 ³	0/4 0/4 0/4 1/4
χ 8650	Δpmi -2426 UK-1 Nutrient Broth	1.7 x 10 ⁹ 1.7 x 10 ⁸ 1.7 x 10 ⁷ 1.7 x 10 ⁶ 1.7 x 10 ⁵	8/8 8/8 7/8 4/4 4/4
χ 8650	Δpmi -2426 UK-1 Nutrient Broth + 0.5% mannose + 0.5% glucose	1.5 x 10 ⁹ 1.5 x 10 ⁸ 1.5 x 10 ⁷ 1.5 x 10 ⁶ 1.5 x 10 ⁵	3/8 7/8 7/8 4/4 4/4
χ 8882	$\Delta relA$ 1123 UK-1	8.0 x 10 ⁷ 8.0 x 10 ⁶ 8.0 x 10 ⁵ 8.0 x 10 ⁴ 8.0 x 10 ³	0/4 1/5 1/4 3/4 4/4
χ 8990	$\Delta relA$ 196:: <i>araC</i> P _{BAD} <i>lacI</i> TT UK-1	1.0 x 10 ⁵ 1.0 x 10 ⁴ 1.0 x 10 ³	1/5 4/5 5/5
χ 8923	$\Delta sopB$ 1925 UK-1	1.2 x 10 ⁷ 1.2 x 10 ⁶ 1.2 x 10 ⁵ 1.2 x 10 ⁴	1/5 2/5 5/5 5/5

[0246] Strain χ 9558 Δpmi -2426 $\Delta(gmd-fcl)$ -26 $\Delta P_{fur81}::TT$ *araC* P_{BAD} *fur* $\Delta P_{crp527}::TT$ *araC* P_{BAD} *crp* $\Delta asdA$ 27::*TT* *araC* P_{BAD} *c2* $\Delta araE$ 25 $\Delta araBAD$ 23 $\Delta relA$ 198::*araC* P_{BAD} *lacI* TT $\Delta sopB$ 1925 $\Delta agfBAC$ 811 was used in Example 3 to deliver heterologous antigens such as the *S. pneumoniae* α -helical domain of the Rx1 PspA antigen encoded by pYA3634 either secreted into the extra-cellular environment or displayed on the vaccine carrier surface. Such approaches are based on observations

that antigens localized on the surface of *Salmonella* cells or extracellularly secreted produce greater immune responses and protection (6, 11-13). In addition, secretion of heterologous antigens by RASV may decrease the toxicity of the protein to the bacterial vector, facilitate bacterial growth and antigen uptake by antigen-presenting cells (APC) and continuously stimulate the host immune system during the colonization of lymphatic tissues by *Salmonella*, so as to enhance the immune response against the heterologous antigens (5, 14).

[0247] *Streptococcus pneumoniae*, a gram-positive human pathogen, causes serious health problems, including community-acquired pneumonia, otitis media, meningitis, and bacteremia in persons of all ages. *S. pneumoniae* is a leading agent of childhood pneumonia worldwide, resulting in about 3 million deaths per year (15). The pneumococcal surface protein A (PspA) and pneumococcal surface protein C (PspC) have been considered as pneumococcal subunit vaccine candidates. PspA and PspC/Hic are expressed in all clinically isolated pneumococcal strains (16-18). Immune responses to PspA and PspC can protect mice against virulent *S. pneumoniae* challenge (6, 17, 19-23).

[0248] Toward this end of making a suitable vaccine to prevent pneumococcal disease in newborns and infants, we have introduced the improved Asd⁺ vector pYA4088 (Example 1) that expresses at high level the longer Rx1 PspA specified by a codon-optimized sequence into χ 9558. In addition to repeating the immunogenicity and protection studies described in Example 3 with superior results, we have demonstrated that this recombinant strain can be orally administered to newborn mice only a few hours old at doses of 10⁸ CFU or more with no deaths, disease symptoms or impairment in growth. Furthermore, when the χ 9558(pYA4088) strain is inoculated intranasally into six-week old BALB/c mice, there is no colonization of the olfactory lobe or brain and no development of meningitis as found after intranasal inoculation of other *Salmonella* strains.

[0249] Based on the above successes, we have generated *S. Typhi* vaccine vectors as listed in Table 1 that have most all of the mutations present in χ 9558. Since there has been a lack of success of recombinant attenuated *S. Typhi* vaccines derived from the widely used Ty2 strain, we have generated recombinant strains that have the *rpoS* mutation in the Ty2 strain replaced with the wild-type *rpoS*

allele and have also derived a vaccine strain from the Chilean clinical isolate ISP1820. χ 9639 is the *S. Typhi* Ty2 derived strain that is RpoS⁻ and has the genotype $\Delta P_{crp527}::TT \text{ araC } P_{BAD} \text{ crp } \Delta P_{fur81}::TT \text{ araC } P_{BAD} \text{ fur } \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::\text{araC } P_{BAD} \text{ lacI } TT \Delta araE25 \Delta tviABCDE10 \Delta agfBAC811 \Delta asdA33$ and χ 9640 is the Ty2 RpoS⁺ derivative with the identical genotype as in χ 9639. χ 9633 is the *S. Typhi* ISP1820 derivative is RpoS⁺ and has the genotype $\Delta P_{crp527}::TT \text{ araC } P_{BAD} \text{ crp } \Delta P_{fur81}::TT \text{ araC } P_{BAD} \text{ fur } \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta sopB1925 \Delta relA198::\text{araC } P_{BAD} \text{ lacI } TT \Delta araE25 \Delta araBAD23 \Delta tviABCDE10 \Delta agfBAC811 \Delta asdA33$. χ 9639 and χ 9640 do not have the $\Delta araBAD23$ mutation present in χ 9633 since the parental Ty2 strain is already unable to use arabinose for growth or as a fermentation substrate. All three strains possess the attenuating $\Delta tviABCDE10$ mutation that eliminates synthesis of the capsular Vi antigen that is unique to *S. Typhi* strains and is not made by *S. Typhimurium*. The $\Delta(gmd-fcl)-26$ and $\Delta agfBAC811$ mutations block synthesis of colanic acid and thin aggregative fimbriae (curli), respectively, that prevent formation of biofilms and preclude persistent colonization of gallstones in the gall bladder. The $\Delta sopB1925$ mutation reduces the potential to induce fluid secretion in the intestinal track and also eliminates a means by which *Salmonella* suppresses induction of immune responses. Thus, this mutation increases the immunogenicity of these *S. Typhi* antigen delivery vaccine vectors.

[0250] We have also designed and constructed vaccine vector derivatives in *S. Paratyphi* A and note that χ 9857 (Table 1) with the genotype $\Delta P_{crp527}::TT \text{ araC } P_{BAD} \text{ crp } \Delta P_{fur81}::TT \text{ araC } P_{BAD} \text{ fur } \Delta pmi-2426 \Delta(gmd-fcl)-26 \Delta agfBAC811 \Delta relA198::\text{araC } P_{BAD} \text{ lacI } TT \Delta sopB1925 \Delta araE25 \Delta araBAD23 \Delta asdA33$ has much the same properties as χ 9558 and the three *S. Typhi* vaccine vectors described above.

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Example 5: Biological Containment

[0251] Live attenuated pathogens such as *Salmonella enterica* have been developed as homologous vaccines to protect against *Salmonella* infections and as carriers of heterologous antigens because of their capacity for efficient mucosal antigen delivery (1, 2). A variety of attenuating mutations 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 *S. Typhimurium*, including *shdA*, *misL* and *ratB*, that reduce environmental shedding in mice without negatively influencing immunogenicity (8). While these mutations lead to a reduction in fecal shedding, it is not clear 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.

[0252] 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.

[0253] In this example, a RASV programmed bacterial lysis system vectoring the β -lactamase-PspA fusion protein was constructed. It was previously shown that this fusion protein is directed to the periplasm, but only about 10 to 20% is released into the extracellular environment (13). This new system combines the features of a previously described antigen expression plasmid with a novel 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.

Materials and methods for Example 5

[0254] Bacterial strains, plasmids, media, and growth conditions: Bacterial strains and plasmids used in this example are listed in Tables 1 and 2, respectively. *S. Typhimurium* strains with *asdA* gene deletions were grown at 37°C in LB broth or on LB agar (14) supplemented with 50 μ g/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, no sodium chloride, was used for *sacB* gene-based counterselection in allelic exchange experiments (15). For mouse inoculation, *Salmonella* strains were grown with aeration after inoculated with a 1/20 dilution of a non-aerated static overnight culture.

[0255] 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 (LPS) profiles of *Salmonella* strains were examined by described methods (16). For detection of PspA in RASV, 12 μ l or 2 μ l of cultures at an OD₆₀₀ of 0.8 were subjected to SDS-PAGE or immunoblot analysis, respectively.

[0256] Construction of a Regulated Programmed Lysis *S. Typhimurium* Vaccine Host-Vector System: The Δ P_{murA7::araC} P_{BAD} *murA* deletion-insertion mutation

was constructed by standard methods and introduced into wild-type strain χ 3761 to yield χ 8645. The $\Delta asdA19::araC$ P_{BAD} $c2$ deletion-insertion mutation was introduced into χ 8645 by P22HT *int* transduction from χ 8290 ($\Delta asdA19::TT$ $araC$ P_{BAD} $c2$). As described in Example 4 and presented in Table 13, these two types of mutations render *Salmonella* totally avirulent. However, as noted in the Background to the Invention, the $\Delta P_{murA7::araC}$ P_{BAD} $murA$ deletion-insertion mutation represents an example of a mutation conferring a regulated delayed attenuation phenotype since a strain with this mutation would be expected to grow and divide a generation or two before undergoing muramic acid-less death by lysis. The $\Delta endA2311$, $\Delta(gmd-fcl)-26$ and $\Delta relA1123$ mutations were added sequentially using suicide vectors (see Table 2) resulting in vaccine strain χ 8937. The presence of mutations and absence of suicide vector sequences were confirmed by PCR using suitable primer sets (Table 14). The primers and steps used to construct plasmids are described as follows. The *E. coli* B/r *araC* P_{BAD} activator-promoter was derived from pBAD18 (17). The *E. coli* K-12 *araC* P_{BAD} activator-promoter was PCR amplified using primers *araC-NsiI* and *EaraBAD-EcoRI* from strain χ 289. The SD-GTG mutation in pYA3530 was introduced into the *asdA* gene by PCR. The ATG-*murA* gene was amplified by PCR from *E. coli* K-12 strain χ 289 (*glnV42* λ^- T3') using primers *EmurA-EcoRI* 5' and *EmurA-EcoRI* 3', then the ATG start codon of the *murA* gene was changed to GTG by PCR. The P22 P_R promoter was derived from plasmid pMEG104. The P_{trc} -5ST1T2- P_{BR} *ori* fragment came from plasmid pYA3342. The fragment including in-frame fusion of the rPspA Rx1 (α -helical region of PspA from amino acid residue 3 to 257 of mature PspA_{Rx1}) to the β -lactamase signal sequence was derived from pYA3634 (18). Expression of the rPspA Rx1 antigen was verified by SDS-PAGE and western blot analysis with the anti-PspA monoclonal antibody Xi126 (19).

Table 14. Primers used in this example

Primer Name	Sequence
A. Construction of plasmid pYA3681	
GTG <i>asd</i>	
GTG <i>asd</i> 5'	cag gaa aaa aac gct gtg aaa aat gtt gg
GTG <i>asd</i> 3'	gtc ctt ttt ttg cga cac ttt tta caa cc
<i>araC</i> P_{BAD} GTG <i>asd</i>	
<i>araC</i> -SmaI	cga ccc ggg atc gat ctg tgc ggt att tca cac cg
<i>asd</i> -SmaI	gca ccc ggg tcg aca gat cct tgg cgg cga gaa ag
<i>araC</i>* P_{BAD} (from χ289)	
E <i>araC</i> -NsiI	cca atg cat aat gtg cct gtc aaa tgg
E <i>araBAD</i> -EcoRI	cgg aat tcg cta gcc caa aaa aac g
<i>MurA</i>	
E <i>murA</i> -EcoRI 5'	cgg aat tct gag aac aaa cta aat gg
E <i>murA</i> -EcoRI 3'	cgg aat tct tat tcg cct ttc aca cgc
GTG <i>murA</i>	
EMGTGRV-NcoI	cat gcc atg gag ctc ggt acc cgg gga t
EMGTG-NcoI-EcoRI	cat gcc atg gaa ttc tga gaa caa act aag tgg ata aat ttc gtg ttc ag
P_{trc}-pBR <i>ori</i> cassette	
P _{trc} -PmeI	agc ttt gtt taa acg gat ctt ccg gaa gac ctt cca ttc
XbaI-pBR	gct cta gac tgt cag acc aag ttt act cat a
Synthetic <i>rrfG</i> TT	
<i>rrfG</i> TT	aac tgc agt cta gat tat gcg aaa ggc cat cct gac gga tgg cct ttt tgt tta aac gga tcc gc
Primer Name	Sequence
B. Construction of plasmid pYA3685	
NcoI-bla-PspA	cat gcc atg ggt att caa cat ttc cgt gtc gcc ctt att c
SmaI-TAA-PspA	tcc ccc ggg cta tta ttc tac att att gtt ttc T
C. Construction of suicide vectors	

ΔrelA1123	
<i>relA</i> C-SphI	aca tgc atg ccc aga tat ttt cca gat ctt cac
<i>relA</i> C-EcoRI	cgg aat tca ccc cag aca gta atc atg tag cgg
<i>relA</i> N-EcoRI	cgg aat tca agg gac cag gcc tac cga ag
<i>relA</i> N-BamHI	cgg gat ccg agg gcg ttc cgg cgc tgg tag aa
Δ(gmd-fcl)-26	
<i>wcaF</i> -XbaI	gct cta gat cct caa ata gtc ccg tta gg
<i>wcaF</i> -SmaI	tcc ccc ggg caa aat att gta tcg ctg g
<i>gmm</i> -SphI	gcacgc atg ctc agg cag gcg taa atc gct ct
<i>gmm</i> -XbaI	cct cta gac aat gtt ttt acg tca gga aga tt
ΔendA2311	
<i>endAN</i> -BamHI	cgg gat ccg cta cga aat ccg cct caa c
<i>endAN</i> -HindIII	ccc aag ctt agc aaa acg agc ccg caa cg
<i>endAC</i> -HindIII	ccc aag ctt cct aca cta gcg gga ttc ttg
<i>endAC</i> -SphI	aca tgc atg ccg cag cgc tca gag

[0257] 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 (LPS) profiles of *Salmonella* strains were examined by described methods (16). For detection of PspA in RASV, 12 μ l or 2 μ l of cultures at an OD₆₀₀ of 0.8 were subjected to SDS-PAGE or immunoblot analysis, respectively.

[0258] 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 pre-warmed LB broth supplemented with or without 0.02% arabinose β -galactosidase activity in supernatant and cell-pellet fractions were assayed at indicated time points as described (20).

[0259] Colonization of Mice with the Regulated Programmed Lysis *Salmonella* Vaccine Strain: Seven-week-old female BALB/c mice (3 mice for each time

point) were deprived of food and water for 4h 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 non-aerated static overnight culture. 1.3×10^9 CFU of $\chi 8937(pYA3681)$ in 20 μ l of phosphate-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 euthanized 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.

[0260] Immunization of Mice: Groups of 5 seven-week-old female BALB/c mice were orally vaccinated with either 1.3×10^9 CFU *S. Typhimurium* vaccine strain $\chi 8937(pYA3685)$ (expressing rPspA Rx1) or 1.1×10^9 CFU host-vector controls $\chi 8937(pYA3681)$ as described above. A second oral dose of 1.2×10^9 CFU $\chi 8937(pYA3685)$ or 1.1×10^9 CFU $\chi 8937(pYA3681)$ was given one 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, 8 after immunization. Serum fractions were stored at -20°C . Vaginal secretion specimens were collected by wash with 50 μ l BSG, solid material was removed by centrifugation and secretion samples were stored at -20°C (21).

[0261] Antigen Preparation: rPspA Rx1 protein and *S. Typhimurium* SOMP were purified as described (13).

[0262] Enzyme linked immunosorbent assay (ELISA): The procedures used for detection of antibody have been described elsewhere (13, 22). Briefly, polystyrene 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated with *S. Typhimurium* SOMP (100 ng/well) or purified rPspA Rx1 (100 ng/well). Antigens suspended in sodium carbonate-bicarbonate coating buffer (pH 9.6) were applied with 100 μ l volumes in each well. Vaginal secretions obtained from the same experimental group were pooled and diluted 1: 10, and sera were diluted 1: 1280 for detection of IgG and 1: 400 for IgG1 and IgG2a, respectively. A 100 μ l 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 Inc., Birmingham) for sera and IgA for vaginal secretions.

[0263] Statistical Analysis: Most data were expressed as means $\pm$ standard error. The means were evaluated with One-way ANOVA and LSD tests for multiple comparisons among groups. $p < 0.05$ was considered statistically significant.

Construction of a Regulated Programmed Lysis System.

[0264] Diaminopimelic acid (DAP) and muramic acid are essential components of the peptidoglycan layer of the bacterial cell wall (23). The *asdA* gene encodes an enzyme essential for DAP synthesis and the *murA* gene encodes the first enzyme in muramic acid synthesis (24, 25). To test the feasibility of an arabinose-regulated *asdA*-based lysis system, we introduced the $\Delta asdA16$ deletion mutation into *S. Typhimurium* UK-1 resulting in strain $\chi 8276$. We then introduced plasmid pYA3450, which carries the *asdA* gene with an ATG start codon transcribed from the P_{BAD} promoter which is activated by the AraC protein in the presence of arabinose (26), into $\chi 8276$ to yield $\chi 8276(pYA3450)$. 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, $\chi 8276(pYA3450)$ also retained wild-type virulence in mice. 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. 37A). However, strain $\chi 8276(pYA3530)$ 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.

[0265] 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 $\Delta P_{murA7}::araC P_{BAD} murA$ mutation into wild-type *S. Typhimurium* resulting in strain $\chi 8645$. 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 (27). 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. 37B). These results confirm that the $\Delta P_{murA7}::araC P_{BAD} murA$ mutation confers a regulated delayed attenuation phenotype since some growth was possible prior to onset of death by lysis.

[0266] 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 the previously described constructs was derived from an *E. coli* B/r strain (17). We discovered that when we substituted the *araC* P_{BAD} promoter-activator from *E. coli* K-12 strain $\chi 289$, transcription from the plasmid was more tightly regulated and arabinose-dependent growth was achieved. 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 (TT) 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. 14).

[0267] The host strain for this system was constructed by introducing a $\Delta asdA$ mutation into the $\Delta P_{murA7}::araC P_{BAD} murA$ mutant strain $\chi 8645$. To facilitate regulation of P22 P_R in plasmid pYA3681, we introduced the $\Delta asdA19$ deletion/insertion mutation, in which the P22 phage C2 repressor gene under transcriptional control of the P_{BAD} promoter was inserted into the $\Delta asdA16$ deletion (Fig. 38A). Three additional mutations designed to enhance lysis and facilitate antigen delivery were also included in this strain (Fig. 38A). The $\Delta(gmd-fcl)-26$ 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 $\Delta relA1123$ 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 semi-aldehyde 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 $\Delta endA$ mutation which eliminates the periplasmic endonuclease I enzyme (32), to increase plasmid survival upon its release into the host cell. The resulting strain, $\chi 8937 (\Delta asdA19::araC P_{BAD} c2 \Delta P_{murA7::araC} P_{BAD} murA \Delta(gmd-fcl)-26 \Delta relA1123 \Delta endA2311)$, requires both arabinose and DAP for growth (Fig. 38B).

[0268] pYA3681 was introduced into *S. Typhimurium* $\chi 8937$. Growth of the resulting strain $\chi 8937(pYA3681)$ required arabinose (Fig. 38B). The plasmid was stably maintained for 50 or more generations when grown in the presence of arabinose and DAP. 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. 39). 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 due to 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 anti-sense 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.

[0269] The regulated lysis vaccine strain $\chi 8937(pYA3681)$ grew well in LB broth supplemented with 0.02% arabinose, but began to die after one hour of incubation

in LB broth without arabinose (Fig. 40A). To evaluate cell lysis, release of the cytoplasmic enzyme β -galactosidase into culture supernatants was used as an indicator. The *atrB13::MudJ* allele which directs constitutive expression of β -galactosidase (13) was transduced into *S. Typhimurium* wild-type as a non-lysis control and into vaccine strain χ 8937, resulting in strains χ 9379 and χ 9380, respectively. We then introduced plasmid pYA3681 into χ 9380 to yield χ 9380(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 χ 9380(pYA3681) occurred only in medium lacking arabinose (Fig. 40B). Conversely, the amount of cell-associated β -galactosidase decreased over time when χ 9380(pYA3681) was grown in medium without arabinose, but no decrease was seen in media containing arabinose. β -galactosidase release was not observed when the wild-type control strain χ 9379 was grown without arabinose. These results are consistent with our expectations for the arabinose-regulated cell lysis phenotype.

[0270] To evaluate virulence, BALB/c mice were orally inoculated with doses in excess of 10^9 CFU of the host-vector strain χ 8937(pYA3681), a dose 50,000 times the LD_{50} of the wild-type parent strain, χ 3761. During the 30 days observation period after dosing, we observed no deaths or signs of illness in any of the mice. Colonization by strain χ 8937(pYA3681) was evaluated in eight-week old female mice orally inoculated with 10^9 CFU. The strain transiently colonized lymphoid tissues (Fig. 40C) and no bacteria were recovered by four weeks post-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 lysis system is attenuated and is efficiently cleared from the host following colonization of lymphoid tissues.

Construction of the rPspA Rx1-expressing Plasmid.

[0271] It was previously shown that a recombinant protein fusing the first 35 amino acids of β -lactamase to the α -helical region of PspA (rPspA Rx1) is highly immunogenic when delivered by a recombinant avirulent *S. Typhimurium* (13). We

utilized 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 257 of mature PspA_{Rx1}) was inserted into pYA3681 to yield pYA3685 (Fig. 41A). The nucleic acid encoding the antigen is constitutively expressed from the P_{trc} promoter. Production of rPspA Rx1 in *S. Typhimurium* χ 8937(pYA3685) grown in media with arabinose was detected by western blot analysis with the anti-PspA monoclonal antibody (Fig. 41B), and we confirmed that the fraction of antigen secreted to the periplasm was similar to that reported previously (13). The strain did not grow on LB agar without arabinose and expression of the recombinant antigen did not interfere with programmed lysis when χ 8937(pYA3685) was grown in LB broth without arabinose (Fig. 41C).

Immune Responses in Mice after Oral Immunization with the Regulated Programmed Lysis Host-vector System.

[0272] 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. 42). 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. 42A). The anti-SOMP IgG response was slower to develop in mice vaccinated with χ 8937(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 χ 8937(pYA3685). However, both χ 8937(pYA3681) and χ 8937(pYA3685) elicited equivalent anti-SOMP IgA responses in vaginal secretions, with no significant differences between groups after two weeks, while rPspA Rx1-specific IgA was detected only in samples from mice immunized with vaccine strain χ 8937(pYA3685) ($p < 0.05$) (Fig. 42B).

IgG isotype analyses

[0273] 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. 43). 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. 43).

Discussion of example 5

[0274] 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 example 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).

[0275] These considerations led us to develop a RASV containment/delivery system capable of releasing antigen by cell lysis within the immunized animal. We utilized 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 sub-micromolar range (37). Studies in our laboratory have shown that in our *Salmonella* strains, P_{BAD} is not activated by 13 μ M (0.0002%) arabinose (S. Wang, personal communication).

[0276] We chose the *asdA* gene as the primary driver of cell lysis, since it is known that, unlike some lethal mutations, a lack of Asd not only results in cell death,

but also cell lysis (38). To further facilitate containment, we also included the *murA* gene in our scheme. 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 anti-sense 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 anti-sense mRNA is transcribed.

[0277] The data of this example show that the system we have devised results in cell lysis in the absence of arabinose and clearance of the strain from host tissues. 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, thereby demonstrating that this system has all the features required for biological containment of a RASV. This plasmid-host system of regulated delayed lysis in vivo depends on regulated delayed shut off in the synthesis of enzymes essential for peptidoglycan synthesis results in complete avirulence in the complete absence of any other attenuating mutations. As such this is another means to confer a regulated delayed attenuation phenotype.

[0278] 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 of time. Strains with these arabinose gene deletions are currently being evaluated for use as antigen or DNA delivery vectors.

[0279] 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.

[0280] Lastly, this system could be modified to provide effective biological containment for genetically engineered bacteria used for a diversity of purposes in addition to vaccines

References for Example 5

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CLAIMS

What is claimed is:

1. A recombinant bacterium capable of the regulated expression of at least one nucleic acid sequence encoding an antigen of interest, and capable of regulated attenuation, wherein the bacterium comprises
 - a. at least one chromosomally integrated nucleic acid sequence encoding a repressor operably linked to a regulatable promoter,
 - b. a vector comprising a nucleic acid sequence encoding at least one antigen of interest operably linked to a promoter regulated by the repressor, such that the expression of the nucleic acid sequence encoding the antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level expression of the nucleic acid sequence encoding the antigen in a host, and
 - c. a regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence of an attenuation protein.
2. The recombinant bacterium of claim 1, wherein the repressor is selected from the group consisting of LacI, C2, and C1.
3. The recombinant bacterium of claim 1, wherein the bacterium comprises at least two chromosomally integrated nucleic acid sequences, each encoding a repressor operably linked to a regulatable promoter.
4. The recombinant bacterium of claim 3, wherein the repressors are independently is selected from the group consisting of LacI, C2, and C1.
5. The recombinant bacterium of claim 1, wherein the regulatable promoter is P_{BAD}, or a promoter regulated by maltose, rhamnose, or xylose.
6. The recombinant bacterium of claim 5, wherein the regulatable promoter is also operably-linked to *araC*.

7. The recombinant bacterium of claim 1, wherein the codons of the nucleic acid sequence encoding the repressor have been modified so as to optimize the expression level of the nucleic acid sequence encoding the repressor.
8. The recombinant bacterium of claim 1, wherein the repressor comprises a modified Shine-Dalgarno sequence and optimized codons so as to optimize the expression level of the nucleic acid sequence encoding the repressor.
9. The recombinant bacterium of claim 1, wherein the vector is a plasmid.
10. The recombinant bacterium of claim 1, wherein the nucleic acid encoding an antigen of interest is operably linked to a promoter regulated by a repressor selected from the group consisting of LacI, C1, and C2.
11. The recombinant bacterium of claim 1, wherein the nucleic acid encoding an antigen of interest is operably linked to a P_{trc} promoter.
12. The recombinant bacterium of claim 1, wherein the antigen is toxic.
13. The recombinant bacterium of claim 1, wherein the vector further comprises a nucleic acid sequence encoding a secretion signal for the antigen of interest.
14. The recombinant bacterium of claim 1, wherein the bacterium elicits a protective immune response in the host.
15. The recombinant bacterium of claim 1, wherein the bacterium comprises more than one means of attenuation.
16. The recombinant bacterium of claim 1, wherein the bacterium further comprises the mutation $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$.
17. The recombinant bacterium of claim 1, wherein the repressor is selected from the group consisting of LacI, C2, and C1; the regulatable promoter is P_{BAD} ; the vector is a plasmid; and the nucleic acid encoding an antigen of interest is operably linked to the P_{trc} promoter.

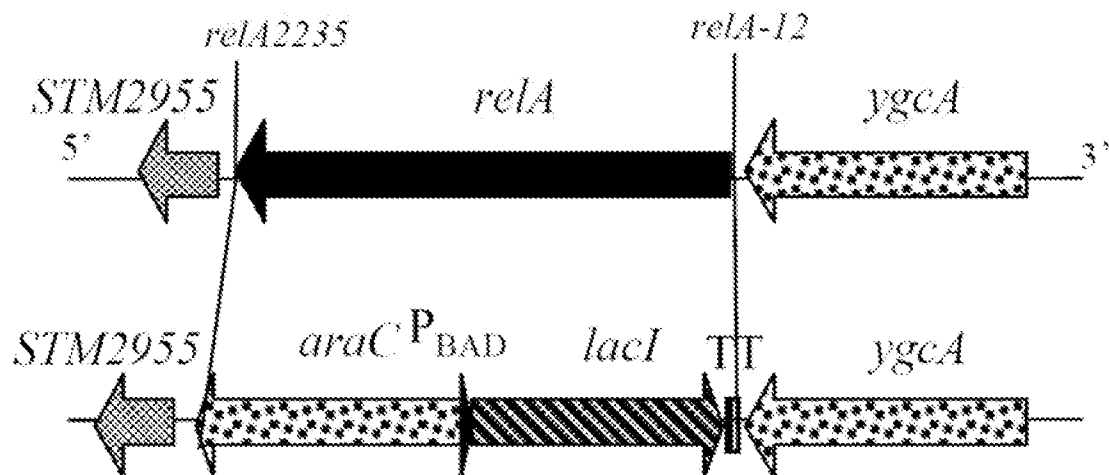
18. A recombinant bacterium capable of regulated expression of at least one nucleic acid sequence encoding an antigen of interest, wherein the bacterium comprises
 - a. at least one chromosomally integrated nucleic acid sequence encoding a repressor operably linked to a regulatable promoter, wherein the nucleic acid sequence encoding the repressor and/or promoter have been modified from the wild-type nucleic acid sequence so as to optimize the expression level of the nucleic acid sequence encoding the repressor, and
 - b. a vector comprising at least one nucleic acid sequence encoding an antigen of interest operably linked to a promoter regulated by the repressor, such that the expression of the nucleic acid sequence encoding the antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level expression of the nucleic acid sequence encoding the antigen in a host.
19. The recombinant bacterium of claim 18, wherein the repressor is selected from the group consisting of LacI, C2, and C1.
20. The recombinant bacterium of claim 18, wherein the bacterium comprises at least two chromosomally integrated nucleic acid sequences, each encoding a repressor operably linked to a regulatable promoter.
21. The recombinant bacterium of claim 20, wherein the repressor is selected from the group consisting of LacI, C2, and C1.
22. The recombinant bacterium of claim 18, wherein the regulatable promoter is P_{BAD}.
23. The recombinant bacterium of claim 22, wherein the regulatable promoter is also operably linked to *araC*.
24. The recombinant bacterium of claim 18, wherein the codons of the nucleic acid sequence encoding the repressor have been modified so as to optimize the expression level of the nucleic acid sequence encoding the repressor.

25. The recombinant bacterium of claim 18, wherein the promoter comprises a modified Shine-Dalgarno sequence so as to optimize the expression level of the nucleic acid sequence encoding the repressor.
26. The recombinant bacterium of claim 18, wherein the vector is a plasmid.
27. The recombinant bacterium of claim 18, wherein the nucleic acid sequence encoding an antigen of interest is operably linked to a promoter regulated by a repressor selected from the group consisting of LacI, C1, and C2.
28. The recombinant bacterium of claim 18, wherein the nucleic acid sequence encoding an antigen of interest is operably linked to the P_{trc} promoter.
29. The recombinant bacterium of claim 18, wherein the antigen is toxic.
30. The recombinant bacterium of claim 18, wherein the vector further comprises a nucleic acid sequence encoding a secretion signal for the antigen of interest.
31. The recombinant bacterium of claim 18, wherein the bacterium elicits a protective immune response in the host.
32. The recombinant bacterium of claim 18, wherein the bacterium is attenuated.
33. The recombinant bacterium of claim 32, wherein the bacterium further comprises a regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence encoding an attenuation protein.
34. The recombinant bacterium of claim 18, wherein the bacterium further comprises the mutation $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$.
35. The recombinant bacterium of claim 18, wherein the repressor is selected from the group consisting of LacI, C2, and C1; the regulatable promoter is P_{BAD}; the vector is a plasmid; and the nucleic acid encoding an antigen of interest is operably linked to the P_{trc} promoter.

36. A recombinant bacterium capable of regulated attenuation, wherein the bacterium comprises a modified regulatable promoter chromosomally integrated so as to replace the native promoter of, and be operably linked to, at least one nucleic acid sequence encoding an attenuation protein.
37. The recombinant bacterium of claim 36, wherein the attenuation protein is synthesized in vitro.
38. The recombinant bacterium of claim 36, wherein the attenuation protein is selected from the group consisting of RpoS, PhoPQ, OmpR, MurA, Fur, and Crp.
39. The recombinant bacterium of claim 36, wherein the native promoter of at least two attenuation proteins is replaced by a modified regulatable promoter.
40. The recombinant bacterium of claim 39, wherein the native promoter of at least three attenuation proteins is replaced by a modified regulatable promoter.
41. The recombinant bacterium of claim 40, wherein the native promoter of at least four attenuation proteins is replaced by a modified regulatable promoter.
42. The recombinant bacterium of claim 36, wherein the regulatable promoter is P_{BAD}.
43. The recombinant bacterium of claim 42, wherein the regulatable promoter is also operably-linked to *araC*.
44. The recombinant bacterium of claim 36, wherein the bacterium further comprises
 - a. at least one chromosomally integrated nucleic acid sequence encoding a repressor operably linked to a regulatable promoter, and
 - b. a vector comprising at least one nucleic acid sequence encoding an antigen of interest operably linked to a promoter regulated by the repressor, such that the expression of the nucleic acid sequence encoding the antigen is repressed during in vitro growth of the bacterium, but the bacterium is capable of high level expression of the nucleic acid sequence encoding the antigen in a host.

45. The recombinant bacterium of claim 44, wherein the repressor is selected from the group consisting of LacI, C2, and C1; the regulatable promoter is P_{BAD}; the vector is a plasmid; and the nucleic acid encoding an antigen of interest is operably linked to the P_{trc} promoter.
46. The recombinant bacterium of claim 36, wherein the bacterium further comprises the mutation $\Delta P_{crp}::TT\ araC\ P_{BAD}\ crp$.
47. A vaccine composition comprising the recombinant bacterium of claim 1.
48. A method for eliciting an immune response in a host, the method comprising administering to the host an effective amount of a vaccine composition comprising a recombinant bacterium of claim 1.

$\Delta relA::araC$ P_{BAD} *lacI* TT



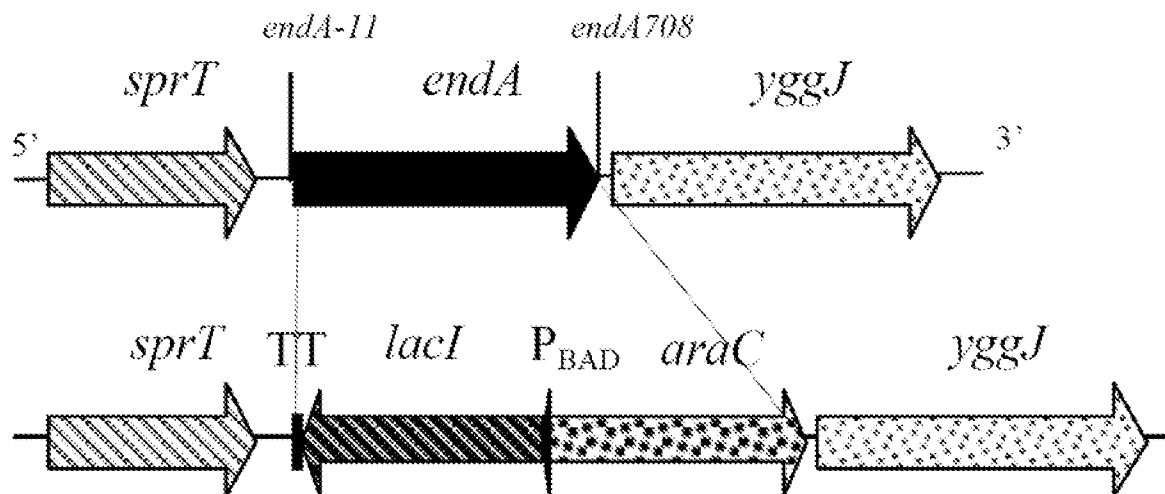
$\Delta relA196::araC$ P_{BAD} AGG ~~G~~ TGGTGAAT ~~G~~ TG-*lacI* TT
SD

$\Delta relA197::araC$ P_{BAD} AGG ~~A~~ TGGTGAAT ATG-*lacI* TT
SD

$\Delta relA198::araC$ P_{BAD} AGG ~~A~~ TGGTGAAT ATG-*lacI** TT
SD

FIG. 1

$\Delta endA::araC$ P_{BAD} *lacI* TT



$\Delta endA19::araC$ P_{BAD} AGGGTGGTGAAT GTG-*lacI* TT
SD

$\Delta endA20::araC$ P_{BAD} AGGATGGTGAAT ATG-*lacI* TT
SD

$\Delta endA21::araC$ P_{BAD} AGGATGGTGAAT ATG-*lacI** TT
SD

FIG. 2

SD-lacI in *ΔendA19* and *ΔrelA196* AGG GTG GTG AAT
 SD-lacI in *ΔendA20* and *ΔrelA197* AGG ATG GTG AAT
 SD-lacI in *ΔendA21* and *ΔrelA198* AGG ATG GTG AAT

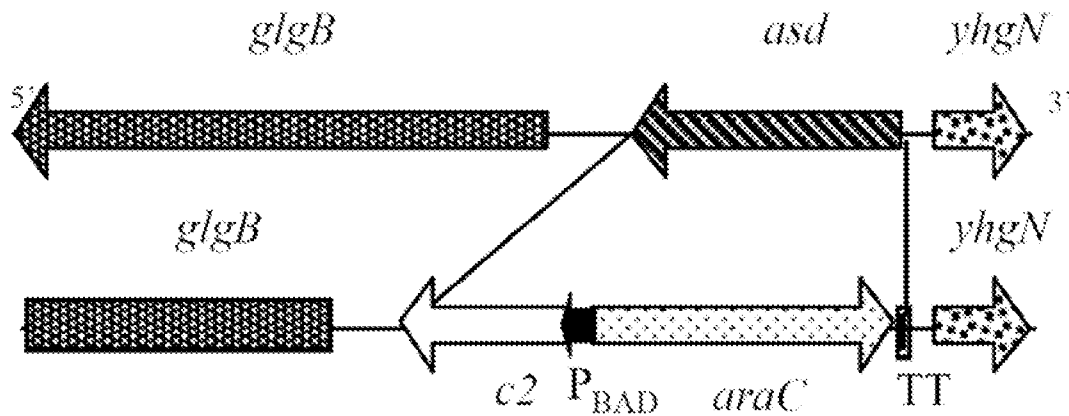
13/1 43/11
 GTG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC TCT TAT CAG ACC GTT
 ATG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC TCT TAT CAG ACC GTT
 ATG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC TCT TAT CAG ACC GTT
 V K P V T L Y D V A E Y A G V S Y Q T V
 73/21 103/31
 TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT GCG AAA ACG CGG GAA AAA GTG GAA GCG
 TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT GCG AAA ACG CGG GAA AAA GTG GAA GCG
 TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT GCG AAA ACG CGT GAA AAA GTG GAA GCG
 S R V V N Q A S H V S A K T R E K V E A
 133/41 163/51
 GCG ATG GCG GAG CTG AAT TAC ATT CCC AAC CGC GTG GCA CAA CAA CTG GCG GGC AAA CAG
 GCG ATG GCG GAG CTG AAT TAC ATT CCC AAC CGC GTG GCA CAA CAA CTG GCG GGC AAA CAG
 GCG ATG GCG GAG CTG AAT TAC ATT CCC AAC CGC GTG GCA CAA CAA CTG GCG GGC AAA CAG
 A M A E L N Y I P N R V A Q Q L A G K Q
 193/61 223/71
 TCG TTG CTG ATT GGC GTT GCC ACC TCC AGT CTG GCC CTG CAC GCG CCG TCG CAA ATT GTC
 TCG TTG CTG ATT GGC GTT GCC ACC TCC AGT CTG GCC CTG CAC GCG CCG TCG CAA ATT GTC
 TCG TTG CTG ATT GGC GTT GCC ACC TCC AGT CTG GCC CTG CAC GCG CCG TCG CAA ATT GTC
 S L L I G V A T S S L A L H A P S Q I V
 253/81 283/91
 GCG GCG ATT AAA TCT CGC GCC GAT CAA CTG GGT GCC AGC GTG GTG GTG TCG ATG GTA GAA
 GCG GCG ATT AAA TCT CGC GCC GAT CAA CTG GGT GCC AGC GTG GTG GTG TCG ATG GTA GAA
 GCG GCG ATT AAA TCT CGC GCC GAT CAA CTG GGT GCC AGC GTG GTG GTG TCG ATG GTA GAA
 A A I K S R A D Q L G A S V V V S M V E
 313/101 343/111
 CGA AGC GGC GTC GAA GCC TGT AAA GCG GCG GTG CAC AAT CTT CTC GCG CAA CGC GTC AGT
 CGA AGC GGC GTC GAA GCC TGT AAA GCG GCG GTG CAC AAT CTT CTC GCG CAA CGC GTC AGT
 CGT AGC GGC GTC GAA GCC TGT AAA GCG GCG GTG CAC AAT CTT CTC GCG CAA CGC GTC AGT
 R S G V E A C K A A V H N L L A Q R V S
 373/121 403/131
 GGG CTG ATC ATT AAC TAT CCG CTG GAT GAC CAG GAT GCC ATT GCT GTG GAA GCT GCC TGC
 GGG CTG ATC ATT AAC TAT CCG CTG GAT GAC CAG GAT GCC ATT GCT GTG GAA GCT GCC TGC
 GGG CTG ATC ATT AAC TAT CCG CTG GAT GAC CAG GAT GCC ATT GCT GTG GAA GCT GCC TGC
 G L I I N Y P L D D Q D A I A V E A A C
 433/141 463/151
 ACT AAT GTT CCG GCG TTA TTT CTT GAT GTC TCT GAC CAG ACA CCC ATC AAC AGT ATT ATT
 ACT AAT GTT CCG GCG TTA TTT CTT GAT GTC TCT GAC CAG ACA CCC ATC AAC AGT ATT ATT
 ACT AAT GTT CCG GCG TTA TTT CTT GAT GTC TCT GAC CAG ACA CCG ATC AAC AGT ATT ATT
 T N V P A L F L D V S D Q T P I N S I I
 493/161 523/171
 TTC TCC CAT GAA GAC GGT ACG CGA CTG GGC GTG GAG CAT CTG GTC GCA TTG GGT CAC CAG
 TTC TCC CAT GAA GAC GGT ACG CGA CTG GGC GTG GAG CAT CTG GTC GCA TTG GGT CAC CAG
 TTC TCC CAT GAA GAC GGT ACG CGT CTG GGC GTG GAG CAT CTG GTC GCA TTG GGT CAC CAG
 F S H E D G T R L G V E H L V A L G H Q
 553/181 583/191
 CAA ATC GCG CTG TTA GCG GGC CCA TTA AGT TCT GTC TCG GCG CGT CTG CGT CTG GCT GGC
 CAA ATC GCG CTG TTA GCG GGC CCA TTA AGT TCT GTC TCG GCG CGT CTG CGT CTG GCT GGC
 CAA ATC GCG CTG TTA GCG GGC CCA TTA AGT TCT GTC TCG GCG CGT CTG CGT CTG GCT GGC
 Q I A L L A G P L S S V S A R L R L A G

FIG. 3

613/201
 TGG CAT AAA TAT CTC ACT CGC AAT CAA ATT CAG CCG ATA GCG GAA CGG GAA GGC GAC TGG
 TGG CAT AAA TAT CTC ACT CGC AAT CAA ATT CAG CCG ATA GCG GAA CGG GAA GGC GAC TGG
 TGG CAT AAA TAT CTC ACT CGC AAT CAA ATT CAG CCG ATC GCG GAA CGT GAA GGC GAC TGG
 W H K Y L T R N Q I Q P I A E R E G D W
 673/221
 AGT GCC ATG TCC GGT TTT CAA CAA ACC ATG CAA ATG CTG AAT GAG GGC ATC GTT CCC ACT
 AGT GCC ATG TCC GGT TTT CAA CAA ACC ATG CAA ATG CTG AAT GAG GGC ATC GTT CCC ACT
 AGT GCC ATG TCC GGT TTT CAA CAA ACC ATG CAA ATG CTG AAT GAG GGC ATC GTT CCG ACT
 S A M S G F Q Q T M Q M L N E G I V P T
 733/241
 GCG ATG CTG GTT GCC AAC GAT CAG ATG GCG CTG GGC GCA ATG CGC GCC ATT ACC GAG TCC
 GCG ATG CTG GTT GCC AAC GAT CAG ATG GCG CTG GGC GCA ATG CGC GCC ATT ACC GAG TCC
 GCG ATG CTG GTT GCC AAC GAT CAG ATG GCG CTG GGC GCA ATG CGC GCC ATT ACC GAG TCC
 A M L V A N D Q M A L G A M R A I T E S
 793/261
 GGG CTG CGC GTT GGT GCG GAT ATC TCG GTA GTG GGA TAC GAC GAT ACC GAA GAC AGC TCA
 GGG CTG CGC GTT GGT GCG GAT ATC TCG GTA GTG GGA TAC GAC GAT ACC GAA GAC AGC TCA
 GGG CTG CGC GTT GGT GCG GAT ATC TCG GTA GTG GGT TAC GAC GAT ACC GAA GAC AGC TCA
 G L R V G A D I S V V G Y D D T E D S S
 853/281
 TGT TAT ATC CCG CCG TTA ACC ACC ATC AAA CAG GAT TTT CGC CTG CTG GGG CAA ACC AGC
 TGT TAT ATC CCG CCG TTA ACC ACC ATC AAA CAG GAT TTT CGC CTG CTG GGG CAA ACC AGC
 TGT TAT ATC CCG CCG TTA ACC ACC ATC AAA CAG GAT TTT CGC CTG CTG GGG CAA ACC AGC
 C Y I P P L T T I K Q D F R L L G Q T S
 913/301
 GTG GAC CGC TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG CCC
 GTG GAC CGC TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG CCC
 GTG GAC CGC TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG CCG
 V D R L L Q L S Q G Q A V K G N Q L L P
 973/321
 GTC TCA CTG GTG AAA AGA AAA ACC ACC CTG GCG CCC AAT ACG CAA ACC GCC TCT CCC CGC
 GTC TCA CTG GTG AAA AGA AAA ACC ACC CTG GCG CCC AAT ACG CAA ACC GCC TCT CCC CGC
 GTC TCA CTG GTG AAA CGT AAA ACC ACC CTG GCG CCG AAT ACG CAA ACC GCC TCT CCG CGC
 V S L V K R K T T L A P N T Q T A S P R
 1033/341
 GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGA CAG GTT TCC CGA CTG GAA AGC GGG CAG
 GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGA CAG GTT TCC CGA CTG GAA AGC GGG CAG
 GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGT CAG GTT TCC CGT CTG GAA AGC GGG CAG
 A L A D S L M Q L A R Q V S R L E S G Q
 1093/361
 TGA
 TGA
 TGA
 *

FIG. 3

$\Delta asdA::TT\ araC\ P_{BAD}\ c2^*$



$\Delta asdA18::TT\ araC\ P_{BAD}\ \underline{AGGAGACTTAACT}\ \underline{ATG}\ AAT-c2$
SD Start codon

$\Delta asdA20::TT\ araC\ P_{BAD}\ \underline{AGGAGACTTAACT}\ \underline{ATG}\ AAT-c2^*$
SD Start codon

$\Delta asdA21::TT\ araC\ P_{BAD}\ \underline{TAAGGAGGTTTAACT}\ \underline{ATG}\ AAT-c2^*$
SD Start codon

$\Delta asdA27::TT\ araC\ P_{BAD^*}\ \underline{TAAGGAGGTTTAACT}\ \underline{ATG}\ AAA-c2^{**}$
SD Start codon

FIG. 4

1/1 31/11
 ATG AAT ACA CAA TTG ATG GGT GAG CGT ATT CGC GCT CGA AGA AAA AAA CTC AAG ATT AGA original
 atg aat aca caa ttg atg ggt gag cgt att cgc gct cga aga aaa aaa ctc aag att cgt optimized
 M N T Q L M G E R I R A R R K K L K I R

61/21 91/31
 CAA GCC GCT CTT GGT AAG ATG GTG GGA GTG TCT AAT GTT GCA ATA TCG CAA TGG GAG CGC
 caa gcc gct ctt ggt aag atg gtg gg^g gtg tct aat gtt gca atc tgc caa tgg gag cgc
 Q A A L G K M V G V S N V A I S Q W E R

121/41 151/51
 TCG GAG ACT GAG CCA AAT GGG GAG AAC CTG TTG GCA CTT TCG AAG GCT CTT CAG TGC TCC
 tcg gag act gag cca aat ggg gag aac ctg ttg gca ctt tgc aag gct ctt cag tgc tcc
 S E T E P N G E N* L L A L S K A L Q C S

181/61 211/71
 CCT GAC TAT TTG CTG AAA GGA GAT TTA AGC CAG ACA AAC GTT GCC TAT CAT AGT AGG CAT
 cct gac tat ttg ctg aaa gg^g gat tta agc cag aca aac gtt gcc tat cat agt cgt cat
 P D Y L L K G D L S Q T N V A Y H S R H

241/81 271/91
 GAG CCA AGA GGA TCA TAC CCT CTT ATC AGT TGG GTA AGC GCA GGG CAA TGG ATG GAA GCT
 gag cca aga gga tca tac cct ctt atc agt tgg gta agc gca ggg caa tgg atg gaa gct
 E P R G S Y P L I S W V S A G Q W M E A

301/101 331/111
 GTA GAA CCT TAT CAC AAG CGC GCG ATA GAG AAC TGG CAC GAC ACC ACT GTA GAT TGT TCA
 gta gaa cct tat cac aag cgc gcg ata gag aac tgg cac gac acc act gta gat tgt tca
 V E P Y H K R A I E N W H D T T V D C S

361/121 391/131
 GAA GAT TCA TTT TGG CTT GAT GTC CAA GGT GAC TCT ATG ACA GCA CCG GCA GGG TTA AGC
 gaa gat tca ttt tgg ctt gat gtc caa ggt gac tct atg aca gca ccg gca ggg tta agc
 E D S F W L D V Q G D S M T A P A G L S

421/141 451/151
 ATT CCA GAA GGA ATG ATA ATT CTG GTT GAT CCC GAA GTC GAA CCA AGA AAC GGC AAG CTG
 att cca gaa gg^g atg atc att ctg gtt gat ccg gaa gtc gaa cca aga aac ggc aag ctg
 I P E G M I I L V D P E V E P R N G K L

481/161 511/171
 GTT GTT GCA AAA TTA GAA GGT GAA AAC GAG GCC ACA TTC AAA AAA TTA GTT ATG GAT GCA
 gtt gtt gca aaa tta gaa ggt gaa aac gag gcc aca ttc aaa aaa tta gtt atg gat gca
 V V A K L E G E N E A T F K K L V M D A

541/181 571/191
 GGC CGA AAG TTT TTA AAA CCA TTA AAC CCA CAA TAT CCG ATG ATA GAA ATC AAC GGA AAC
 ggc cga aag ttt tta aaa cca tta aac cca caa tat ccg atg ata gaa atc aac gg^g aac
 G R K F L K P L N P Q Y P M I E I N G N

601/201 631/211
 TGC AAA ATC ATT GGC GTA GTT GTT GAC GCA AAA CTC GCA AAT CTT CCA TAA
 tgc aaa atc att ggc gta gtt gtt gac gca aaa ctc gca aat ctt cca taa
 C K I I G V V V D A K L A N L P *

FIG. 5

P_{BAD}-35 -10

Original: TTT ATC CAT AAG ATT AGC GGA TCC TAC CTG ACG CTT TTT ATC GCA ACT CTC TAC TGT

Modified: TTT ATC CAT AAG ATT AGC GGA TCC TAC CTG ACG CTT TTT ATC GCA ACT CTC TAT AAT

improved SD

TTC TCC ATA CCC GTT TTT TTG GGC TAG CCT CGA GGG TACC TAA GGA GGT TTA ACT

TTC TCC ATA CCC GTT TTT TTG GGC TAG CCT CGA GGG TACC TAA GGA GGT TTA ACT

c2 (codon improved)

ATG AAT ACA CAA TTG ATG GGT GAG CGT ATT CGC GCT CGT CGT AAA AAA CTC AAG ATT CGT

M N T Q L M G E R I R A R R K K L K I R

▼

ATG AAA ACA CAA TTG ATG GGT GAG CGT ATT CGC GCT CGT CGT AAA AAA CTC AAG ATT CGT

K

FIG. 6

$\Delta P_{\text{crp527}}::\text{TT } \textit{araC} \text{ P}_{\text{BAD}} \textit{crp}$

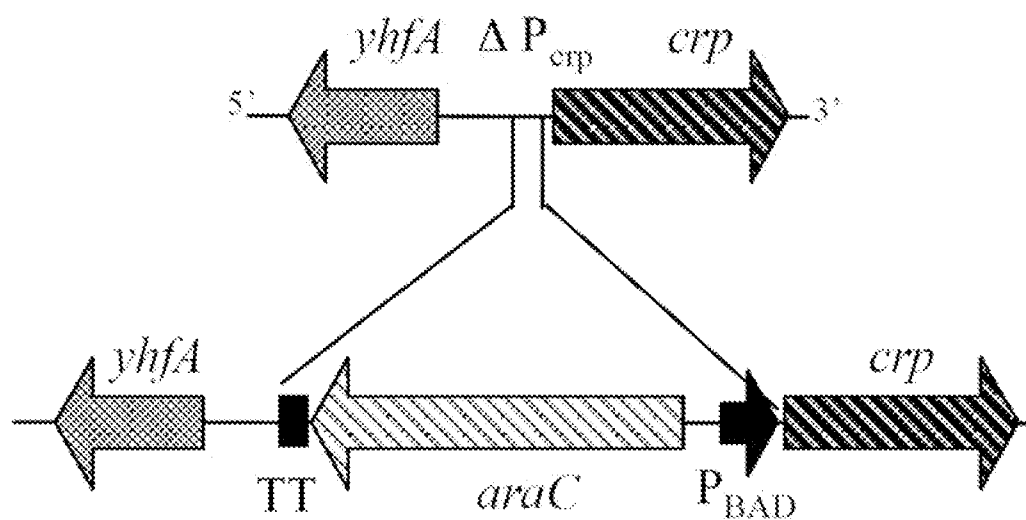
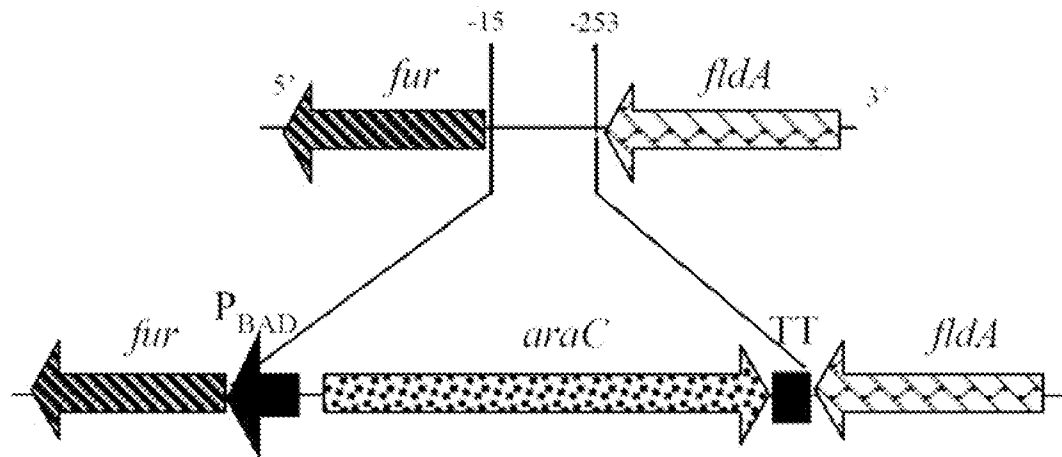


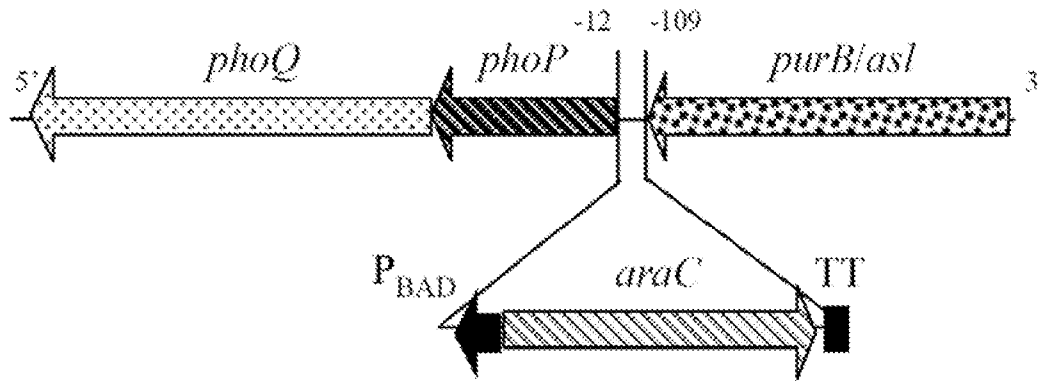
FIG. 7

$\Delta P_{fur}::TT\ araC\ P_{BAD}\ fur$



- a. $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ $\xrightarrow{fur}$
AG GAC AGA TTC CGC **ATG** ACT GAC
 SD M T D
- b. $\Delta P_{fur77}::TT\ araC\ P_{BAD}\ fur$ $\xrightarrow{fur}$
AG GAC AGA TTC CGC **GTG** ACT GAC
 SD V T D
- c. $\Delta P_{fur81}::TT\ araC\ P_{BAD}\ fur$ $\xrightarrow{fur}$
AA GGC AGA TTC CGC **GTG** ACT GAC
 SD V T D

FIG. 8

$$\Delta P_{\text{phoPQ}}::TT\text{ }araC\text{ }P_{\text{BAD}}\text{ }phoPQ$$


- ΔP_{phoPQ107}::TT araC P_{BAD} phoPQ : AGGGA GAAGAG ATG ATG CGC GTA
M M R
- ΔP_{phoPQ173}::TT araC P_{BAD} phoPQ : AGGGA GAAGAG GTG GTG CGC GTA
V V R
- ΔP_{phoPQ177}::TT araC P_{BAD} phoPQ: AAGGC GAAGAG GTG GTG CGC GTA
V V R
- ΔP_{phoPQ174}::TT araC P_{BAD} phoPQ: AAGGC GAAGAG GTG ATG CGC GTA
V M R
- ΔP_{phoPQ175}::TT araC P_{BAD} ΔGAG-phoPQ: AAGGC GAG ATG ATG CGC GTA
M M R
- ΔP_{phoPQ176}::TT araC P_{BAD} ΩCTC-phoPQ: AAGGC CTCGAAGAG ATG ATG CGC GTA
M M R

FIG. 9

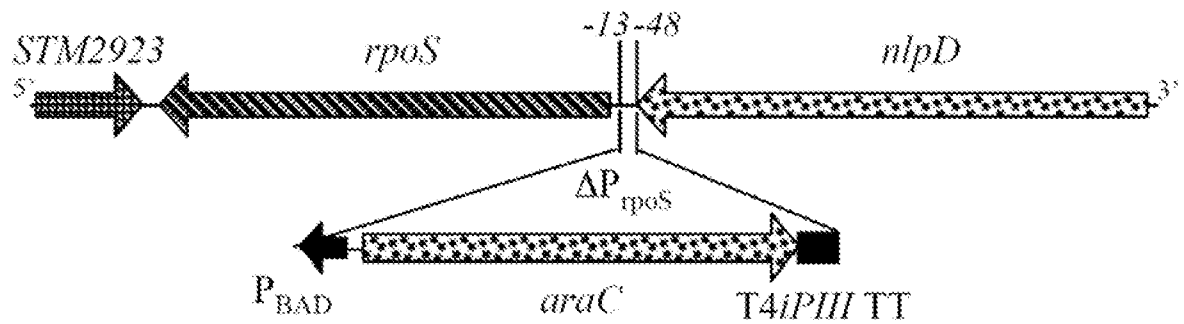
$$\Delta P_{rpoS183}::TT \text{ } araC \text{ } P_{BAD} \text{ } rpoS$$


FIG. 10

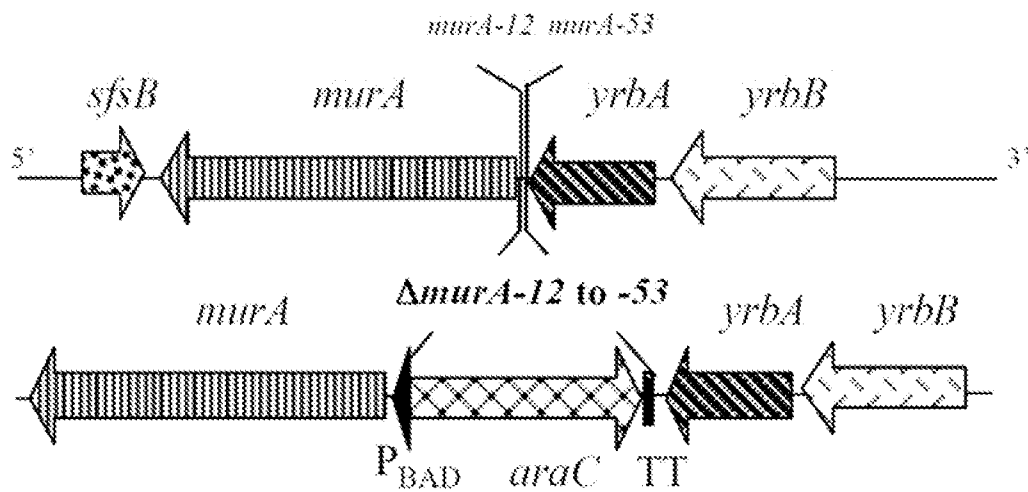
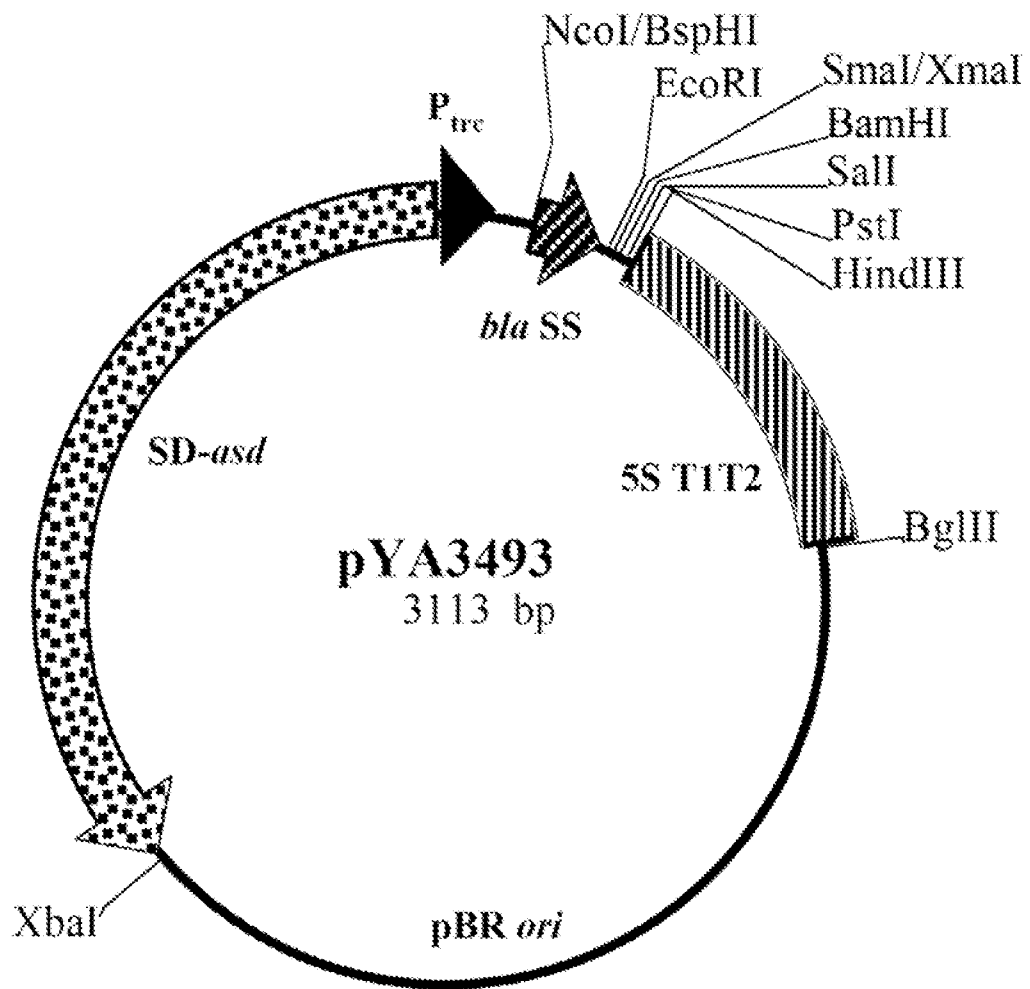
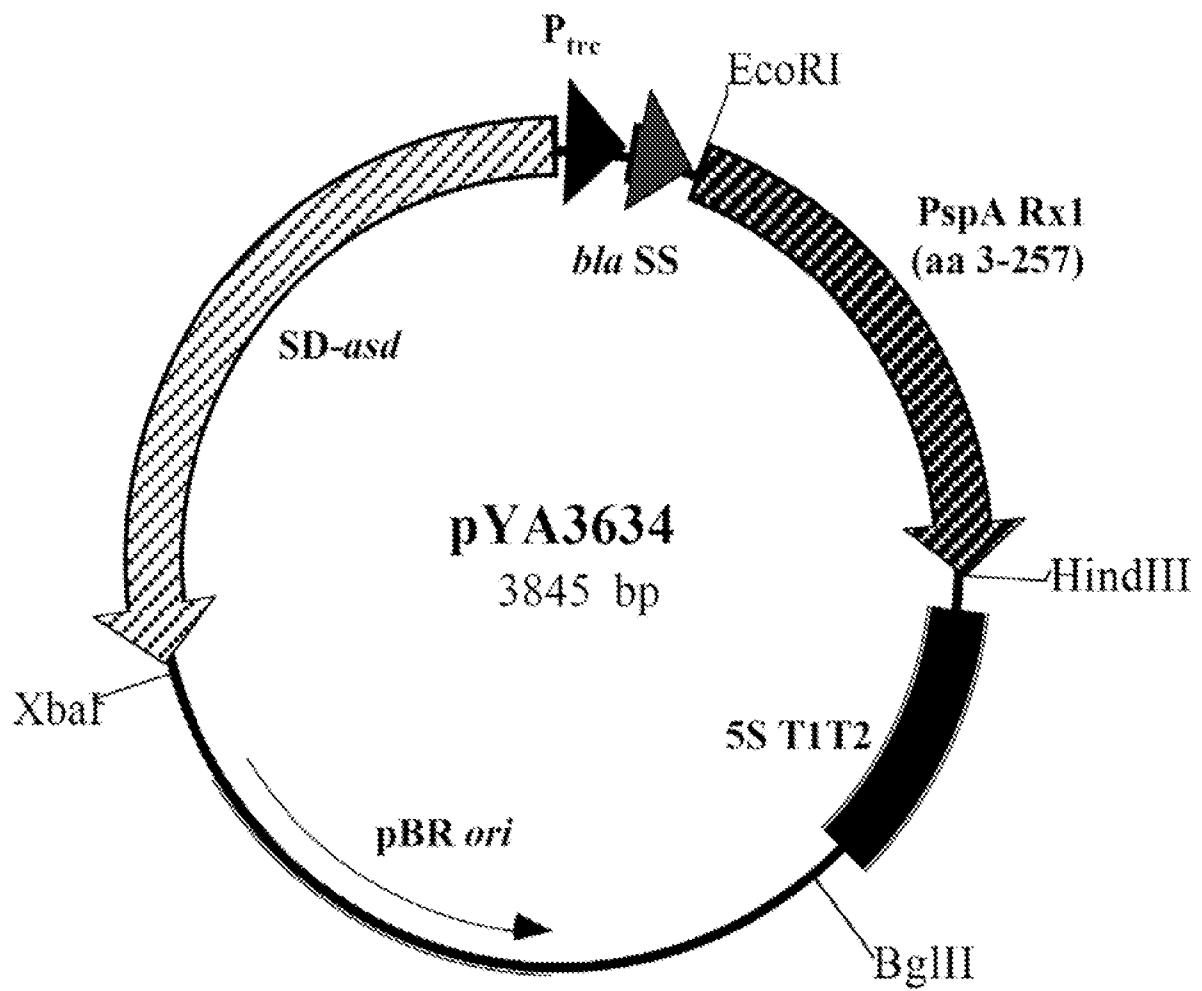
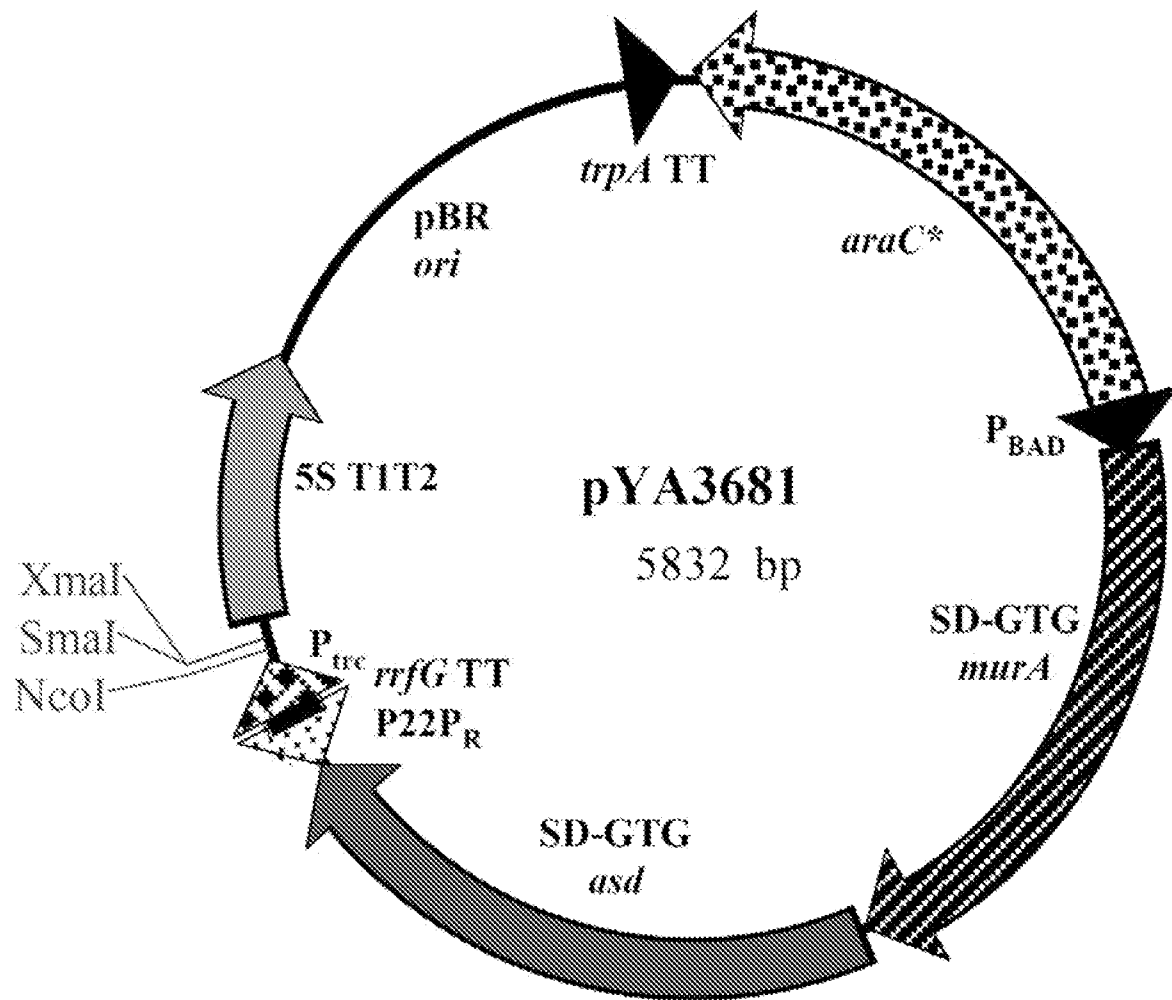
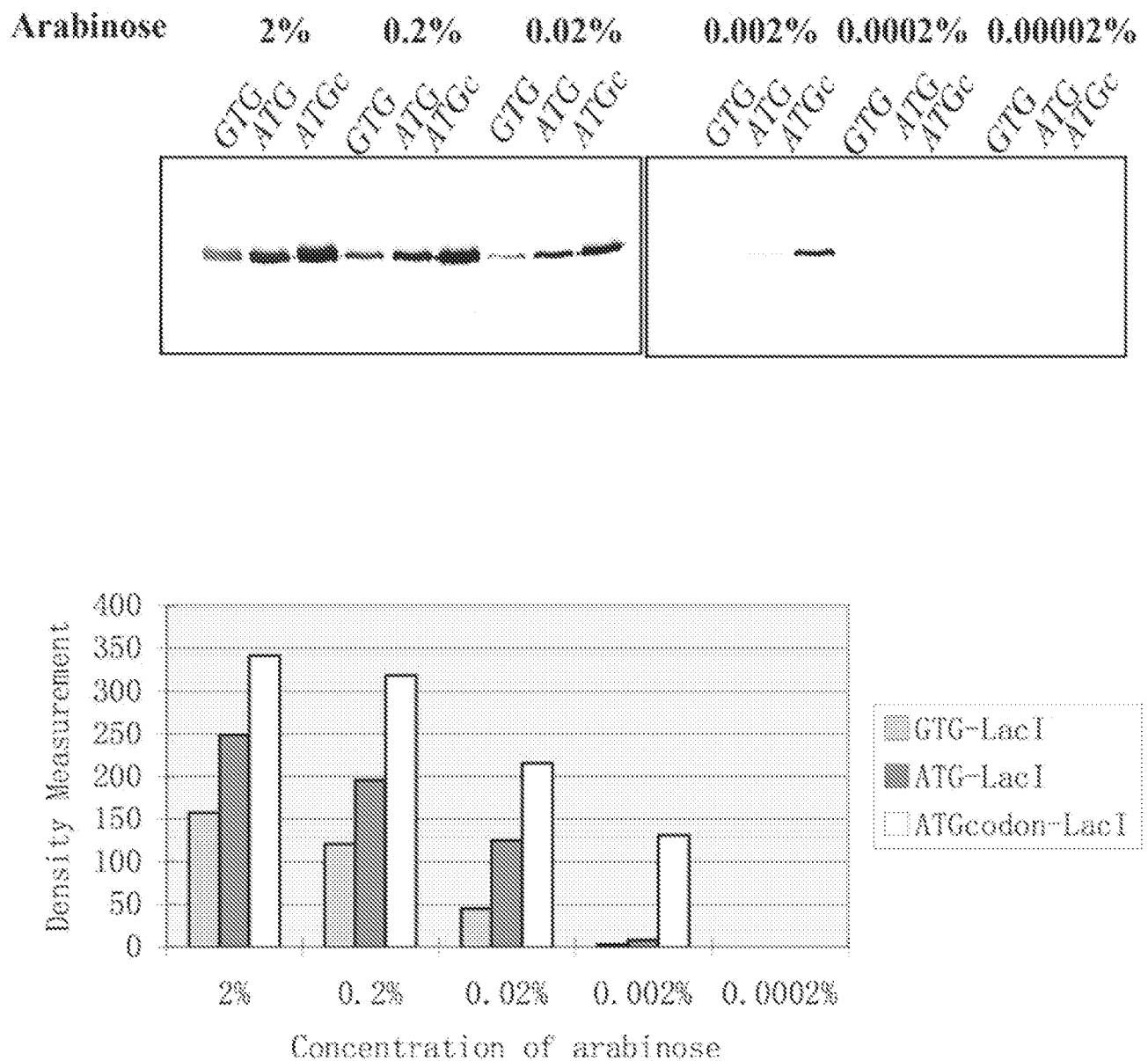
$$\Delta P_{murA12}::TT \text{ } araC \text{ } P_{BAD} \text{ } murA$$


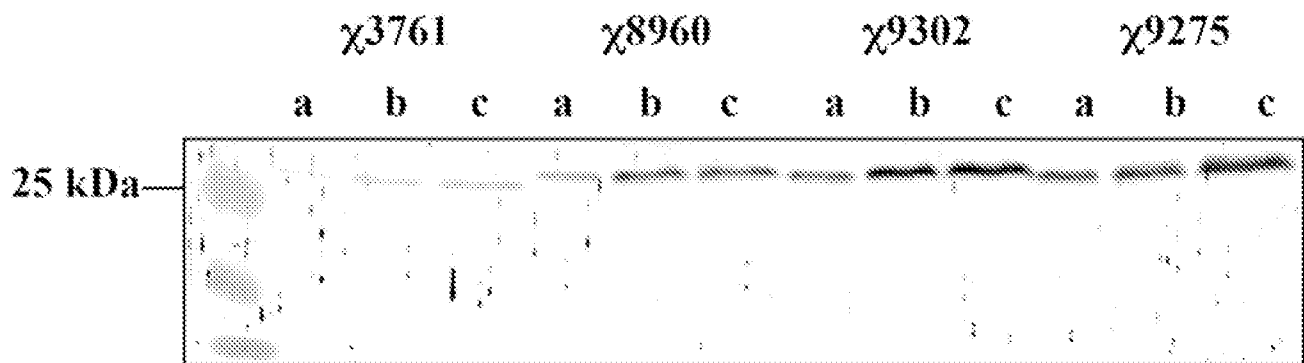
FIG. 11

**FIG. 12**

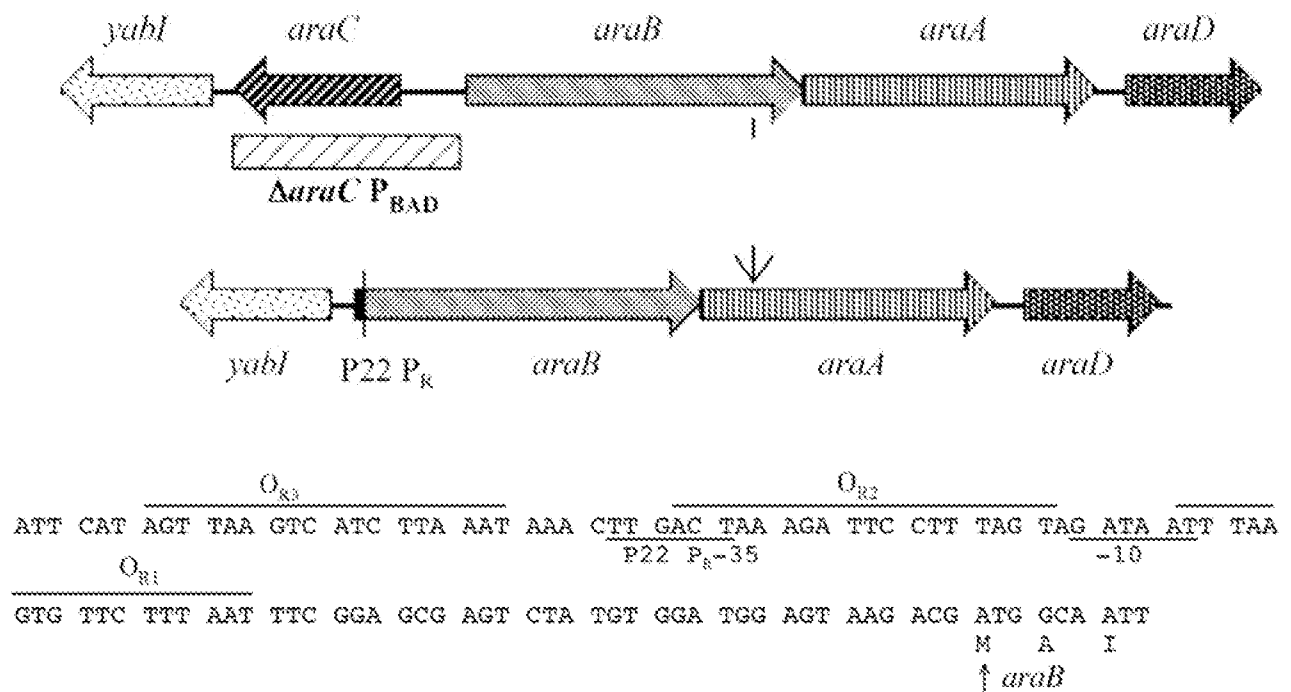
**FIG. 13**

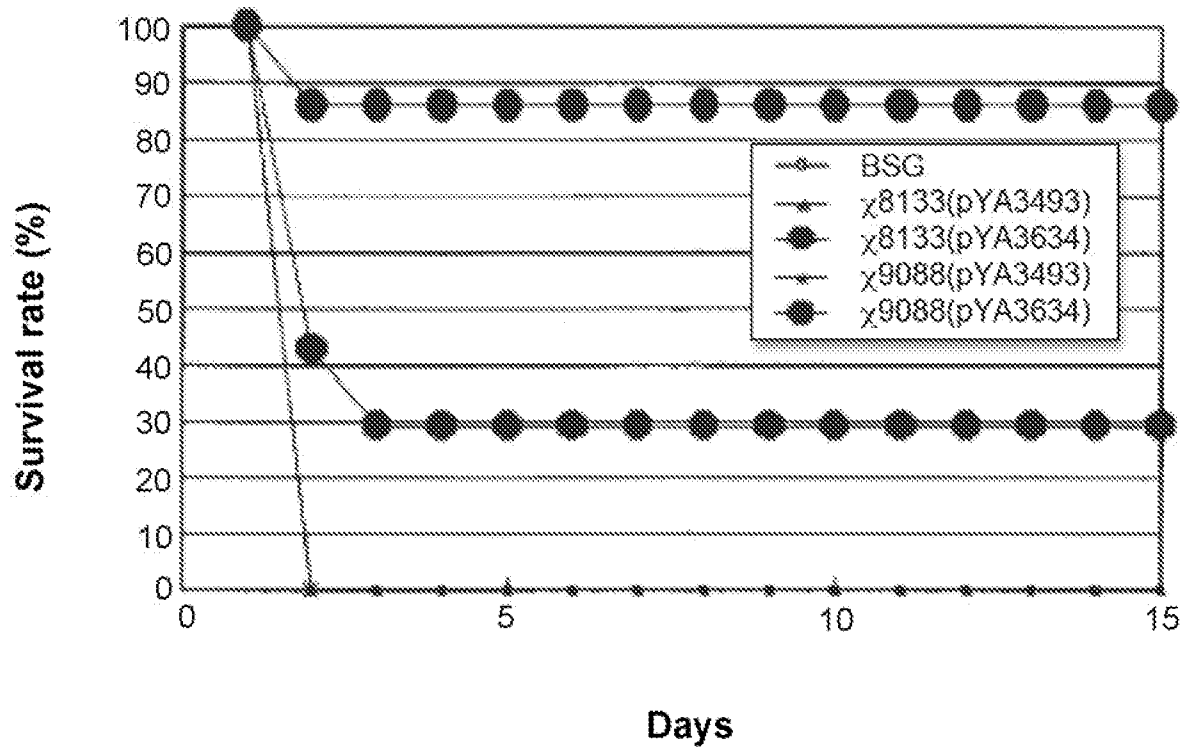
**FIG. 14**

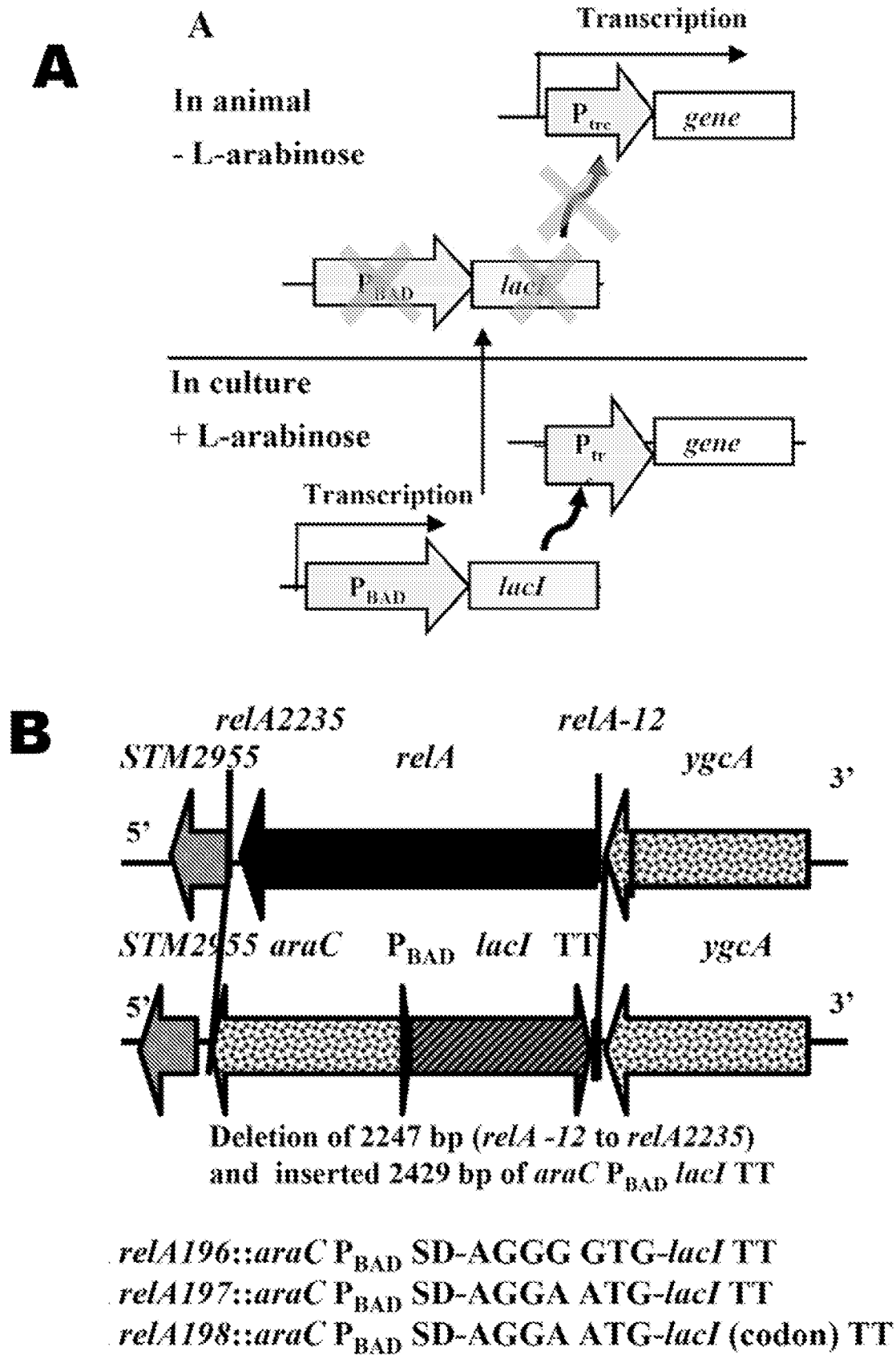
**FIG. 15**

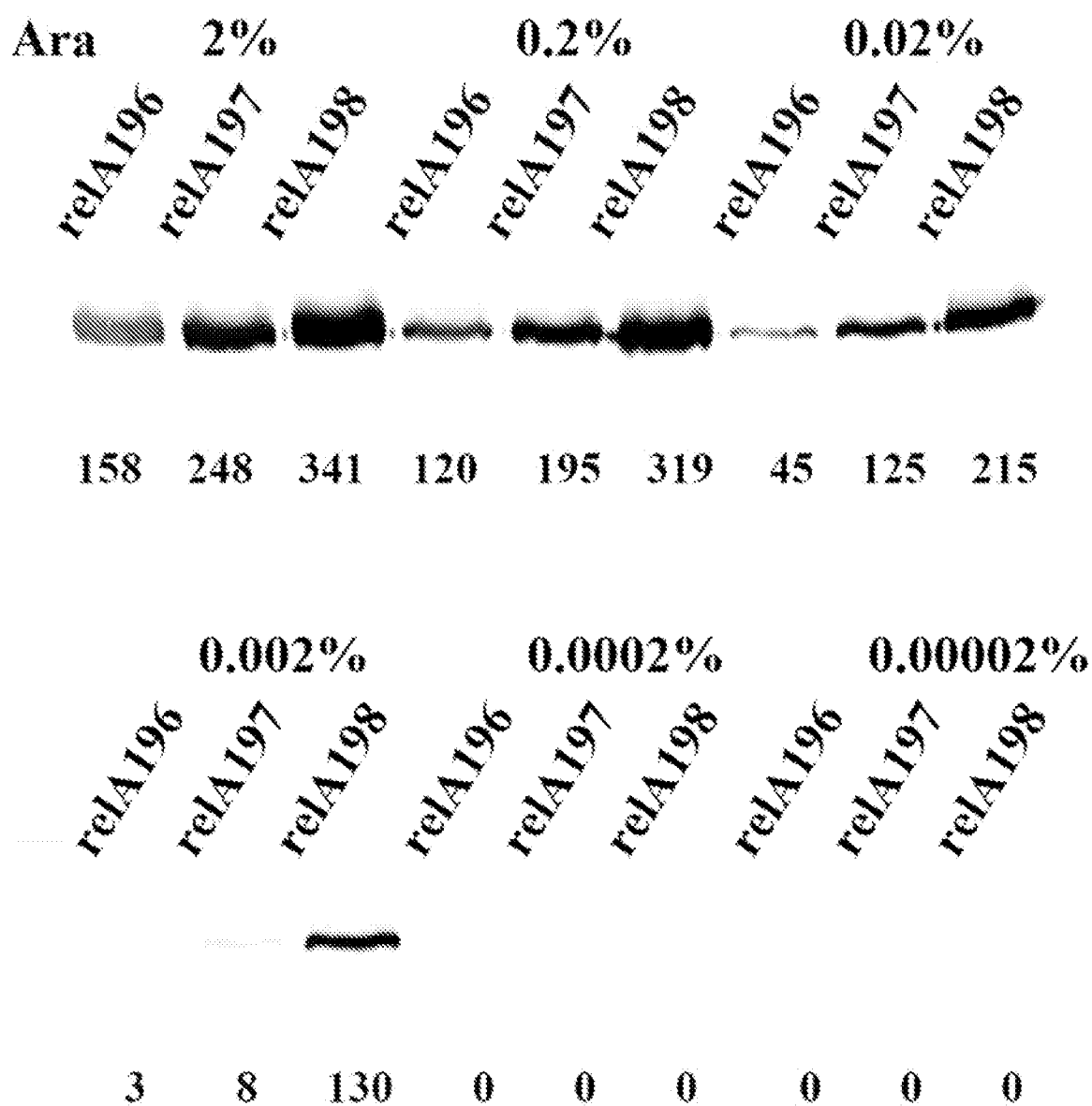
**FIG. 16**

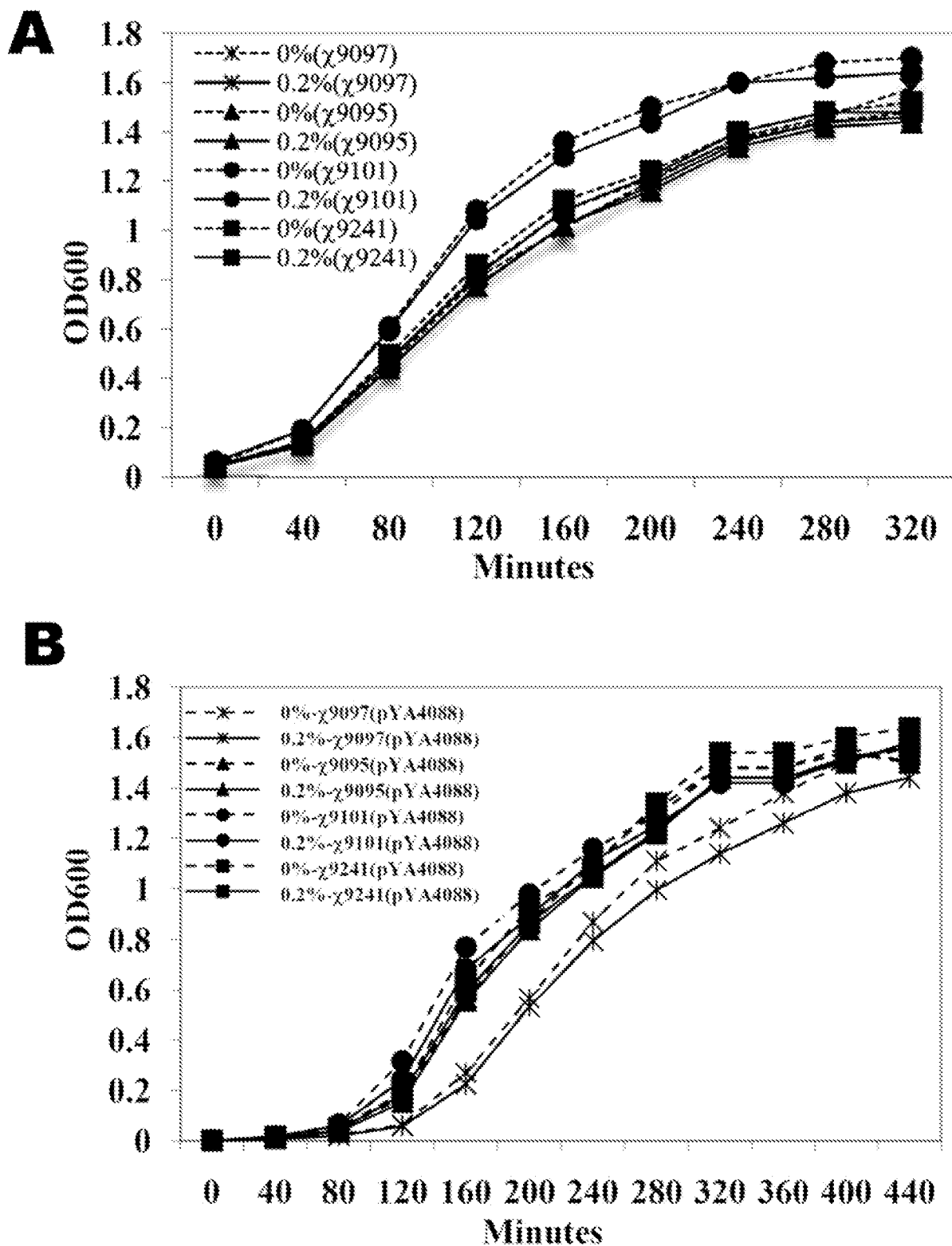
$\Delta(araC P_{BAD})-5::P_R araBAD$

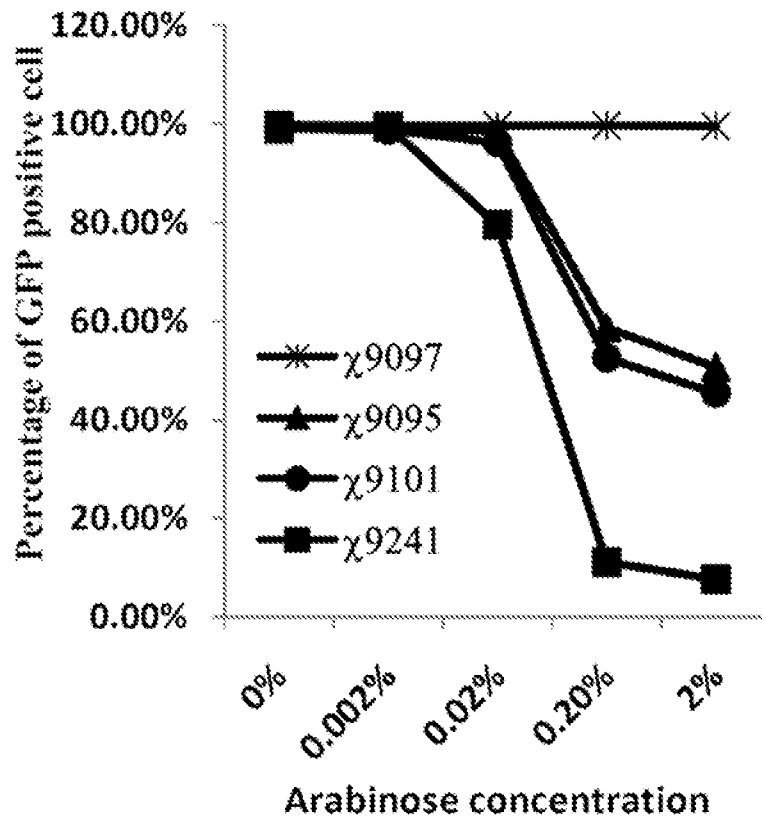
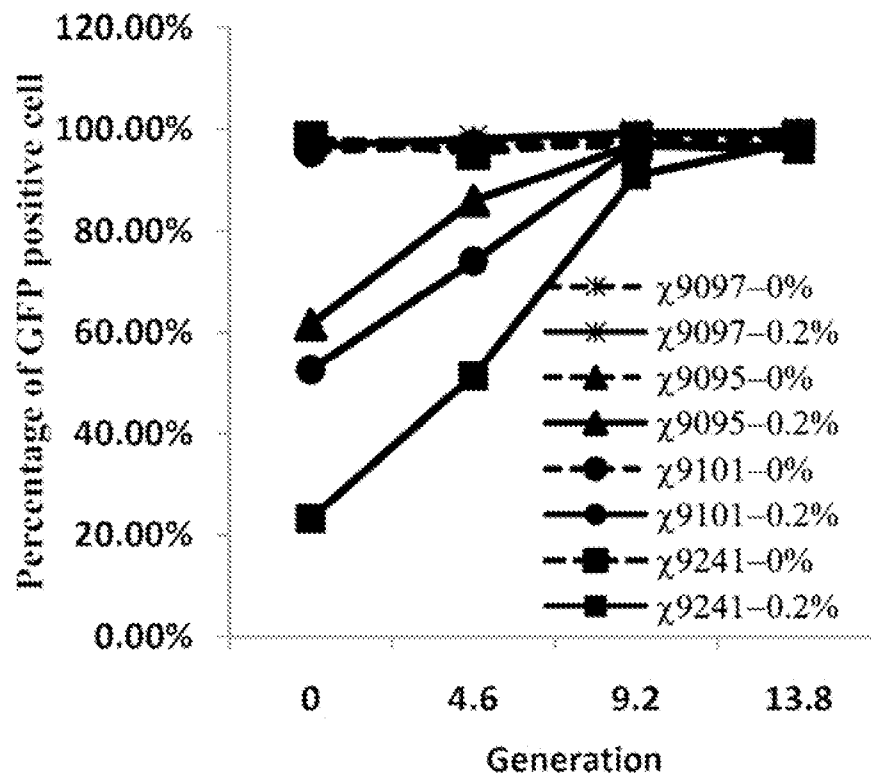
**FIG. 17**

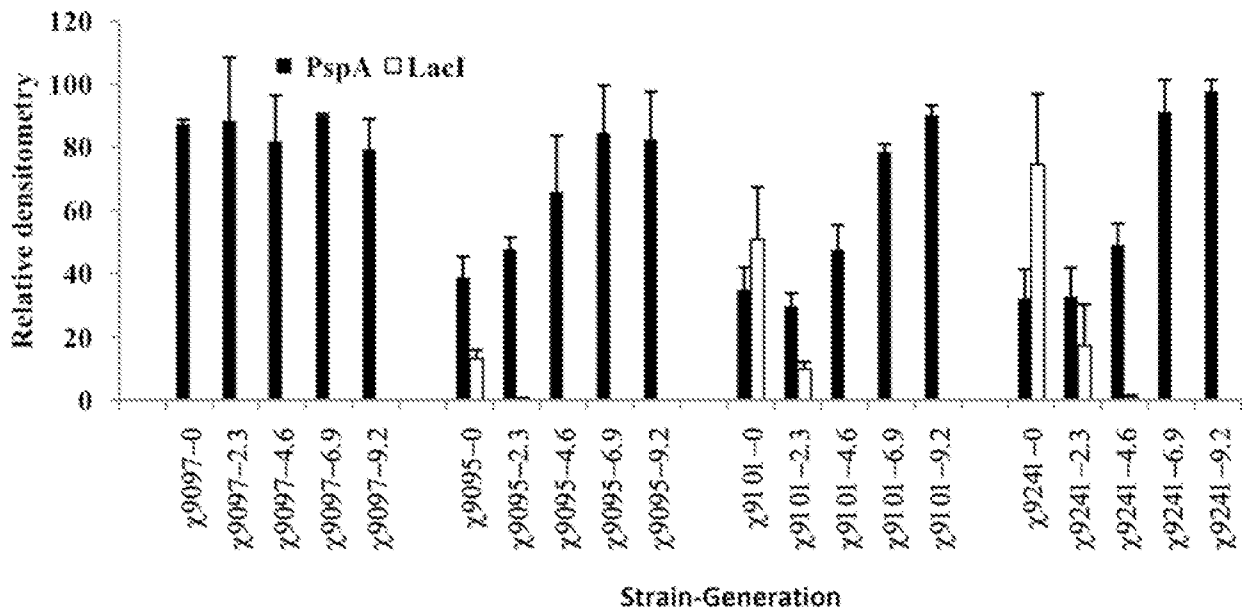
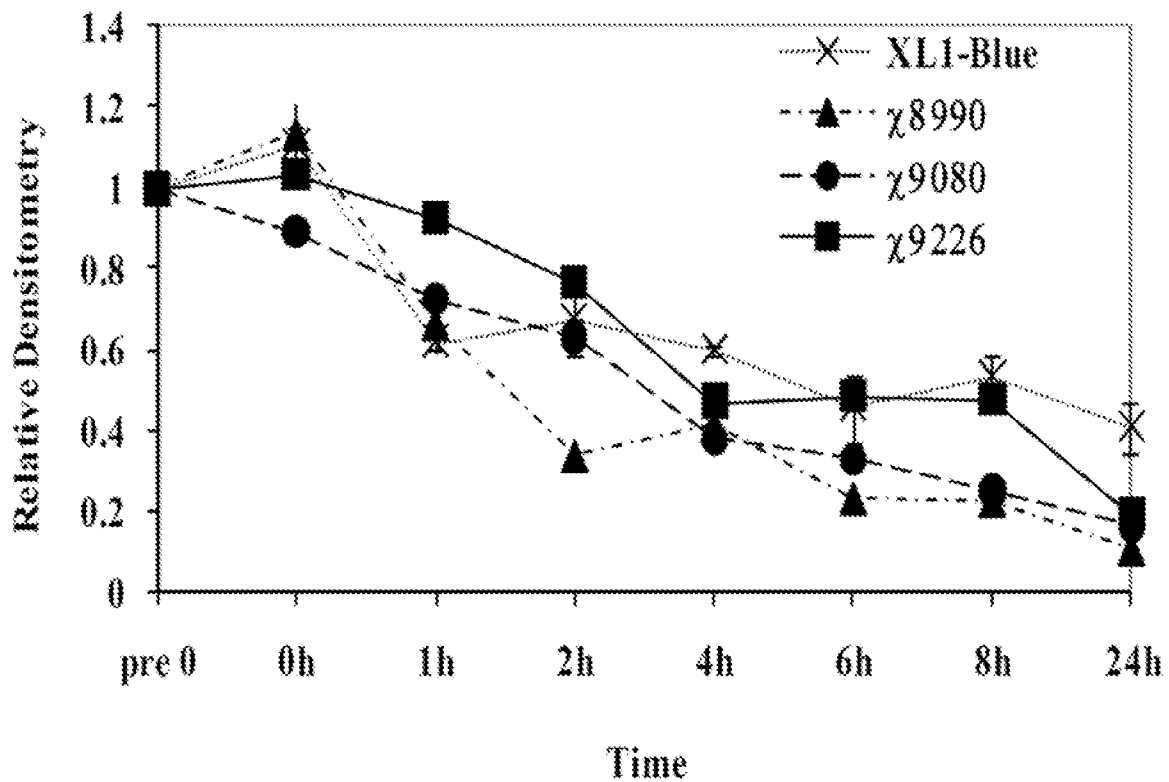
**FIG. 18**

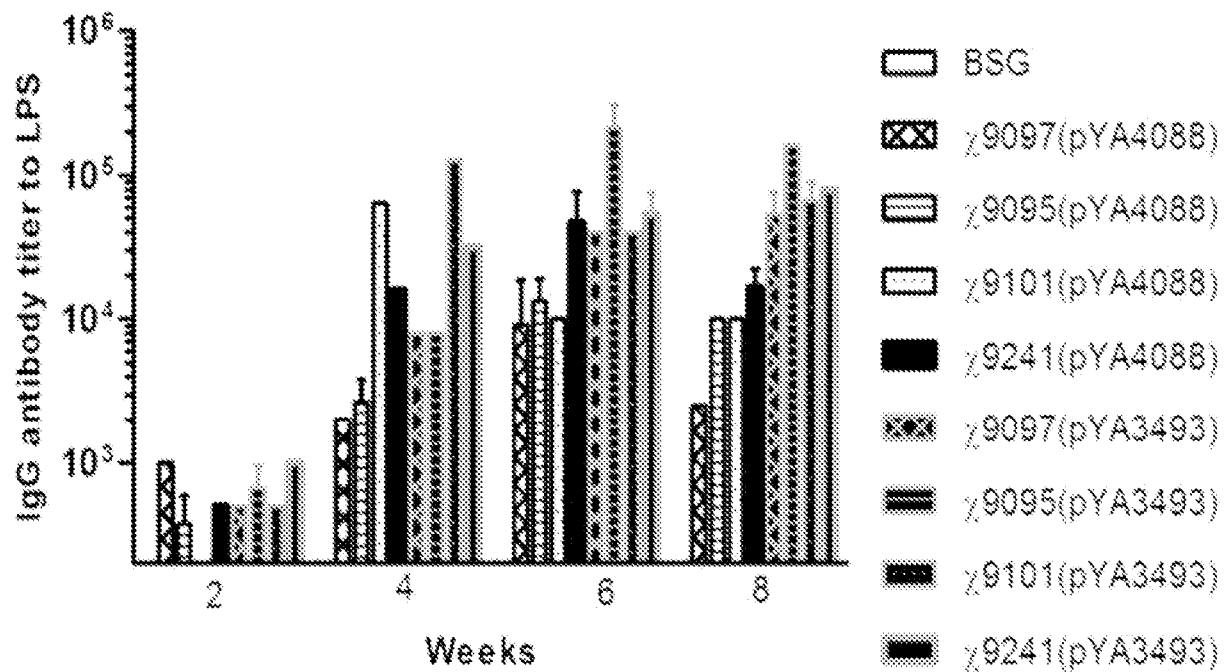
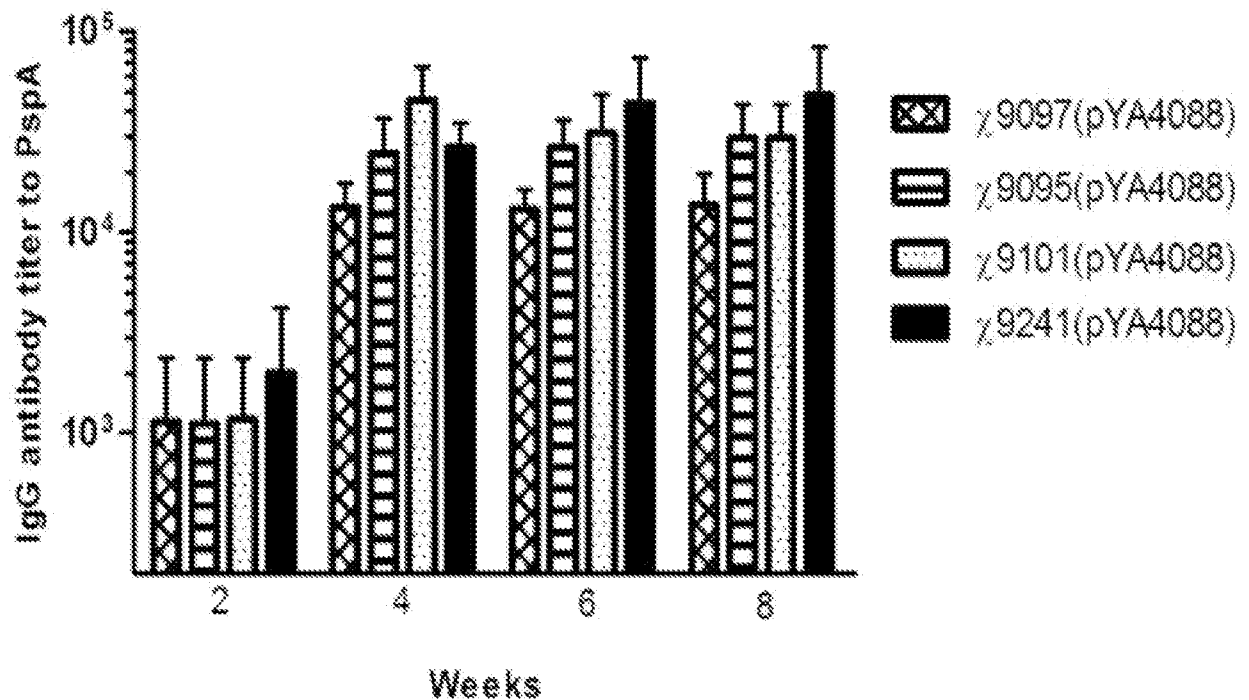
**FIG. 19**

**FIG. 19C**

**FIG. 20**

A**B****FIG. 21**

**FIG. 21C****FIG. 22**

A**B****FIG. 23**

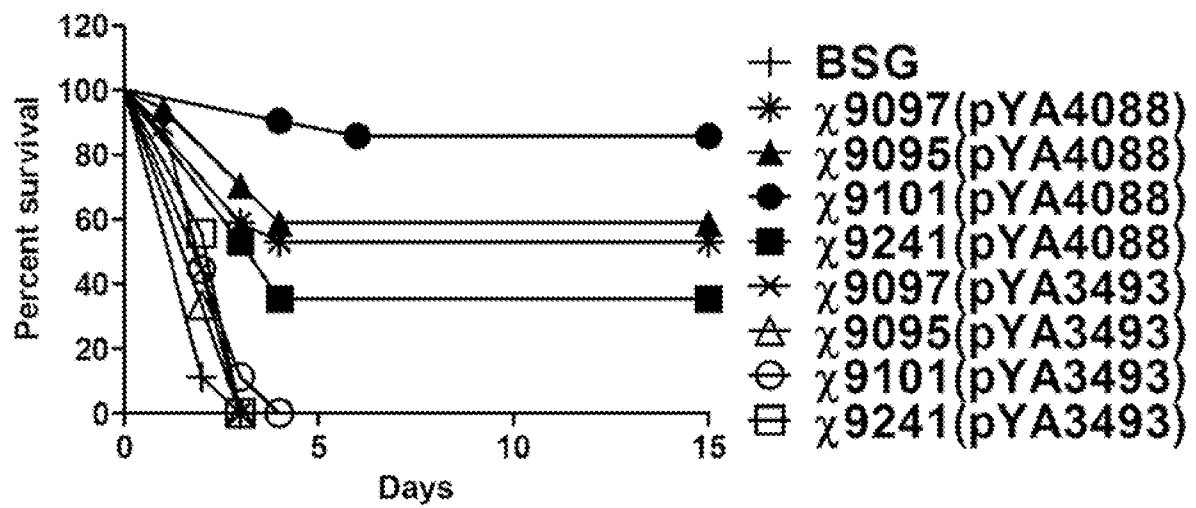


FIG. 24

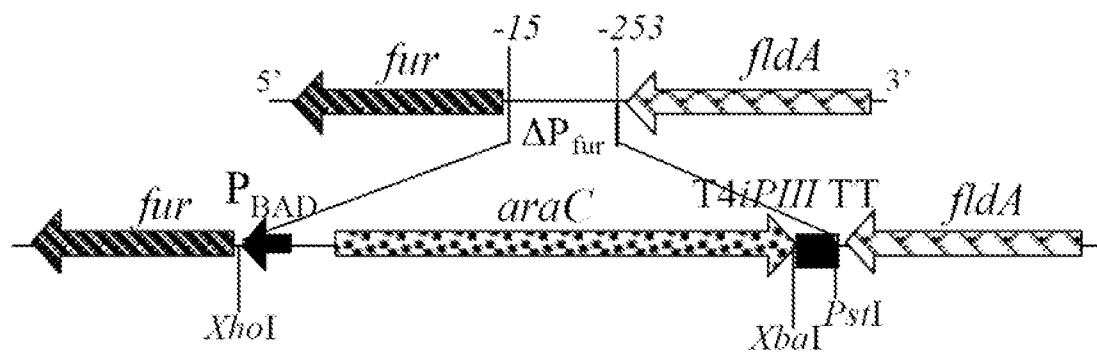
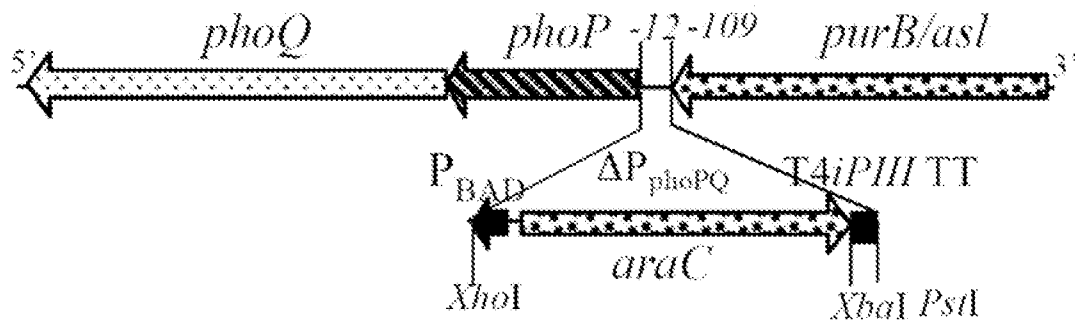
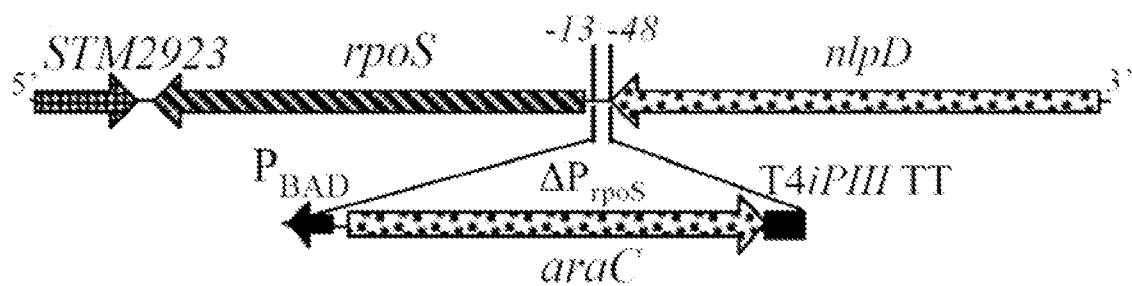
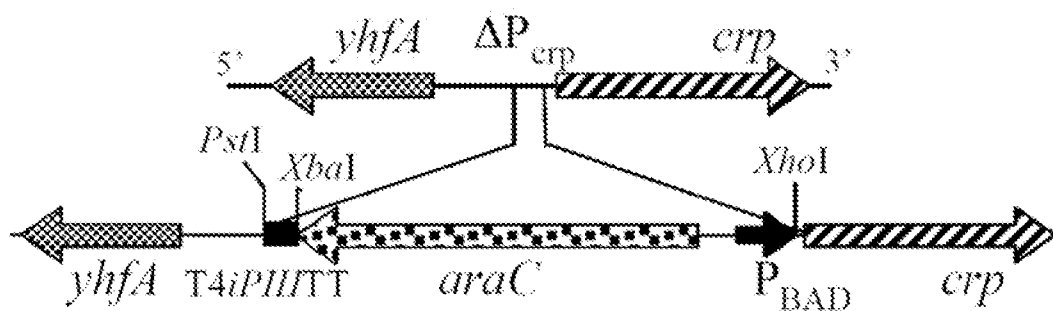
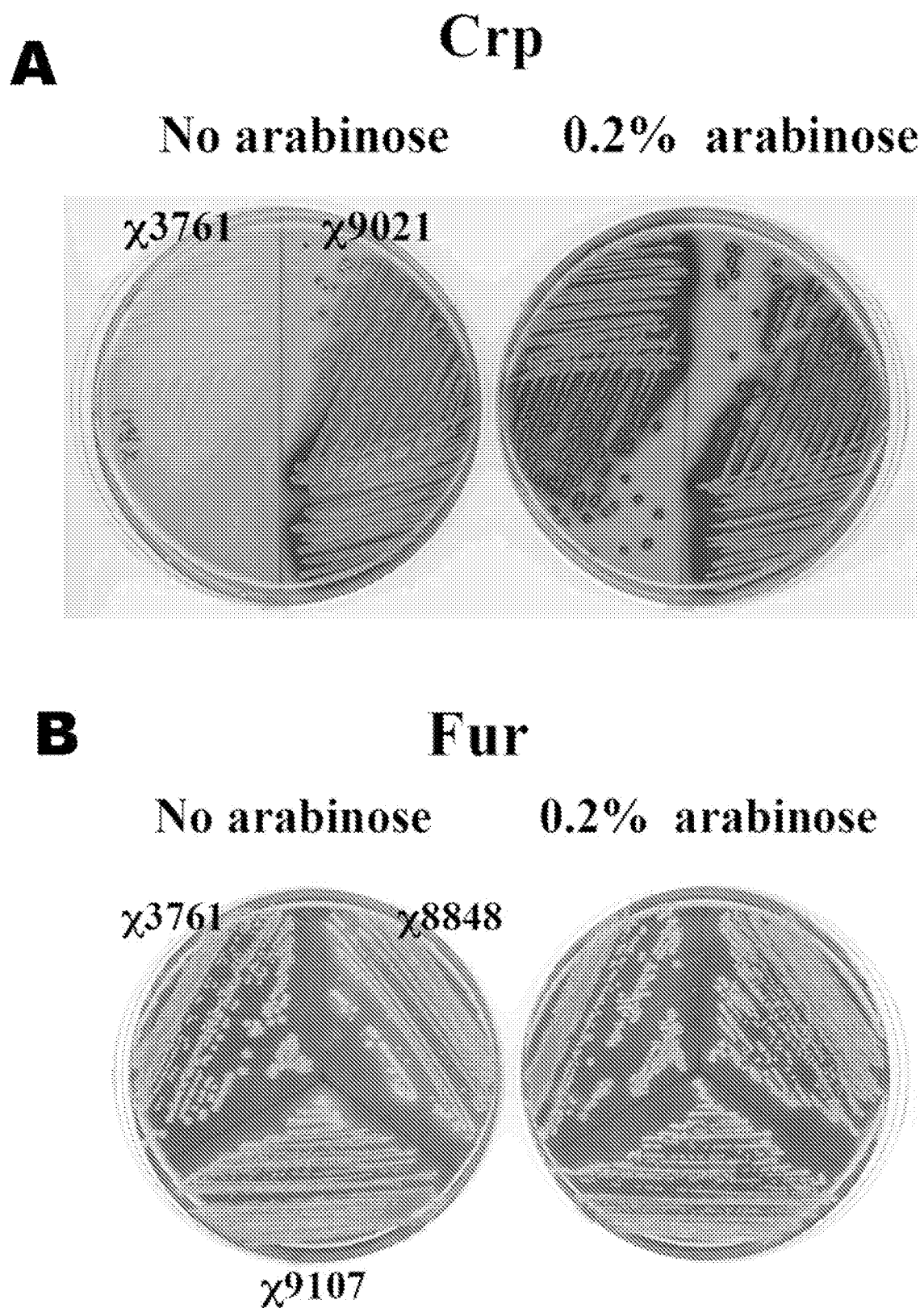
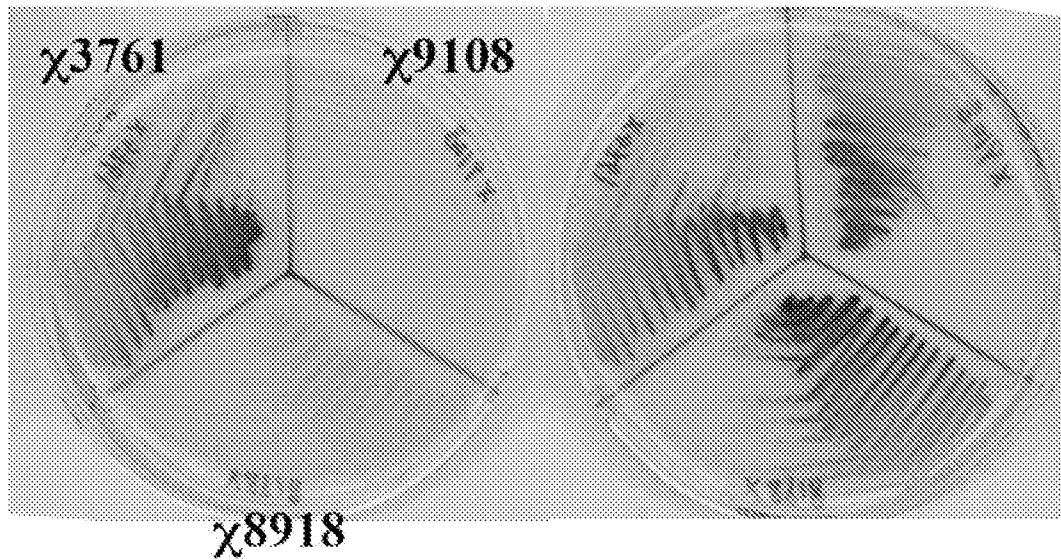
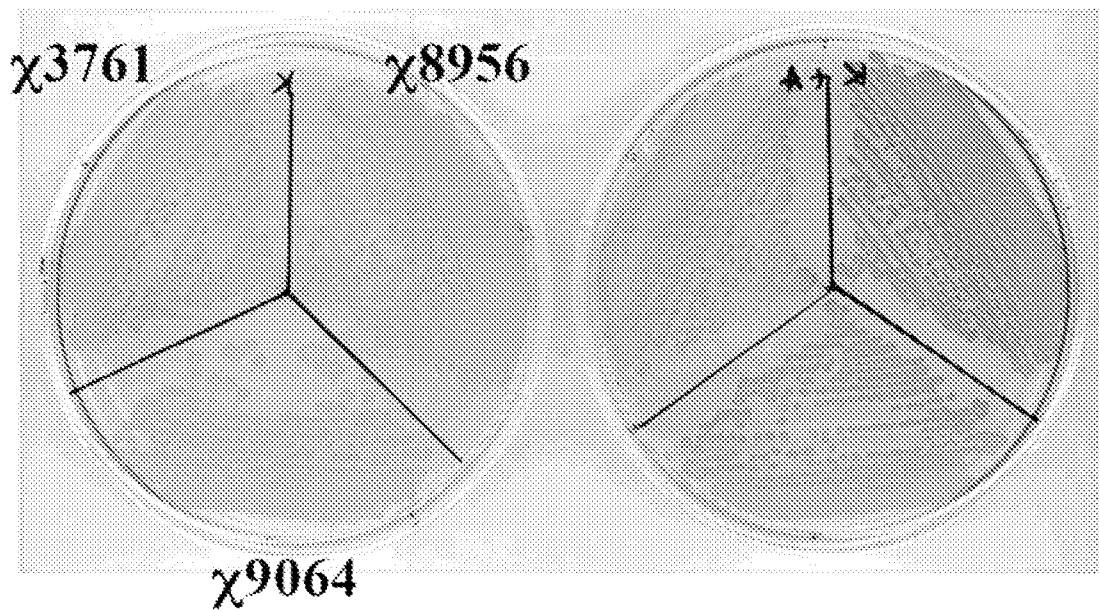
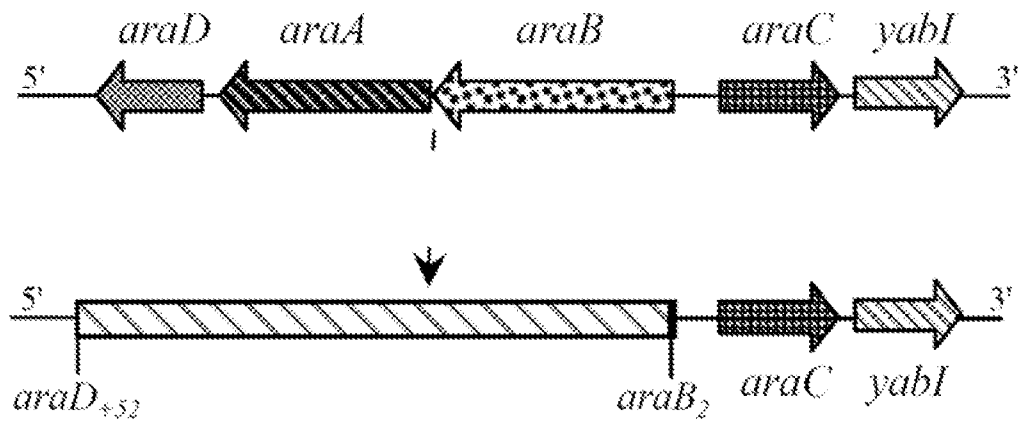
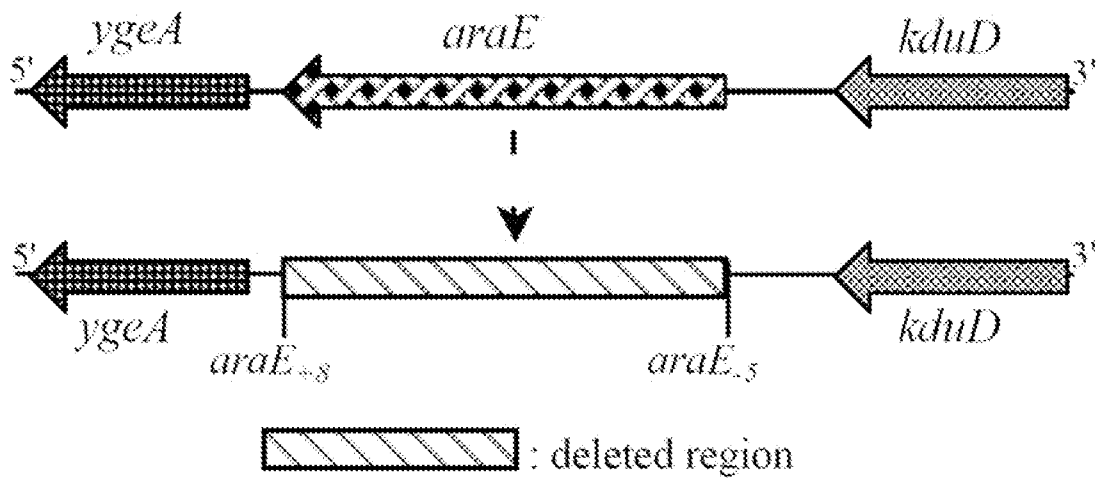
$$\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$$


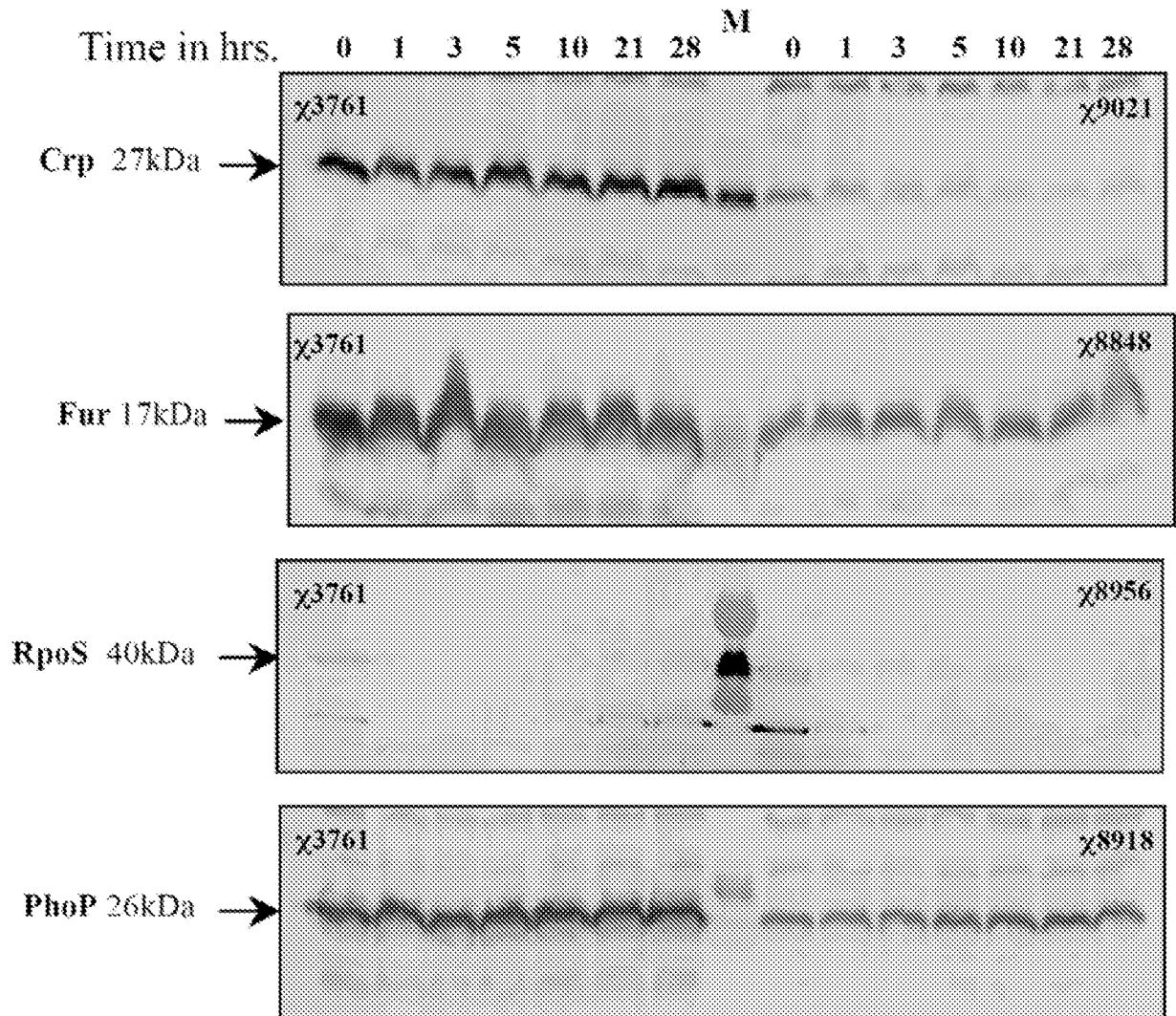
FIG. 25A

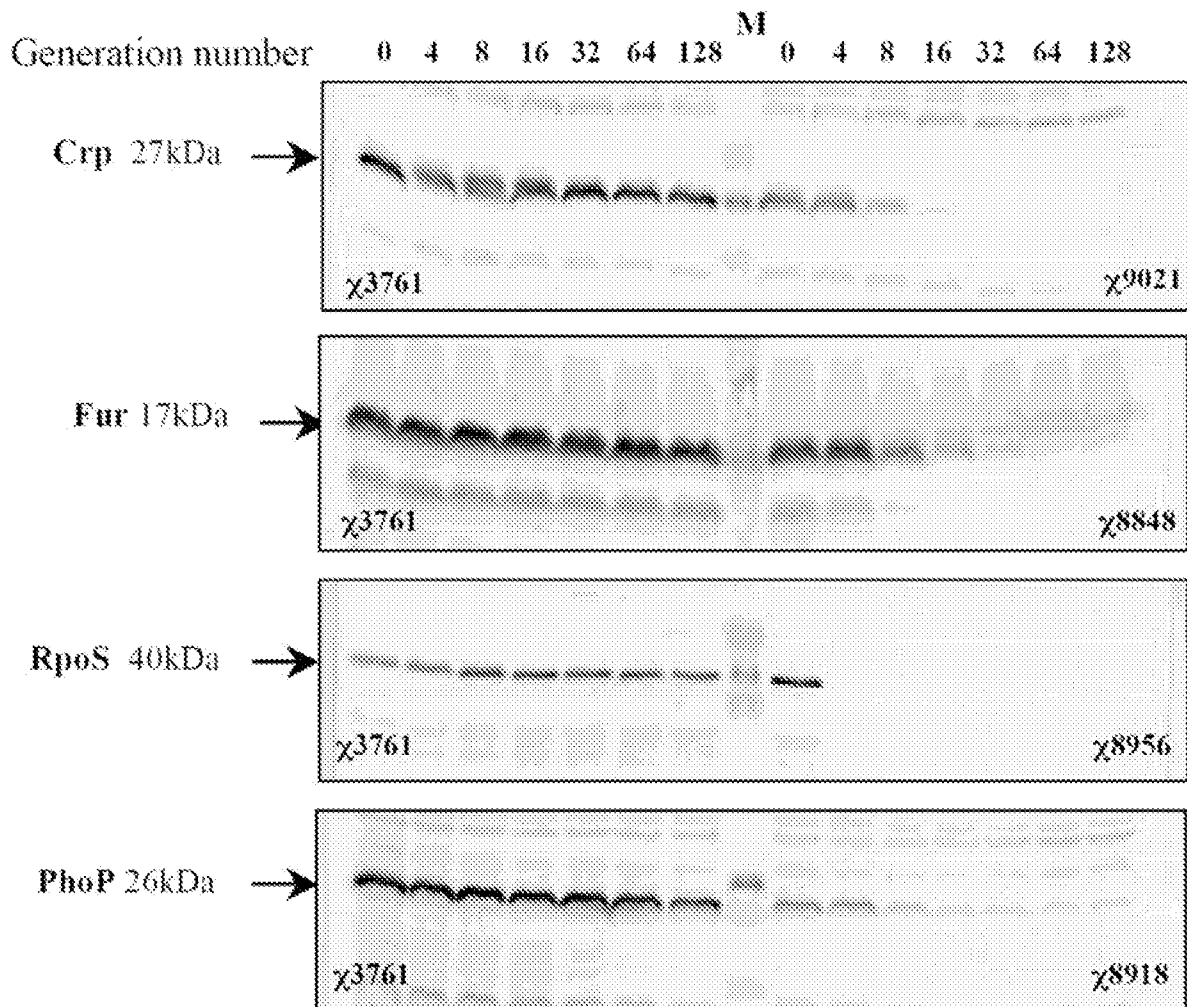
B $\Delta P_{phoPQ107}::TT\ araC\ P_{BAD}\ phoPQ$ **C** $\Delta P_{rpoS183}::TT\ araC\ P_{BAD}\ rpoS$ **D** $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$ **FIG. 25**

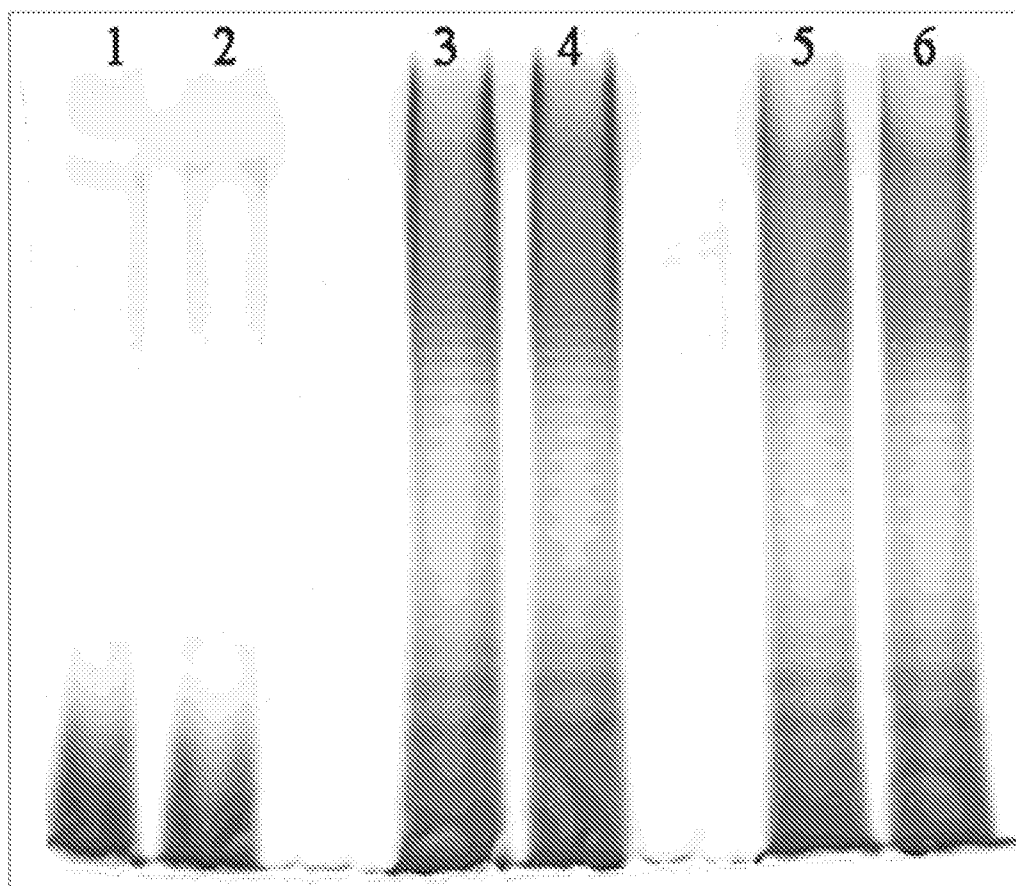
**FIG. 26**

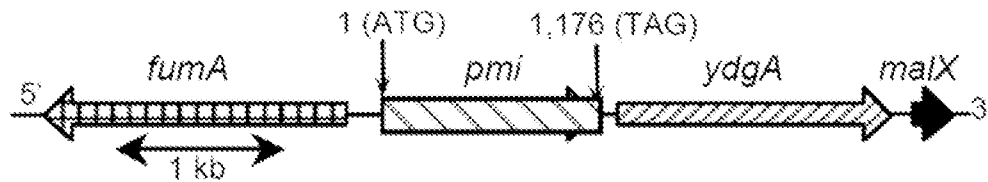
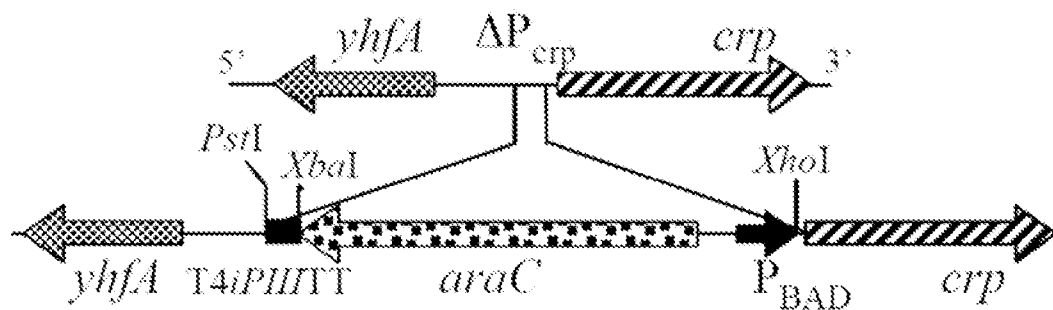
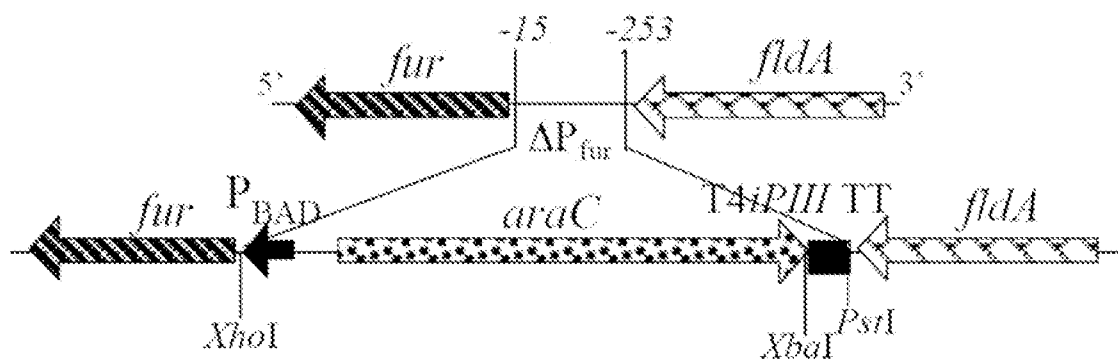
C**PhoP****No arabinose****0.2% arabinose****D****RpoS****No arabinose****0.2% arabinose****FIG. 26**

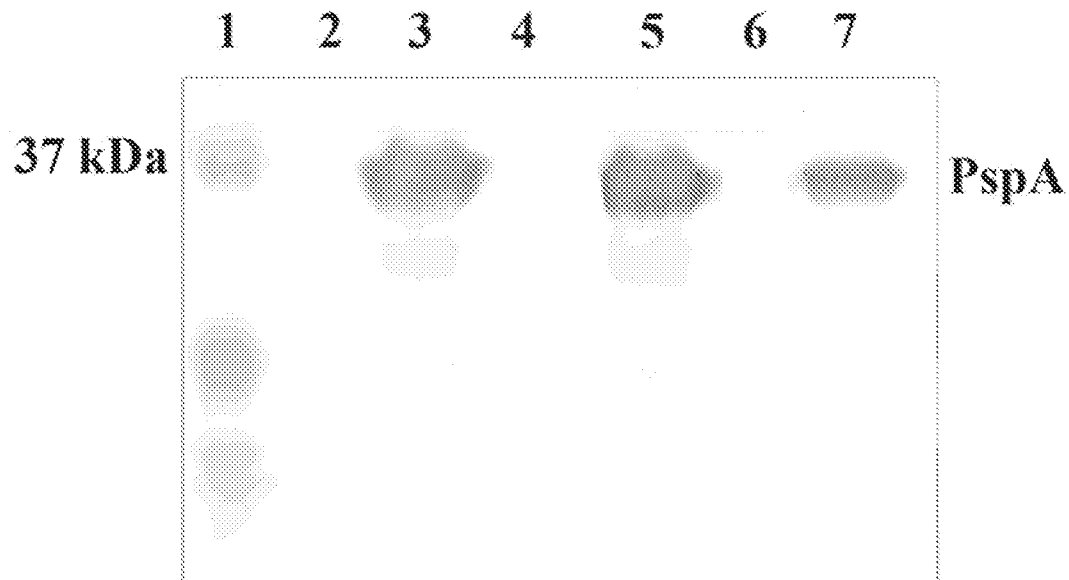
A***ΔaraBAD23*****B*****ΔaraE25*****FIG. 27**

**FIG. 28**

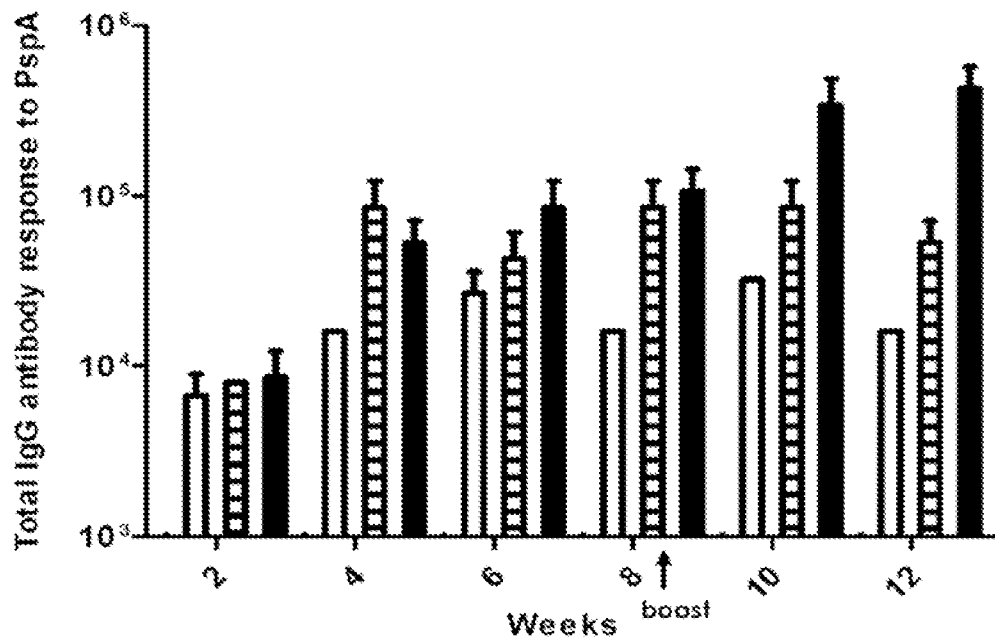
**FIG. 29**

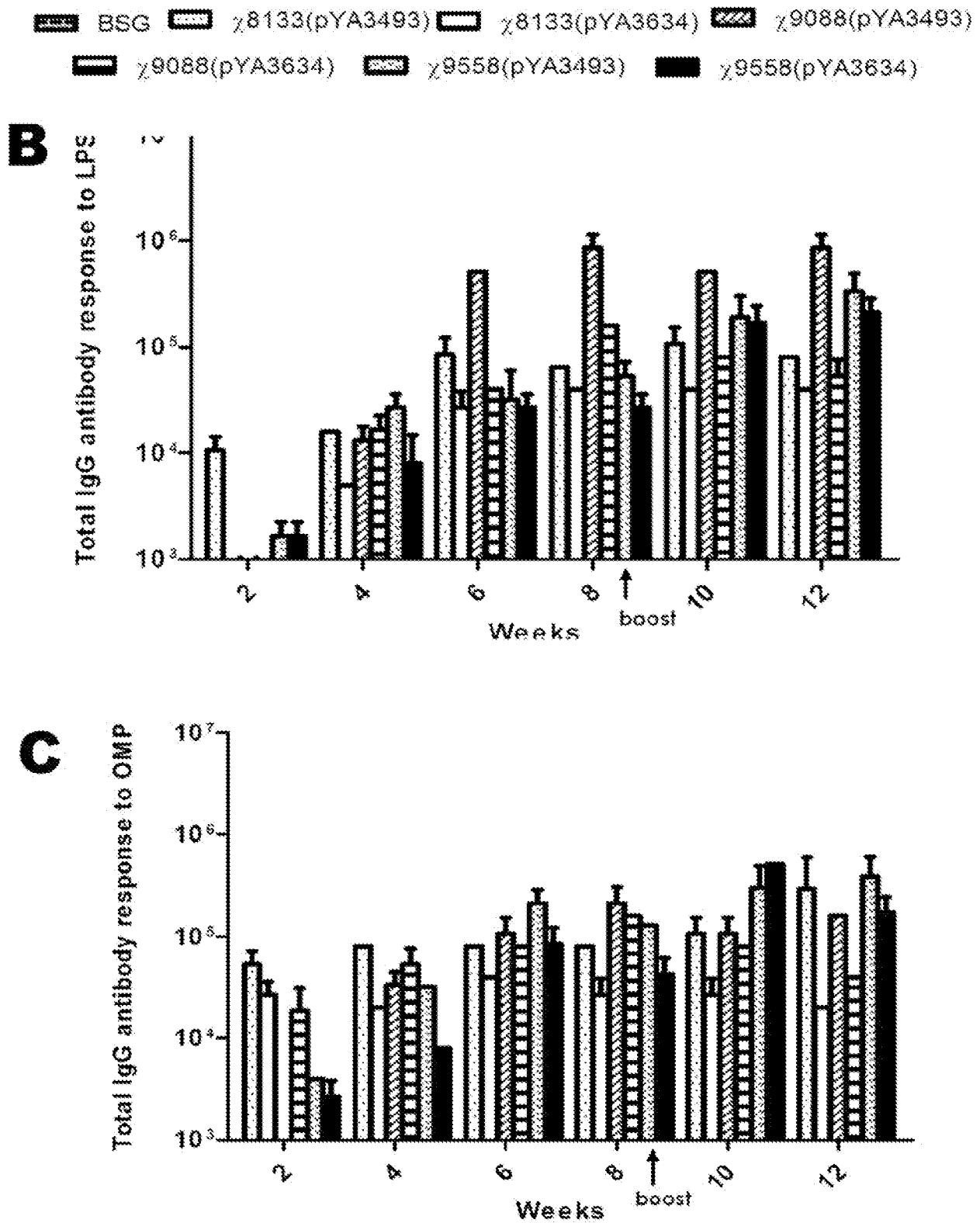
**FIG. 30**

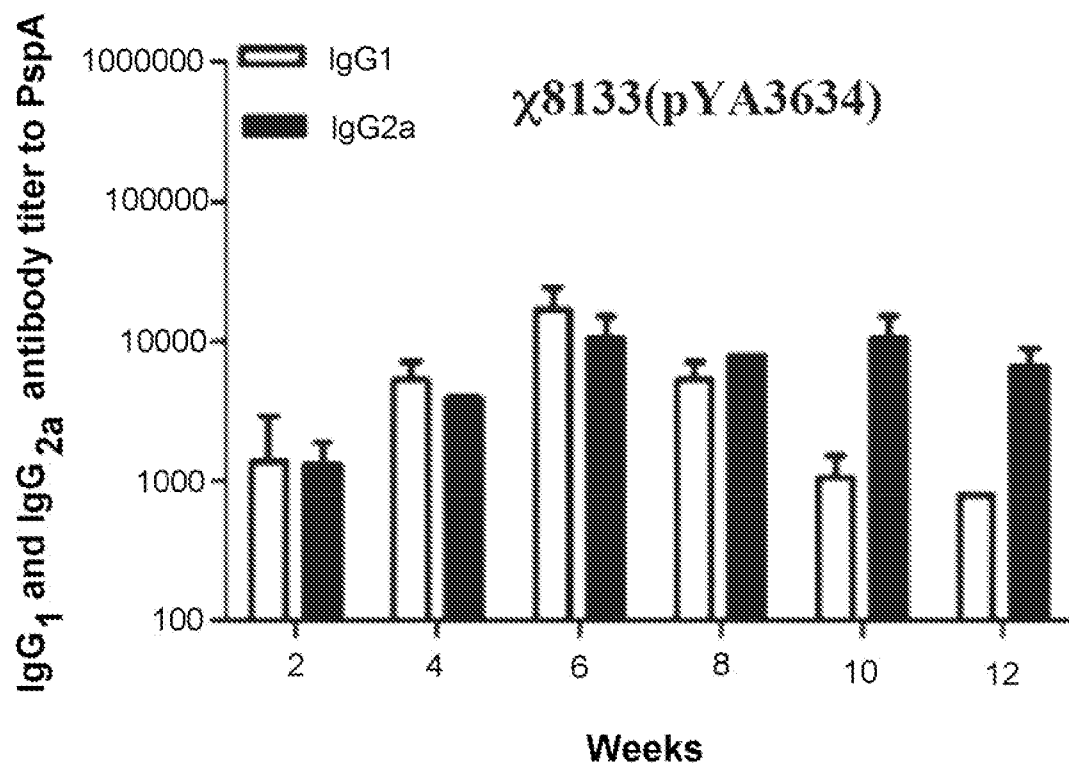
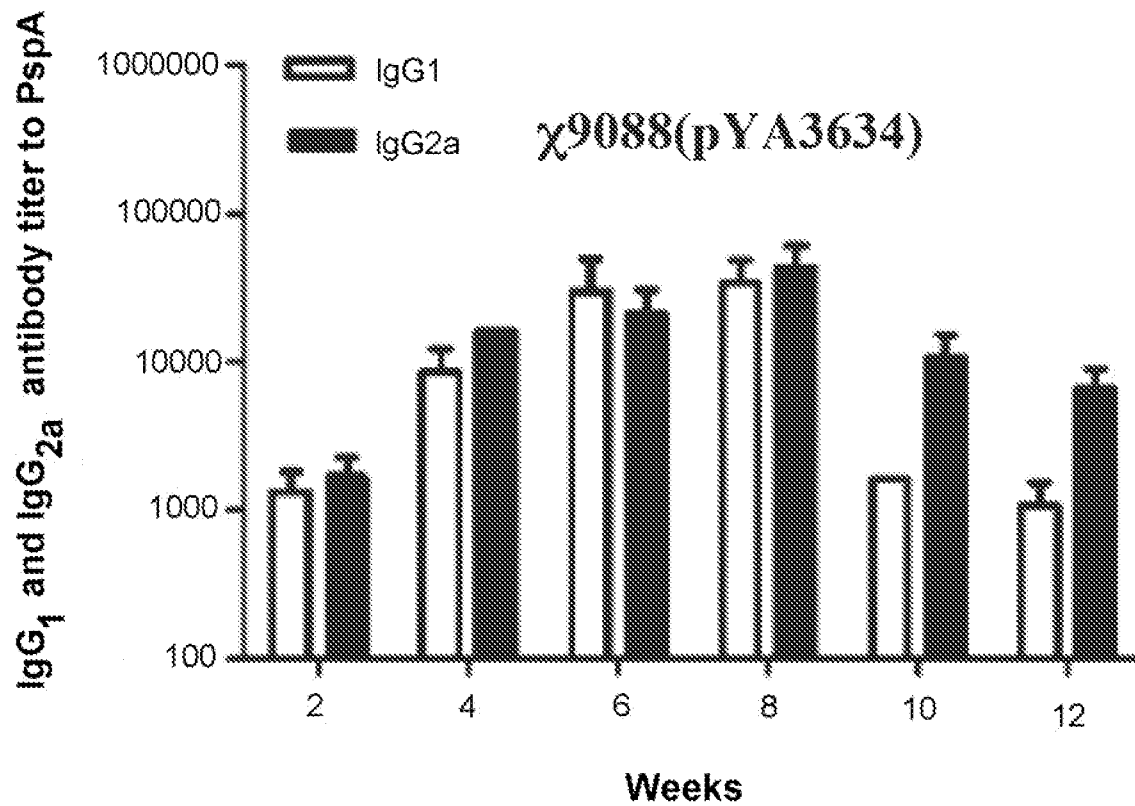
$\Delta pmi-2426$  $\Delta P_{crp527}::TT\ araC\ P_{BAD}\ crp$  $\Delta P_{fur33}::TT\ araC\ P_{BAD}\ fur$ **FIG. 31**

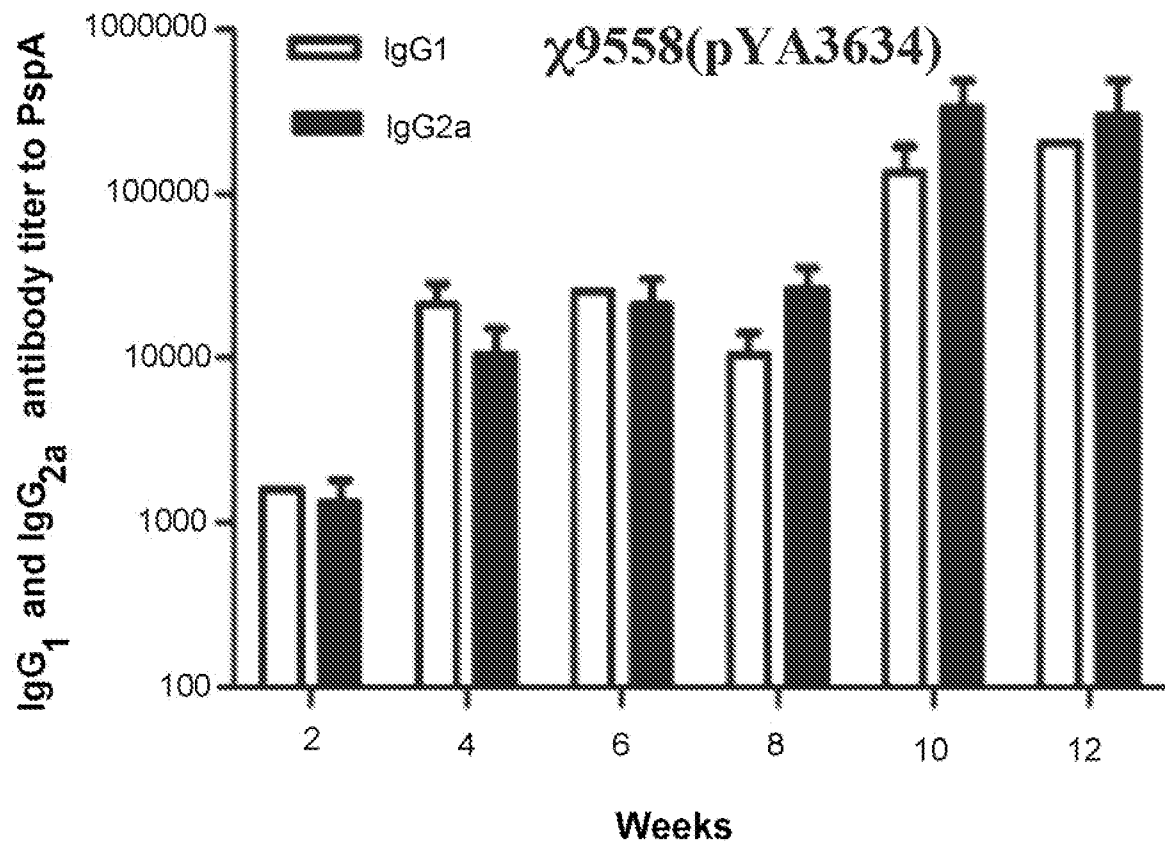
**FIG. 32**

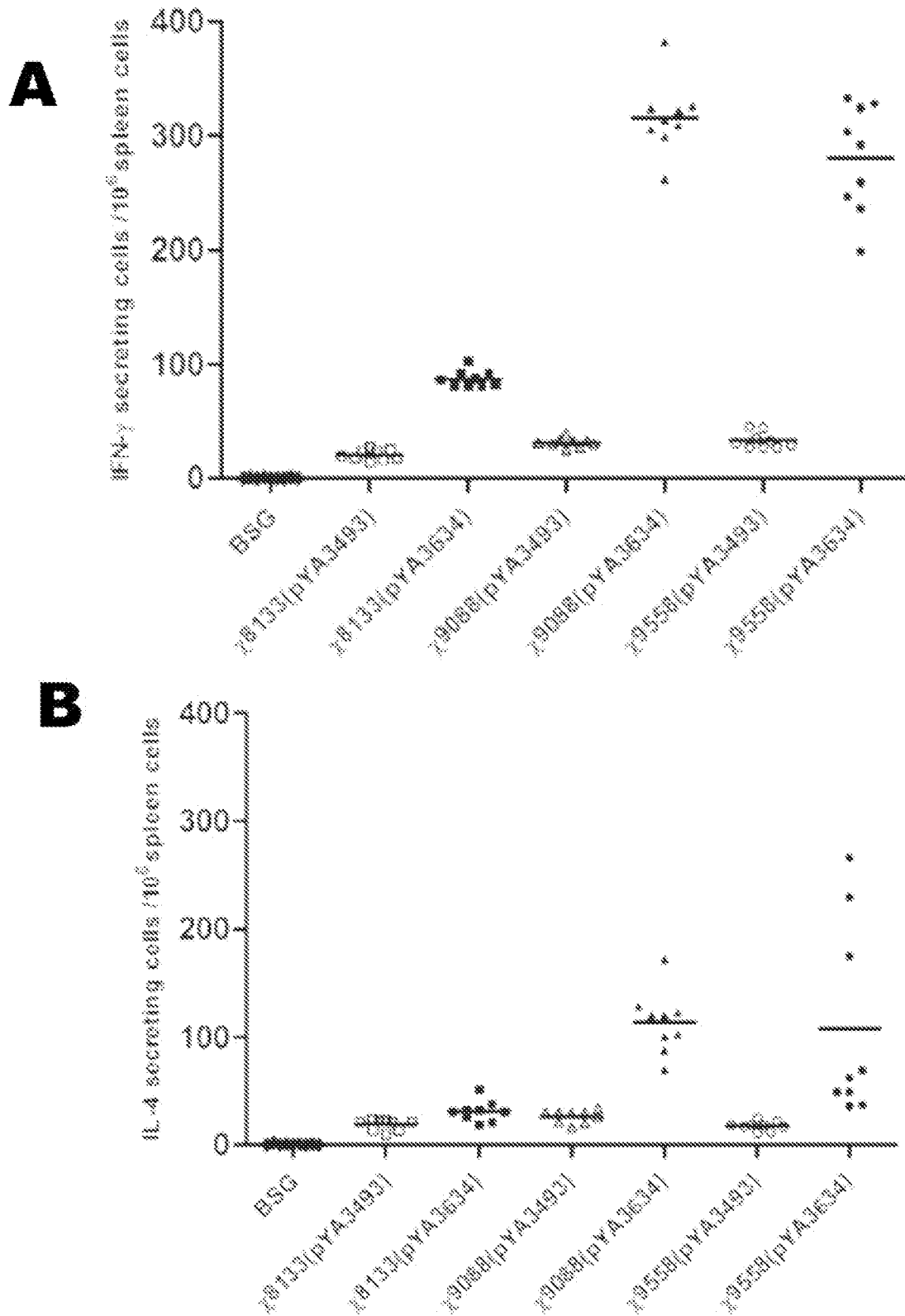
BSG χ 8133(pYA3493) χ 8133(pYA3634) χ 9088(pYA3493)
 χ 9088(pYA3634) χ 9558(pYA3493) χ 9558(pYA3634)

**FIG. 33A**

**FIG. 33**

A**B****FIG. 34**

**FIG. 34C**

**FIG. 35**

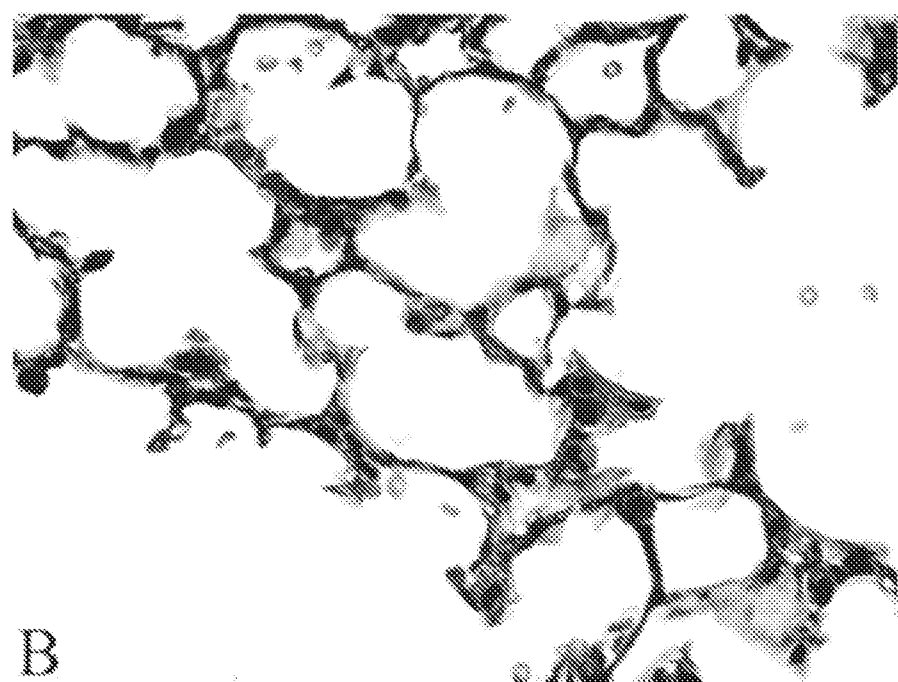
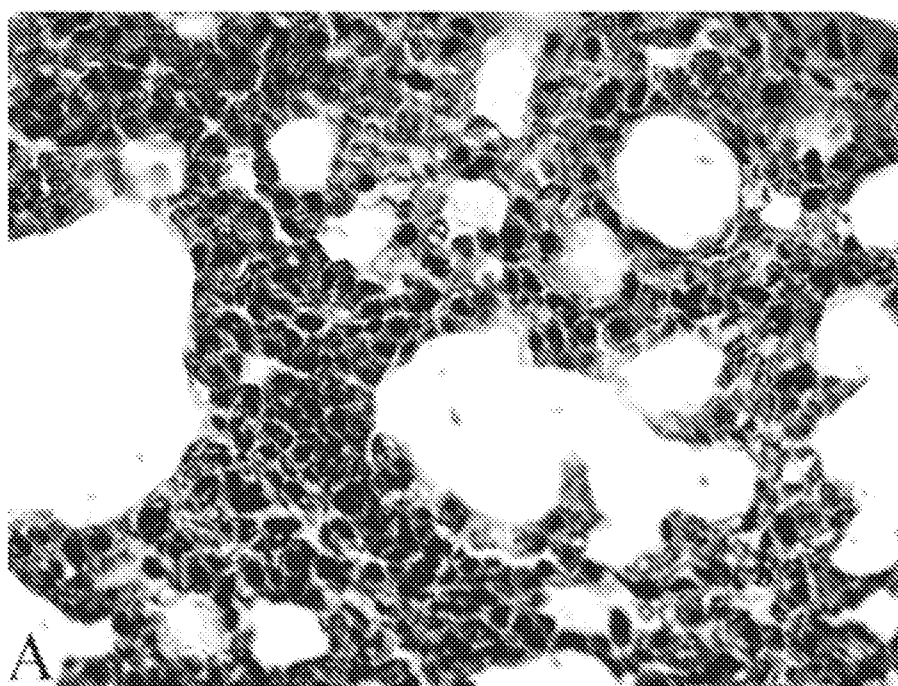
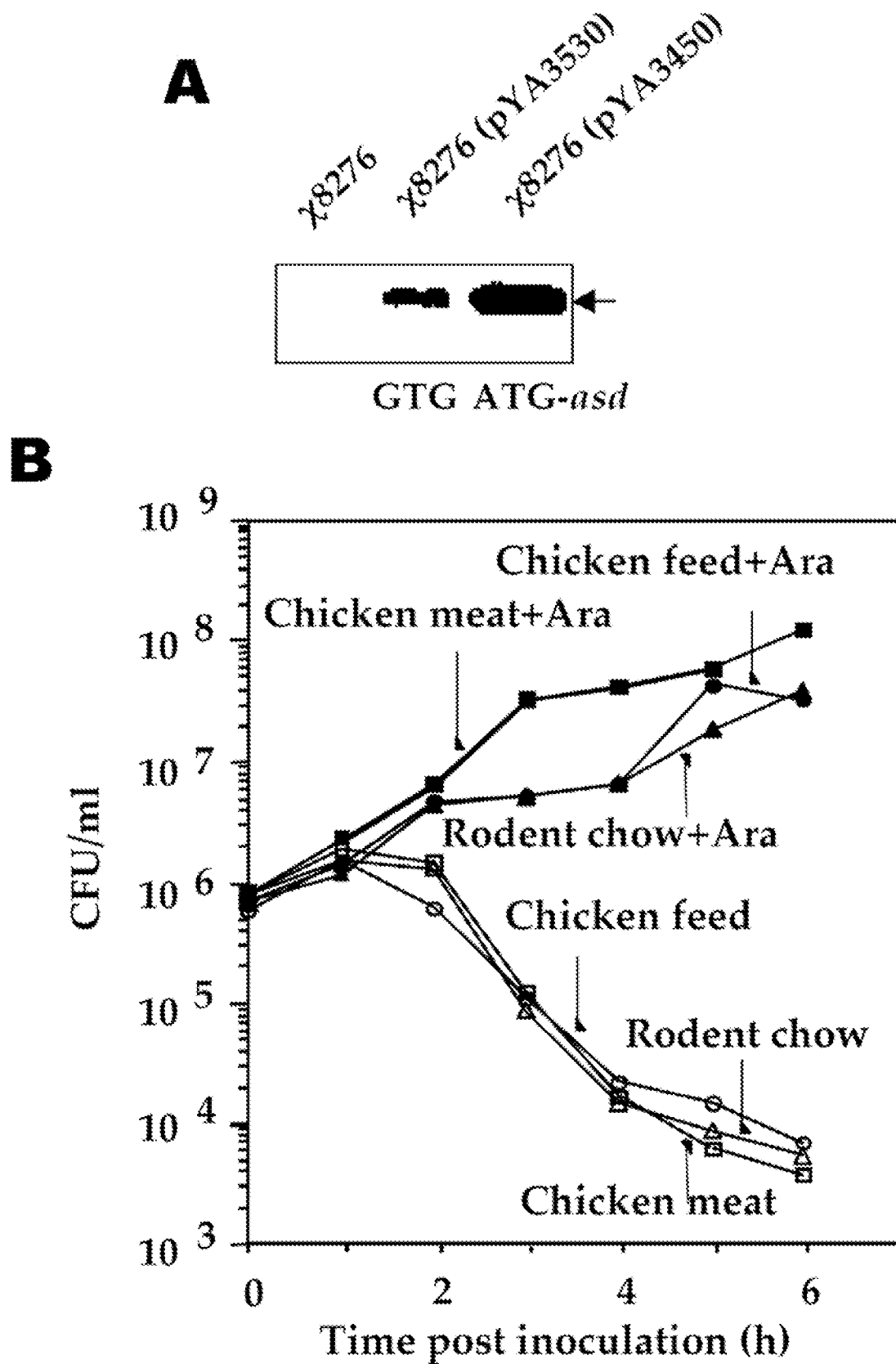
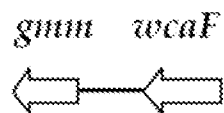
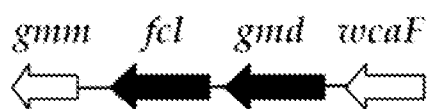
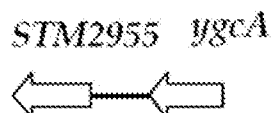
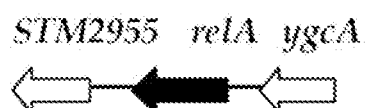
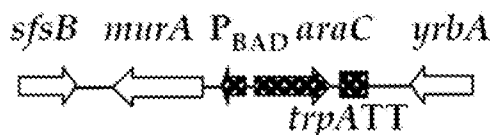
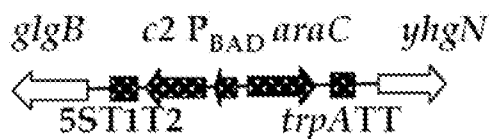


FIG. 36

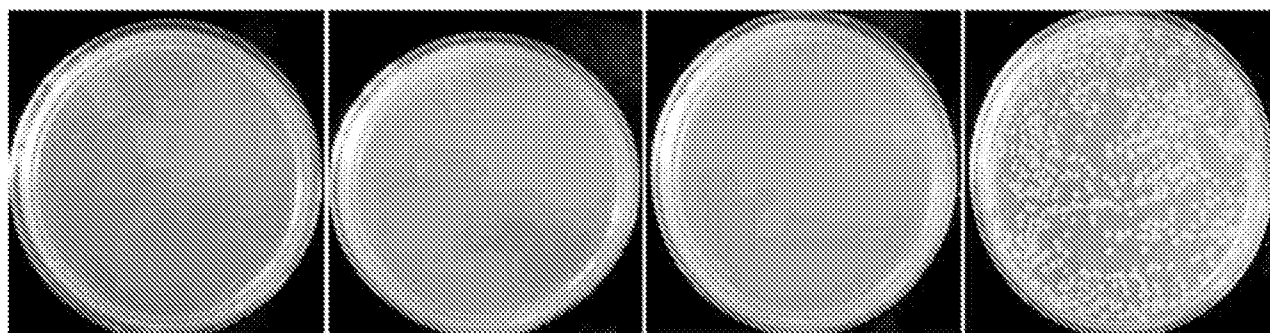
**FIG. 37**

Chromosomal regions
in *S. Typhimurium* UK-1Chromosomal mutations
in χ 8937

■ Deleted region

■ Inserted fragment

FIG. 38A

χ 8937

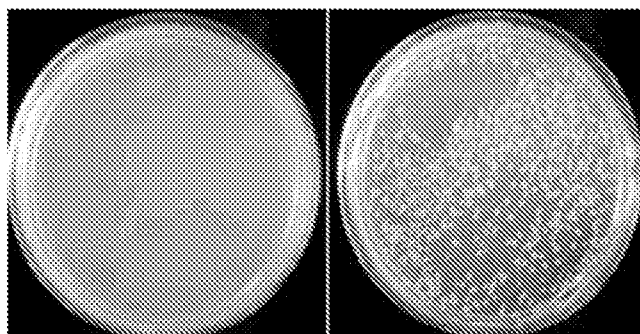
- Ara

+ Ara

+DAP

+DAP

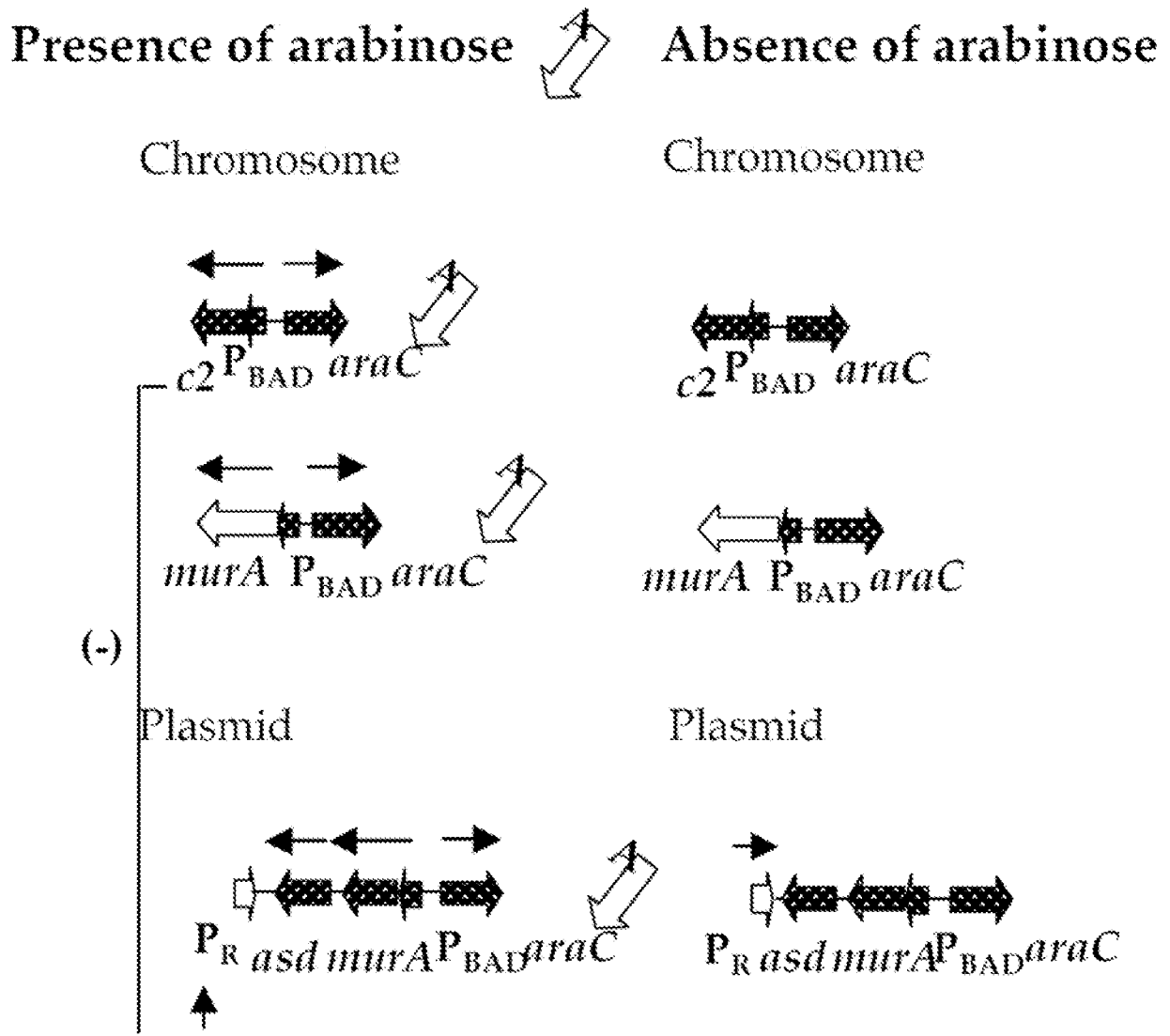
+Ara

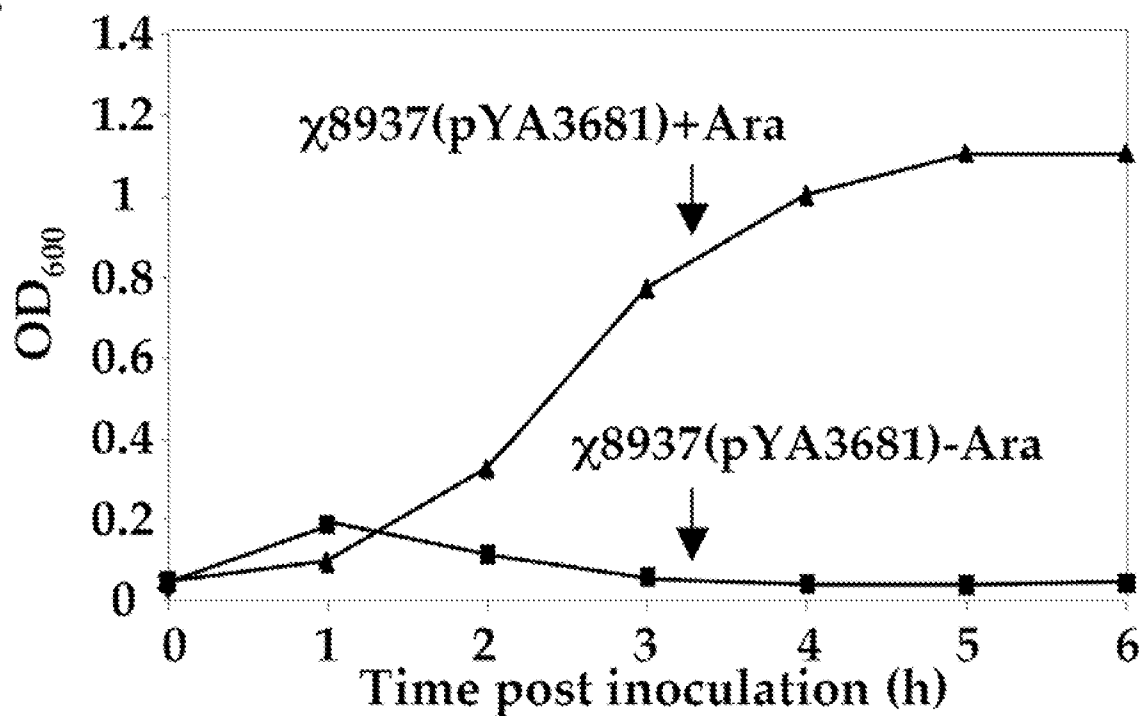
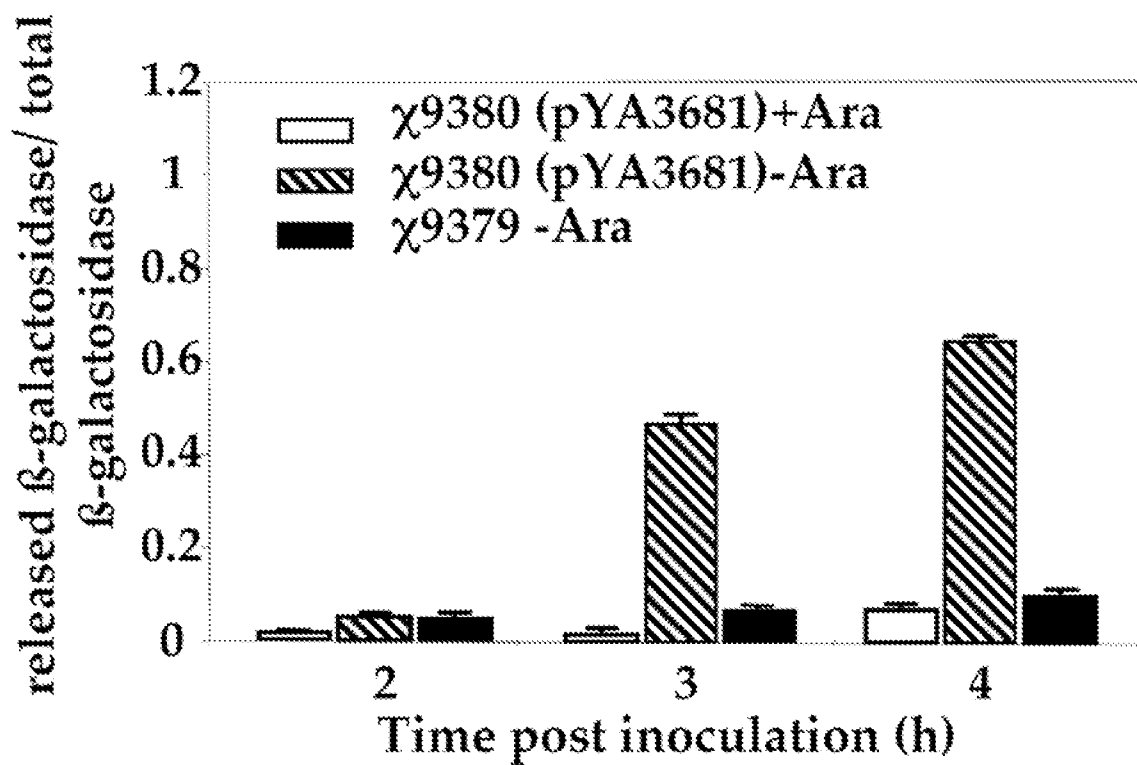
 χ 8937 (pYA3681)

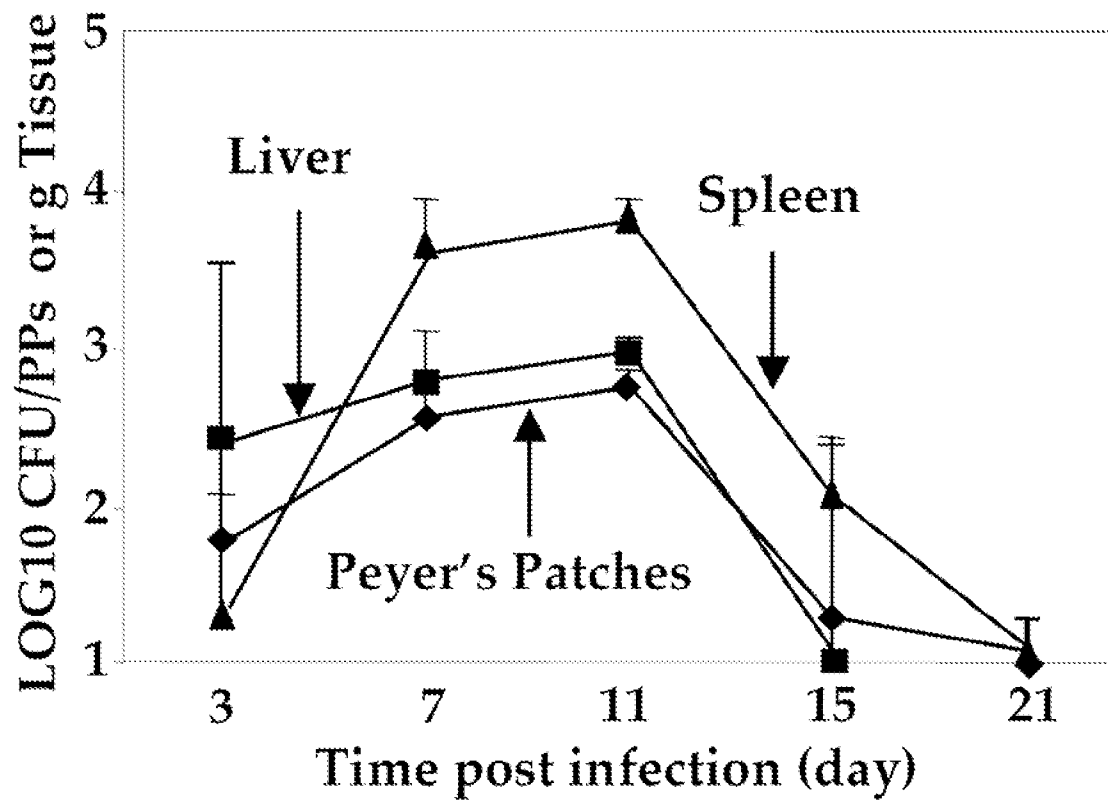
- Ara

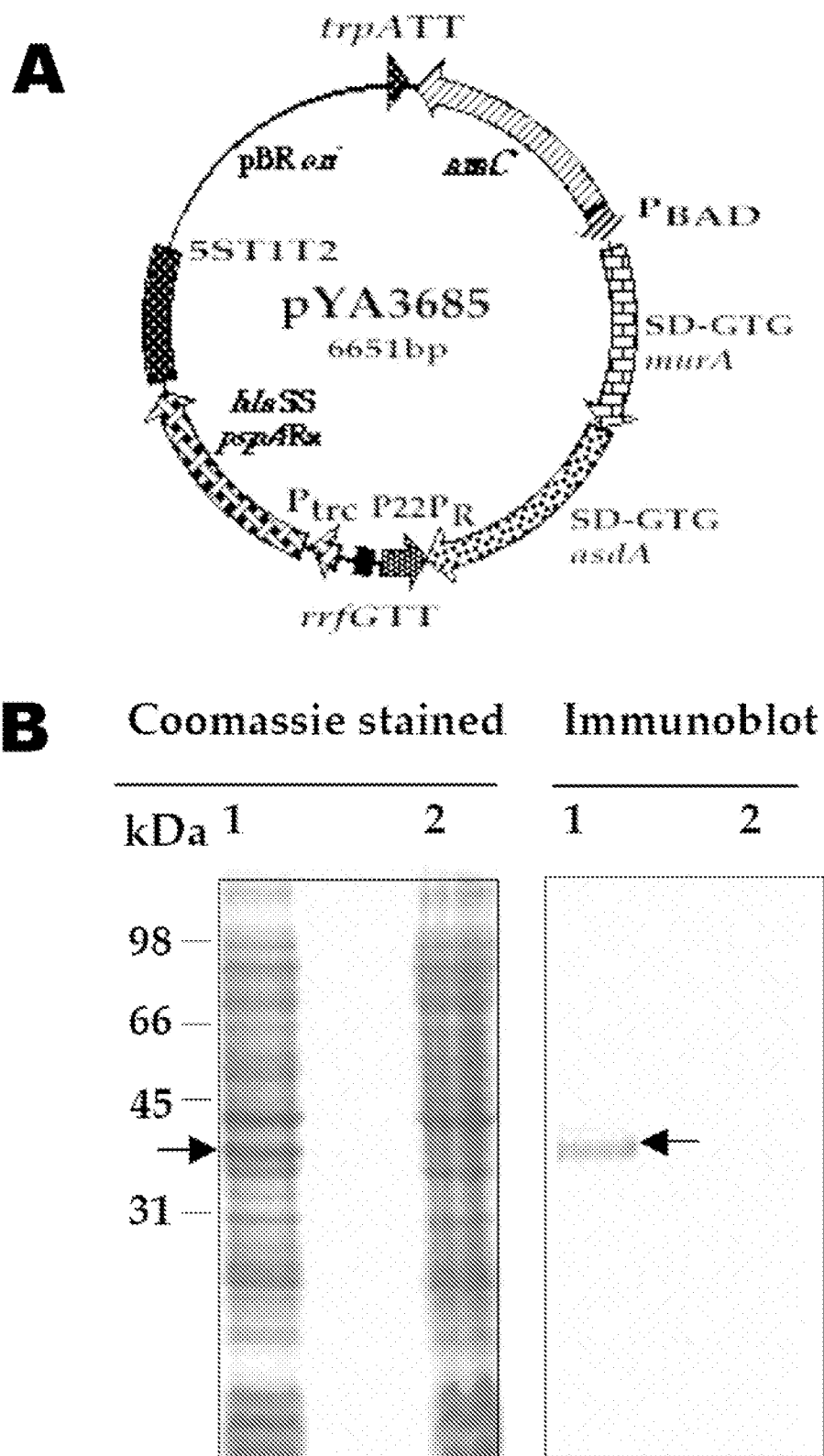
+ Ara

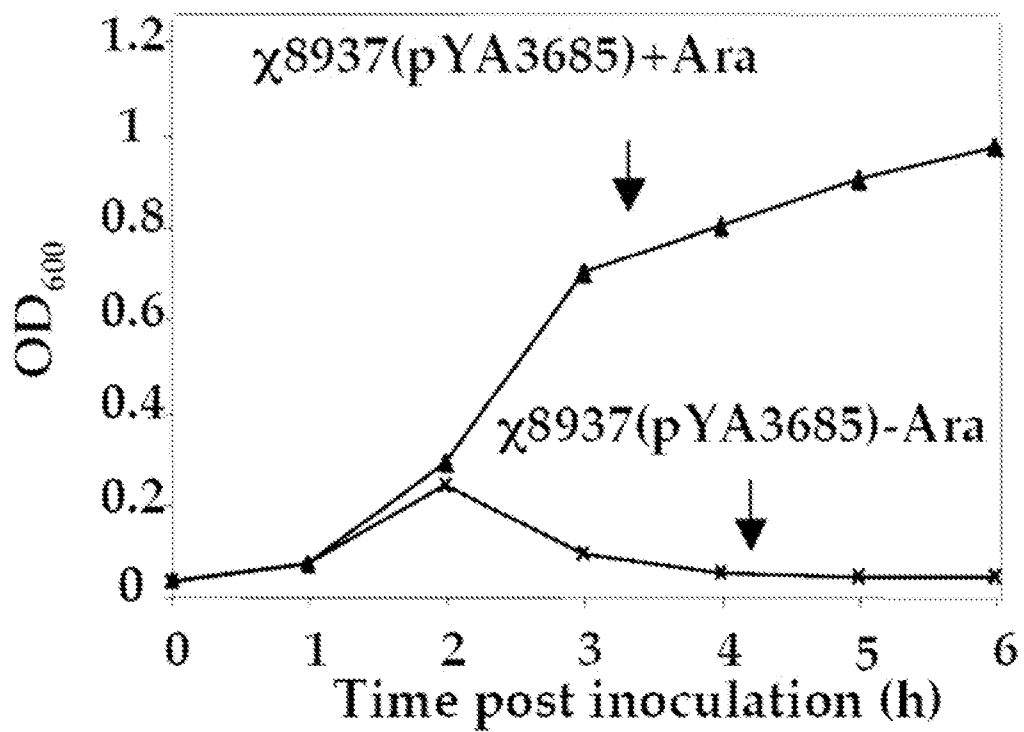
FIG. 38B

**FIG. 39**

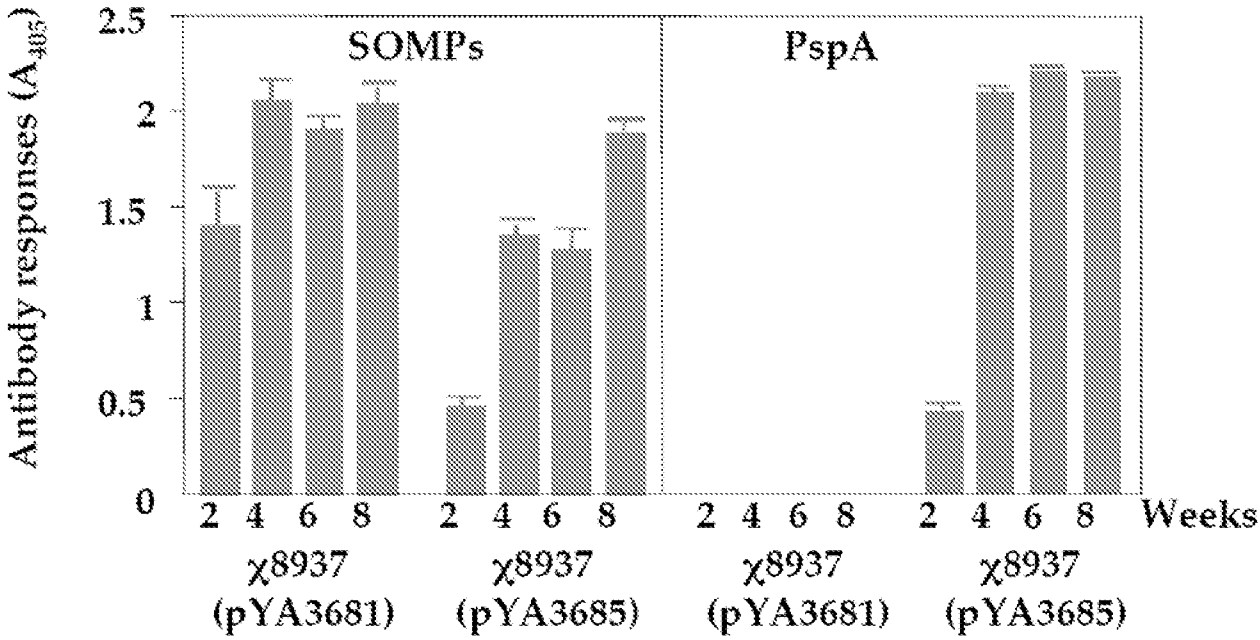
A**B****FIG. 40**

**FIG. 40C**

**FIG. 41**

**FIG. 41C**

A



B

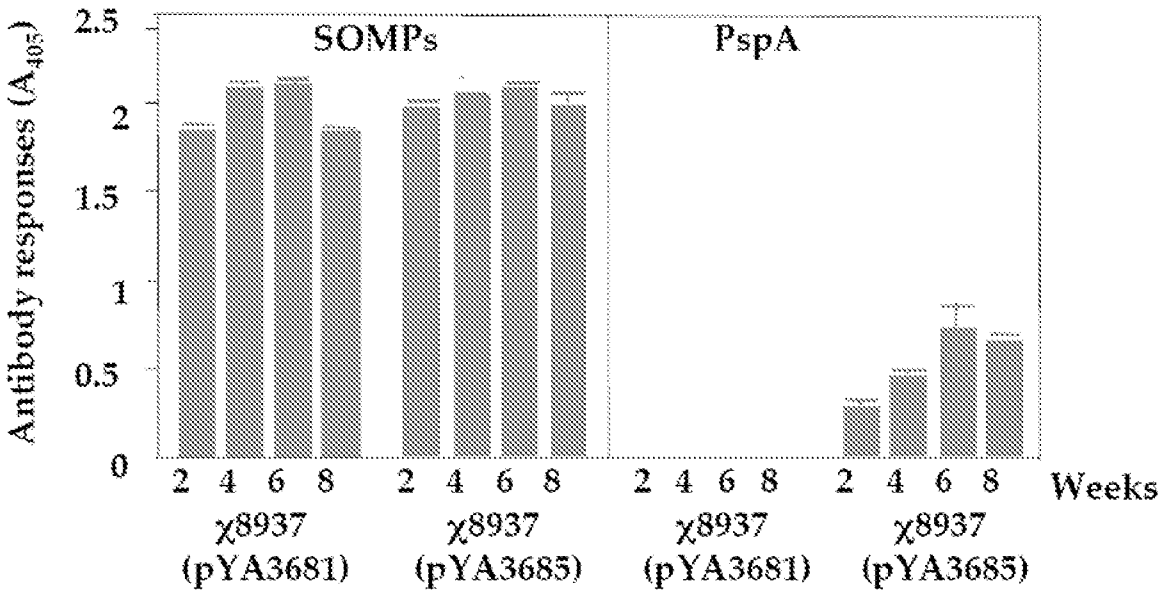
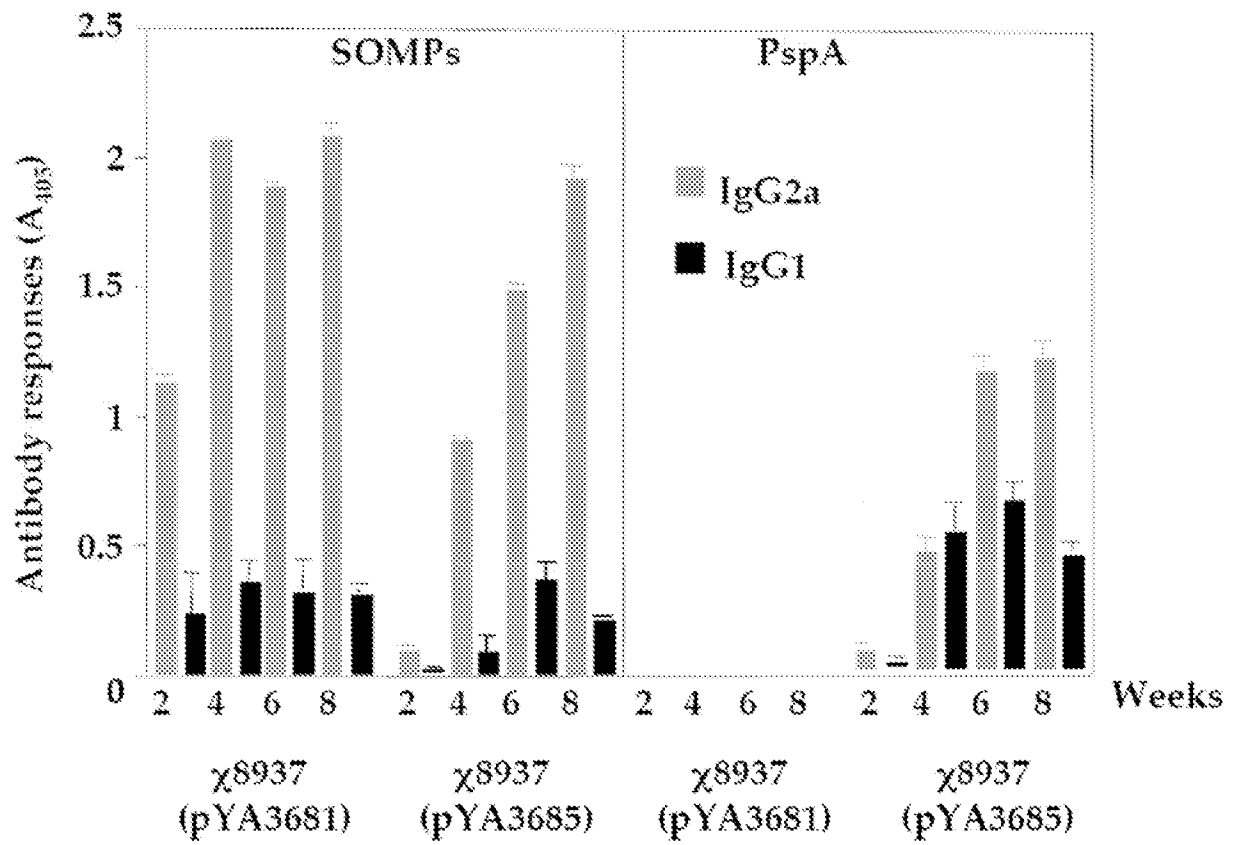


FIG. 42

**FIG. 43**