Assessment of attenuated *Salmonella* vaccine strains in controlling experimental *Salmonella* Typhimurium infection in chickens

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Abstract

Salmonella hold considerable promise as vaccine delivery vectors for heterologous antigens in chickens. Such vaccines have the potential additional benefit of also controlling *Salmonella* infection in immunized birds. As a way of selecting attenuated strains with optimal immunogenic potential as antigen delivery vectors, this study screened 20 novel *Salmonella* Typhimurium vaccine strains, differing in mutations associated with delayed antigen synthesis and delayed attenuation, for their efficacy in controlling colonization by virulent *Salmonella* Typhimurium, as well as for their persistence in the intestine and the spleen. Marked differences were observed between strains in these characteristics, which provide the basis for selection for further study as vaccine vectors.

Résumé

La bactérie Salmonella *est considérée comme un vecteur vaccinal prometteur pour la livraison d'antigènes hétérologues chez les poulets. De tels vaccins ont le potentiel bénéfique supplémentaire de limiter les infections par* Salmonella *chez les oiseaux immunisés. Comme moyen de sélectionner les souches atténuées avec le potentiel immunogène optimal comme vecteur de livraison d'antigènes, la présente étude a examiné 20 souches vaccinales nouvelles de* Salmonella *Typhimurium, qui différaient en mutation associées avec une synthèse antigénique retardée et une atténuation retardée, pour leur efficacité à limiter la colonisation par du* Salmonella *Typhimurium virulent, ainsi que pour leur persistance dans l'intestin et la rate. Des différences marquées furent observées entre les souches pour ces caractéristiques, fournissant ainsi des éléments de sélection pour des études ultérieures comme vecteurs vaccinal.*

(Traduit par Docteur Serge Messier)

Introduction

Salmonellosis is one of the most important food-borne zoonotic diseases throughout the world, and poultry products represent an important source of human infection. Chicken meat and eggs have been found to be common food sources of *Salmonella enterica* infection (1). Among the 2500 serovars of *Salmonella*, Enteritidis and Typhimurium have been responsible for the majority of food-borne salmonellosis in humans in the past, although the pattern of dominant serovar varies over time and geographically (2,3).

Effective control of food-borne pathogens, including *Salmonella*, is a major challenge to the poultry industry. Control of *Salmonella* infection in chicken focuses partly on farm management, although vaccination is sometimes used, for example in Europe to control *S.* Enteritidis infection (4). For vaccine prevention, both killed virulent and live attenuated *Salmonella* strains are used (4,5). Although killed and attenuated *Salmonella* vaccines reduce intestinal colonization, and egg and meat contamination, the level of protection is variable, dependent in part on the serovars involved (6,7). Live attenuated *Salmonella* vaccines have been shown to induce both humoral and cell-mediated responses similar to those observed after natural infections (8,9), and have the benefits of ease of administration and low cost. *Salmonella* have promise as delivery vectors for control of bacterial and viral infections in chickens (10–21). In recent years, improvements of attenuated *Salmonella* as vaccine vehicles have included regulated delayed attenuation (22) and regulated antigen synthesis (23) such that strains express protective antigens at high levels and stimulate strong and lasting immune responses without producing tissue damage, while improving their immunogenicity and safety (23–27).

We have been interested in assessing the value of attenuated *Salmonella* as vectors for the control of necrotic enteritis (7,16,17,21). If *Salmonella* vectored vaccines are developed that can effectively control necrotic enteritis, one additional benefit might be the reduction of colonization by *Salmonella*, so that these vaccines could have an additional benefit to public health. In an earlier study of a *Salmonella* vaccine vectored control of necrotic enteritis, the unexpectedly disappointing results obtained were attributed to the poor viability of the vector in chickens (7,21). The strain induced only a weak protective effect against *Salmonella* colonization. There was an association between the efficacy of vaccine strains in protecting against necrotic enteritis and their efficacy in protecting against *Salmonella* colonization (7,16,21). We concluded (7) that the optimal choice of an attenuated *S.* Typhimurium vaccine vector for delivery

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of heterologous antigens in chickens should be based partly on its value in protecting against colonization with serovars within the serogroup B or D.

The purpose of the study reported here was to screen a large number (n = 20) of novel *Salmonella* vaccine vector strains for their efficacy in controlling colonization by a virulent strain of *Salmonella* Typhimurium, as the first step in identifying potentially improved vaccine vectors for delivery of heterologous antigens likely to give protection against necrotic enteritis.

Materials and methods

Chickens

White Leghorn mixed-gender fertile specific-pathogen-free (SPF) eggs, obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario), were hatched at the Arkell Poultry Research Station, University of Guelph. Two separate experiments, A and B, were carried out under similar conditions. Ten birds in each group were housed in Horsfall isolators to prevent cross-contamination. All chickens were fed an antibiotic–free chicken starter containing 20% protein. Experiments were approved and monitored by the University of Guelph Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Bacterial strains

Salmonella vaccine strains and the *Salmonella* Typhimurium wild type are listed in Table I. All *Salmonella* vaccine strains, except χ3985, had a mutation in the *asd* gene (28). Briefly, mutants involved genes including combinations of: *ara*, affecting arabinose utilization (a carbohydrate not available *in vivo*) and arabinose-controlled expression of other genes; ΔP_{crp} *araC* P_{BAD} *crp*, conferring arabinose-dependent expression of the *crp* gene that encodes the catabolite regulatory protein (29); ΔP_{fur} *araC* P_{BAD} *fur*, giving differential expression of the *fur* gene dependent on arabinose and causing up-regulation of all Fur-regulated genes for iron and partially for manganese uptake *in vivo* after colonization of lymphoid tissues; *gmd-fcl*, eliminating 2 enzymes in colanic acid synthesis pathway for conversion of GDP-mannose to GDP-fucose; *lacI*, the gene encoding the LacI repressor to confer regulated delayed *in vivo* synthesis of recombinant protective proteins; Δ*pabA*, requirement for *p*-aminobenzoic acid; $P_{phoPQ177}$::TT *araC* P_{BAD} *phoPQ*, conferring arabinose-dependent expression of the global virulence regulator genes *phoP* and *phoQ*; *pmi*, phosphomannose isomerase, conferring mannose-dependent LPS O-antigen synthesis and loss of O-antigen *in vivo* due to a lack of free mannose; P_{rfc174}::TT *araC* P_{BAD} *rfc* and $\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH* conferring arabinose-dependent LPS O-antigen synthesis through regulation of either *rfc*, encoding O-antigen polymerase or *rfaH*, encoding an antitermination factor required for transcription of the *rfb* operon; and Δ*relA*, uncoupling growth from necessary protein synthesis.

Growth curves

All *Salmonella* vaccine strains carrying the Δ*asd* mutations were complemented by an *asd*+ plasmid (pYA3342), so that they could grow in the absence of 2-6-diaminopimelic acid (DAP) *in vitro* and *in vivo* (30). Complemented *Salmonella* vaccine vector strains and wild type *S.* Typhimurium were grown in LB broth (Difco, Detroit, Michigan, USA) only or in LB broth containing 0.05% L-arabinose. Mannose was not added to the media for this experiment. Each overnight culture was adjusted to an optical density at 600 nm (OD_{600}) to 0.5, and 0.5 mL was then inoculated into 50 mL pre-warmed LB or LB containing 0.05% L-arabinose. The OD_{600} was measured every 60 min.

Growth of *Salmonella* for vaccination of chickens

The vaccine strains and wild type strain were grown in LB broth supplemented with 0.05% L-arabinose and, for strains carrying a Δ*pmi* mutation, 0.1% mannose (Table I). A 100 mL volume of pre-warmed LB broth was inoculated with 2 mL fresh overnight culture and incubated at 37°C until an OD_{600} of 0.8 was achieved. Cells were pelleted by centrifugation at 8000 × *g* for 15 min at 4°C. The pellet was suspended with 2 mL phosphate buffered saline, pH 7.2, with 1% gelatin (BSG), and serial dilutions of suspended *Salmonella* strains were plated onto unsupplemented LB agar to determine the numbers of colony-forming units (CFU). Vaccine strains were diluted to 1×10^9 CFU/mL and the wild type challenge strain was diluted to 2×10^6 CFU/mL.

Vaccination procedure

Two studies were done with the different vaccine strains. The first study (study A) involved 100 birds and the second, study B, involved 120 birds. For these studies, 1-day-old SPF white Leghorn chickens were randomly divided into groups of 10 chickens, which were housed individually in heated Horsfall isolators equipped with high efficiency particulate air filters. Groups were deprived of food and water for 4 h before crop inoculation (gavage) for vaccination or challenge; food and water were returned 1 h later. All, except one group in each study, were vaccinated at 1 d of age and given a booster of the same dose at 7 d of age. The vaccine dose was 1×10^8 CFU of the vaccine strain suspended in BSG, delivered directly into the crop by injection with a 1 mL syringe. One control group was vaccinated on the same schedule with 100 µL BSG. Five birds of each group (10 birds in the control group) were challenged orally at 14 d of age with 2×10^5 CFU of the wild type strain, SA2004-4003.

Enumeration of vaccine strains in spleen, feces, and cecum

Sample collection and processing was similar to that described previously (7). Pooled fecal samples were collected after vaccination on day 14 of age. Five birds per group were euthanized on day 14 of age and processed as described (31). The spleen and cecum were collected aseptically from each bird into sterile pre-weighed sample tubes, weighed, and kept on ice. The spleen was homogenized 1:10 (w/v) in selenite cystine broth (SCB, Difco) and serially diluted in BSG before plating onto *Salmonella-Shigella* agar (Difco). Colorless colonies with black centers of typical *Salmonella* were counted. Day 14 cecal content and the pooled day 14 fecal sample were serially diluted as 1:10 (w/v) in SCB, incubated for 36 to 48 h at 37°C, and 100 µL aliquots were plated onto brilliant green agar (Difco), and incubated at 37°C for 24 h. Pink colonies were considered to be positive and subcultured on MacConkey

Table I. Salmonella strains used in this study

Strains	Genotypes	Description of strains
χ3985	Δ*crp-11* Δ[*zhb* ::Tn*10*] Δ*cya-12* Δ[*zid-62* ::Tn*10*]	Curtiss et al (22); Hassan and Curtiss (29)
χ3987	Δ*crp-11* Δ[*zhb* ::Tn*10*] Δ*cya-12* Δ[*zid-62* ::Tn*10*] Δ*asdA1* Δ[*zhf*::Tn*10*] (χ3985 derivative, Δ*asdA1*)	Wyszynska et al. (13)
χ9373	Δ*pmi-2426* Δ *(gmd-fcl)-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23*Δ*relA198*::*araC* P_{BAD} *lacI* TT	Li et al. (18)
χ9088	Δ*pmi-2426* Δ *(gmd-fcl)-26*ΔΔP_{fur33}::TT *araC* P_{BAD} *fur*	Curtiss et al. (24)
χ9241	Δ*pabA1516*Δ*pabB232* Δ*asdA16* Δ*araBAD23*Δ*relA198*::*araC* P_{BAD} *lacI* TT	Wang et al. (23)
χ9852	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT $\Delta P_{rfaH178}$::TT *araC* P_{BAD} *rfaH*	Kong et al. (19)
χ9853	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT P_{rfc174}::TT *araC* P_{BAD} *rfc*	Kong et al. (27)
χ9885	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*rfc-48*	Kong et al. (27)
χ9894	$P_{phoPQ177}$::TT *araC* P_{BAD} *phoPQ* Δ*araBAD23*Δ*asdA33*ΔΔP_{crp527}::TT *araC* P_{BAD} *crp*	Kader R, Curtiss R, III
χ9945	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23*Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*rfaH49*	Kong et al. (19)
χ11304	Mutation details proprietary	Kong Q, Shi Z, Curtiss R, III
χ11426	Δ*asdA33* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	Brenneman K, Curtiss R, III
χ11434	Δ*asdA33* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{fur81}::TT *araC* P_{BAD} *fur*	Brenneman K, Curtiss R, III
χ11442	Δ*asdA33*Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* Δ*pmi-2426*	Brenneman K, Curtiss R, III
χ11437	Δ*asdA33* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{crp527}::TT *araC* P_{BAD} *crp* ΔP_{fur81}::TT *araC* P_{BAD} *fur*	Brenneman K, Curtiss R, III
χ11443	Δ*asdA33*Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{fur81}::TT *araC* P_{BAD} *fur* Δ*pmi-2426*	Brenneman K, Curtiss R, III
χ11444	Δ*asdA33*Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*pmi-2426*	Brenneman K, Curtiss R, III
χ11445	Δ*pmi-2426* Δ*(gmd-fcl)-26* ΔP_{fur33}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	Wanda S-Y, Curtiss R, III
χ11447	Mutation details proprietary	Maddux J, Shi Z, Curtiss R, III
χ11464	Δ*asdA33*Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*araBAD23* ΔP_{crp527}::TT *araC* P_{BAD} *crp* ΔP_{fur81}::TT *araC* P_{BAD} *fur* Δ*pmi-2426*	Brenneman K, Curtiss R, III
Salmonella Typhimurium	Strain SA 2004-4003; Nalidixic acid resistant; wild type virulent challenge strain.	

agar plates supplemented with 0.5% maltose to verify their colorless phenotype.

Enumeration of the wild type challenge strain in tissues and cecum

After challenge with strain SA 2004-4003 at 2 wk of age, a pooled fecal sample was collected on day 28 of age. Five birds per group were also euthanized on day 28. The spleen and cecal contents collection and processing methods for these birds were as described for the vaccine strains, except that 100 µL aliquots of samples serially diluted in SCB were immediately plated onto brilliant green agar and *Salmonella-Shigella* agar supplemented with 30 µg/mL nalidixic acid, then incubated at 37°C for 24 h. To monitor day 28 fecal shedding of the challenge strain, the feces were processed as described for the vaccine strains except that 100 µL aliquots of samples serially diluted in SCB were immediately plated onto brilliant green agar (Difco) supplemented with 30 µg/mL nalidixic acid and incubated for 24 h.

Statistical analysis

The CFU data were analyzed for significance using a 2-tailed Student's *t*-test. The null hypothesis was rejected if $P < 0.05$.

Results

Growth of vaccine strains *in vitro*

Eighteen of the 20 vaccine strains have been modified for delayed attenuation and for heterologous antigen expression controlled by arabinose. Therefore, 2 parallel experiments were conducted under the same conditions, except that in the second study 0.05%

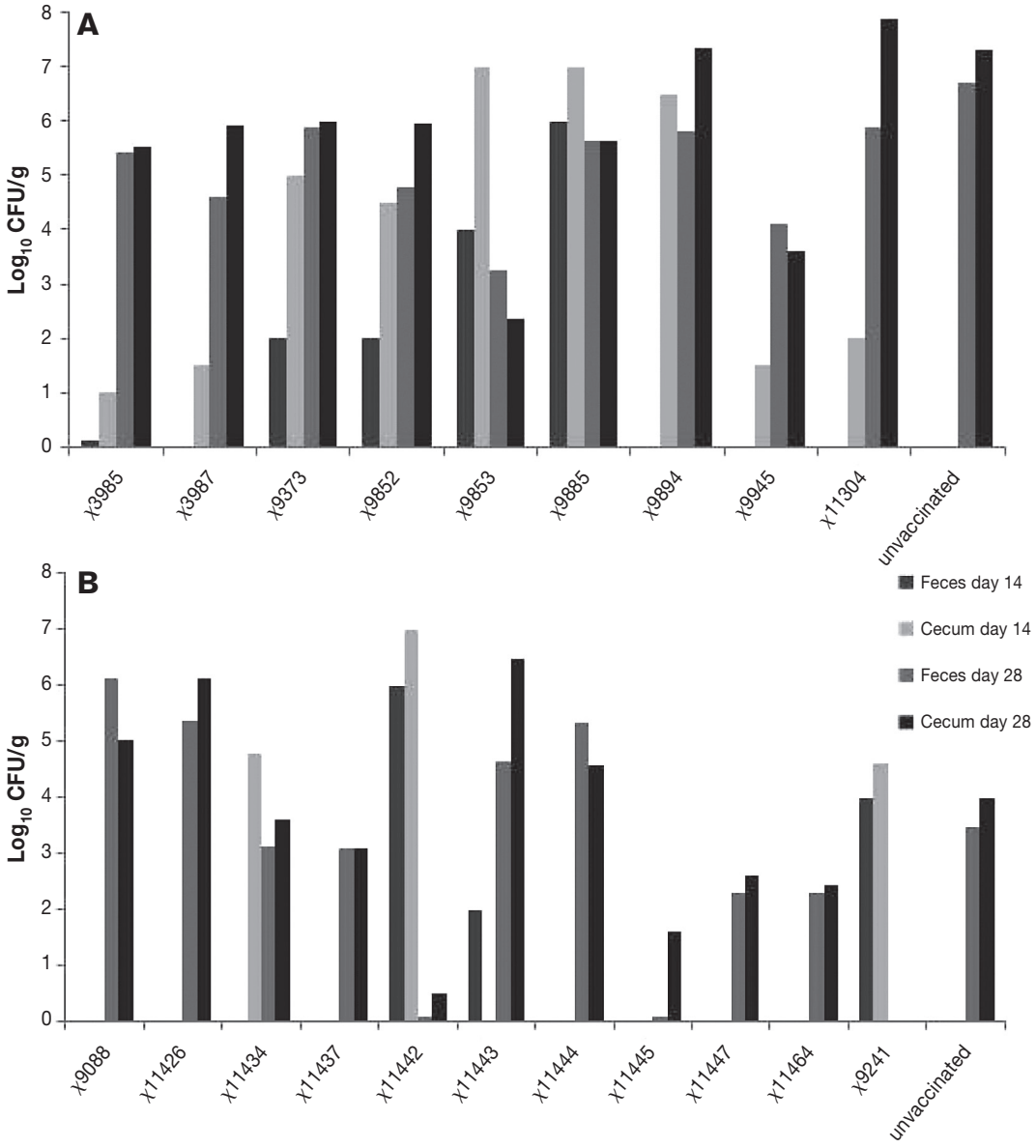

Figure 1. Isolation of *S.* Typhimurium vaccine strains (day 14) and wild-type challenge strain (day 28) from pooled feces and ceca (CFU/g) of inoculated birds (5 birds/group). Strain genotypes are given in Table I.

L-arabinose was added to LB broth. No difference was noted in the presence and absence of arabinose. The growth characters of the 20 strains showed division into 3 types: fast, intermediate, and slow growers. Two strains (χ11304, χ11464) were slow, 9 strains (χ3985, χ3987, χ9373, χ9894, χ11426, χ11434, χ11437, χ11444, χ11447) were intermediate, and 9 strains (χ9088, χ9241, χ9852, χ9853, χ9885, χ9945, χ11442, χ11443, χ11445) were fast growers (data not shown).

Vaccine strains shedding in feces

Fecal *Salmonella* vaccine shedding on day 14 after immunization is summarized in Figure 1. Four of the 20 vaccines strains (χ9241, χ9853, χ9885, χ11442) shed at levels higher than 10^4 CFU/g, shedding of 13 strains was undetected, and the remaining 3 strains (χ9375, χ9852, χ11443) showed intermediate levels of shedding.

Vaccine strains colonization of the cecum and spleen

The colonizing ability of the 12 strains that were found in the cecum varied from $\geq 10^7$ to $10^{0.5}$ CFU/g (Figure 1). The order from highest to lowest was χ11442, χ9885, χ9853, χ9894, χ11434, χ9241, χ9375, χ9852, χ11304, χ3987, χ9945, and χ3985. Cecal colonization of vaccine strains was more marked in birds in study A (9/9) than in study B (3/11). Eight of 20 vaccine strains were not detected in the cecum on day 14 after primary immunization (Figure 1), all of which were detected in study B (Figure 1). In general, slight or absent fecal shedding on day 14 after immunization corresponded with low numbers in the cecum (Figure 1). A pattern generally reflecting high cecal colonization by the different vaccine strains was observed in

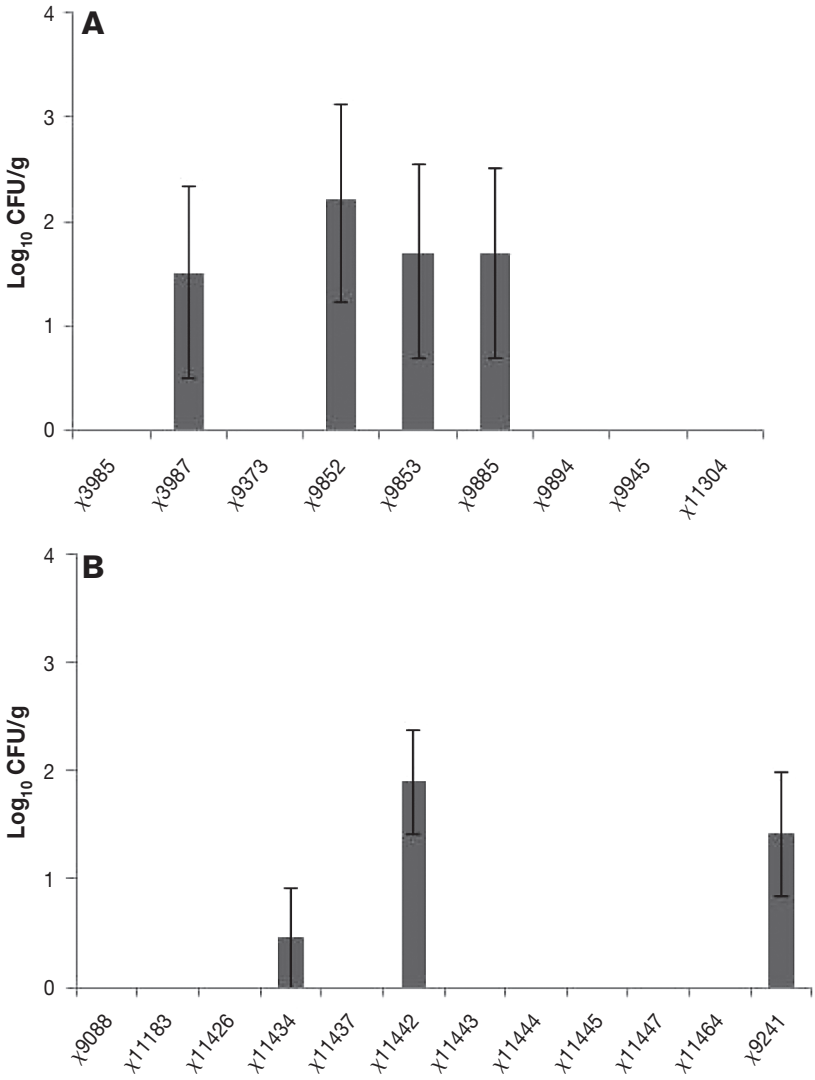

Figure 2. Isolation of *Salmonella enterica* serovar Typhimurium vaccine strains in $\log_{10}$ colony-forming units from the spleen on day 14 after primary immunization (5 birds each group).

the spleen (Figure 2). Seven of the 20 vaccine strains were found in the spleen, representing 7 of the 12 vaccine strains present in the cecum at day 14 (Figure 1). The order from the highest to lowest was χ9852, χ11442, χ9885, χ9853, χ3987, χ9241, and χ11434 (Figure 1).

Challenge strain shedding in feces

The virulent challenge strain was isolated from unvaccinated birds at about 3 logs higher in study A than in study B (Figure 1), and the numbers of wild type bacteria in the feces and cecum were correspondingly higher on day 28 in the immunized birds in study A (Figure 1).

On day 28 after immunization, 3 of the 20 vaccine strains showed reductions in fecal shedding of the wild type challenge strain of approximately $2\log_{10}$ CFU (χ9945, χ11447, χ11464), 1 strain (χ9853) of approximately $3\log_{10}$ CFU, and 3 strains (χ9241, χ11442, χ11445) of approximately $4\log_{10}$ CFU or greater compared to the non-vaccinated control birds (Figure 1).

Challenge strain colonization in cecum and spleen

On day 28 after immunization, numbers of the challenge strain in the cecum were generally similar to those in the feces (Figure 1). Three of the 20 vaccine strains showed cecal reductions of the challenge strain of approximately $2\log_{10}$ CFU (χ11437, χ11447, χ11464), 3 strains (χ9853, χ9945, χ11445) of approximately $3\log_{10}$ CFU, and 2 strains (χ9241, χ11442) of approximately $4\log_{10}$ CFU compared to non-vaccinated control birds (Figure 1). Three of these (χ9241, χ9853, χ11442) showed statistically significant reductions in cecal colonization compared to the non-vaccinated but challenged control chickens (Figure 1).

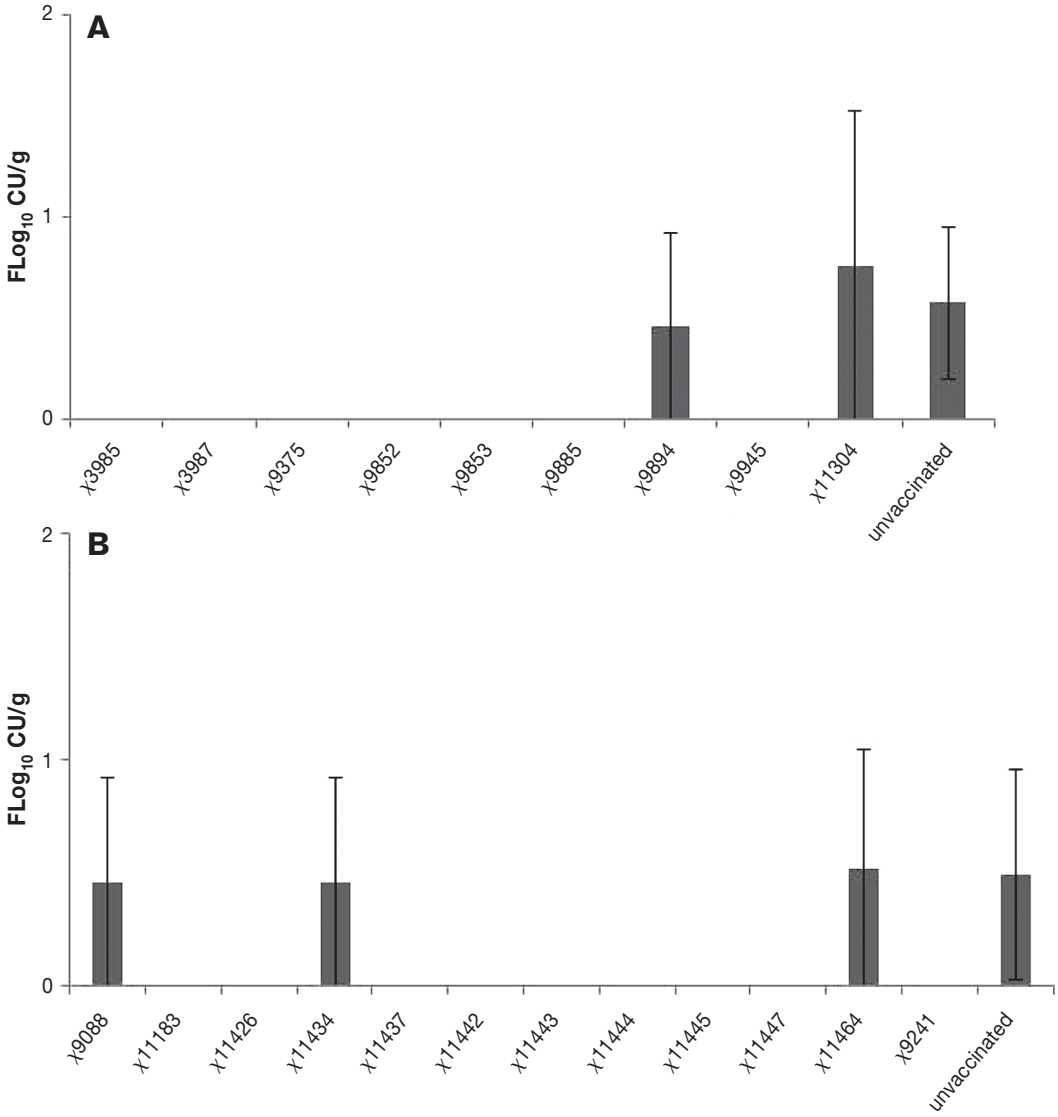

Figure 3. Isolation of wild type challenge strain of *Salmonella enterica* serovar Typhimurium in log_{10} colony-forming units from the spleen on day 28, 14 d after challenge (5 birds each group).

The challenge strain was found in small but variable numbers in the spleen in 7 groups 28 d after initial immunization (Figure 3).

Discussion

There were large differences between the 20 novel genetically modified vaccine strains in persistence in the intestinal tract of vaccinated birds and in protection against colonization by wild type *Salmonella*. Among these strains, immunization with χ9241, χ9853, χ9945, χ11442, and χ11445 produced the greatest reductions in fecal shedding and cecal colonization of wild type *S.* Typhimurium. Three of these (χ9241, χ9853, χ11442) showed significant reductions in challenge strain cecal colonization (Figure 1).

The growth curve showed that 11 strains grew slower than the wild type. Two of the slowest growing strains (χ11304, χ11464) showed very limited ability either to persist in the intestine or to invade the spleen (Figures 1, 2, and 3). These slow-growing strains produced poor protection against wild type intestinal colonization and invasion of the spleen (Figures 1, 2, and 3). These strains also grew slowly *in vitro,* indicating that they are much attenuated. On this basis, these would likely be poor choices as vectors for heterogenous antigens.

In contrast with the strains that grew poorly *in vitro,* there was no clear pattern discernible between intermediate and fast growers in their ability to persist in the intestine and spleen and to protect against colonization by the wild type challenge strain. For example, one strain that grew fast *in vitro,* strain χ11442, colonized the intestine well, persisted in the spleen of immunized birds, and reduced intestinal and splenic colonization by the wild type challenge strain. Strain χ9088 also grew fast *in vitro,* but colonized the intestine and cecum poorly, showed no persistence in the spleen, and produced very poor protection against challenge strain infection. The shedding of vaccine strains in feces on day 14 after immunization was generally consistent with their numbers in the cecum on day 14

(Figure 1), and generally corresponded with their presence in the spleen on day 14 (Figure 2). The vaccine strains found in the spleen (Figure 2) and in the cecum on day 14 after immunization (Figure 1) were, generally, most likely to prevent the presence of the wild type challenge strain in the spleen on day 28 (Figure 3). Notably, 3 of the 4 best vaccine strains (χ9241, χ9853, χ11442) were isolated from the spleen on day 14 after immunization; χ11445 was the exception. With this and one other exception (χ9852), these strains were also most effective in reducing wild type intestinal colonization and presence in the spleen. We did not examine the efficiency of plating of the vaccine strains on brilliant green agar or their growth in SCB; mutations can affect plating efficiency in or on selective media. There was, however, no notable lack of growth of a vaccine strain in feces and cecum with a discrepant ability to control wild type colonization (Figure 1), suggesting that the growth of mutants was efficient.

There was a marked difference in wild type colonization in nonimmunized controls between birds in study A and study B; the reason for this difference is unclear. We, therefore, compared the two groups by examining $\log_{10}$ reduction in CFU in these birds, although this approach may underestimate the efficacy of immunization by some strains in study A, for example χ9945. Unexpectedly, χ3985 and χ3987, which is a DAP mutant of χ3985, were not as effective in preventing wild type colonization of ceca as previously reported (31,32). This discrepancy might be related to the 10-fold reduction in vaccine dose used in the current study and/or to the high challenge that birds in study A experienced, to differences in the challenge strains, or to differences in immunization and challenge schedules.

In general, strains with mutations in *pabA* and *pabB* appeared to colonize better than strains with other mutations and give better protection against wild type colonization. However, there was no clear pattern across the range of mutants assessed. Details of the mutations in the 2 least immunogenic strains (χ11304, χ11447), which were the poorest growers, were proprietary, so we cannot comment further on the relationship between specific mutations and colonizing ability. *lacI,* the gene encoding the LacI repressor confer regulated delayed *in vivo* synthesis of recombinant protective proteins under the control of the arabinose promoter, was present in 15 of the 20 candidate vaccine strains (Table I). Once the strain is present in the intestine, where arabinose is absent, then antigen expression is no longer repressed (23). There is evidence that LacI synthesis may itself be attenuating (33). At low copy, without control of an antigen, LacI may have a slight detrimental effect on growth, but when an antigen gene under the control of LacI is present then enhanced immunity has been demonstrated (34). There was, however, no clear pattern here of the effect of *lacI* on immunogenicity of the vaccine strains tested, and no effect on growth of *araC* P_{BAD} *lacI* TT strains in the presence or absence of arabinose. The lack of apparent effect of arabinose in enhancing growth of the arabinose-regulated (ΔP_{crp} *araC* P_{BAD} *crp*) mutants compared to the unregulated Δ*crp* mutant χ3985 was unexpected, but may be explained by other genotypic differences.

The ability of vaccine strains to persist in the intestine and to invade the spleen is critical for an effective immune response against *Salmonella* (20). However, high colonization in the cecum is associated with invasion of the spleen and liver, raising potential food safety issues for consumers. Selection of vaccine strains should be based on efficacy as antigen delivery vectors for the target infection and their potential hazard for consumers. This study indicated that χ9241, χ9853, and χ11442 among other strains, are potential vaccine candidates for control of *Salmonella* infection in chickens, with χ11445 having an apparent advantage in not persisting in the spleen.

Our goal is to develop an oral *Salmonella* vaccine against chicken necrotic enteritis while also controlling *Salmonella* infection in chickens. A previous study indicated that there was an association between the efficacy of vaccine strains in protecting necrotic enteritis and in protecting against *Salmonella* colonization (7,16,21). The current study was designed to make the selection of potential vaccine vectors less empirical by selecting them on the basis of immunogenicity against *Salmonella*. Further work using strains selected on this basis from the 20 strains examined here as vectors for *Clostridium perfringens* antigens is, however, required to determine whether this hypothesis is correct. The strains may behave differently if they express cloned antigens, since they are designed for regulated expression of antigens.

Acknowledgments

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