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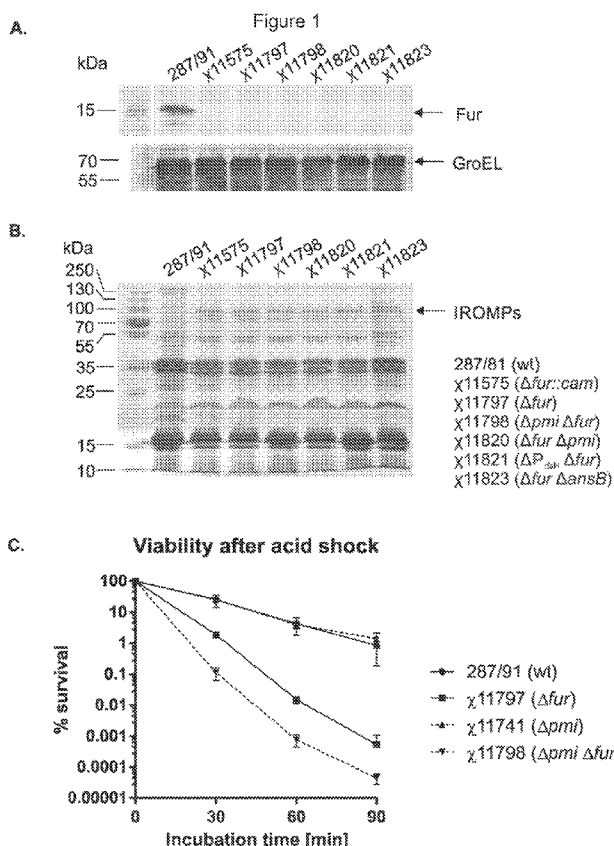
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[Continued on next page]

## (54) Title: LIVE SALMONELLA VACCINE AND METHODS TO PREVENT FOWL TYPHOID



(57) Abstract: We constructed *S. Gallinarum* strains deleted for the global regulatory gene *fur* (Fig. 1) and evaluated their virulence and protective efficacy in Rhode Island Red chicks and Brown Leghorn layers. The *fur* deletion mutant was a virulent and, when delivered orally to chicks, elicited excellent protection against lethal *S. Gallinarum* challenge. We also examined the effect of a *pmi* mutant and a combination of *fur* deletions with mutations in the *pmi* and *rfaH* genes, which affect O-antigen synthesis, and *ansB*, whose product inhibits host T cell responses. The Δ*Afur* Δ*pmi* and Δ*fur* Δ*ansB* double mutants were attenuated, but not protective when delivered orally to chicks. However, a Δ*pmi* Δ*fur* strain was substantially immunogenic when administered intramuscularly. Altogether our results show that the *fur* gene is essential for virulence of *S. Gallinarum* and the *fur* mutant is effective as a live recombinant vaccine against fowl typhoid.



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## **LIVE SALMONELLA VACCINE AND METHODS TO PREVENT FOWL TYPHOID**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

**[0001]** This application claims priority to U.S. Provisional Patent Application No. 61/987,820 filed on May 2, 2014.

### **STATEMENT OF FEDERALLY SPONSORED RESEARCH OF DEVELOPMENT**

**[0002]** This invention was made with government support under 0965511 awarded by The National Science Foundation. The government has certain rights in the invention.

### **BACKGROUND OF THE INVENTION**

**[0003]** *S. Gallinarum* causes fowl typhoid, recognized worldwide as a socially and economically important disease. It is a septicemic disease mainly affecting chickens and turkeys, although natural infections in a number of wild birds, including ducks, pheasants ostriches, peacocks and quail have been reported. Fowl typhoid has been essentially eradicated in many developed countries while it remains an important economic problem in many other areas of the world, including Tanzania, Zambia, Libya, Nigeria, and Morocco.

**[0004]** In Tanzania for example, it is the most important disease affecting commercial chickens, where it is a particularly important pathogen for commercial layers. Fowl typhoid is a significant economic problem in Mexico and Central and South America. In the countries listed above, many of which have a high ambient temperature, the possibilities of disease prevention by a combination of hygiene and housing improvements are limited.

**[0005]** There remains nothing in current data indicating the magnitude or economic consequences of *S. Gallinarum* infection. Currently, there is only one live vaccine in use for the prevention of fowl typhoid. In the 1950s, a rough (lacking the complete lipopolysaccharide (LPS) O-antigen) live vaccine, *S. Gallinarum* strain 9R, was developed. LPS is a unique lipid found in the outer membrane of all gram negative bacteria, such as *Salmonella*. LPS is composed of a hydrophobic domain called lipid A, a nonrepeating “core” oligosaccharide and a distal polysaccharide called O-antigen. While this vaccine is protective, there are several drawbacks.

[0006] First, the 9R vaccine possesses an uncharacterized attenuation lesion(s) and, despite its apparent safety, the risk of reversion to wildtype is unknown. The second drawback is that it is administered subcutaneously to birds at 6 weeks of age, followed by two booster inoculations at 14 and 16 weeks of age, meaning that every immunized bird must be physically handled three times and materials for injection (needles, syringes, etc) must be available, adding to the expense of vaccination.

[0007] Therefore, improvements in a vaccine for fowl typhoid and methods for providing a new fowl typhoid vaccine are desirable.

### SUMMARY OF THE INVENTION

[0008] The disclosure herein relates to live *Salmonella Gallinarum* vaccine with defined mutations that can be applied orally or by intramuscular injection to poultry for the prevention of fowl typhoid.

[0009] While *Salmonella* fur deletions have been noted as being attenuated, to varying degrees, the general consensus of previous work is that they are not immunogenic in healthy animals. This work represents the first time that a fur mutant has been shown to be both highly attenuated and substantially immunogenic.

[0010] In an embodiment, a strain of *S. Gallinarum* bacteria includes a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein and wherein the strain is attenuated and immunogenic in fowl.

[0011] In an embodiment, a strain of *S. Gallinarum* bacteria includes a mutation that prohibits the strain from synthesizing phosphomannose isomerase and wherein the strain is attenuated and immunogenic in fowl.

[0012] In a further embodiment, a vaccine for inoculation against *S. Gallinarum* infection includes a pharmaceutically acceptable carrier and a strain of *S. Gallinarum* that further includes a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein.

[0013] In another embodiment, a method of making a vaccine includes providing a strain of *S. Gallinarum* including a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein, wherein the strain is attenuated and immunogenic and incorporating the strain into a pharmaceutically acceptable carrier.

[0014] In another embodiment, a method of vaccinating fowl against typhoid includes the administration of a strain of *S. Gallinarum*, which includes a mutation that prohibits

the strain from synthesizing a functional ferric uptake regulator protein, in a pharmaceutically acceptable carrier.

**[0015]** In yet another embodiment, a method of vaccinating fowl against typhoid includes the administration of a strain of *S. Gallinarum*, which includes a mutation that prohibits the strain from synthesizing phosphomannose isomerase, in a pharmaceutically acceptable carrier.

**[0016]** These and other aspects of the invention will be apparent upon reference to the following detailed description and figures. To that end, any patent and other documents cited herein are hereby incorporated by reference in their entirety.

### BRIEF DESCRIPTION OF THE DRAWINGS

**[0017]** Figure 1 illustrates phenotype characterization of *S. Gallinarum*  $\Delta fur$  mutants.

**A.** Fur production in *S. Gallinarum* wild-type (287/91),  $\Delta fur-453::cam$  ( $\chi 11575$ ) and  $\Delta fur-712$  vaccine strains ( $\chi 11798$ ,  $\chi 11820$ ,  $\chi 11821$ ,  $\chi 11823$ ). Whole-cell lysates were obtained from overnight cultures, electrophoresed on a 12% SDS-PAGE gel, transferred onto nitrocellulose and probed with anti-Fur serum. The blot was also probed with anti-GroEL antibodies to serve as a loading control. **B.** IROMPs production in *S. Gallinarum* vaccine strains. OMPs were obtained by Sarkosyl-extraction from overnight cultures, electrophoresed on a 10% SDS-PAGE gel and stained with Coomassie blue. **C.** Effect of acid shock on viability of *S. Gallinarum* *fur* mutants. Strains 287/91 (wt),  $\chi 11741$  ( $\Delta pmi-2426$ ),  $\chi 11797$  ( $\Delta fur-712$ ) and  $\chi 11798$  ( $\Delta pmi-2426 \Delta fur-712$ ) were grown in LB to early logarithmic phase, washed in E medium (pH 7.0) and then challenged with E medium (pH 3.0). Survival was monitored by plating samples on LB agar. The data shown are means and SEM from four independent experiments. A statistical analysis was carried out using two-way ANOVA followed by Tukey's multiple comparison test. All possible pairs of data within each time point except 287/91 vs.  $\chi 11741$  were significantly different ( $P < 0.01$ ).

**[0018]** Figure 2 Illustrates a Colonization of spleen and liver in birds that survived the challenge with wild-type *S. Gallinarum*. Survivors from Expt. 3 (Table 2) were euthanized nineteen days post infection and spleens and livers were collected to recover viable *S. Gallinarum* from each tissue. Organs were homogenized, diluted and plated on LB agar. Negative samples were additionally enriched using RV broth and plated on SS agar.

### DETAILED DESCRIPTION OF THE INVENTION

[0019] *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S. Gallinarum*) is a host-adapted pathogen that causes fowl typhoid – an important disease of poultry. Fowl typhoid is a septicemic disease with a typically short course and significant morbidity and mortality, which can reach as high as 100%. The disease occurs primarily in mature flocks, although birds of all ages may be infected. Resistance to *S. Gallinarum* also varies with the species and breed. Among chickens, heavier breeds such as Rhode Island Red are more susceptible than lighter breeds such as white leghorns. Fowl typhoid has been eradicated from commercial poultry in many developed countries including the USA and Canada through isolation and removal of contaminated flocks and implementing biosecurity and hygiene management.

[0020] Nevertheless, it still constitutes a considerable economic problem for poultry growers, both small backyard farmers and larger commercial operations in many parts of the world such as Central and South America, Africa and Asia, where control measures are insufficient and the climate favors spread of *S. Gallinarum* in the environment.

[0021] We introduce a live attenuated *S. Gallinarum* vaccine for fowl typhoid that can be applied orally, by coarse spray or by injection in one or two doses. Fowl typhoid is a devastating disease of poultry caused by *Salmonella enterica* serovar Gallinarum. This disease is currently controlled in the developed world by culling of diseased flocks, good husbandry practices and vaccination. However, in the developing world, this disease is still rampant and accounts for economic losses ranging from 10% of all poultry death due to disease and reduced egg output in survivors. The current vaccine for fowl typhoid is an injectable live *Salmonella Gallinarum* vaccine that requires 3 doses.

[0022] Fur (ferric uptake regulator) acts as a repressor of many genes whose products are involved in iron, zinc and manganese acquisition and uptake. One notable class of Fur-regulated proteins is the iron regulated outer membrane proteins (IROMPs), which serve as receptors for iron siderophore complexes. The genes for these proteins are repressed by Fur when iron is abundant and are up regulated when iron is limiting. Animal hosts restrict iron from invading bacteria during infection, a phenomenon known as “nutritional immunity”. Thus, mechanisms for iron acquisition are important to the pathogenicity of many microorganisms including *Salmonella* sp.

[0023] Fur can also act as a transcriptional activator by enhancing RNAP recruitment, regulating production of small RNAs or functioning as an antirepressor. In *Salmonella*, Fur also modulates expression of genes involved in acid shock and adaptation and oxidative stress resistance. It further plays a role in regulation of the *Salmonella* pathogenicity island 1 (SPI1) genes (e.g. *hilA* and *hilD*) necessary for invasion. *S. Typhimurium* strains with an arabinoseregulated *fur* genotype (*fur* expressed in vitro in presence of arabinose, not expressed in vivo where arabinose is not available) were partially attenuated and substantially immunogenic in mice. The same study also showed that the attenuation of *S. Typhimurium* arabinose regulated *fur* mutants is correlated with the level of the Fur expression.

[0024] Furthermore, an *S. Enteritidis*  $\Delta fur$  strain was partially attenuated and immunization of mice with this strain resulted in decrease of bacterial load in systemic organs after challenge with the wildtype strain. A *fur* deletion was also employed to improve the safety of a *S. Typhimurium*  $\Delta ssaV$  mutant. The  $\Delta ssaV \Delta fur$  double mutant was safe and immunogenic in immunocompromised mice. The *pmi* gene encodes phosphomannose isomerase that facilitates the interconversion of fructose6phosphate into mannose6phosphate, which is subsequently converted into GDPmannose – a substrate for incorporation into LPS O-antigen side chains. Thus  $\Delta pmi$  mutants cannot produce O-antigen unless an exogenous source of mannose is present.

[0025] In the context of a vaccine,  $\Delta pmi$  strains are grown in vitro in the presence of mannose and synthesize a complete O-antigen, a requirement for optimal host colonization. The O-antigen is subsequently lost after several generations of growth in animal tissues, which are devoid of free nonphosphorylated mannose. *S. Typhimurium* *pmi* mutants are substantially immunogenic and partially attenuated in mice.

[0026] The vaccine described herein is comprised of *S. Gallinarum* strain(s) with deletions in the global regulatory gene *fur* and/or *pmi*. The *fur* mutant is fully attenuated and protective when administered orally or by injection. This was demonstrated in two chicken breeds: Rhode Island Red and brown leghorn. A *fur pmi* double mutant is protective when administered by intramuscular injection into brown leghorns.

[0027] Vaccination of chickens seems to be the most effective strategy to control fowl typhoid in developing countries where *S. Gallinarum* is endemic. The rough *S. Gallinarum* 9R strain is the most widely used vaccine. While somewhat effective, a number of drawbacks have been noted: variability in protective efficacy between breeds;

persistence in immunized chickens leading to transmission through eggs and residual virulence in some breeds. Moreover, the means of attenuation is not well defined genetically. Until recently, the attenuation of this strain was believed to be due solely to a defect in lipopolysaccharide (LPS) synthesis. However, recent comparative analysis of its proteome and transcriptome has showed that 9R may also be impaired for the regulation of several virulence factors.

**[0028]** In our efforts to develop safe and efficacious fowl typhoid vaccine candidates we have been examining mutations in global virulence regulators and genes that affect O-antigen synthesis, with emphasis on genes required for virulence in *S. Typhimurium*. For example, modification or deletion of the global regulator gene *crp* in *S. Gallinarum* results in a strain that is safe and efficacious against challenge with virulent *S. Gallinarum*.

**[0029]** Conversely, mutations in *rfc* (*wzy*), required for complete O-antigen synthesis, are attenuating in *S. Typhimurium*, but have no effect on the virulence of *S. Gallinarum* when delivered by the oral route. In addition, an arabinose-regulated *rfaH* construction that results in arabinose-regulated O-antigen synthesis was partially attenuating in *S. Typhimurium*, but was not attenuating in *S. Gallinarum*. These results demonstrate that it is not possible to make accurate predictions regarding the virulence and immunogenicity of *S. Gallinarum* mutants in chickens based on *S. Typhimurium* mutant data in mice. We decided to expand our approach and explore additional genes involved in global regulation or O-antigen synthesis.

**[0030]** Fur (ferric uptake regulator) acts as a repressor of many genes whose products are involved in iron, zinc and manganese acquisition and uptake. One notable class of Fur-regulated proteins is the iron-regulated outer membrane proteins (IROMPs), which serve as receptors for iron-siderophore complexes. The genes for these proteins are repressed by Fur when iron is abundant and are up regulated when iron is limiting. Animal hosts restrict iron from invading bacteria during infection, a phenomenon known as “nutritional immunity”. Thus, mechanisms for iron acquisition are crucial to the pathogenicity of many microorganisms including *Salmonella* sp. Fur can also act as a transcriptional activator by enhancing RNAP recruitment, regulating production of small RNAs or functioning as an antirepressor. In *Salmonella*, Fur also modulates expression of genes involved in acid shock and adaptation and oxidative stress resistance and it

plays a role in regulation of the Salmonella pathogenicity island 1 (SPI-1) genes (e.g. *hilA* and *hilD*) necessary for invasion.

**[0031]** *S. Typhimurium* strains with an arabinose-regulated *fur* genotype (*fur* expressed in vitro in presence of arabinose, not expressed in vivo where arabinose is not available) were partially attenuated and substantially immunogenic in mice. The same study also showed that the attenuation of *S. Typhimurium* arabinose-regulated *fur* mutants is correlated with the level of the *fur* expression. Furthermore, an *S. Enteritidis*  $\Delta fur$  strain was attenuated and immunization of mice with this strain resulted in decrease of bacterial load in systemic organs after challenge with the wild-type strain. A *fur* deletion was also employed to improve the safety of a *S. Typhimurium*  $\Delta$ ssaV mutant. The  $\Delta$ ssaV  $\Delta fur$  double mutant was safe and immunogenic in immunocompromised mice.

**[0032]** The *pmi* gene encodes phosphomannose isomerase that facilitates the interconversion of fructose-6-phosphate into mannose-6-phosphate, which is subsequently converted into GDP-mannose – a substrate for incorporation into LPS O-antigen side chains. Thus  $\Delta pmi$  mutants cannot produce O-antigen unless an exogenous source of mannose is present. In the context of a vaccine,  $\Delta pmi$  strains are grown in vitro in the presence of mannose and synthesize a complete O-antigen, a requirement for optimal host colonization. The O-antigen is subsequently lost after several generations of growth in animal tissues, which are devoid of free non-phosphorylated mannose. *S. Typhimurium* *pmi* mutants are substantially immunogenic and partially attenuated in mice.

**[0033]** The primary focus of this work is to evaluate the virulence and immunogenicity of *S. Gallinarum* strains with deletions in the global regulatory gene *fur* and/or in *pmi*. We also examined the impact of the *fur* deletion in combination with several other mutations. Strains were screened for virulence and protective efficacy in two chicken breeds: Rhode Island Red and brown leghorn, which are differently susceptible to fowl typhoid. Our results showed that immunization with an *S. Gallinarum* *fur* mutant provided excellent protection against challenge with virulent *S. Gallinarum* in both breeds.

#### **Materials and methods**

**[0034]** **Bacterial strains, plasmids, media and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1.

**Table 1.** Bacterial strains and plasmids used in this study.

| Name                                | Relevant characteristics                                                                                                                                                                                                             | Source       |
|-------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| <b><i>E. coli</i> strains</b>       |                                                                                                                                                                                                                                      |              |
| $\chi 7213$                         | <i>thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1</i><br>RP4-2-Tc::Mu[ $\lambda$ pir] $\Delta asdA4 \Delta(zhf-2::Tn10)$ ; used<br>for conjugational transfer of suicide plasmids                                                       |              |
| $\chi 7232$                         | <i>endA1 hsdR17 (r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) glnV44 thi-1 recA1 gyrA</i><br><i>relA1</i> $\Delta(lacZYA-argF)U169 \lambda$ pir <i>deoR</i> ( $\phi 80dlac$<br>$\Delta(lacZ)M15$ ); used for general cloning |              |
| <b><i>S. Gallinarum</i> strains</b> |                                                                                                                                                                                                                                      |              |
| $\chi 4173$                         | wild-type challenge strain                                                                                                                                                                                                           |              |
| 287/91                              | wild-type vaccine parent strain                                                                                                                                                                                                      |              |
| $\chi 11575$                        | $\Delta fur-453::cam$                                                                                                                                                                                                                | 287/91       |
| $\chi 11386$                        | $\Delta P_{rfaH178::TT} araC P_{BAD} rfaH$                                                                                                                                                                                           | 287/91       |
| $\chi 11741$                        | $\Delta pmi-2426$                                                                                                                                                                                                                    | 287/91       |
| $\chi 11797$                        | $\Delta fur-712$                                                                                                                                                                                                                     | 287/91       |
| $\chi 11798$                        | $\Delta pmi-2426 \Delta fur-712$                                                                                                                                                                                                     | $\chi 11741$ |
| $\chi 11820$                        | $\Delta fur-712 \Delta pmi-2426$                                                                                                                                                                                                     | $\chi 11797$ |
| $\chi 11821$                        | $\Delta P_{rfaH178::TT} araC P_{BAD} rfaH \Delta fur-712$                                                                                                                                                                            | $\chi 11386$ |
| $\chi 11822$                        | $\Delta ansB1235$                                                                                                                                                                                                                    | 287/91       |
| $\chi 11823$                        | $\Delta fur-712 \Delta ansB1235$                                                                                                                                                                                                     | $\chi 11797$ |
| <b>Plasmids</b>                     |                                                                                                                                                                                                                                      |              |
| pKD46                               | $\lambda$ red expression vector                                                                                                                                                                                                      |              |
| pRE112                              | <i>sacB mobRP4</i> ; R6K ori; Cm <sup>R</sup>                                                                                                                                                                                        |              |
| pYA3546                             | suicide vector for introduction $\Delta pmi-2426$                                                                                                                                                                                    |              |
| pYA5239                             | suicide vector for introduction $\Delta fur-712$                                                                                                                                                                                     | pRE112       |
| pYA5272                             | suicide vector for introduction $\Delta ansB1235$                                                                                                                                                                                    | pRE112       |

**Working Example 1**

**[0035]** Escherichia coli and S. Gallinarum strains were routinely cultured at 37°C in LB broth or on LB agar. Cultures of S. Gallinarum mutants were supplemented with 0.05% mannose (Sigma-Aldrich, St. Louis, MO) (for  $\Delta pmi-2426$ ), 0.2% arabinose (Sigma-Aldrich) (for  $\Delta P_{rfaH178::TT} araC P_{BAD} rfaH$ , hereafter  $\Delta P_{rfaH178}$ ) or chloramphenicol (15  $\mu$ g/ml; Sigma-Aldrich) (for  $\Delta fur-453::cam$ ). Carbohydrate-free nutrient broth (NB) was used for growth when determining LPS profiles. Strains were grown in NB without mannose (for pmi strains) or arabinose (for  $\Delta P_{rfaH178}$  strains) overnight and subcultured (1:100) into fresh NB with or without the appropriate sugar

for a second passage. LB agar without sodium chloride and with 7.5% sucrose (Sigma-Aldrich) was employed for *sacB*-based counterselection. MacConkey plates with 1% mannose were used to indicate sugar fermentation.

**[0036]** For animal experiments, *S. Gallinarum* strains were cultured in LB broth with appropriate supplements. Overnight cultures were diluted 1:100 and grown with shaking (200 rpm) to an optical density at 600 nm of ~0.8. Then, bacteria were centrifuged at  $5,000 \times g$  for 15 min at room temperature and resuspended in phosphate-buffered saline (PBS) or buffered saline with 0.01% gelatin (BSG). LB or Salmonella Shigella (SS) agar plates were used to enumerate *S. Gallinarum* recovered from chicken tissues. Rappaport-Vassiliadis R10 (RV) broth was employed to enrich samples for *S. Gallinarum*. All media were purchased from BD Difco (Franklin Lakes, NJ) unless otherwise indicated.

**[0037]** General DNA procedures. DNA manipulations, including plasmid and genomic DNA isolation, restriction enzyme digestions, ligations and other DNA-modifying reactions, were carried out as described by Sambrook and Russell or were performed according to the manufacturers' instructions (New England Biolabs, Ipswich, MA; Qiagen, Valencia, CA; Promega, Madison, WI). Synthesis of primers (Table 2) and DNA sequencing were performed by Integrated DNA Technologies (Coralville, IA) and DNA Laboratory at Arizona State University (Tempe, AZ), respectively. Polymerase chain reactions (PCR) were carried out with KlenTaq LA polymerase (DNA Polymerase Technology, St. Louis, MO), possessing proofreading activity. Recombinant plasmids were introduced into *E. coli* and *S. Gallinarum* cells by transformation or electroporation, respectively.

**[0038]** Construction of *S. Gallinarum* vaccine strains. All vaccine candidates were derived from *S. Gallinarum* strain 287/91. The *fur* deletion/insertion mutation  $\Delta fur-453::cam$  was constructed via the  $\lambda$  red recombination method. Flanking sequences were based on the *S. Gallinarum* 287/91 genome using primers AM-115 and AM-116 (Table 2).

**Table 2.** Primers used in this study.

| Name    | Sequence (5' → 3')                                                                                | Orientation | Restriction site |
|---------|---------------------------------------------------------------------------------------------------|-------------|------------------|
| AM-115  | <b>TCTAATGAAGTGAATCGTTTAGCA</b><br><b>ACAGGACAGATTCCGCGTGTAGGCT</b><br>GGAGCTGCTTC (SEQ ID NO. 1) | forward     | Ø                |
| AM-116  | <b>AAAAGCCAACCGGGCGGTTGGCTC</b><br><b>TTCGAAAGATTTACACCATATGAAT</b><br>ATCCTCCTTAG (SEQ ID NO. 2) | reverse     | Ø                |
| fur-1F  | TATAGAGCT <u>C</u> TCTGCCTGTTCTGCT<br><b>ATG</b> (SEQ ID NO. 3)                                   | forward     | SacI             |
| fur-1R  | <b>GGCGCAGATATAACGCTGCGCCGC</b><br><b>ATAAGATTAGGC</b> (SEQ ID NO. 4)                             | reverse     | Ø                |
| fur-2F  | CAGCGTTATATCTGCGCCCTTTCGAA<br><b>GAGCCAACCG</b> (SEQ ID NO. 5)                                    | forward     | Ø                |
| fur-2R  | TATAGGTACCGCCAGTTGTTTCAGGT<br><b>GTG</b> (SEQ ID NO. 6)                                           | reverse     | KpnI             |
| ansB-1F | TATAGAGCT <u>C</u> GCCGCTCATGCAGAT<br><b>TAC</b> (SEQ ID NO. 7)                                   | forward     | SacI             |
| ansB-1R | <b>TTACTTCAGGCTGCCAACCAGCGC</b><br><b>TTTGCGGCTATC</b> (SEQ ID NO. 8)                             | reverse     | Ø                |
| ansB-2F | GTTGGCAGCCTGAAGTAATGATAAT<br><b>GCCCCGGTCGG</b> (SEQ ID NO. 9)                                    | forward     | Ø                |
| ansB-2R | TATAGGTACCCCAATACGCGTCCGC<br><b>TTC</b> (SEQ ID NO. 10)                                           | reverse     | KpnI             |

Nucleotides underlined denote restriction enzyme sites used for cloning. Nucleotides bolded are complementary to the *S. Gallinarum* 287/91 chromosome.

**[0039]** All other gene replacements were introduced by conjugational transfer of suicide plasmids using donor *E. coli* strain  $\chi$ 7213.

**[0040]** To construct the  $\Delta$ fur-712 deletion, fur flanking regions were amplified from the *S. Gallinarum* 287/91 genome by two-step PCR. Firstly, 644 bp and 663 bp DNA fragments flanking fur gene were amplified with fur-1F/-1R and fur-2F/-2R primer sets (Table 2), respectively. Thereafter, the mix of PCR products was used as a template in the next amplification reaction with fur-1F and fur-2R primers. The 1.3 kb DNA fragment was digested with SacI/KpnI restriction enzymes and cloned into suicide plasmid vector pRE112. The resulting suicide plasmid, pYA5239, carried a deletion of the entire fur gene including 251 bp promoter region. The  $\Delta$ fur-712 mutation was introduced by allelic exchange into *S. Gallinarum* strains 287/91,  $\chi$ 11741 and  $\chi$ 11386 to

generate  $\chi$ 11797 ( $\Delta$ fur-712),  $\chi$ 11798 ( $\Delta$ pmi-2426  $\Delta$ fur-712) and  $\chi$ 11821 ( $\Delta$ PrfaH178::TT araC PBAD rfaH  $\Delta$ fur-712), respectively.

**[0041]** The  $\Delta$ ansB1235 deletion was constructed as described above using ansB-1F/-1R and ansB-2F/-2R primer pairs (Table 2). The resulting suicide plasmid, pYA5272, carried a deletion of the entire ansB gene including the 188 bp promoter sequence. The  $\Delta$ ansB1235 mutation was introduced into *S. Gallinarum* strains 287/91 and  $\chi$ 11797 to generate  $\chi$ 11822 ( $\Delta$ ansB1235) and  $\chi$ 11823 ( $\Delta$ fur-712  $\Delta$ ansB1235), respectively.

**[0042]** As *S. Typhimurium* and *S. Gallinarum* share >99% sequence similarity in the flanking region surrounding pmi, previously constructed suicide plasmid pYA3546 carrying *S. Typhimurium* DNA sequences was used to create *S. Gallinarum*  $\Delta$ pmi-2426 mutants (25, 32). Plasmid pYA3546 was introduced by conjugation into *S. Gallinarum* strains 287/91 and  $\chi$ 11797 to generate  $\chi$ 11741 ( $\Delta$ pmi-2426) and  $\chi$ 11820 ( $\Delta$ fur-712  $\Delta$ pmi-2426), respectively.

**[0043]** All mutations were verified by PCR. We confirmed Fur production, or lack thereof, by western blotting. The  $\Delta$ pmi mutation was confirmed by white colony phenotype on mannose-MacConkey agar. LPS profiles were examined by silver staining in 12% polyacrylamide gels as described previously.

**[0044]** Isolation of outer membrane proteins (OMPs). OMPs were isolated using the Sarkosyl-extraction method.

**[0045]** SDS-PAGE and western blotting. SDS-PAGE and western blotting procedures were done by standard techniques. Blots were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyl phosphate (Amresco, Solon, OH) as a substrate, using rabbit polyclonal anti-Fur serum or anti-GroEL antibodies (Sigma-Aldrich) as primary antibodies and mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary antibodies.

**[0046]** Acid shock assay. Acid resistance was evaluated essentially as previously described, with a few modifications. Strains were grown aerobically in LB broth with appropriate supplements until they reached an optical density of  $\sim 0.4$ . Culture aliquots were centrifuged (10 min  $5000 \times g$ ) at room temperature and bacterial pellets were washed with E medium (pH 7.0). Thereafter, cells were centrifuged again and resuspended at a density of  $\sim 0.5 \times 10^9$  CFU/ml in E medium (pH 3.0). Acid challenge was conducted at 37°C, and samples were collected immediately after resuspension and

in 30-minute intervals. Samples were serially diluted and plated onto LB agar to assess bacterial viability.

**[0047]** Animal supply and housing. Animal experiments were performed using two breeds of chickens: Rhode Island Reds and Brown Leghorns. Straight run Rhode Island Red chicks were obtained from Randall Burkey Co. (Boerne, TX) or Murray McMurray Hatchery (Webster City, IA) one or two days after hatch. Birds were housed in separate cages for each group and given water and feed ad libitum. All animal experiments were carried out in compliance with Institutional Animal Care and Use Committee (IACUC) and Animal Welfare Act at Arizona State University.

**[0048]** Female Brown Leghorn chickens were hatched in-house. Chickens were feed Purina Lab Chow 5065, water and feed available ad libitum. Six-week old chickens were distributed among several isolators and tagged.

**[0049]** Determination of lethal dose, 50% (LD50). Strains were grown and harvested as described above in section "Bacterial strains, plasmids, media and growth conditions". Bacterial pellets were resuspended in PBS or BSG and adjusted to achieve a dose of ~10<sup>2</sup> to ~10<sup>8</sup> CFU in a volume of 100 µl for orally inoculating chicks. The virulence of wild-type strain, 287/91, and its derivatives were assessed in three- or five-day-old Rhode Island Red chicks. Birds were observed for fowl typhoid symptoms for three weeks post inoculation. Deaths were recorded daily. The LD50 was calculated using the Reed and Muench method.

**[0050]** Immunization and challenge regimen. For Rhode Island Reds, three- or five-day-old chicks were inoculated orally with 100 µl of PBS containing ~1 × 10<sup>8</sup> CFU of the appropriate *S. Gallinarum* strain and boosted with the same strain and dose two weeks later. No food was provided for ~5-6 h prior to immunizations or challenge. Groups of birds inoculated with buffer (PBS or BSG) served as controls. At four weeks of age (i.e. two weeks post booster), all birds were orally challenged with ~1 × 10<sup>7</sup> CFU of heterologous *S. Gallinarum* strain  $\chi$ 4173. Note that in the case of fur::cam deletion/insertion strain  $\chi$ 11575, all chicks survived the virulence study described above. They were then treated as vaccinated chicks, boosted with ~1 × 10<sup>8</sup> CFU of  $\chi$ 11575 and challenged.

**[0051]** Chickens were observed for fowl typhoid symptoms for 3 weeks post challenge. Deaths were recorded daily. At the end of the observation period, surviving birds were euthanized and their organs were inspected for lesions. Spleens and livers

were collected and homogenized. Dilutions of the homogenate were made in BSG and plated onto LB agar plates for enumeration of *Salmonella* present in each tissue. Enrichment with RV broth and subsequent plating onto SS agar plates was carried out for organ samples in which no *Salmonella* was detected by direct plating.

**[0052]** For experiments with Brown Leghorns, groups of 15 or 16 seven-week-old pullets were immunized orally ( $\sim 2 \times 10^7 - 2 \times 10^8$  CFU in most cases) or intramuscularly ( $\sim 2 \times 10^4$  or  $\sim 2 \times 10^7$  CFU) of the appropriate *S. Gallinarum* strain. A group of non-vaccinated birds was used as a control. At ten weeks of age (i.e. three weeks post immunization) all birds were orally challenged with  $\sim 2 \times 10^8$  CFU of homologous *S. Gallinarum* strain 287/91. Birds were monitored for 3 weeks post-challenge. Then, surviving birds were euthanized and necropsies performed to determine the presence of tissue lesions.

**[0053]** Statistical analyses. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). The significance of differences between the obtained values was appraised using two-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's tests. P values  $< 0.05$  were considered significant.

#### Results

**[0054]** Screening for *S. Gallinarum* immunogenic mutants. To evaluate the impact of a fur deletion in *S. Gallinarum*, we constructed strain  $\chi 11575$ , harboring the  $\Delta fur-453::cam$  deletion/insertion. As expected, Fur was not detected in  $\chi 11575$  by western blot analysis (Fig. 1A). Then we screened for production of IROMPs after growth in LB, a medium in which iron is not limiting ( $\sim 7.6 \mu M$  iron). Under these conditions, IROMPs were not detected in parent strain 287/91, but were easily detectable in  $\chi 11575$  (Fig. 1B). The three distinct bands with approximate molecular masses of 83, 78 and 74 kDa correspond to the predicted molecular masses of the Fur-regulated IROMPs FepA, IroN and Cir, respectively (Fig. 1B). The protein pattern is in an agreement with previous observations of *S. Typhimurium* outer membrane preparations from wild-type cells grown under iron-limiting conditions or from a fur mutant grown in the relatively iron-rich medium, NB.

**[0055]** Strain  $\chi 11575$  was then screened for virulence in Rhode Island Red chicks. Birds were given orally graded doses of bacteria and monitored for three weeks. The strain was fully attenuated with no deaths occurring at the highest dose tested ( $LD_{50} > \sim 1 \times 10^8$  CFU) (Table 3).

**Table 3.** Attenuation of *S. Gallinarum* mutants in Rhode Island Red chickens.

| Strain       | Genotype                            | LD <sub>50</sub> (CFU) |
|--------------|-------------------------------------|------------------------|
| 287/91       | wild type                           | $6.7 \times 10^4$      |
| $\chi$ 11575 | $\Delta fur-453::cam$               | $>1 \times 10^8$       |
| $\chi$ 11797 | $\Delta fur-712$                    | $>0.9 \times 10^8$     |
| $\chi$ 11741 | $\Delta pmi-2426$                   | $1.0 \times 10^7$      |
| $\chi$ 11822 | $\Delta ansB1235$                   | $1.3 \times 10^4$      |
| $\chi$ 11821 | $\Delta P_{rfaH178} \Delta fur-712$ | $>1.2 \times 10^8$     |

**[0056]** Encouraged by these results, we evaluated the ability of  $\chi$ 11575 to confer protection against challenge with virulent *S. Gallinarum*. The same chicks used in the virulence assay were boosted two weeks after the first inoculation with  $\sim 1 \times 10^8$  CFU of  $\chi$ 11575 and challenged two weeks later with  $\sim 1 \times 10^7$  CFU of heterologous wild-type strain  $\chi$ 4173. All the birds, even those primed with the lowest dose ( $\sim 1 \times 10^2$  CFU) of  $\chi$ 11575, survived challenge with virulent *S. Gallinarum* strain (Table 4), suggesting that an *S. Gallinarum* fur mutant is a viable vaccine candidate. However, strain  $\chi$ 11575 contains a chloramphenicol resistance cassette in the chromosome, precluding its use as a vaccine. Thus, we constructed *S. Gallinarum* strain  $\chi$ 11797, carrying the unmarked  $\Delta fur-712$  deletion (Table 1). We confirmed the absence of detectable Fur in this strain (Fig.1A) and production of IROMPs following growth in LB was indistinguishable from  $\chi$ 11575 (Fig. 1B).

**[0057]** In *S. Typhimurium*, fur mutants display an acid-sensitive phenotype. To determine acid resistance of *S. Gallinarum* fur mutants,  $\chi$ 11797 ( $\Delta fur-712$ ) and parent strain 287/91 were cultured in LB to early logarithmic phase of growth and then challenged at pH 3.0. The percentage of viable cells during low-pH challenge declined more rapidly for  $\chi$ 11797 than for 287/91 (Fig. 1C). After 30 min of pH 3.0 exposure, survival of the mutant was significantly lower (2.0%) compared to that of the wild type (25.1%;  $P < 0.01$ ). After 90 min of challenge only 0.001% of  $\chi$ 11797 cells survived compared to 0.847% of the wild type, corresponding to a  $\sim 880$ -fold (2.9 log) difference in the number of viable cells ( $P < 0.0001$ ).

**[0058]** Virulence and protective efficacy of *S. Gallinarum*  $\Delta fur-712$  mutant in Rhode Island Red chickens. We determined the virulence of *S. Gallinarum*  $\chi$ 11797 ( $\Delta fur-712$ ) in five-day-old Rhode Island Red chicks. As expected, strain  $\chi$ 11797 was fully attenuated ( $LD_{50} > 0.9 \times 10^8$  CFU) (Table 3). In contrast, parent strain 287/91 was highly virulent, with an  $LD_{50}$  of  $6.7 \times 10^4$  CFU, consistent with previous results.

**[0059]** Strain  $\chi$ 11797 was then evaluated for its ability to induce protective immunity against challenge with a virulent *S. Gallinarum* strain. Two independent protection experiments were performed on five-day-old Rhode Island Red chickens. Birds were primed and boosted orally two weeks later with identical doses of  $\sim 1 \times 10^8$  CFU of  $\chi$ 11797. In each study a control group was given a sterile buffer instead of vaccine. At four weeks of age, all birds were challenged with  $\sim 1 \times 10^7$  CFU of heterologous virulent strain  $\chi$ 4173. In both studies immunization with strain  $\chi$ 11797 provided significant protection compared to non-immunized control birds (Table 4).

**Table 4.** Protective efficacy of *S. Gallinarum fur* mutants in Rhode Island Red chickens<sup>a</sup>.

| Strain                                          | Genotype                                  | Exp | Prime (CFU)                 | Boost (CFU)          | Hepato-megaly          | Spleno-megaly           | Heart lesions / pericarditis | Alive / total | Percent survival  |
|-------------------------------------------------|-------------------------------------------|-----|-----------------------------|----------------------|------------------------|-------------------------|------------------------------|---------------|-------------------|
| <b>Single <i>fur</i></b>                        |                                           |     |                             |                      |                        |                         |                              |               |                   |
| $\chi$ 11575                                    | $\Delta fur-453::cam$                     | 1   | Range of doses <sup>b</sup> | $\sim 1 \times 10^8$ | NT <sup>c</sup>        | NT                      | NT                           | 20/20         | 100% <sup>d</sup> |
| $\chi$ 11797                                    | $\Delta fur-712$                          | 2   | $1.0 \times 10^8$           | $1.2 \times 10^8$    | NT                     | NT                      | NT                           | 10/11         | 91% <sup>d</sup>  |
|                                                 |                                           | 3   | $1.2 \times 10^8$           | $1.1 \times 10^8$    | 1/13 (8%) <sup>d</sup> | 2/13 (15%) <sup>d</sup> | 5/13 (38%)                   | 12/13         | 92% <sup>d</sup>  |
| <b><i>fur</i> combined with other mutations</b> |                                           |     |                             |                      |                        |                         |                              |               |                   |
| $\chi$ 11798                                    | $\Delta pmi-2426$                         | 2   | $1.0 \times 10^8$           | $1.0 \times 10^8$    | NT                     | NT                      | NT                           | 2/9           | 22%               |
| $\chi$ 11820                                    | $\Delta fur-712$<br>$\Delta pmi-2426$     | 3   | $1.2 \times 10^8$           | $1.3 \times 10^8$    | 10/12 (83%)            | 9/12 (75%)              | 3/12 (25%)                   | 4/12          | 33%               |
| $\chi$ 11821                                    | $\Delta P_{rfaH178}$<br>$\Delta fur-712$  | 3   | $1.1 \times 10^8$           | $1.1 \times 10^8$    | 9/12 (75%)             | 8/12 (67%)              | 3/12 (25%)                   | 4/12          | 33%               |
| $\chi$ 11823                                    | $\Delta fur-712$<br>$\Delta ansB123$<br>5 | 3   | $1.0 \times 10^8$           | $0.9 \times 10^8$    | 7/12 (58%)             | 8/12 (67%)              | 3/12 (25%)                   | 6/12          | 50%               |
| <b>Controls</b>                                 |                                           |     |                             |                      |                        |                         |                              |               |                   |
| BSG                                             | -                                         | 1   | -                           | -                    | NT                     | NT                      | NT                           | 2/20          | 10%               |
| PBS                                             | -                                         | 2   | -                           | -                    | NT                     | NT                      | NT                           | 2/11          | 18%               |
| PBS                                             | -                                         | 3   | -                           | -                    | 10/12 (83%)            | 10/12 (83%)             | 1/12 (8%)                    | 2/12          | 17%               |

<sup>a</sup> Three- or five-day-old chicks were immunized orally with the indicated dose of *S. Gallinarum* and boosted two weeks later. At four weeks of age all birds were challenged with  $\sim 1 \times 10^7$  CFU of heterologous *S. Gallinarum* wild-type strain ( $\chi$ 4173).

<sup>b</sup> These birds were survivors of the virulence assay so the chicks received  $1 \times 10^2$ ,  $10^4$ ,  $10^6$  or  $10^8$  CFU as a priming dose. The boost was  $1 \times 10^8$  CFU for all birds.

<sup>c</sup> not tested

<sup>d</sup>  $P < 0.01$  compared to control

**[0060]** In both experiments, >90% of the vaccinated chickens survived compared to only 17-18% survival in the control groups ( $P < 0.001$ ).

**[0061]** Additionally, in one of the protection studies (Experiment 3 in Table 4), internal organs from all animals were inspected for lesions and bacterial loads after

challenge. Birds that died from challenge were necropsied immediately and survivors were euthanized three weeks post challenge and necropsies performed at that time. In Rhode Island Reds that died of fowl typhoid, characteristic lesions included splenomegaly and hepatomegaly and, in some animals, some bronzing of the liver was noted. No other gross lesions were detected in chickens that did not survive the challenge. In contrast spleens and livers in birds vaccinated with  $\chi$ 11797 were, for the most part, not enlarged or congested (Table 4). However, we found nodules in the hearts and observed acute pericarditis in 38% of immunized birds. Furthermore, nineteen days post-challenge, spleen and liver samples were collected from all surviving birds to enumerate *S. Gallinarum* colonization in each tissue (Fig. 2). In birds vaccinated with  $\chi$ 11797 ( $\Delta$ fur-712), the *S. Gallinarum* challenge strain was not detectable in 50% of spleen and or 42% of liver samples. The bacterial loads were significantly lower (max.  $1.4 \times 10^4$  CFU/g for spleen;  $7.9 \times 10^2$  CFU/g for liver) in the remaining, *S. Gallinarum* positive tissues than in the non-vaccinated birds that succumbed to the infection, where counts were typically around  $1 \times 10^6$  CFU per gram of tissue (data not shown).

### Working Example 2

**[0062]** Immunogenicity of *S. Gallinarum* double mutants. We next examined two distinct genetic strategies for enhancing the immunogenicity of  $\chi$ 11797. It is well established that Salmonella O-antigen is required for efficient colonization of the chicken host. Mutations that result in the gradual loss of O-antigen in vivo can be used in Salmonella vaccine strains to enhance induction of high-antibody titers to outer membrane proteins. Thus, we investigated the possibility that introduction of a  $\Delta$ pmi or an arabinose-regulated *rfaH* mutation could enhance the immunogenicity of  $\chi$ 11797.

**[0063]** It is likely that all successful pathogens have various means to suppress host immune responses. An example of this in *S. Typhimurium* is *ansB*. The product of this gene – L-asparaginase II – suppresses host T cell responses important for clearance of a *S. Typhimurium* infection and *S. Typhimurium*  $\Delta$ ansB mutants are attenuated for virulence in mice. Thus, as an alternative approach for enhancing immunogenicity, we examined the effect of  $\Delta$ ansB on virulence and immunogenicity alone or when combined with a  $\Delta$ fur mutation.

**[0064]** We constructed single mutant strains  $\chi$ 11741 ( $\Delta$ pmi-2426) and  $\chi$ 11822 ( $\Delta$ ansB1235) and double mutant strains:  $\chi$ 11798 ( $\Delta$ pmi-2426  $\Delta$ fur-712),  $\chi$ 11820 ( $\Delta$ fur-712  $\Delta$ pmi-2426),  $\chi$ 11821 ( $\Delta$ PrfaH178::TT *araC* PBAD *rfaH*  $\Delta$ fur-712) and  $\chi$ 11823

( $\Delta$ fur-712  $\Delta$ ansB1235). The absence of detectable Fur was verified in the double mutants (Fig. 1A) and IROMP synthesis was not affected by combining  $\Delta$ pmi-2426,  $\Delta$ PrfaH178 or  $\Delta$ ansB123 with  $\Delta$ fur-712 (Fig. 1B). Analysis of the LPS profiles of both pmi mutants ( $\chi$ 11741,  $\chi$ 11798) and the  $\Delta$ PrfaH178 double mutant strains  $\chi$ 11821 indicated that full length O-antigen was produced by both pmi strains and the  $\Delta$ PrfaH178 mutant only when mannose or arabinose, respectively, was added to the growth medium (data not shown).

**[0065]** We also evaluated acid resistance of the  $\Delta$ pmi mutant strains. Interestingly, strain  $\chi$ 11798 ( $\Delta$ pmi-2426  $\Delta$ fur-712) was more sensitive to low pH than  $\chi$ 11797 ( $\Delta$ fur-712), even though it was grown in presence of mannose prior to challenge (Fig. 1C). At every time point during challenge, the survival rate of strain  $\chi$ 11798 was significantly less than that of strain  $\chi$ 11797 ( $P < 0.001$ ). It is unlikely that the addition of mannose to strain  $\chi$ 11798 was responsible for the increase in acid sensitivity because strain  $\chi$ 11741 ( $\Delta$ pmi-2426), when grown in LB with mannose, displayed a survival profile identical to wild-type strain 287/91 and we observed no change in survival to acid challenge when mannose was added during growth of  $\chi$ 11797 ( $\Delta$ fur-712) (data not shown).

**[0066]** Since an adequate level of attenuation is critical for designing safe and efficacious vaccines, we examined the virulence of *S. Gallinarum* strains  $\chi$ 11741 and  $\chi$ 11822, harboring single  $\Delta$ pmi or  $\Delta$ ansB mutations, respectively. The  $\Delta$ pmi mutant  $\chi$ 11741 was partially attenuated, similar to the phenotype observed for *S. Typhimurium*, while  $\Delta$ ansB mutant  $\chi$ 11822 was fully virulent (Table 3). Strain  $\chi$ 11821 ( $\Delta$ PrfaH178  $\Delta$ fur-712), a derivative of hypervirulent strain  $\chi$ 11386 ( $\Delta$ PrfaH178), was also tested. Introduction of  $\Delta$ fur-712 into  $\chi$ 11386 resulted in complete loss of virulence.

**[0067]** The *S. Gallinarum* double mutants were then tested for protective efficacy in Rhode Island Reds. Note that while strains  $\chi$ 11820 and  $\chi$ 11798 have the same genotype, the mutations were introduced in different orders, with the  $\Delta$ fur-712 mutation introduced first, before  $\Delta$ pmi-2426, in strain  $\chi$ 11820 and second in strain  $\chi$ 11798. Birds immunized with either  $\chi$ 11820 ( $\Delta$ fur-712  $\Delta$ pmi-2426) or  $\chi$ 11798 ( $\Delta$ pmi-2426  $\Delta$ fur-712) were not protected (33% and 22% survival, respectively) (Table 4). A lack of protection was also observed for birds vaccinated with  $\chi$ 11821 ( $\Delta$ PrfaH178  $\Delta$ fur-712) (33% survival). Vaccination with  $\chi$ 11823 ( $\Delta$ fur-712  $\Delta$ ansB1235) resulted in 50% protection, but this result was not significantly different from non-vaccinated controls.

[0068] Protective efficacy of *S. Gallinarum* vaccine strains in brown leghorn chickens. Two vaccine strains:  $\chi$ 11797 ( $\Delta$ fur-712) and  $\chi$ 11798 ( $\Delta$ pmi-2426  $\Delta$ fur-712) were also tested for protection immunity in Brown Leghorn chickens. In this study, seven-week-old female chickens were vaccinated with a single dose of vaccine by intramuscular ( $\sim 2 \times 10^4$  or  $2 \times 10^7$  CFU) or oral ( $\sim 2 \times 10^7$  CFU) routes and challenged three weeks later with the virulent wild-type vaccine parent strain 287/19. As shown in Table 5, intramuscular immunization with a high dose ( $2.6 \times 10^7$  CFU) of strain  $\chi$ 11797 provided protection to all vaccinated birds.

**Table 5.** Protective efficacy of *S. Gallinarum* *fur* mutants in female Brown Leghorn chickens.

| Strain       | Genotype                           | Route | Prime (CFU)       | Hepato-megaly          | Spleno-megaly           | Heart lesions / pericarditis | Alive / total | Percent survival  |
|--------------|------------------------------------|-------|-------------------|------------------------|-------------------------|------------------------------|---------------|-------------------|
| $\chi$ 11797 | $\Delta$ fur-712                   | IM    | $2.6 \times 10^4$ | 0/16 (0%) <sup>a</sup> | 3/16 (19%) <sup>a</sup> | 2/16 (13%)                   | 16/16         | 100% <sup>b</sup> |
|              |                                    | IM    | $2.6 \times 10^7$ | 1/16 (6%) <sup>a</sup> | 2/16 (12%) <sup>a</sup> | 3/16 (19%)                   | 15/16         | 100% <sup>b</sup> |
| $\chi$ 11798 | $\Delta$ pmi-2426 $\Delta$ fur-712 | Oral  | $2.6 \times 10^7$ | 14/16 (88%)            | 15/16 (94%)             | 2/16 (13%)                   | 8/16          | 50%               |
|              |                                    | IM    | $2.2 \times 10^7$ | 1/16 (6%) <sup>a</sup> | 4/16 (25%) <sup>a</sup> | 5/16 (31%)                   | 16/16         | 100% <sup>a</sup> |
| no vaccine   | -                                  | -     | -                 | 11/15 (73%)            | 11/15 (73%)             | 5/16 (31%)                   | 6/16          | 38%               |

<sup>a</sup> Seven-week-old birds were immunized orally or intramuscularly (IM) with the indicated dose of *S. Gallinarum* vaccine strain.

<sup>b</sup>  $P < 0.01$  compared to control

[0069] Moreover, enlargement of spleen or liver was observed only in 6 and 12% of vaccinated birds post-challenge, respectively. Interestingly, a single low dose ( $2.6 \times 10^4$  CFU) of  $\chi$ 11797 delivered intramuscularly was also highly protective (100% survival; splenomegaly and hepatomegaly detected in 0 and 19% of birds, respectively). In comparison, only 38% of non-vaccinated birds survived the challenge and spleen and liver lesions were observed in most of them (73%). On the other hand, when delivered orally, strain  $\chi$ 11797 did not protect Brown Leghorns from wild-type challenge in this model. A survival rate of 50% for Brown Leghorns vaccinated with this route was not significantly different than that of non-vaccinated birds. Moreover, the percentage of birds in this group with organ lesions was similar to the control. In contrast, when administrated intramuscularly, strain  $\chi$ 11798 provided significant protection against fowl

typhoid (100% survival,  $P < 0.001$ ; lower percentage of birds with lesions relative to control;  $P < 0.01$ ).

### Discussion

[0070] In our study we found that deletion of the *fur* gene in *S. Gallinarum* resulted in a completely avirulent strain that is highly efficacious as a live vaccine and can protect chickens against fowl typhoid when delivered orally in Rhode Island Red chickens or intramuscularly in Brown Leghorn chickens. These results differ from observations of an *S. Typhimurium* mutant, which is commonly used as a model for typhoid fever-like infections. While *S. Typhimurium*  $\Delta fur$  mutants were attenuated when delivered orally or intraperitoneally in mice, they were not found to be substantially immunogenic.

[0071] However, the level of attenuation conferred to *S. Typhimurium* by a *fur* mutation appears to be strain dependent. When delivered orally, derivatives of *S. Typhimurium* SL1344 are only attenuated about 10-fold, while *S. Typhimurium* UK-1 *fur* mutants are attenuated 1000-fold or more. Differences in *rpoS* alleles can influence the acid tolerance response of both wild type and *fur* mutants of *Salmonella* and may therefore affect other phenotypic aspects of *fur* mutants.

[0072] Alternatively, it is possible that undefined differences between strains may also affect virulence and immunogenicity. In addition, differences in immunogenicity between *fur* mutants of *S. Typhimurium* and *S. Gallinarum* may also be explained by the fact that the disease caused by *S. Typhimurium* in mice is not exactly the same as that caused by *S. Gallinarum* in chickens. Support for this view comes from observations that mutations that completely attenuate *S. Typhimurium* are often insufficiently attenuating for *S. Typhi* in humans. Since *S. Gallinarum* and *S. Typhi* are strictly host-adapted serovars, mechanisms of their pathogenesis are different from the broad-host range *S. Typhimurium*. Unfortunately, the molecular basis of host specificity as well as the mechanisms determining which type of disease is caused in which animal species are still poorly understood.

[0073] Adequate balance between the level of attenuation and immunogenicity is crucial for designing effective live vaccines, but is often difficult to achieve. As we suggest above, the same means of attenuation may result in different levels of attenuation, reactogenicity and/or immunogenicity depending on serovars or strains used for their construction. Protection from disease may also be influenced by the route of

administration as well as genetic properties or age of particular breeds such as Rhode Island Red or Brown Leghorn chickens.

[0074] Deletions of *fur* have been introduced into fish pathogens *Pseudomonas fluorescens* and *Edwardsiella ictaluri* to generate live attenuated vaccines. A *fur* mutant of *P. fluorescens* was attenuated and able to elicit protection in Japanese flounders against *P. fluorescens*, as well as cross-protection against *Aeromonas hydrophila*. The authors of that study suggest that the observed cross-protection was related, at least in part, to constitutive production of IROMPs by the *P. fluorescens fur* mutant. Similarly, arabinose-regulated *fur* mutants of *S. Typhimurium* induce antibodies that recognize the IROMPs present in the outer membranes of a number of *S. enterica* serovars and *E. coli*. Since our *S. Gallinarum fur* mutants constitutively synthesize IROMPs (Fig. 1B), it will be interesting to determine how well an *S. Gallinarum*  $\Delta fur$  mutant such as  $\chi 11797$  protects chickens against other *Salmonella* serovars, in particular *S. Enteritidis* and *S. Typhimurium*. This will be a topic for future study.

[0075] The  $\Delta pmi$  mutant strain  $\chi 11741$  was moderately attenuated, with an oral LD<sub>50</sub> about 2.5 logs higher than its wild-type parent, 287/91 (Table 3). This modest reduction in virulence is similar to the situation seen in *S. Typhimurium*, where a 3.3-log increase in oral LD<sub>50</sub> (for mice) was observed for a *pmi* mutant grown with mannose. The partial virulence of  $\chi 11741$  makes this mutant unsuitable for use as a stand-alone vaccine strain. The idea behind combining  $\Delta pmi$  and  $\Delta fur$  in the same strain was that the loss of O-antigen over time would enhance presentation of the IROMPs to the host immune system.

[0076] Because it has been argued that the lack of immunogenicity of *S. Typhimurium fur* mutants is due to an inability to colonize the gut associated lymphoid tissue (GALT), we considered it a plus that the *pmi* mutant was not fully attenuated and should therefore have a minimal impact on its immunogenicity. We felt that this would reduce the possibility that the double mutant would be over attenuated. However, while the double mutants  $\chi 11798$  and  $\chi 11820$  were attenuated, neither strain was protective when administered orally (Table 4). We used a similar strategy by combining *fur* with the arabinose-regulated *rfaH* mutation,  $\Delta PrfaH178$  mutation, which is not attenuating, and in fact appears to be hypervirulent. Once again, this combination was also not protective (Table 4).

[0077] In contrast to our results in Rhode Island Red chicks, *S. Gallinarum*  $\Delta$ pmi  $\Delta$ fur mutant  $\chi$ 11798 was substantially immunogenic when used to intramuscularly immunize seven-week-old Brown Leghorns (Table 5). Thus, it may be that because the double mutant is more sensitive to low pH than the  $\Delta$ fur strain (Fig. 1C), it does not survive as well during passage through the low pH environment of the proventriculus. If this is the case, pH sensitivity may also help to explain our conflicting results with fur mutant  $\chi$ 11797, which was protective when orally administered to chicks (Table 4), but was less effective when orally administered to older layers (Table 5).

[0078] A recent study showed that the proventricular pH in chickens changes during the first few weeks of life, ranging from a pH of about 5 at two days of age to about 3 to 3.5 by fifteen days of age. Thus, it is possible that survival of strain  $\chi$ 11797 was greater in chicks than in the older birds used in our study. When we bypassed the gastric compartment by intramuscular injection,  $\chi$ 11797 was able to elicit a protective response (Table 5). The increased acid sensitivity of  $\chi$ 11798 could account for its lack of immunogenicity in chicks. An alternative interpretation of these results is that because fur, pmi and rfaH all affect outer membrane structure/composition, overexpression of outer membrane proteins (*e.g.*, IROMPs) in the absence of complete O-antigen has a negative influence on immunogenicity, perhaps due to destabilization of outer membrane integrity *in vivo*. Of course, it is possible that other factors, including possible iron toxicity, could have played a role.

[0079] Recently it was shown that *S. Typhimurium* utilizes a product of ansB gene – L-asparaginase II – to inhibit host T cell responses essential to clearance of Salmonella infection. A canonical function of L-asparaginase II is hydrolyzing L-asparagine to L-aspartate and ammonia. However, beyond the metabolic function, the enzyme plays a role in virulence. Production of L-asparaginase II by Salmonella leads to depletion of exogenous L-asparagine, a metabolite required for T cell proliferation. While an *S. Typhimurium* ansB mutant was attenuated for virulence in mice, this was not the case for *S. Gallinarum* in chicks (Table 3) and introduction of a  $\Delta$ ansB mutation into the  $\Delta$ fur mutant strain  $\chi$ 11797 abrogated, rather than enhanced, its immunogenicity (Table 4).

[0080] One of the primary goals of our research is to develop a safe and effective orally administered fowl typhoid vaccine for birds. Oral administration of vaccines is, in general, easier to perform than injection and more likely to induce mucosal responses.

The results of this study indicate that  $\Delta$ fur mutant  $\chi$ 11797 is safe (Table 3). It is effective in chicks (Table 4), but is not as effective for use as an oral vaccine in older birds (Table 5), though it is substantially immunogenic when delivered by the intramuscular route. It is possible that the problem of efficacy in older birds can be rectified by introduction of a mutation that allows for regulated delayed fur expression, as has been demonstrated to be effective in the *S. Typhimurium* mouse model. We used a similar strategy to regulate expression of *crp* in *S. Gallinarum* with promising results. If this strategy is effective with *fur*, it may allow us to take advantage of a second mutation in *pmi*, as we described above.

**[0081]** In conclusion, this study demonstrated that the *fur* gene is essential for virulence of *S. Gallinarum* and a *fur* deletion resulted in complete attenuation of *S. Gallinarum*. Further, a  $\Delta$ fur mutant is protective against fowl typhoid when used as a live recombinant vaccine following intramuscular administration, or by oral route in young birds.

**[0082]** The claims are not intended to be limited to the embodiments, examples, materials and methods described above.

**CLAIMS**

1. A strain of *S. Gallinarum* bacteria comprising:  
a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein, wherein the strain is attenuated and immunogenic in fowl.
2. The strain of claim 1 further comprising a mutation that prohibits the strain from synthesizing phosphomannose isomerase.
3. A strain of *S. Gallinarum* bacteria comprising:  
a mutation that prohibits the strain from synthesizing phosphomannose isomerase, wherein the strain is attenuated and immunogenic in fowl.
4. A vaccine for inoculation against *S. Gallinarum* infection comprising:  
a pharmaceutically acceptable carrier; and  
a strain of *S. Gallinarum* including a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein.
5. The vaccine of claim 4 further comprising a mutation that prohibits the strain from synthesizing phosphomannose isomerase.
6. The vaccine of claim 4 wherein the pharmaceutically acceptable carrier is suitable for intramuscular administration or oral administration.
7. A method of making a vaccine comprising:  
providing a strain of *S. Gallinarum* including a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein, wherein the strain is attenuated and immunogenic; and  
incorporating the strain into a pharmaceutically acceptable carrier.
8. The method of claim 7 further comprising a mutation that prohibits the strain from synthesizing phosphomannose isomerase.
9. The method of claim 7 wherein the pharmaceutically acceptable carrier is suitable for intramuscular administration or oral administration.

10. A method of vaccinating fowl against typhoid, comprising the administration of a strain of *S. Gallinarum*, which includes a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein, in a pharmaceutically acceptable carrier.

11. A method of vaccinating fowl against typhoid, comprising the administration of a strain of *S. Gallinarum*, which includes a mutation that prohibits the strain from synthesizing phosphomannose isomerase, in a pharmaceutically acceptable carrier.

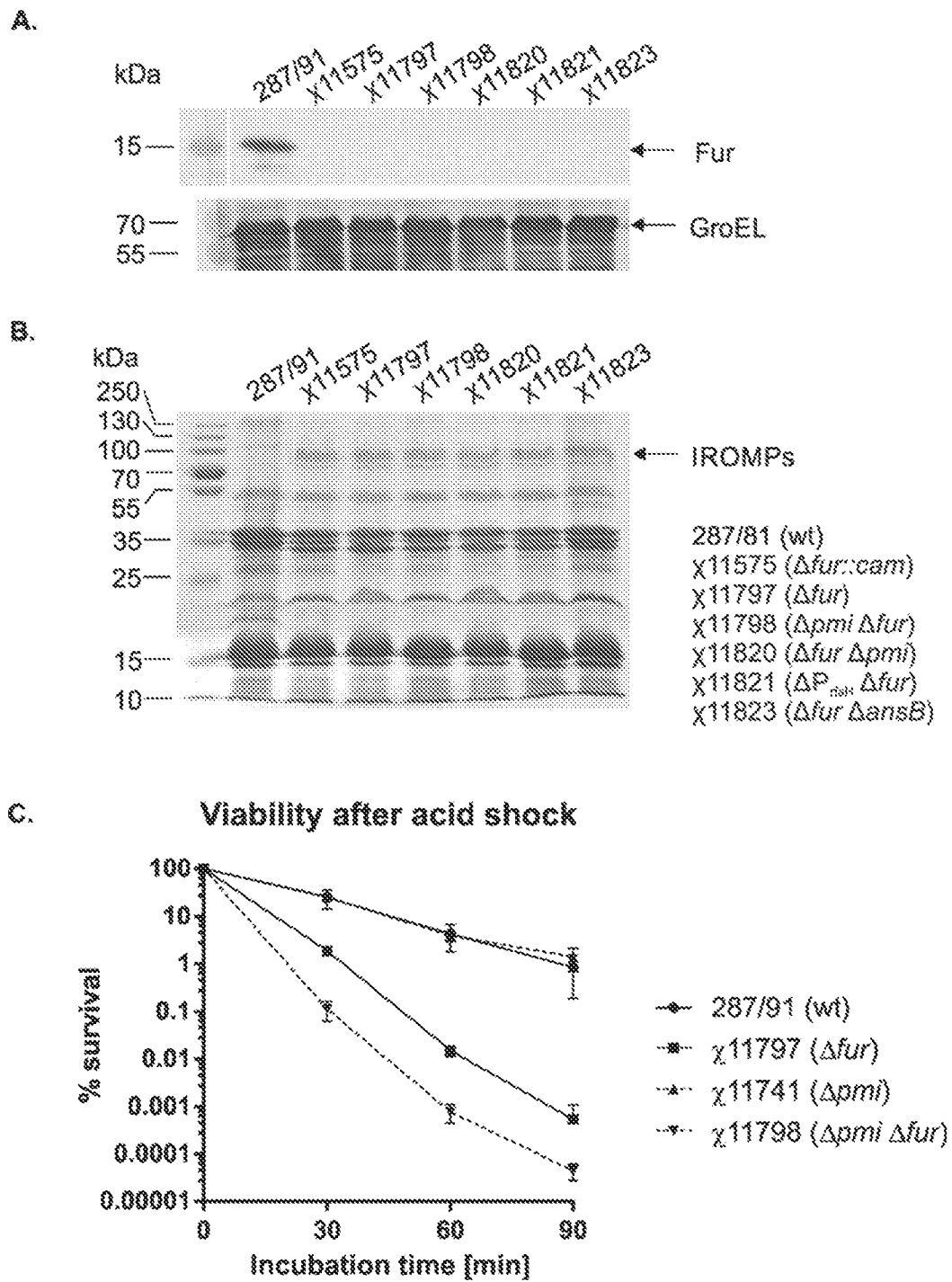


Figure 1

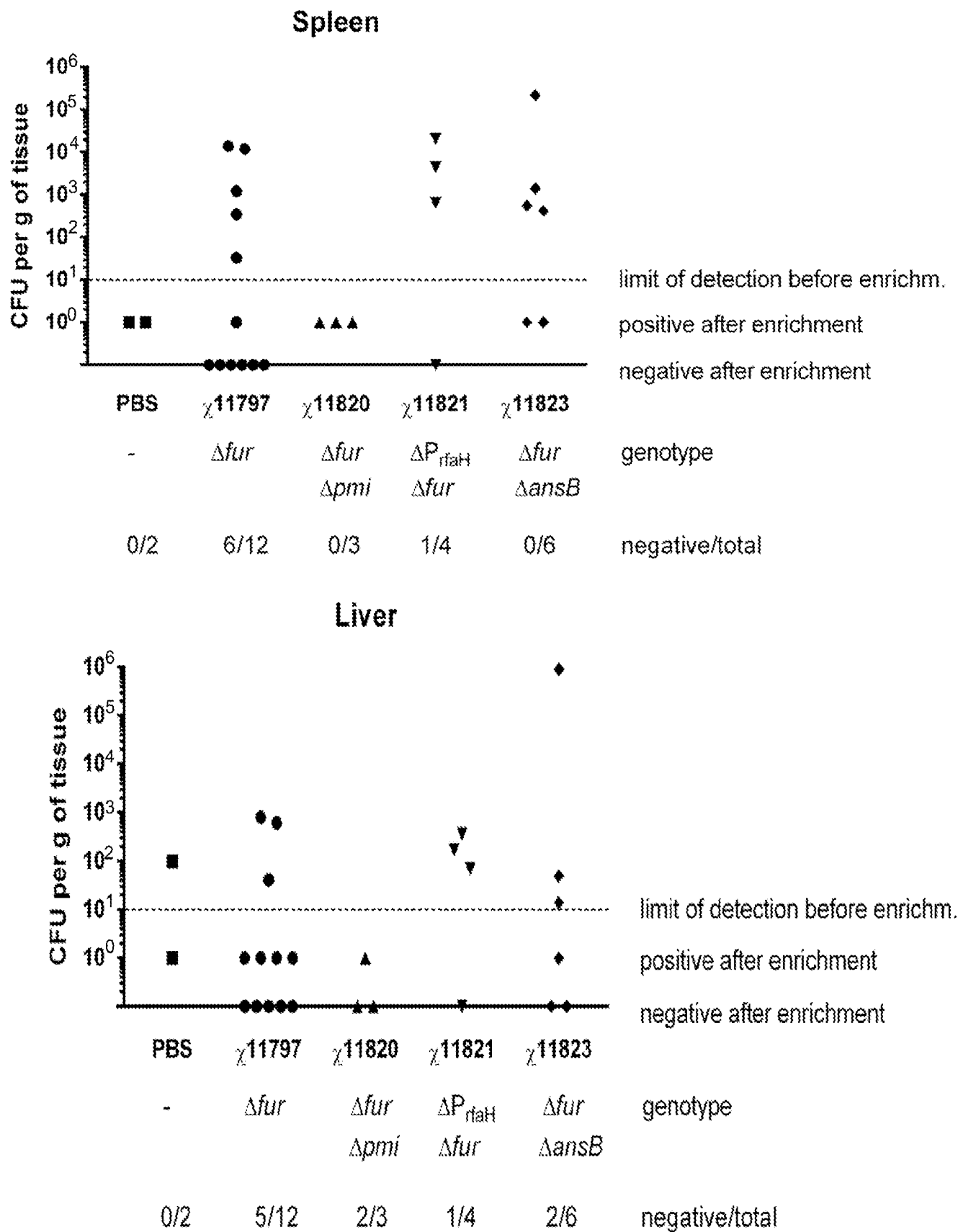


Figure 2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27148

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/112, A01N 63/00, C12N 1/20 (2015.01)

CPC - A61K 39/0275, C12N 15/86, A61K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
USPC- 424/258.1Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
IPC(8)- A61K 39/112, A01N 63/00, C12N 1/20 (2015.01)  
USPC- 424/258.1, 424/93.2, 435/252.3; CPC- A61K 39/0275, C12N 15/86, A61K 38/00Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
IPC(8)- A61K 39/112, A01N 63/00, C12N 1/20 (2015.01)  
USPC- 424/258.1, 424/93.2, 435/252.3

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                 | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | US 2013/0004537 A1 (Curtiss et al.) 03 January 2013 (03.01.2013) Abstract, para [0002]-[0006], [0013]-[0014], [0020], [0026], [0032], [0202]                                       | 1-6                   |
| A         | Matsuda et al., Safety and efficacy of a virulence gene-deleted live vaccine candidate for fowl typhoid in young chickens, Avian Pathology, 40:3, 309-314 (17 June 2011); Abstract | 1-6                   |

☐ Further documents are listed in the continuation of Box C.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

10 August 2015 (10.08.2015)

Date of mailing of the international search report

03 SEP 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

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PCT OSP: 571-272-7774

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/27148

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-6, directed to a vaccine.

Group II, claims 7-9, directed to a method of making a vaccine.

Group III, claims 10-11, directed to a method of vaccinating fowl against typhoid.

See supplemental box

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-6

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box III, Lack of unity of invention:

The inventions listed as Groups I, II and III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

**Special technical features**

Group I has the special technical feature of a vaccine for inoculation against *S. Gallinarum* infection, that is not required by Groups II or III.

Group II has the special technical feature of a method of making a vaccine, that is not required by Groups I or III.

Group III has the special technical feature of a method of vaccinating fowl against typhoid comprising the administration of a strain of *S. Gallinarum*, that is not required by Groups I or II.

**Common technical features:**

Group I (a vaccine), II (a method of making a vaccine) and III (a method of using a vaccine) are related to each other, and Groups I-III share the common technical feature of a strain of *S. Gallinarum* bacteria, which includes a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein in a vaccine for inoculation against *S. Gallinarum* infection comprising a pharmaceutically acceptable carrier. Groups I and III further share the common technical feature of a strain of *S. Gallinarum*, which includes a mutation that prohibits the strain from synthesizing phosphomannose isomerase.

However, these shared technical features do not represent a contribution over prior art, because these shared technical features are obviated by US 2013/0004537 A1 to Curtiss et al. (hereinafter Curtiss).

Curtiss discloses a vaccine for inoculation against *S. Gallinarum* infection (Abstract, para [0026]: *S. Gallinarum* strains carrying regulated *rfaH* or *rfc* mutations; para [0032],[0202]: Regulated O Antigen Expression in Avian Host-Restricted Serovar *Salmonella enterica* Serovar *Gallinarum* *S. Gallinarum* is a poultry pathogen that causes fowl typhoid) comprising: a pharmaceutically acceptable carrier (para [0101]: a recombinant bacterium of the invention may optionally comprise one or more possible additives, such as carriers; para [0103]); and a strain of *Salmonella* including a mutation that prohibits the strain from synthesizing a functional ferric uptake regulator protein (para [0058]: 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; para [0077]: Strains with the .DELTA.P.sub.fur and/or the .DELTA.P.sub.phoPQ mutations are attenuated at oral doses of 10.sup.9 CFU [Note: *fur* is ferric uptake regulator]).

Curtiss further discloses a strain of *S. Gallinarum* bacteria (Abstract, para [0026]: *S. Gallinarum* strains; para [0032],[0202]: Regulated O Antigen Expression in Avian Host-Restricted Serovar *Salmonella enterica* Serovar *Gallinarum* *S. Gallinarum* is a poultry pathogen that causes fowl typhoid), comprising: a mutation that prohibits the strain from synthesizing phosphomannose isomerase (para [0118]: One means to achieve regulated delayed attenuation is the deletion of certain genes essential for O-antigen synthesis such as *pmi* (*manA*); para [0153]: *Pmi* is a phosphomannose isomerase . . . the deletion mutant is unable to synthesize the O-antigen due to unavailability of mannose, which is a component of O-antigen; para [0202]: Regulated O Antigen Expression in Avian Host-Restricted Serovar *Salmonella enterica* Serovar *Gallinarum* *S. Gallinarum* is a poultry pathogen that causes fowl typhoid).

Although Curtiss discloses a strain of *Salmonella enterica* Serovar *Gallinarum* and vaccines comprising attenuated *Salmonella* strains with the .DELTA.P.sub.fur and/or .DELTA.P.pmi; Curtiss fails to provide explicitly a specific example of *Gallinarum* strain with the .DELTA.P.sub.fur and/or .DELTA.P.pmi. However, it would have been obvious to a person having ordinary skill in the art to use methods of Curtiss to prepare a vaccine comprising *Gallinarum* strains with the .DELTA.P.sub.fur and/or .DELTA.pmi, because Curtiss suggests such vaccines with other *Salmonella* strains, and further it would be routine experimentation to implement the procedures and protocols of Curtiss to *Gallinarum* as suggested by Curtiss, and further because where the general conditions of the claim are disclosed in the prior art, discovering the optimum or workable ranges involves only routine skill in the art.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I-III lack unity of invention under PCT Rule 13.