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A Live *Salmonella enterica* Serovar Enteritidis Vaccine Allows Serological Differentiation between Vaccinated and Infected Animals▽

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Three precisely defined deletion mutants of *Salmonella enterica* serovar Enteritidis were constructed, a guanine auxotrophic Δ*guaB* mutant, a nonflagellated Δ*fliC* mutant, and an auxotrophic and nonflagellated Δ*guaB* Δ*fliC* double mutant. All three mutants were less invasive than the wild-type strain in primary chicken cecal epithelial cells and the human epithelial cell line T84 and less efficiently internalized in the chicken macrophage cell line HD11. The Δ*fliC* mutant was pathogenic in orally infected BALB/c mice, while the Δ*guaB* mutant was attenuated and conferred protection against a challenge with the pathogenic parent strain. The Δ*guaB* Δ*fliC* double mutant was totally asymptomatic and conferred better protection than the Δ*guaB* mutant. This indicates that the major flagellar protein flagellin is not required for efficient vaccination of BALB/c mice against *Salmonella* infection. The Δ*guaB* Δ*fliC* mutant was also safe for vaccination of 1-day-old chickens. After two immunizations, it induced statistically significant protection against infection of the internal organs of the birds by a virulent *S. enterica* serovar Enteritidis challenge strain but not against intestinal colonization. These data demonstrate that nonflagellated attenuated *Salmonella* mutants can be used as marker vaccines.

Human salmonellosis is mainly caused by consumption of food such as eggs, milk, and meat contaminated with salmonellae (12). Worldwide, salmonellosis is a serious medical and veterinary problem and raises great concern in the food industry. Vaccination is potentially an effective tool for the prevention of salmonellosis. Whole-cell killed vaccines and subunit vaccines were used with variable results for the prevention of *Salmonella* infection in humans and animals (21). Recent advances in the genetics of *Salmonella* led to the development of attenuated *Salmonella* vaccine strains with single or multiple defined mutations in the bacterial genome (21). Live attenuated *Salmonella* vaccines were also used successfully as carriers for the delivery of heterologous antigens to the immune system (21).

A major drawback of vaccination as a disease control measure is that immunized animals produce antibodies against the vaccine strain and can therefore no longer be distinguished from field-exposed animals by serological tests. Flagellin, the major structural protein of flagella, is used for the serotyping of *Salmonella*. Purified flagellin induces a high systemic, humoral, and mucosal immune response in C3H/HeJ mice (38). While mice immunized intraperitoneally with flagellated *Salmonella* show a strong systemic (immunoglobulin G IgG) response against flagellin, mice immunized mucosally with the strain did not (34). Nevertheless, flagella elicit a strong immune response in chickens (27, 44) and are useful serological markers that carry the serotype-specific H-antigenic determinants (17) in the central variable domain of the protein (41). Therefore, deletion of *fliC*, the gene that codes for flagellin (FliC) should allow serological differentiation between animals immunized with the Δ*fliC* vaccine strain and animals infected by wild-type *S. enterica* serovar Enteritidis. An enzyme-linked immunosorbent assay kit (FlockChek *Salmonella enterica* serovar Enteritidis antibody test kit from IDEXX Laboratories) is available to detect antibodies against H-antigenic determinants of the FliC flagellin of *S. enterica* serovar Enteritidis (H:g,m). Specific antibodies against *S. enterica* serovar Enteritidis FliC were detected in sera of spray-inoculated young chickens but not in sera of young chickens inoculated orally with *S. enterica* serovar Enteritidis (27).

The attenuation of *guaB* mutants of *S. enterica* serovars Dublin and Typhimurium was previously described (22). *S. enterica* serovar Typhi Δ*guaBA* mutants are attenuated and induce strong serum O- and H-antigen responses in mice inoculated intranasally (46).

The present study shows that an *S. enterica* serovar Enteritidis *guaB* mutant is attenuated but retains some residual virulence in inoculated mice. Protection against the homologous parent strain was observed in BALB/c mice after a challenge with the parent *S. enterica* serovar Enteritidis strain. Moreover, a Δ*guaB* Δ*fliC* double-deletion mutant of *S. enterica* serovar Enteritidis was more attenuated than the Δ*guaB* single mutant in BALB/c mice and conferred better protection of immunized

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TABLE 1. Nucleotide sequences of primers used for PCR amplification or sequencing

Primer	Sequence
P1	5′ GTGTAGGCTGGAGCTGCTTC 3′
P2	5′ CATATGAATATCCTCCTTAG 3′
GuaB2	5′ CGTTCAGGCGCAACAGGCCGTTGT 3′
GuaB3	5′ GGCTGCGATTGGCGAGGTAGTA 3′
GuaB4	5′ GGTGATCCCGGGCGTCAAACGTCAGGG CTTCTTTA 3′
GuaB5	5′ TTGACGCCCGGGATCACCAAAGAGTCC CCGAACTA 3′
GuaB6	5′ GCAACAACTCCTGCTGGTTA 3′
GuaB7	5′ AGACCGAGGATCACTTTATC 3′
GuaB10	5′ AGGAAGTTTGAGAGGATAA 3′
FliCP1	5′ ATGGCACAAGTCATTAATACAAACAGC CTGTCGCTGGTTGACCCAGAATAATGTG TAGGCTGGAGCTGCTTC 3′
FliCP2	5′ CGCATTAACGCAGTAAAGAGAGGACGT TTTGCGGAACCTGGTTMGCCTGCGCCAC ATATGAATATCCTCCTTAG 3′
FliC1	5′ ATGGCACAAGTCATTAATACAAACAG 3′
FliC2	5′ CGCATTAACGCAGTAAAGAGAGGAC 3′
FliC3	5′ TATCGGCAATCTGGAGGCAA 3′

mice against a challenge with the wild-type strain. The double mutant was safe for 1-day-old chicks when administered by the intratracheal or oral gavage route. Vaccination of chicks with the Δ*guaB* Δ*fliC* double-deletion mutant protected them against colonization of the internal organs by virulent *S. enterica* serovar Typhimurium but not against intestinal colonization.

The lack of flagellin may be exploited both as a marker for the live vaccine strain and as a serological marker facilitating the differentiation between vaccinated chickens and chickens infected with an *S. enterica* serovar Enteritidis field strain.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Enteritidis phage type 4 strain 76Sa88, isolated from a turkey (8), was used in all experiments. MiniTn*5lacZ2* insertion mutants of *S. enterica* serovar Enteritidis 76Sa88 Rif^r were obtained essentially as previously described (7). *S. enterica* serovar Enteritidis 76Sa88 contains only one gene that codes for flagellin, *fliC*.

Construction of *guaB* and *fliC* deletion mutants. The *guaB* and *fliC* genes of *S. enterica* serovar Enteritidis 76Sa88 were deleted by homologous recombination of PCR fragments, with the Red recombinase system of bacteriophage Lambda (5). The sequences of the primers used are shown in Table 1.

To construct an *S. enterica* serovar Enteritidis *guaB* deletion mutant, overlap PCR (13) was performed with two fragments that were amplified, respectively, with primer sets GuaB3-GuaB4 and GuaB5-GuaB2 and with *S. enterica* serovar Enteritidis genomic DNA as the template. A nested PCR with primer set GuaB6-GuaB7 was subsequently carried out to amplify a linear fragment in which an 861-bp internal segment of the coding sequence of *guaB* was replaced with an SmaI site. This PCR fragment was cloned into the SmaI site of pUC18 with the Sureclone ligation kit (Pharmacia Biotech). A chloramphenicol resistance gene with flanking FLP recombinase target (FRT) sites, amplified with plasmid pKD3 DNA as the template and primer set P1-P2 (5), was ligated into the SmaI site of the cloned Δ*guaB* fragment. The PCR fragment (Δ*guaB*::*catFRT*) generated on this clone with primer set GuaB6-GuaB7 was introduced by electroporation (32) into *S. enterica* serovar Enteritidis, previously transformed with pKD46 (5), after induction of the Red recombinase system with 0.2% arabinose.

For the construction of a Δ*fliC* deletion mutant and a Δ*guaB* Δ*fliC* double mutant of *S. enterica* serovar Enteritidis, a recombinant fragment harboring the kanamycin resistance gene with flanking FRT sites (*kanFRT*) and extensions homologous to the initial 50 (1 to 50) and the terminal 50 (1468 to 1518) nucleotides of the *fliC* coding sequence, was amplified with pKD4 plasmid DNA as the template and primers FliCP1 and FliCP2. The Δ*fliC* mutants were obtained by electroporation of the resulting PCR fragment into *S. enterica* serovar Enteritidis(pKD46) and *S. enterica* serovar Enteritidis Δ*guaB*::*catFRT*(pKD46) as previously described (5).

P22 transduction. To avoid additional undesirable mutations, the substitution mutations containing the selectable resistance genes were transduced into a wild-type *S. enterica* serovar Enteritidis 76Sa88 background with bacteriophage P22 HT *int* (6). Antibiotic resistance genes were subsequently eliminated with pCP20 (5), and the deletions were confirmed by PCR and phenotypic characterization as described further.

Virulence in and protection of mice. Seven-week-old female BALB/c mice were orally inoculated to evaluate the virulence and efficacy as a vaccine of the Δ*guaB* and Δ*guaB* Δ*fliC* deletion mutants. A Δ*fliC* mutant was also included to study the effect of inactivation of the *fliC* gene on the virulence of the wild-type strain. For the virulence assay, mice each received about 10^8 CFU of the bacterial strains that were cultured overnight in Luria-Bertani (LB) broth (25) and suspended in milk. This dose corresponds to approximately 10^5 times the 50% lethal dose of the wild-type strain (30). As a positive control, mice were inoculated with wild-type pathogenic *S. enterica* serovar Enteritidis strain 76Sa88. Noninfected control mice were inoculated with milk. The efficacy of the mutants was determined in vaccinated mice 3 weeks after the initial immunization by oral challenge with about 10^8 CFU of wild-type *S. enterica* serovar Enteritidis strain 76Sa88 per mouse. The challenged mice were observed for 21 days for death and clinical signs. The animal experiments were performed by following all relevant national and institutional guidelines.

Invasion and phagocytosis assays. Invasion experiments were performed with the human colon carcinoma cell line T84 and with chicken primary cecal epithelial cells as previously described (43). Phagocytosis assays (29) were performed with the chicken macrophage cell line HD11 (2).

Briefly, cells of the human colon carcinoma cell line T84 were seeded in 96-well cell culture plates at a density of 10^5 cells/well in cell culture medium (Dulbecco modified Eagle medium plus 10% fetal calf serum and 2% L-glutamine without antibiotics) and grown overnight. The bacteria were grown overnight at 37°C in 5 ml of LB broth (25) on a shaker platform, subcultured 1:100 in fresh LB broth (5 ml), and grown to late log phase at 37°C without shaking. After 5 h of incubation, the bacterial suspensions were centrifuged and resuspended in Dulbecco modified Eagle medium with 10% fetal calf serum. The number of CFU per milliliter was determined by plating on brilliant green (BG) agar at 37°C. The suspensions were kept at 4°C until they were used in the assay, diluted to a density corresponding to a multiplicity of infection of 10, and added to the cultured cells. To ensure close contact between the bacteria and the cells, the plates were centrifuged for 10 min at 1,500 rpm and incubated for 1 h at 37°C under 5% CO_2. The cells were subsequently rinsed three times with Hanks' balanced salt solution (Life Technologies, Paisley, Scotland). Cell culture medium with gentamicin (50 μg/ml) was added, and the plates were incubated for 1 h at 37°C under 5% CO_2. Thereafter, the cells were rinsed three times with phosphate-buffered saline (PBS) and lysed with 1% Triton X-100 (Sigma, St. Louis, MO) in distilled water. From this lysate, a 10-fold dilution series was plated on BG agar plates. Invasion and phagocytosis assays with chicken primary cells and chicken macrophage cells (HD11) were performed essentially as described above, with some modifications (29, 43). All assays were performed in duplicate, with three repeats of each experiment. Percentages of invasion and phagocytosis by defined *S. enterica* serovar Enteritidis mutants, relative to invasion and phagocytosis by the *S. enterica* serovar Enteritidis wild-type strain (100%), were calculated. Analysis of variance was performed with SPSS 12.0 software.

Safety assessment with chickens. The safety of the Δ*guaB* and Δ*guaB* Δ*fliC* isogenic mutant strains was evaluated with chickens in two experiments by inoculation of 1-day-old chicks (SPAFAS). In the first experiment, the safety of the *S. enterica* serovar Enteritidis Δ*guaB* strain was determined with four groups of 10 1-day-old chicks. Chicks in groups 1 and 2 were inoculated by the intratracheal route and by oral gavage, respectively. Birds in control groups 3 and 4 were inoculated with PBS, respectively, by the intratracheal and oral gavage routes. Mortality was recorded for 38 days.

In the second experiment, the safety of the *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* mutant was also evaluated with four groups of 1-day-old chicks. Similar to the first experiment, 10 chicks in groups 1 and 2 were inoculated by the intratracheal route and by oral gavage, respectively. Ten birds in group 3 were inoculated by the intratracheal route with the Poulvac ST *aroA S. enterica* serovar Typhimurium vaccine (Fort Dodge Animal Health). Five birds in group 4 were inoculated with PBS by the intratracheal route. The body weight of each bird from all four groups was measured at 21 days postinoculation. Body weight was compared among groups in an analysis-of-variance model with body weight as

the dependent variable and treatment included as an independent variable. The estimated body weight and its 95% confidence interval were constructed. Group comparisons were made with Tukey's test for multiple comparisons. The frequency distribution of the continuous outcome (body weight) was assessed by PROC UNIVARIATE. All analysis of data was performed with the SAS system (SAS Institute Inc.). The level of significance was set at $P < 0.05$.

Efficacy assessment with chickens. One-day-old specific-pathogen-free white Leghorn chicks were randomly divided into five groups. Birds in groups 1 and 1A were vaccinated with *S. enterica* serovar Enteritidis Δ*guaB* by coarse spray on the first day. At 2 weeks of age, these birds received a second vaccine dose by drinking water (group 1) or by oral gavage (group 1A). Birds in groups 2 and 2A were vaccinated with *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* by coarse spray on the first day. At 2 weeks of age, these birds received a second vaccine dose by drinking water (group 2) or by oral gavage (group 2A). Birds in group 3 were administered PBS by spray on the first day.

At 6 weeks of age, the birds from all groups were challenged by oral gavage with virulent *S. enterica* serovar Enteritidis PT4 (nalidixic acid resistant) and observed for clinical signs and death.

At 7 days postchallenge, all surviving birds were necropsied. Tissue samples of approximately 1 g each of the spleen, kidney, and liver from each bird were obtained aseptically. These were pooled in sterile swirl pack bags containing 10 ml BG tetrathionate broth (BGTB; prepared with BG broth and tetrathionate broth base obtained from Difco Laboratories, Becton, Dickinson and Company, Sparks, MD), macerated for 30 s in a Stomacher blender, and incubated at 42°C for 24 h. Also, 10-mm lengths of the duodenum (bottom of the duodenal loop below the pancreas), jejunum (region of yolk sac diverticulum), and ileum (anterior to the ileocecal junction) were aseptically collected from each bird, flushed internally and externally with sterile PBS, pooled in sterile swirl pack bags containing 10 ml BGTB medium, macerated for 30 s in a Stomacher blender, and incubated at 42°C. After 24 h of incubation, a loopful of culture from each swirl pack bag was streaked onto BG and xylose-lysine-tergitol 4 agar plates (26) supplemented with 100 μg/ml nalidixic acid. The swirl pack bags were incubated further for another 24 h. If *Salmonella* isolation was negative after 48 h, 1 ml culture from each bag was transferred to a tube containing 10 ml BGTB medium. The tube was incubated for 24 h, and the culture was streaked onto BG and xylose-lysine-tergitol 4 agar plates containing 100 μg/ml nalidixic acid. When *S. enterica* serovar Enteritidis PT4 grew on either of the plates, the pool was considered positive.

For assessing *Salmonella* colonization of the cecum, approximately 1 g of cecal content collected from each bird was placed in a swirl pack bag containing 100 ml of sterile PBS and mixed thoroughly. A 0.1-ml sample of the suspension was plated in duplicate on BG agar plates supplemented with 100 μg/ml nalidixic acid. When *S. enterica* serovar Enteritidis PT4 grew on either of the plates, the pool was considered positive. An agglutination test with group D *Salmonella* antiserum was performed with at least one colony from all positive plates to confirm the presence of a group D *Salmonella*.

Data analysis was performed with the SAS system (SAS Institute Inc.). For statistical analysis, the experimental unit was the individual bird. The outcome was *Salmonella* isolation, and the study tested the null hypothesis that there would be no difference in *Salmonella* isolation among groups. *Salmonella* isolation (percentage or number of organisms recovered) was compared between each group and its control group by chi-square analysis. When expected cell sizes were too small, comparisons were made by Fisher's exact test. Group comparisons were made by the bootstrap method (PROC MULTTEST). The relative risk and its 95% confidence interval were constructed for each comparison.

RESULTS

A *guaB* mutant is attenuated in mice and induces protection. In a set of miniTn*5lacZ2* insertion mutants of *S. enterica* serovar Enteritidis 76Sa88 Rif^r, several auxotrophic mutants were identified. One of these mutants was characterized as a *guaB* mutant, since it grows on minimal A medium (25) complemented with 0.3 mM guanine, guanosine, xanthine, or xanthosine but not on medium complemented with hypoxanthine or inosine. The *guaB* gene (47) encodes the enzyme IMP dehydrogenase (EC 1.1.1.205), which converts IMP to XMP, the penultimate step in the biosynthetic pathway of GMP. The insertion of the miniTn*5lacZ2* transposon into the *guaB* gene of the mutant was confirmed by PCR (data not shown).

Three mice at 7 weeks of age were orally inoculated with the *guaB* insertion mutant by administering 3.5×10^8 CFU per mouse. All three mice remained healthy during the 21-day observation period and were then challenged by oral administration of 2.7×10^8 CFU of wild-type strain *S. enterica* serovar Enteritidis 76Sa88 per mouse. All three mice survived the challenge. As a positive control, wild-type strain *S. enterica* serovar Enteritidis 76Sa88 (4.4×10^8 CFU) was orally given to three BALB/c mice of the same age that were not previously immunized. All three mice died within 6 days. These results demonstrate that the *guaB* mutant is highly attenuated in mice and that oral immunization of mice with this mutant protects them against infection by wild-type *S. enterica* serovar Enteritidis.

Construction of *guaB* and *fliC* deletion mutants. A *guaB* deletion mutant was constructed to avoid reversion of the miniTn*5lacZ2* insertion mutant and to remove the kanamycin resistance gene of miniTn*5lacZ2*. After electroporation of a linear fragment (containing a Δ*guaB*::*catFRT* mutation) in *S. enterica* serovar Enteritidis, chloramphenicol-resistant *S. enterica* serovar Enteritidis Δ*guaB*::*catFRT* mutants that require supplementation of minimal A medium (25) with 0.3 mM guanine were selected. The replacement of *guaB* with the chloramphenicol resistance cassette was further confirmed by PCR with primer combinations GuaB6-GuaB7, GuaB6-P2, GuaB7-P1, and P1-P2.

To delete the flagellin gene *fliC*, an internal 1,416-bp segment (bp 51 to 1467) of the *fliC* coding sequence (AY649742.1) was replaced with the kanamycin resistance gene in *S. enterica* serovar Enteritidis(pKD46) and *S. enterica* serovar Enteritidis 76Sa88 Δ*guaB*::*catFRT*(pKD46). By homologous recombination, *S. enterica* serovar Enteritidis Δ*fliC*::*kanFRT* and the double mutant *S. enterica* serovar Enteritidis Δ*guaB*::*catFRT* Δ*fliC*::*kanFRT* were generated. Isogenic strains were obtained after P22 transduction of the antibiotic resistance-encoding substitution mutations to a wild-type background. The antibiotic resistance genes were subsequently excised with plasmid pCP20 (5). The colonies were tested for carbenicillin, chloramphenicol, and/or kanamycin sensitivity to ascertain the loss of the plasmid pCP20 and the elimination of the antibiotic resistance genes. Both the *S. enterica* serovar Enteritidis Δ*guaB* and *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* mutants required guanine (0.3 mM) for growth on minimal A medium. The *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* and *S. enterica* serovar Enteritidis Δ*fliC* mutants were nonmotile on LB medium containing 0.4% agar. The deletion mutants were confirmed by PCR with primer combinations GuaB6-GuaB7 for *guaB* and FliC1-FliC2 for *fliC*. Sequencing (33) of the resulting fragments with primers GuaB10 and FliC3, respectively, confirmed the presence of the P1-FRT-P2 scar sequence.

The Δ*guaB* Δ*fliC* double mutant is safe and confers full protection on mice. To study the effect of the deletion of the *fliC* gene on the immunogenicity of the *S. enterica* serovar Enteritidis Δ*guaB* vaccine strain, virulence and protection tests with both mutants were carried out with 7-week-old female BALB/c mice. Two independent experiments were performed (Table 2). Mice infected with the *S. enterica* serovar Enteritidis Δ*guaB* mutant showed typical *Salmonella* disease symptoms (reduced activity, untidy coat, and curved back), and 1 out of 10 died in the first experiment, while no disease symptoms

TABLE 2. Evaluation of virulence and efficacy of Δ*guaB* and Δ*guaB* Δ*fliC* mutants of *S. enterica* serovar Enteritidis in mice[a]

S. enterica serovar Enteritidis 76Sa88 strain	Immunization			Challenge		
	Dose (CFU/mouse)	No. of survivors/ total (no. of days until death)	Disease symptoms[b]	Dose (CFU/mouse)	No. of survivors/ total (no. of days until death)	Disease symptoms[c]
Wild type	2.1×10^8	0/5 (7, 7, 8, 8, 9)	Severe symptoms from day 5 onward			
	1.4×10^8	0/3 (8, 9, 9)	Severe symptoms from day 6 onward			
Δ*guaB*	5.1×10^8	9/10 (13)	Mild-to-severe symptoms from day 7 until day 17	1.6×10^8	4/4	None
	1.9×10^8	3/3	None	1.5×10^8	2/3 (10)	None
Δ*guaB* Δ*fliC*	4.3×10^8	10/10	None	1.6×10^8	5/5	None
	3.2×10^8	3/3	None	1.5×10^8	3/3	None
None (no infection)		11/11	None	1.6×10^8	0/5 (7, 8, 8, 8, 9)	Severe symptoms from day 6 onward
		4/4	None	1.5×10^8	0/2 (9, 18)	Severe symptoms from day 6 onward

[a] Seven-week-old female BALB/c mice were orally immunized with *S. enterica* serovar Enteritidis mutants; a wild-type-infected control and a noninfected negative control were also included. A challenge by oral infection with wild-type *S. enterica* serovar Enteritidis was performed 3 weeks after immunization. Infection doses, survival rates, and observations over a period of 21 days are shown.
[b] Days counted after infection.
[c] Days counted after challenge. Two independent experiments were performed.

were observed in the second experiment. The Δ*guaB* Δ*fliC* double mutant did not induce disease symptoms in both experiments. All mice inoculated with the wild-type *S. enterica* serovar Enteritidis strain died within 9 days after infection, while the noninfected control mice remained healthy during the observation period of 21 days.

One mouse immunized with the Δ*guaB* mutant died after challenge with the wild-type *S. enterica* serovar Enteritidis 76Sa88 strain. All other immunized mice survived the challenge without observable disease symptoms. All nonimmunized control mice died after challenge. These data show that both mutants are attenuated and confer protection against a challenge with the corresponding wild-type parent strain.

Additional mutations, due to unwanted recombination events caused by the Red recombinase, can influence the results. Therefore, the experiment was repeated with isogenic strains that were constructed by P22 transduction.

A Δ*fliC* mutant was also included to study the effect of inactivation of the *fliC* gene on the virulence of the prototrophic wild-type strain. This Δ*fliC* mutant remained as virulent as the wild-type strain under these conditions. Data obtained from the Δ*guaB* and Δ*guaB* Δ*fliC* transductants (Table 3) confirmed the observations made in the first experiments. The Δ*guaB* Δ*fliC* double mutant is more attenuated in BALB/c mice and confers better protection against challenges with high doses of the wild-type strain than the Δ*guaB* mutant.

Immunization with Δ*guaB* and Δ*guaB* Δ*fliC* mutants generates high anti-lipopolysaccharide (LPS) IgG titers. Fifty-four

TABLE 3. Evaluation of virulence and efficacy of isogenic deletion mutants of *S. enterica* serovar Enteritidis in mice[a]

S. enterica serovar Enteritidis 76Sa88 strain	Immunization			Challenge[b]	
	Dose (CFU/mouse)	No. of survivors/ total (no. of days until death)	Disease symptoms[c]	No. of survivors/ total (no. of days until death)	Disease symptoms[d]
Wild type	3.7×10^8	0/3 (7, 8, 9)	Severe symptoms from day 5 onward		
Δ*fliC*	1.4×10^8	0/3 (6, 8, 8)	Severe symptoms from day 4 onward		
Δ*guaB*	7.6×10^8	5/5	Mild symptoms from day 11 until day 18	2/5 (8, 8, 19)	Severe symptoms from day 5 onward
Δ*guaB* Δ*fliC*	1.2×10^8	5/5	Reduced activity from day 11 until day 13	5/5	None
None (no infection)		4/4	None	0/4 (8, 8, 8, 9)	Severe symptoms from day 5 onward

[a] Seven-week-old female BALB/c mice were orally immunized with *S. enterica* serovar Enteritidis mutants; a wild-type-infected control and a noninfected negative control were also included. A challenge by oral infection with wild-type *S. enterica* serovar Enteritidis was performed 3 weeks after immunization. Infection doses, survival rates, and observations over a period of 21 days are shown.
[b] Dose administered: 3.1×10^8 CFU.
[c] Days counted after infection.
[d] Days counted after challenge.

TABLE 4. Percentages of invasion and phagocytosis by Δ*guaB*, Δ*fliC*, and Δ*guaB* Δ*fliC* mutants relative to invasion and phagocytosis by the *S. enterica* serovar Enteritidis 76Sa88 wild-type strain (100%)[a]

S. enterica serovar Enteritidis 76Sa88 mutant strain	Relative % invasion of T84 intestinal epithelial cells (SD)	Relative % invasion of primary chicken intestinal epithelial cells (SD)	Relative % phagocytosis of HD11 macrophages (SD)
Δ*guaB*	17.04 (5.09)	16.71 (6.68)	8.82 (1.86)
Δ*fliC*	14.53 (3.35)	6.7 (2.46)	9.85 (7.32)
Δ*guaB* Δ*fliC*	5.16 (0.07)	0.82 (0.24)	12.83 (4.37)

[a] Invasion assays were performed with the human colon carcinoma cell line T84 and isolated primary chicken cecal epithelial cells. Phagocytosis assays were performed with the chicken HD11 macrophage cell line. Experiments were performed in duplicate with three repeats of each experiment.

days following initial oral immunization of BALB/c mice with approximately 10^8 CFU per mouse of, respectively, the *S. enterica* serovar Enteritidis Δ*guaB* and *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* mutants, blood samples were collected from the tail arteries of five mice. Anti-LPS IgG titers were determined by means of an enzyme-linked immunosorbent assay with the use of 0.5 μg of *S. enterica* serovar Enteritidis LPS (Sigma) per well for coating. Comparison between sera of mice immunized with *S. enterica* serovar Enteritidis Δ*guaB* and mice immunized with *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* showed that, in both cases, comparable anti-LPS serum IgG responses were elicited. A second and third boost did not enhance the anti-LPS serum IgG levels (data not shown).

Reduced invasiveness and phagocytosis of *S. enterica* serovar Enteritidis mutants. The isogenic Δ*guaB* and Δ*fliC* single mutants and the Δ*guaB* Δ*fliC* double mutant were less invasive than the wild-type *S. enterica* serovar Enteritidis strain in the human colon carcinoma cell line T84 and in isolated primary chicken cecal epithelial cells (Table 4). Internalization of these mutants in the chicken macrophage cell line HD11 was also reduced compared to that of wild-type strain (Table 4).

***The* Δ*guaB* Δ*fliC* mutant is safe in 1-day-old chicks.** Ten 1-day-old chicks were inoculated intratracheally with the *S. enterica* serovar Enteritidis Δ*guaB* mutant strain (group 1) at 1.3×10^8 CFU per chick. One bird died during inoculation. Two birds died at 2 days postinoculation, and three birds died, respectively, at days 3, 5, and 13 postinoculation. Out of the 10 birds inoculated with the same dose of this strain by oral gavage (group 2), 1 chick died because of inoculation trauma during oral gavage and 1 bird died at day 5 postinoculation. No birds in the PBS-treated groups (groups 3 and 4) died. These results indicate that the *S. enterica* serovar Enteritidis Δ*guaB* mutant strain is not safe in chickens when administered at 1.3×10^8 CFU per bird at 1 day of age by the intratracheal or oral gavage route.

One-day-old chicks that were inoculated with the *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* mutant strain at 2.5×10^7 CFU per chick by the intratracheal or oral gavage route survived the 21-day observation period, and no deaths were recorded. Also, chicks inoculated with Poulvac ST (a licensed commercial vaccine), used as an intratracheal procedural control, and chicks inoculated with PBS survived the inoculation. Twenty-one days after inoculation, the average weight and standard deviation of the birds in groups 2, 3, and 4 were, respectively, 0.224 ± 0.018 kg, 0.200 ± 0.019 kg, and 0.205 ± 0.009 kg. The body weights of the chicks inoculated with the *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* mutant are not statistically significantly different from the body weights of the chicks vaccinated with the Poulvac ST vaccine or the body weights of the chicks inoculated with PBS. The average body weight (0.181 ± 0.030 kg) of the birds in group 1, which received the vaccine strain by intratracheal administration, was statistically significantly lower ($P = 0.0009$) than the average body weight of birds that were immunized with the same strain by oral gavage. It can be concluded that *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* is safe in 1-day-old chicks after inoculation with 2.5×10^7 CFU per bird by the intratracheal or oral gavage route.

Immunization with the Δ*guaB* Δ*fliC* mutant protects chickens against infection of the internal organs by *S. enterica* serovar Enteritidis. Immunization of chicks with *S. enterica* serovar Enteritidis Δ*guaB* and *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* and oral challenge with a virulent nalidixic acid-resistant *S. enterica* serovar Enteritidis PT4 strain were performed as described in Materials and Methods and in Table 5.

The vaccine strains were administered at 1 day of age by coarse spray and at 2 weeks of age by drinking water or oral gavage. The vaccines were deemed safe because no birds died and no adverse effects were observed in the postvaccination period, confirming the results of the previous experiment.

Four weeks post the second vaccination, the birds were challenged with 8.5×10^7 CFU of a virulent nalidixic acid-resistant *S. enterica* serovar Enteritidis PT4 strain. One week after the challenge, samples of organs (kidney, liver, and spleen), intestines (duodenum, jejunum, and ileum), and cecal contents were tested for the presence of the *S. enterica* serovar Enteritidis challenge strain. The results summarized in Table 5 show that the vaccine candidates *S. enterica* serovar Enteritidis Δ*guaB* and *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* protect the organs against infection but do not protect the intestines and ceca against colonization by pathogenic *S. enterica* serovar Enteritidis.

DISCUSSION

Virulence and efficacy studies carried out with the *guaB*::miniTn*5lacZ2* insertion mutant of *S. enterica* serovar Enteritidis 76Sa88 showed that the mutant was attenuated and conferred partial protective immunity against infection with the wild-type parent strain after oral immunization of BALB/c mice. These results were confirmed with the corresponding deletion mutant.

The lack of guanine biosynthesis in the Δ*guaB* mutant could be a limiting factor for gene expression, resulting in decreased

TABLE 5. Results of virulent *S. enterica* serovar Enteritidis PT4 challenge strain isolation from vaccinated chickens after challenge[a]

Group	Vaccine strain	Vaccination protocol	*S. enterica* serovar Enteritidis challenge strain isolation[b]					
			Organ pool		Intestine pool		Cecal content	
			No. positive/ total (%)	*P* value	No. positive/ total (%)	*P* value	No. positive/ total (%)	*P* value
1	Δ*guaB*	Spray, OG[c]	2/9 (22)	0.000054	9/9 (100)		5/9 (56)	0.30
1A	Δ*guaB*	Spray, DW[c]	10/24 (42)	0.00016	24/24 (100)		14/24 (58)	0.22
2	Δ*guaB* Δ*fliC*	Spray, OG[d]	4/10 (40)	0.0014	10/10 (100)		8/10 (80)	0.68
2A	Δ*guaB* Δ*fliC*	Spray, DW[d]	15/26 (58)	0.0068	25/26 (96)	0.25	16/26 (62)	0.32
3	None (no vaccination)		30/34 (88)		34/34 (100)		25/34 (74)	

[a] Chicks were vaccinated by coarse spray at 1 day of age and boosted via oral gavage (OG) or drinking water (DW) after 2 weeks. The number and percentage of chicks that were *Salmonella* positive upon necropsy and enrichment culture at 7 days after challenge are indicated.
[b] Organ pool: kidney, liver, and spleen. Intestine pool: duodenum, jejunum, and ileum.
[c] Doses administered per chick: 3.9×10^6 CFU (spray) and subsequently 2.8×10^6 CFU (drinking water or oral gavage).
[d] Doses administered per chick: 2.6×10^6 CFU (spray) and subsequently 2.3×10^6 CFU (drinking water or oral gavage).

invasion and in attenuation of the mutant strain. Invasion and survival of the bacteria in animal cells require expression of genes located on SPI-1 and SPI-2. The expression level of 20% of *Salmonella* genes is altered upon entry into cultured macrophages, with 384 genes being up-regulated (10). The effect could also be explained at the level of phagosome-lysosome formation, as bacterial protein synthesis is involved in the inhibition of phagosome-lysosome fusion (16).

A major drawback of vaccination as a disease control measure is that the immunized animals produce antibodies against the vaccine strain and can therefore no longer be distinguished by serological tests from animals infected by wild-type strains. This can, in principle, be avoided by introducing a mutation in a gene that encodes an antigen of the vaccine strain. Flagellin (FliC), the major structural protein of flagella, is a dominant antigen that is used for serotyping of *Salmonella* (41). Bacterial flagellin in monomeric form is recognized by Toll-like receptor 5 (TLR5) (3, 23, 37), which is expressed on different murine cell types and in chicken heterophils (19). Binding of flagellin to TLR5 can activate expression of inflammatory (9, 28, 36, 39, 48) and antiapoptotic mediators (49). TLR signaling activates nuclear factor κB and mitogen-activated protein kinase pathways via the adaptor molecule MyD88 (myeloid differentiation factor 88) (1) and activates the transcription of genes that encode immune modulators (40). Mammalian macrophages respond to cytosolic flagellin through members of the NOD-like receptor family (11). Flagellin is the ligand for Ipaf, a NOD-like receptor protein that is known to be involved in caspase I activation and interleukin-1β secretion in macrophages (24). Since flagellin is a target of the innate and adaptive immune response (14, 23, 31), the influence of the inactivation of the flagellin gene on the efficacy of the *S. enterica* serovar Enteritidis Δ*guaB* vaccine strain was investigated. Deletion of the *fliC* gene in the *S. enterica* serovar Enteritidis Δ*guaB* strain reduced its residual pathogenicity but did not reduce the efficacy of protection after challenge of immunized BALB/c mice, as shown in three independent experiments. These results confirm that FliC is not a dominant protective antigen (18) in BALB/c mice. The Δ*fliC* single mutant of *S. enterica* serovar Enteritidis remains virulent in mice. This confirms and extends the previous work on *S. enterica* serovar Typhimurium demonstrating that flagella are not necessary for pathogenicity in mice (35). Aflagellate *Salmonella* mutants are impaired in the ability to activate expression of proinflammatory and antiapoptotic effector molecules in murine models of salmonellosis but are potent activators of epithelial caspases and subsequent apoptosis (45). This correlates with a delayed but stronger mucosal inflammation at later stages of infection, as well as an elevated extraintestinal and systemic bacterial load, culminating in a more severe clinical outcome.

Chicken TLR5 (chTLR5) is expressed in a broad range of tissues. Exposure of cells expressing chTLR5 to flagellin induced upregulation of chicken interleukin-1β. Aflagellate *S. enterica* serovar Typhimurium *fliM* mutants showed an enhanced ability to establish a systemic infection in chicks and induced less interleukin-1β expression and polymorphonuclear cell infiltration of the gut. These results suggested that recognition of flagellin by chTLR5 contributes to the protection against systemic salmonellosis (15).

As shown in the different in vitro assays, the Δ*guaB* and Δ*fliC* single mutants and, in particular, the Δ*guaB* Δ*fliC* double mutant are less capable than the wild-type strain to invade the human epithelial cell line T84 and primary chicken cecal epithelial cells. Also, the internalization of the mutants in the chicken macrophage cell line HD11 is lower than that of the wild-type strain. These data are in line with the observation that the ability of a *fliC* mutant of *S. enterica* serovar Enteritidis to invade Caco-2 cells was reduced about 50 times compared to that of the corresponding wild-type strain, while bacterial adherence was not significantly different (42). Aflagellate *S. enterica* serovar Enteritidis showed a significant reduction in the invasion of human epithelial Hep-2 cells, compared to flagellate cells, and induced fewer membrane ruffles than the wild-type strain (20). Flagella are also required for efficient invasion of tissue cultures by *S. enterica* serovar Typhimurium (35).

Inoculation of 1-day-old chicks by the intratracheal or oral gavage route confirmed that the Δ*guaB* Δ*fliC* mutant was more attenuated than the Δ*guaB* mutant in chickens, resulting in a safe vaccine strain. The vaccine candidates *S. enterica* serovar Enteritidis Δ*guaB* and *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* protect the organs against infection but do not protect against colonization of the intestines and ceca after a challenge with a virulent *S. enterica* serovar Enteritidis strain. Also, in other studies using attenuated *Salmonella* vaccines in chickens, the conclusion was made that protection against infection of internal organs is easier to achieve than effective protection

against intestinal colonization (4). Although vaccination with the *S. enterica* serovar Enteritidis mutants did not significantly reduce the number of birds carrying the wild-type strain in the intestine and cecum after a challenge, the possibility that a lower excretion rate would be associated with vaccination cannot be excluded. This would reduce the transmission of the pathogen but could also interfere with the bacteriological detection of *Salmonella* infections.

Our results show that the inactivation of flagellin does not reduce the efficacy of the vaccine strain and thus can be further tested as a marker in attenuated vaccines. The resulting absence of antiflagellin antibodies in sera of immunized animals can be used as a serological marker to distinguish between field-infected animals and animals vaccinated with *S. enterica* serovar Enteritidis live-vaccine strains. The antibody response against the *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* vaccine strain cannot be distinguished from the response against *S. enterica* serovar Gallinarum and its biotype Pullorum. However, these host-restricted pathogens are not problematic for public health, normally induce typical clinical symptoms in their animal hosts, and can be distinguished from the *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* vaccine strain by PCR (replacement of the *guaB* and *fliC* genes in the vaccine strain) and bacteriological culture techniques (guanine auxotrophy of the vaccine strain and natural nutrient requirements of the host-restricted serovars). In the same way, the nonmotile and guanine auxotrophic *S. enterica* serovar Enteritidis Δ*guaB* Δ*fliC* vaccine strain can easily be differentiated from wild-type *S. enterica* serovar Enteritidis field strains. By transduction or Red-mediated recombination, *guaB* and flagellin mutations can easily be introduced into other important *S. enterica* serovars. Possibly, this will allow the production of useful attenuated vaccine strains.

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