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Evaluation of Protective Efficacy of Live Attenuated *Salmonella enterica* Serovar Gallinarum Vaccine Strains against Fowl Typhoid in Chickens

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Salmonella enterica **serovar Gallinarum is the etiological agent of fowl typhoid, which constitutes a considerable economic problem for poultry growers in developing countries. The vaccination of chickens seems to be the most effective strategy to control the disease in those areas. We constructed *S.* Gallinarum strains with a deletion of the global regulatory gene *fur* and evaluated their virulence and protective efficacy in Rhode Island Red chicks and Brown Leghorn layers. The *fur* deletion mutant was avirulent and, when delivered orally to chicks, elicited excellent protection against lethal *S.* Gallinarum challenge. It was not as effective when given orally to older birds, although it was highly immunogenic when delivered by intramuscular injection. 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 *S.* Gallinarum Δ*pmi* mutant was only partially attenuated, and the Δ*ansB* mutant was fully virulent. The Δ*fur* Δ*pmi* and Δ*fur* Δ*ansB* double mutants were attenuated but not protective when delivered orally to the chicks. However, a Δ*pmi* Δ*fur* strain was highly immunogenic when administered intramuscularly. All together, our results show that the *fur* gene is essential for the virulence of *S.* Gallinarum, and the *fur* mutant is effective as a live recombinant vaccine against fowl typhoid.**

Salmonella enterica serovar Gallinarum biovar Gallinarum is a host-adapted pathogen that causes fowl typhoid, an important disease of poultry (1). Fowl typhoid is a septicemic disease that typically has a short course and significant morbidity and mortality, which can reach as high as 100% (2). 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 (1). Fowl typhoid has been eradicated from commercial poultry in many developed countries, including the United States and Canada, through the isolation and removal of contaminated flocks and the implementation of biosecurity and hygiene management (3). 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 the spread of *S.* Gallinarum in the environment (1, 2).

The 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: its variability in protective efficacy between breeds, its persistence in immunized chickens leading to transmission through eggs, and the residual virulence in some breeds (4, 5). 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 (5). However, a recent comparative analysis of its proteome and transcriptome showed that 9R may also be impaired in the regulation of several virulence factors (6).

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 an emphasis on the genes required for virulence in *S. enterica* serovar Typhimurium (7). For example, the 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 (7, 8). Conversely, mutations in *rfc* (*wzy*), required for complete O-antigen synthesis, are attenuating in *S.* Typhimurium (9, 10) but have no effect on the virulence of *S.* Gallinarum when delivered by the oral route (7). In addition, an arabinose-regulated *rfaH* construction that results in arabinose-regulated O-antigen synthesis was partially attenuating in *S.* Typhimurium (11) but was not attenuating in *S.* Gallinarum (7). Based on these results, we decided to expand our approach and explore additional genes involved in global regulation or O-antigen synthesis.

The ferric uptake regulator (Fur) protein acts as a repressor of many genes whose products are involved in iron, zinc, and manganese acquisition and uptake (12, 13). 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

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strains		
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4–2-Tc::Mu[λ*pir*] Δ*asdA4* Δ(*zhf-2*::Tn10); used for conjugational transfer of suicide plasmids	31
χ7232	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* λ*pir deoR* (φ80d*lac* Δ(*lacZ*)M15); used for general cloning	31
S. Gallinarum strains		
χ4173	Wild-type challenge strain	7
287/91	Wild-type vaccine parent strain	29
χ11575	Δ*fur-453*::*cam*	287/91
χ11386	$\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH*	287/91; 7
χ11741	Δ*pmi-2426*	287/91
χ11797	Δ*fur-712*	287/91
χ11798	Δ*pmi-2426* Δ*fur-712*	χ11741
χ11820	Δ*fur-712* Δ*pmi-2426*	χ11797
χ11821	$\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH* Δ*fur-712*	χ11386
χ11822	Δ*ansB1235*	287/91
χ11823	Δ*fur-712* Δ*ansB1235*	χ11797
Plasmids		
pKD46	λ red expression vector	30
pRE112	*sacB mobRP4*; R6K ori; Cm^r	53
pYA3546	Suicide vector for introduction Δ*pmi-2426*	25, 32
pYA5239	Suicide vector for introduction Δ*fur-712*	pRE112
pYA5272	Suicide vector for introduction Δ*ansB1235*	pRE112

is abundant and are upregulated when iron is limiting (12). Animal hosts restrict iron from invading bacteria during infection, a phenomenon known as nutritional immunity (14). Thus, the mechanisms for iron acquisition are crucial in the pathogenicity of many microorganisms, including *Salmonella* spp. Fur can also act as a transcriptional activator by enhancing RNA polymerase recruitment, regulating the production of small RNAs, or functioning as an antirepressor (12). In *Salmonella*, Fur also modulates the expression of genes involved in acid shock, adaptation (15, 16), and oxidative stress resistance (17, 18), and it plays a role in the regulation of the *Salmonella* pathogenicity island 1 (SPI-1) genes (e.g., *hilA* and *hilD*) necessary for invasion (19–21).

S. Typhimurium strains with an arabinose-regulated *fur* genotype (*fur* expressed *in vitro* in the presence of arabinose, not expressed *in vivo* where arabinose is not available) were partially attenuated and highly immunogenic in mice (22). The same study also showed that the attenuation of *S.* Typhimurium arabinose-regulated *fur* mutants is correlated with the level of *fur* expression. Furthermore, an *S. enterica* serovar Enteritidis Δ*fur* strain was attenuated, and the immunization of mice with this strain resulted in a decrease in the bacterial load in systemic organs after challenge with the wild-type strain (23). A *fur* deletion was also employed to improve the safety of an *S.* Typhimurium Δ*ssaV* mutant. The Δ*ssaV* Δ*fur* double mutant was safe and immunogenic in immunocompromised mice (24).

The *pmi* gene encodes phosphomannose isomerase, which 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, Δ*pmi* mutants cannot produce O antigen unless an exogenous source of mannose is present. In the context of a vaccine, Δ*pmi* strains are grown *in vitro* in the presence of mannose and synthesize a complete O antigen, a requirement for optimal host colonization (25). The O antigen is subsequently lost after several generations of growth in animal tissues, which are devoid of free nonphosphorylated mannose (25). *S.* Typhimurium *pmi* mutants are highly immunogenic and partially attenuated in mice (9).

The primary focus of this work was 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 (1). Our results show that immunization with an *S.* Gallinarum *fur* mutant provided excellent protection against challenge with virulent *S.* Gallinarum in both breeds.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S.* Gallinarum strains were routinely cultured at 37°C in LB broth (26) or on LB agar. Cultures of *S.* Gallinarum mutants were supplemented with 0.05% mannose (Sigma-Aldrich, St. Louis, MO) (for Δ*pmi-2426*), 0.2% arabinose (Sigma-Aldrich) (for $\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH*, here $\Delta P_{rfaH178}$), or 15 μg/ml chloramphenicol (Sigma-Aldrich) (for Δ*fur-453*::*cam*). Carbohydrate-free nutrient broth (NB) was used for growth when determining LPS profiles. The 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

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Orientation	Restriction site[b]
AM-115	**TCTAATGAAGTGAATCGTTTAGCAACAGGACAGATTCCGC**GTGTAGGCTGGAGCTGCTTC	Forward	∅
AM-116	**AAAAGCCAACCGGGCGGTTGGCTCTTCGAAAGATTTACAC**CATATGAATATCCTCCTTAG	Reverse	∅
fur-1F	TATAGAGCTC**TCTGCCTGTTCTGCTATG**	Forward	SacI
fur-1R	**GGCGCAGATATAACGCTGCGCCGCATAAGATTAGGC**	Reverse	∅
fur-2F	CAGCGTTATATCTGCGCCC**CTTTCGAAGAGCCAACCG**	Forward	∅
fur-2R	TATAGGTACC**GCCAGTTGTTCAGGTGTG**	Reverse	KpnI
ansB-1F	TATAGAGCTC**GCCGCTCATGCAGATTAC**	Forward	SacI
ansB-1R	**TTACTTCAGGCTGCCAACCAGCGCTTTGCGGCTATC**	Reverse	∅
ansB-2F	GTTGGCAGCCTGAAGTAA**TGATAATGCCCCGGTCGG**	Forward	∅
ansB-2R	TATAGGTACC**CCAATACGCGTCCGCTTC**	Reverse	KpnI

[a] Underlined nucleotides denote restriction enzyme sites used for cloning. Nucleotides in bold type are complementary to the *S.* Gallinarum 287/91 chromosome.
[b] Ø, the primer does not encode an engineered restriction site.

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.

For the animal experiments, *S.* Gallinarum strains were cultured in LB broth with the appropriate supplements. Overnight cultures were diluted 1:100 and grown with shaking (200 rpm) to an optical density at 600 nm of ~0.8. Next, bacteria were centrifuged at 5,000 × *g* for 15 min at room temperature and resuspended in phosphate-buffered saline (PBS) or buffered saline with 0.01% gelatin (BSG) (27). LB or *Salmonella Shigella* (SS) agar plates were used to enumerate *S.* Gallinarum organisms recovered from chicken tissues. Rappaport-Vassiliadis R10 (RV) broth was employed to enrich the samples for *S.* Gallinarum. All media were purchased from BD Difco (Franklin Lakes, NJ), unless otherwise indicated.

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 (28) or were performed according to the manufacturers' instructions (New England BioLabs, Ipswich, MA; Qiagen, Valencia, CA; Promega, Madison, WI). The synthesis of primers (Table 2) and DNA sequencing were performed by Integrated DNA Technologies (Coralville, IA) and the DNA Laboratory at Arizona State University (Tempe, AZ), respectively. PCRs 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.

Construction of *S.* Gallinarum vaccine strains. All vaccine candidates were derived from *S.* Gallinarum strain 287/91 (29). The *fur* deletion/insertion mutation Δ*fur-453*::*cam* was constructed via the λ red recombination method (30). The flanking sequences were based on the *S.* Gallinarum 287/91 genome using primers AM-115 and AM-116 (Table 2). All other gene replacements were introduced by the conjugational transfer of suicide plasmids using donor *E. coli* strain χ7213 (31).

To construct the Δ*fur-712* deletion, *fur* flanking regions were amplified from the *S.* Gallinarum 287/91 genome by two-step PCR. First, 644-bp and 663-bp DNA fragments flanking the *fur* gene were amplified with the 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 a 251-bp promoter region. The Δ*fur-712* mutation was introduced by allelic exchange into *S.* Gallinarum strains 287/91, χ11741, and χ11386 to generate χ11797 (Δ*fur-712*), χ11798 (Δ*pmi-2426* Δ*fur-712*), and χ11821 ($\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH* Δ*fur-712*), respectively.

The Δ*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 Δ*ansB1235* mutation was introduced into *S.* Gallinarum strains 287/91 and χ11797 to generate χ11822 (Δ*ansB1235*) and χ11823 (Δ*fur-712* Δ*ansB1235*), respectively.

As *S.* Typhimurium and *S.* Gallinarum share >99% sequence similarity in the flanking region surrounding *pmi*, the previously constructed suicide plasmid pYA3546 carrying *S.* Typhimurium DNA sequences was used to create *S.* Gallinarum Δ*pmi-2426* mutants (25, 32). Plasmid pYA3546 was introduced by conjugation into *S.* Gallinarum strains 287/91 and χ11797 to generate χ11741 (Δ*pmi-2426*) and χ11820 (Δ*fur-712* Δ*pmi-2426*), respectively.

All mutations were verified by PCR. We confirmed Fur production, or lack thereof, by Western blotting. The Δ*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 (33).

Isolation of outer membrane proteins. Outer membrane proteins (OMPs) were isolated using the Sarkosyl extraction method (34).

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting procedures were done by standard techniques. The 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 (22) or anti-GroEL antibodies (Sigma-Aldrich) as primary antibodies, and mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) as secondary antibodies.

Acid shock assay. Acid resistance was evaluated essentially as previously described, with a few modifications (35). Strains were grown aerobically in LB broth with appropriate supplements until they reached an optical density of ~0.4. The culture aliquots were centrifuged (10 min at 5,000 × *g*) at room temperature, and bacterial pellets were washed with E medium (pH 7.0). Thereafter, the cells were centrifuged again and resuspended at a density of ~0.5×10^9 CFU/ml in E medium (pH 3.0). Acid challenge was conducted at 37°C, and samples were collected immediately after resuspension and at 30-min intervals. The samples were serially diluted and plated onto LB agar to assess bacterial viability.

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) 1 or 2 days after hatch. The 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 the Institutional Animal Care and Use Committee (IACUC) and the Animal Welfare Act at Arizona State University.

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

Determination of LD_{50}. The strains were grown and harvested as described above in "Bacterial strains, plasmids, media, and growth conditions." Bacterial pellets were resuspended in PBS or BSG and adjusted to

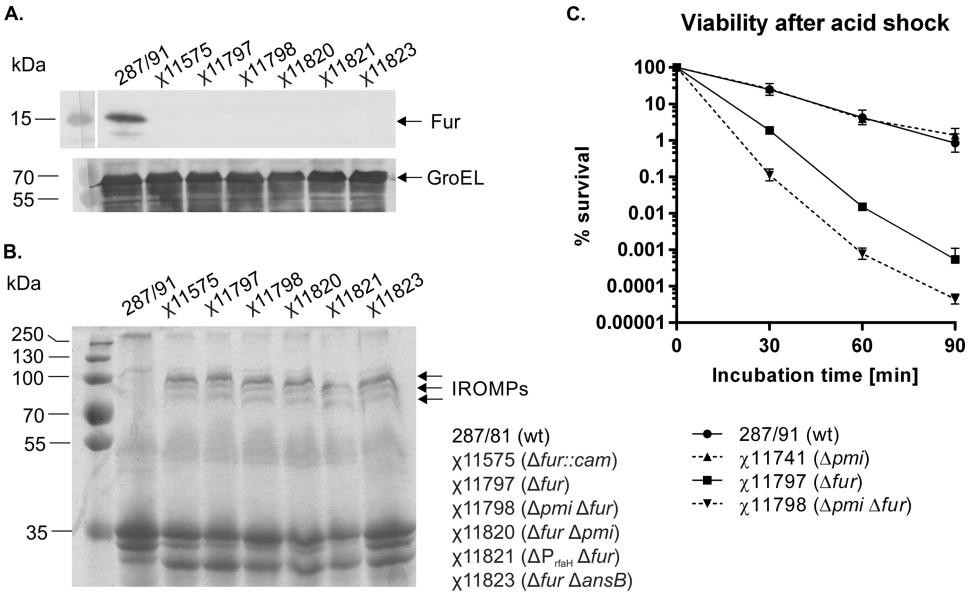

FIG 1 Phenotype characterization of *S.* Gallinarum Δ*fur* mutants. (A) Fur production in *S.* Gallinarum wild-type (287/91), Δ*fur-453*::*cam* (χ11575), and Δ*fur-712* vaccine strains (χ11798, χ11820, χ11821, and χ11823). Whole-cell lysates were obtained from overnight cultures, electrophoresed on a 12% SDS-PAGE gel, transferred onto nitrocellulose, and probed with anti-Fur serum (top). The blot was also probed with anti-GroEL antibodies (bottom) to serve as a loading control. (B) IROMP 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. wt, wild type. (C) Effect of acid shock on viability of *S.* Gallinarum *fur* mutants. Strains 287/91 (wt), χ 11741 (Δ*pmi-2426*), χ11797 (Δ*fur-712*), and χ11798 (Δ*pmi-2426* Δ*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 the mean and standard error of the mean (SEM) values 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 versus χ11741 were significantly different ($P < 0.01$).

achieve a dose of ~10^2 to ~10^8 CFU in a volume of 100 μl for orally inoculating chicks. The virulence of the wild-type strain, 287/91, and its derivatives were assessed in 3- or 5-day-old Rhode Island Red chicks. The birds were observed for fowl typhoid symptoms for 3 weeks postinoculation. Deaths were recorded daily. The 50% lethal dose (LD_{50}) was calculated using the Reed and Muench method (36).

Immunization and challenge regimen. For Rhode Island Reds, 3- or 5-day-old chicks were inoculated orally with 100 μl of PBS containing ~1×10^8 CFU of the appropriate *S.* Gallinarum strain and boosted with the same strain and dose 2 weeks later. No food was provided for ~5 to 6 h prior to immunizations or challenge. Groups of birds inoculated with buffer (PBS or BSG) served as controls. At 4 weeks of age (i.e., 2 weeks after the booster), all birds were orally challenged with ~1×10^7 CFU of heterologous *S.* Gallinarum strain χ4173. Note that in the case of *fur*::*cam* deletion/insertion strain χ11575, all chicks survived the virulence study described above. They were then treated as vaccinated chicks, boosted with ~1×10^8 CFU of χ11575, and challenged.

The chickens were observed for fowl typhoid symptoms for 3 weeks postchallenge. Deaths were recorded daily. At the end of the observation period, each surviving bird was euthanized, and its 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 the *Salmonella* organisms 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* organisms were detected by direct plating.

For experiments with Brown Leghorns, groups of 15 or 16 seven-week-old pullets were immunized orally (~2×10^7 CFU) or intramuscularly (~2×10^4 or ~2×10^7 CFU) with the appropriate *S.* Gallinarum strain. A group of nonvaccinated birds was used as a control. At 10 weeks of age (i.e., 3 weeks postimmunization), all birds were orally challenged with ~2×10^8 CFU of homologous *S.* Gallinarum strain 287/91. The birds were monitored for 3 weeks postchallenge. Next, each surviving bird was euthanized, and necropsies were performed to determine the presence of tissue lesions.

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 of <0.05 were considered significant.

RESULTS

Screening for *S.* Gallinarum immunogenic mutants. To evaluate the impact of a *fur* deletion in *S.* Gallinarum, we constructed strain χ11575, harboring the Δ*fur-453*::*cam* deletion/insertion. As expected, Fur was not detected in χ11575 by Western blot analysis (Fig. 1A). Next, we screened for the production of IROMPs after growth in LB, a medium in which iron is not limiting (~7.6 μM iron [37]). Under these conditions, IROMPs were not detected in parent strain 287/91 but were easily detectable in χ11575 (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 (38) (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 (38) or from a *fur* mutant grown in the relatively iron-rich medium NB (25).

Strain χ11575 was then screened for virulence in Rhode Island Red chicks. The birds were given orally graded doses of bacteria and monitored for 3 weeks. The strain was fully attenuated, with no deaths occurring at the highest dose tested (LD_{50}, >~1×10^8 CFU) (Table 3). Encouraged by these results, we evaluated the ability of χ11575 to confer protection against challenge with vir-

TABLE 3 Attenuation of *S.* Gallinarum mutants in Rhode Island Red chicks

Strain	Genotype	LD_{50} (CFU)
287/91	Wild type	6.7×10^4
χ11575	Δ*fur-453*::*cam*	$>\sim 1 \times 10^8$
χ11797	Δ*fur-712*	$>0.9 \times 10^8$
χ11741	Δ*pmi-2426*	1.0×10^7
χ11822	Δ*ansB1235*	1.3×10^4
χ11821	$\Delta P_{rfaH178}$ Δ*fur-712*	$>1.2 \times 10^8$

ulent *S.* Gallinarum. The same chicks used in the virulence assay were boosted 2 weeks after the first inoculation with $\sim 1 \times 10^8$ CFU of χ11575 and challenged 2 weeks later with $\sim 1 \times 10^7$ CFU of heterologous wild-type strain χ4173. All the birds, even those primed with the lowest dose ($\sim 1 \times 10^2$ CFU) of χ11575, survived challenge with a virulent *S.* Gallinarum strain (Table 4), suggesting that an *S.* Gallinarum *fur* mutant is a viable vaccine candidate. However, strain χ11575 contains a chloramphenicol resistance cassette in the chromosome, precluding its use as a vaccine. Thus, we constructed *S.* Gallinarum strain χ11797, carrying the unmarked Δ*fur-712* deletion (Table 1). We confirmed the absence of detectable Fur in this strain (Fig. 1A), and the production of IROMPs following growth in LB was indistinguishable from that in χ11575 (Fig. 1B).

In *S.* Typhimurium, *fur* mutants display an acid-sensitive phenotype (39). To determine the acid resistance of *S.* Gallinarum *fur* mutants, χ11797 (Δ*fur-712*) and parent strain 287/91 were cultured in LB to the 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 χ11797 than for 287/91 (Fig. 1C). After 30 min of pH 3.0 exposure, the survival of the mutant was significantly lower (2.0%) than that of the wild type (25.1%; $P < 0.01$). After 90 min of challenge, only 0.001% of the χ11797 cells survived compared to 0.847% of the wild-type cells, corresponding to an ~880-fold (2.9-log) difference in the number of viable cells ($P < 0.0001$).

Virulence and protective efficacy of *S.* Gallinarum Δ*fur-712* mutant in Rhode Island Red chickens. We determined the virulence of *S.* Gallinarum χ11797 (Δ*fur-712*) in 5-day-old Rhode Island Red chicks. As expected, strain χ11797 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 6.7×10^4 CFU, consistent with previous results (7).

Strain χ11797 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 5-day-old Rhode Island Red chickens. The birds were primed and boosted orally 2 weeks later with identical doses of $\sim 1 \times 10^8$ CFU of χ11797. In each study, a control group was given a sterile buffer instead of vaccine. At 4 weeks of age, all birds were challenged with $\sim 1 \times 10^7$ CFU of heterologous virulent strain χ4173. In both studies, immunization with strain χ11797 provided significant protection compared to nonimmunized control birds (Table 4). In both experiments, >90% of the vaccinated chickens survived compared to only 17 to 18% survival in the control groups ($P < 0.001$).

Additionally, in one of the protection studies (Table 4, experiment 3), the internal organs from all animals were inspected for lesions and bacterial loads after challenge. The birds that died from challenge were necropsied immediately, and each survivor was euthanized 3 weeks postchallenge, with 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

TABLE 4 Protective efficacy of *S.* Gallinarum *fur* mutants in Rhode Island Red chickens[a]

Strain	Genotype	Expt	Prime (CFU)	Boost (CFU)	No. with condition/total no. (%) for: Hepatomegaly	Splenomegaly	Heart lesions/pericarditis	No. of chickens alive/total no.	% survival
Single *fur*									
χ11575	Δ*fur-453*::*cam*	1	Various[b]	$\sim 1 \times 10^8$	NT[c]	NT	NT	20/20	100[d]
χ11797	Δ*fur-712*	2	1.0×10^8	1.2×10^8	NT	NT	NT	10/11	91[d]
		3	1.2×10^8	1.1×10^8	1/13 (8)[d]	2/13 (15)[d]	5/13 (38)	12/13	92[d]
fur combined with other mutations									
χ11798	Δ*pmi-2426* Δ*fur-712*	2	1.0×10^8	1.0×10^8	NT	NT	NT	2/9	22
χ11820	Δ*fur-712* Δ*pmi-2426*	3	1.2×10^8	1.3×10^8	10/12 (83)	9/12 (75)	3/12 (25)	4/12	33
χ11821	$\Delta P_{rfaH178}$ Δ*fur-712*	3	1.1×10^8	1.1×10^8	9/12 (75)	8/12 (67)	3/12 (25)	4/12	33
χ11823	Δ*fur-712* Δ*ansB1235*	3	1.0×10^8	0.9×10^8	7/12 (58)	8/12 (67)	3/12 (25)	6/12	50
Controls									
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

[a] Three- or 5-day-old chicks were immunized orally with the indicated dose of *S.* Gallinarum and boosted 2 weeks later. At 4 weeks of age, all birds were challenged with $\sim 1 \times 10^7$ CFU of heterologous *S.* Gallinarum wild-type strain (χ4173).

[b] These birds were survivors of the virulence assay, so the chicks received 1×10^2, 10^4, 10^6, or 10^8 CFU as a priming dose. The boost was 1×10^8 CFU for all birds.

[c] NT, not tested.

[d] $P < 0.01$ compared to control.

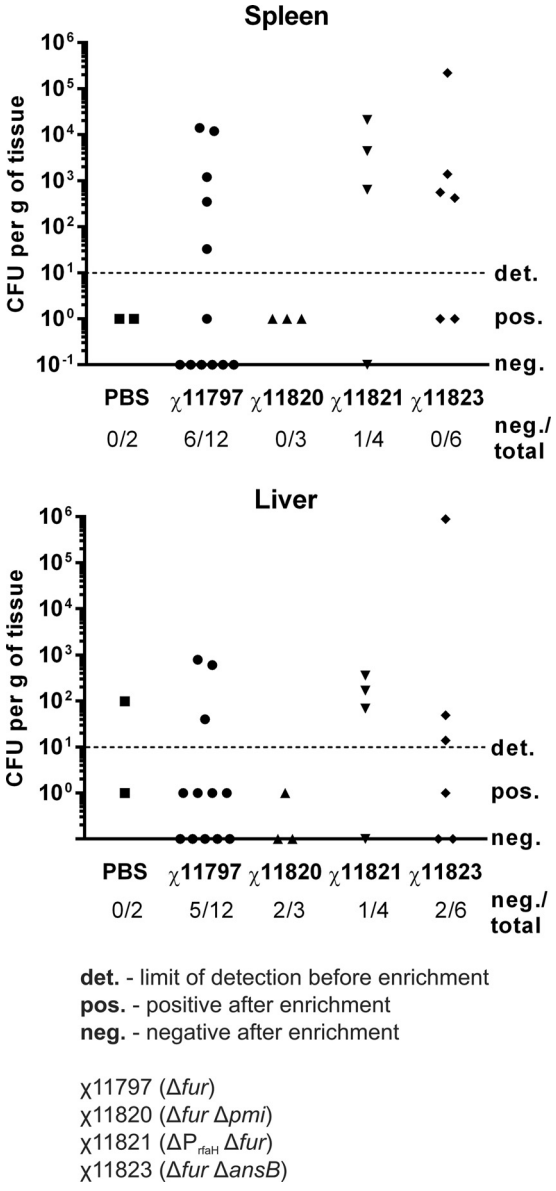

FIG 2 Colonization of spleen and liver in birds that survived the challenge with wild-type *S.* Gallinarum. Survivors from experiment (expt.) 3 (Table 4) were euthanized 19 days postinfection, and spleens and livers were collected to recover viable *S.* Gallinarum from each tissue. The organs were homogenized, diluted, and plated on LB agar. Negative samples were additionally enriched using RV broth and plated on SS agar.

challenge. In contrast, spleens and livers in birds vaccinated with χ11797 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 the immunized birds. Furthermore, 19 days postchallenge, spleen and liver samples were collected from all surviving birds to enumerate *S.* Gallinarum colonization in each tissue (Fig. 2). In birds vaccinated with χ11797 (Δ*fur-712*), the *S.* Gallinarum challenge strain was not detectable in 50% of the spleen and 42% of the liver samples. The bacterial loads were significantly lower (maximum [max], 1.4×10^4 CFU/g for spleen; 7.9×10^2 CFU/g for liver) in the remaining *S.* Gallinarum-positive tissues than in the nonvaccinated birds that succumbed to the infection, where counts were typically around 1×10^6 CFU per g of tissue (data not shown).

Immunogenicity of *S.* Gallinarum double mutants. We next examined two distinct genetic strategies for enhancing the immunogenicity of χ11797. It is well established that *Salmonella* O antigen is required for efficient colonization of the chicken host (40, 41). Mutations that result in the gradual loss of O antigen *in vivo* can be used in *Salmonella* vaccine strains to enhance the induction of high-antibody titers to outer membrane proteins (25). Thus, we investigated the possibility that the introduction of a Δ*pmi* or an arabinose-regulated *rfaH* mutation might enhance the immunogenicity of χ11797.

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 Δ*ansB* mutants are attenuated for virulence in mice (42). Thus, as an alternative approach to enhance immunogenicity, we examined the effect of Δ*ansB* on virulence and immunogenicity alone or when combined with a Δ*fur* mutation.

We constructed χ11741 (Δ*pmi-2426*) and χ11822 (Δ*ansB 1235*) single mutant strains and χ11798 (Δ*pmi-2426* Δ*fur-712*), χ11820 (Δ*fur-712* Δ*pmi-2426*), χ11821 ($\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH* Δ*fur-712*), and χ11823 (Δ*fur-712* Δ*ansB1235*) double mutant strains. The absence of detectable Fur was verified in the double mutants (Fig. 1A), and IROMP synthesis was not affected by combining Δ*pmi-2426*, $\Delta P_{rfaH178}$, or Δ*ansB123* with Δ*fur-712* (Fig. 1B). An analysis of the LPS profiles of both *pmi* mutants (χ11741 and χ11798) and the $\Delta P_{rfaH178}$ χ11821 double mutant strain indicated that full-length O antigen was produced by both *pmi* strains and the $\Delta P_{rfaH178}$ mutant only when mannose or arabinose, respectively, was added to the growth medium (data not shown).

We also evaluated the acid resistance of the Δ*pmi* mutant strains. Interestingly, strain χ11798 (Δ*pmi-2426* Δ*fur-712*) was more sensitive to low pH than χ11797 (Δ*fur-712*), even though it was grown in the presence of mannose prior to challenge (Fig. 1C). At every time point during challenge, the survival rate of strain χ11798 was significantly lower than that of strain χ11797 ($P < 0.001$). It is unlikely that the addition of mannose to strain χ11798 was responsible for the increase in acid sensitivity because strain χ11741 (Δ*pmi-2426*), when grown in LB with mannose, displayed a survival profile identical to that of wild-type strain 287/91, and we observed no change in survival to acid challenge when mannose was added during the growth of χ11797 (Δ*fur-712*) (data not shown).

Since an adequate level of attenuation is critical for designing safe and efficacious vaccines, we examined the virulence of *S.* Gallinarum strains χ11741 and χ11822, harboring single Δ*pmi* or Δ*ansB* mutations, respectively. The Δ*pmi* χ11741 mutant was partially attenuated, similar to the phenotype observed for *S.* Typhimurium (9), while Δ*ansB* χ11822 mutant was fully virulent (Table 3). Strain χ11821 ($\Delta P_{rfaH178}$ Δ*fur-712*), a derivative of hypervirulent strain χ11386 ($\Delta P_{rfaH178}$) (7), was also tested. The introduction of Δ*fur-712* into χ11386 resulted in a complete loss of virulence.

The *S.* Gallinarum double mutants were then tested for protective efficacy in Rhode Island Reds. Note that while strains χ11820 and χ11798 have the same genotype, the mutations were intro-

TABLE 5 Protective efficacy of *S.* Gallinarum *fur* mutants in female Brown Leghorn chickens[a]

Strain	Genotype	Route	Prime (CFU)	No. with condition/total no. (%) for:			No. of chickens alive/total no.	% survival
				Hepatomegaly	Splenomegaly	Heart lesions/pericarditis		
χ11797	Δ*fur-712*	i.m.	2.6×10^4	0/16 (0)[b]	3/16 (19)[b]	2/16 (13)	16/16	100[b]
		i.m.	2.6×10^7	1/16 (6)[b]	2/16 (12)[b]	3/16 (19)	15/16	100[b]
		Oral	2.6×10^7	14/16 (88)	15/16 (94)	2/16 (13)	8/16	50
χ11798	Δ*pmi-2426* Δ*fur-712*	i.m.	2.2×10^7	1/16 (6)[b]	4/16 (25)[b]	5/16 (31)	16/16	100[b]
No vaccine				11/15 (73)	11/15 (73)	5/16 (31)	6/16	38

[a] Seven-week-old birds were immunized orally or intramuscularly (i.m.) with the indicated dose of *S.* Gallinarum vaccine strain. Three weeks later, all birds were challenged orally with 2×10^8 CFU of *S.* Gallinarum strain 287/91. The health of the birds was monitored for 3 weeks postchallenge.
[b] $P < 0.01$ compared to control.

duced in a different order, with the Δ*fur-712* mutation introduced first, and then Δ*pmi-2426*, in strain χ11820 and second in strain χ11798. Birds immunized with either χ11820 (Δ*fur-712* Δ*pmi-2426*) or χ11798 (Δ*pmi-2426* Δ*fur-712*) were not protected (33% and 22% survival, respectively) (Table 4). A lack of protection was also observed for birds vaccinated with χ11821 ($\Delta P_{rfaH178}$ Δ*fur-712*) (33% survival). Vaccination with χ11823 (Δ*fur-712* Δ*ansB1235*) resulted in 50% protection, but this result was not significantly different from that with the nonvaccinated controls.

Protective efficacy of *S.* Gallinarum vaccine strains in Brown Leghorn chickens. Two vaccine strains, χ11797 (Δ*fur-712*) and χ11798 (Δ*pmi-2426* Δ*fur-712*), were also tested for protection immunity in Brown Leghorn chickens. In this study, 7-week-old female chickens were vaccinated with a single dose of vaccine by intramuscular (~2×10^4 or ~2×10^7 CFU) or oral (~2×10^7 CFU) routes and challenged 3 weeks later with the virulent wild-type vaccine parent strain 287/91. As shown in Table 5, intramuscular immunization with a high dose (2.6×10^7 CFU) of strain χ11797 provided protection to all vaccinated birds. Moreover, enlargement of the spleen or liver was observed only in 6 and 12% of vaccinated birds postchallenge, respectively. Interestingly, a single low dose (2.6×10^4 CFU) of χ11797 delivered intramuscularly was also highly protective (100% survival; splenomegaly and hepatomegaly detected in 0 and 19% of birds, respectively). In comparison, only 38% of the nonvaccinated birds survived the challenge, and spleen and liver lesions were observed in most of them (73%). On the other hand, when delivered orally, strain χ11797 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 from that of nonvaccinated birds. Moreover, the percentage of birds in this group with organ lesions was similar to that in the control. In contrast, when administered intramuscularly, strain χ11798 provided significant protection against fowl typhoid (100% survival, $P < 0.001$; lower percentage of birds with lesions relative to control; $P < 0.01$).

DISCUSSION

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 Δ*fur* mutants were attenuated when delivered orally (43) or intraperitoneally (44) in mice, they were not found to be highly immunogenic (45). 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 attenuated only about 10-fold (43, 44), while *S.* Typhimurium strain UK-1 *fur* mutants are attenuated 1,000-fold (43) or more (22). Differences in *rpoS* alleles can influence the acid tolerance response of both wild-type and *fur* mutants of *Salmonella* (46) and may therefore affect other phenotypic aspects of *fur* mutants. 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. enterica* serovar Typhi in humans (47, 48). Since *S.* Gallinarum and *S.* Typhi are strictly host-adapted serovars, the mechanisms of their pathogenesis are different from those of the broad-host range *S.* Typhimurium. Unfortunately, the molecular basis of host specificity and the mechanisms determining which type of disease is caused in which animal species are still poorly understood (49).

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 the serovars or strains used for their construction. Protection from disease may also be influenced by the route of administration as well as the genetic properties or age of particular breeds, such as Rhode Island Red or Brown Leghorn chickens. Deletions of *fur* have been introduced into the fish pathogens *Pseudomonas fluorescens* (50) and *Edwardsiella ictaluri* (51) 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* (50). 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* and *E. coli* serovars (25). Since our *S.* Gallinarum *fur* mutants constitutively synthesize IROMPs (Fig. 1B), it will be interesting to determine how well an *S.* Gallinarum Δ*fur* mutant, such as χ11797, protects chickens against other *Salmonella* serovars, in particular *S.* Enteritidis and *S.* Typhimurium. This is a topic for future study.

The Δ*pmi* mutant strain χ11741 was moderately attenuated, with an oral LD_{50} about 2.5 logs higher than that of its wild-type parent, 287/91 (Table 3). This modest reduction in virulence is similar to a situation seen in *S.* Typhimurium, in which a 3.3-log increase in oral LD_{50} (for mice) was observed for a *pmi* mutant grown with mannose (9). The partial virulence of χ11741 makes this mutant unsuitable for use as a stand-alone vaccine strain. The idea behind combining Δ*pmi* and Δ*fur* in the same strain was that the loss of O antigen over time would enhance the presentation of the IROMPs to the host immune system. 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) (45), 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 overattenuated. However, while the χ11798 and χ11820 double mutants 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, $\Delta P_{rfaH178}$, which is not attenuating and in fact appears to be hypervirulent (7). Once again, this combination was also not protective (Table 4).

In contrast to our results in Rhode Island Red chicks, the *S.* Gallinarum Δ*pmi* Δ*fur* χ11798 mutant was highly immunogenic when used to intramuscularly immunize 7-week-old Brown Leghorns (Table 5). Thus, it may be that because the double mutant is more sensitive to low pH than the Δ*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 the *fur* χ11797 mutant, which was protective when orally administered to chicks (Table 4) but was less effective when orally administered to older layers (Table 5). 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 2 days of age to about 3 to 3.5 by 15 days of age (52). Thus, it is possible that the survival of strain χ11797 was greater in chicks than in the older birds used in our study. When we bypassed the gastric compartment by intramuscular injection, the χ11797 mutant was able to elicit a protective response (Table 5). The increased acid sensitivity of χ11798 might 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, the overexpression of outer membrane proteins (e.g., IROMPs) in the absence of complete O antigen has a negative influence on immunogenicity, perhaps due to the destabilization of outer membrane integrity *in vivo*. Of course, it is possible that other factors, including possible iron toxicity, have played a role (45).

Recently, it was shown that *S.* Typhimurium utilizes a product of *ansB* gene, L-asparaginase II, to inhibit host T-cell responses essential for the clearance of *Salmonella* infection (42). 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. The production of L-asparaginase II by *Salmonella* leads to the 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 the introduction of an Δ*ansB* mutation into the Δ*fur* χ11797 mutant strain abrogated, rather than enhanced, its immunogenicity (Table 4). Thus, our study did not identify a role for *ansB* in *S.* Gallinarum infection or pathogenicity in chicks.

One of the primary goals of our research was 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 the Δ*fur* χ11797 mutant is safe (Table 3). It is effective in chicks (Table 4) but is unsuitable for use as an oral vaccine in older birds (Table 5), though it is highly immunogenic when delivered by the intramuscular route. It is possible that the problem of efficacy in older birds can be rectified by the introduction of a mutation that allows for regulated delayed *fur* expression, as has been demonstrated to be effective in the *S.* Typhimurium mouse model (25). We used a similar strategy to regulate the expression of *crp* in *S.* Gallinarum, with promising results (7). If this strategy is effective with *fur*, it may allow us to take advantage of a second mutation in *pmi*, as we described above.

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

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