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 ΔguaB mutant, a nonflagellated ΔfliC mutant, and an auxotrophic and nonflagellated ΔguaB ΔfliC 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 ΔfliC mutant was pathogenic in orally infected BALB/c mice, while the ΔguaB mutant was attenuated and conferred protection against a challenge with the pathogenic parent strain. The ΔguaB ΔfliC double mutant was totally asymptomatic and conferred better protection than the ΔguaB mutant. This indicates that the major flagellar protein flagellin is not required for efficient vaccination of BALB/c mice against *Salmonella* infection. The ΔguaB ΔfliC 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 also used successfully as carriers of *Salmonella* vaccines (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 ΔfliC 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 ΔguaB4 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 ΔguaB ΔfliC double-deletion mutant of *S. enterica* serovar Enteritidis was more attenuated than the ΔguaB single mutant in BALB/c mice and conferred better protection of immunized...
TABLE 1. Nucleotide sequences of primers used for PCR amplification or sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1</td>
<td>5'TTGAAGACGCTGTCGCTC 3'</td>
</tr>
<tr>
<td>P2</td>
<td>5'CAATGAAATCCCTCTTTAG 3'</td>
</tr>
<tr>
<td>GuaB2</td>
<td>5'CGATCACTGCTGTTGTATG 3'</td>
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<td>GuaB3</td>
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</tr>
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<td>GuaB4</td>
<td>5'TTATCGGCAATCTGGAGGCAA 3'</td>
</tr>
<tr>
<td>GuaB5</td>
<td>5'TTGCGGCGGCGATCGTGGG 3'</td>
</tr>
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<td>GuaB6</td>
<td>5'CGGCGTGGTTCCTCCTTGT 3'</td>
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<tr>
<td>GuaB7</td>
<td>5'TTTGCGGAACCTGGTTMGCCTGCGCCAC 3'</td>
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<tr>
<td>GuaB10</td>
<td>5'CGCATTAACGCAGTAAAGAGAGGAC 3'</td>
</tr>
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<td>FliCP1</td>
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</tr>
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<td>FliCP2</td>
<td>5'GCCATTACACCGCAACAGAAGAGACGCTTTCGAACCTGGTTATGACTA 3'</td>
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<tr>
<td>FliC1</td>
<td>5'ATGCGCAACAGTATTAATAACAC 3'</td>
</tr>
<tr>
<td>FliC2</td>
<td>5'CGGATTAACGCCAAGTAAGAGAGGAC 3'</td>
</tr>
<tr>
<td>FliC3</td>
<td>5'TATCGGCAATCTGGAGGCAA 3'</td>
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MATERIALS AND METHODS

Bacterial strains. S. enterica serovar Enteritidis phage type 4 strain 76Sa88, isolated from a turkey (8), was used in all experiments. MiniTn5lacZ2 isolated from a turkey (8), was used in all experiments. MiniTn5lacZ2

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.

Invasion and phagoctosis 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).

Safety assessment with chickens. The safety of the ΔguaB ΔfliC 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 ΔguaB ΔfliC 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 ΔguaB ΔfliC 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 Pouliéve ST and 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 template and primers FliCP1 and FliCP2. The ΔfliC mutants were obtained by electroporation of the resulting PCR fragment into S. enterica serovar Enteritidis(pKD46) and S. enterica serovar Enteritidis ΔguaB::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 ΔguaB and ΔguaB ΔfliC deletion mutants. A ΔfliC 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 10^6 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 10^6 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 phagoctosis 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 over-night 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 with 100% aeration. After 5 h of incubation, the bacterial suspensions were centrifuged and reus-

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% CO2. The cells were subsequently rinsed three times with Hank's balanced salt solution (Life Technologies, Paisley, Scotland). Cell culture me-

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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 *Salmonella enterica serovar Enteritidis* ΔguaB 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 *Salmonella enterica serovar Enteritidis* ΔguaB ΔfliC 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 *Salmonella enterica* serovar Enteritidis PT4 (naldixic 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 swab stick 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 swab stick 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 swab stick bag was streaked onto BG and xylose-lysine-tergitol 4 agar plates containing 100 μg/ml nalidixic acid. The swab stick 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 *Salmonella 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 swab stick 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 *Salmonella enterica* serovar Enteritidis PT4 grew on either of the plates, the pool was considered positive. An agglutination test with at least one colony from all positive plates to pool was considered positive. An agglutination test with group D *Salmonella enterica* serovar Enteritidis (nalidixic acid resistant) and *Salmonella enterica* serovar Enteritidis, chloramphenicol-resistant *S. enterica* serovar Enteritidis ΔguaB ΔcatFRT 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 ΔguaB::catFRT (pKD46). By homologous recombination, *S. enterica* serovar Enteritidis ΔfliC::kanFRT and the double mutant *S. enterica* serovar Enteritidis ΔguaB::catFRT ΔfliC::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 carbencillin, 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 ΔguaB and *S. enterica* serovar Enteritidis ΔguaB ΔfliC mutants required guanine (0.3 mM) for growth on minimal A medium. The *S. enterica* serovar Enteritidis ΔguaB ΔfliC and *S. enterica* serovar Enteritidis ΔfliC mutants were nonmotile on LB medium containing 0.4% agar. The deletion mutations were confirmed by PCR with primer combinations GuaB6-GuaB7 for *guaB* and FliCl-FliC2 for *fliC*. Sequencing (33) of the resulting fragments with primers GuaB10 and GuaB11, respectively, confirmed the presence of the P1-FRT-P2 scar sequence.

**RESULTS**

A *guaB* mutant is attenuated in mice and induces protection. In a set of miniTslacZ2 insertion mutants of *S. enterica* serovar Enteritidis 76Sa88 Rif°, 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 miniTslacZ2 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 $3.5 \times 10^6$ CFU per mouse. All three mice remained healthy during the 21-day observation period and were then challenged by oral administration of $2.7 \times 10^8$ 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 \times 10^6 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 miniTslacZ2 insertion mutant and to remove the kanamycin resistance gene of miniTslacZ2. After electroporation of a linear fragment (containing a *guaB::catFRT* mutation) in *S. enterica* serovar Enteritidis, chloramphenicol-resistant *S. enterica* serovar Enteritidis ΔguaB::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.

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The Δ*guaB* Δ*flfC* 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
were observed in the second experiment. The ΔguaB ΔfliC 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 ΔguaB mutant died after challenge with the wild-type S. enterica serovar Enteritidis 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 ΔfliC mutant was also included to study the effect of inactivation of the fliC gene on the virulence of the prototrophic wild-type strain. This ΔfliC mutant remained as virulent as the wild-type strain under these conditions. Data obtained from the ΔguaB and ΔguaB ΔfliC transductants (Table 3) confirmed the observations made in the first experiments. The ΔguaB ΔfliC 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 ΔguaB mutant.

### Immunization with ΔguaB and ΔguaB ΔfliC mutants generates high anti-lipopolysaccharide (LPS) IgG titers
Fifty-four

### Table 2. Evaluation of virulence and efficacy of ΔguaB and ΔguaB ΔfliC mutants of S. enterica serovar Enteritidis in mice

<table>
<thead>
<tr>
<th>S. enterica serovar Enteritidis 76Sa88 strain</th>
<th>Immunization</th>
<th>Challenge</th>
<th>Disease symptoms</th>
<th>Disease symptoms</th>
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</thead>
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<tr>
<td>Wild type</td>
<td>Dose (CFU/mouse)</td>
<td>No. of survivors/ total (no. of days until death)</td>
<td>Dose (CFU/mouse)</td>
<td>No. of survivors/ total (no. of days until death)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe symptoms from day 5 onward</td>
<td></td>
<td>Severe symptoms from day 5 onward</td>
</tr>
<tr>
<td>ΔguaB</td>
<td>5.1 × 10^8</td>
<td>9/10 (13) Mild-to-severe symptoms from day 7 until day 17</td>
<td>1.6 × 10^8</td>
<td>4/4 None</td>
</tr>
<tr>
<td>ΔguaB ΔfliC</td>
<td>4.3 × 10^8</td>
<td>10/10 None</td>
<td>1.6 × 10^8</td>
<td>5/5 None</td>
</tr>
<tr>
<td>None (no infection)</td>
<td>11/11 None</td>
<td>4/4 None</td>
<td>1.5 × 10^8</td>
<td>0/2 (9, 18) Severe symptoms from day 6 onward</td>
</tr>
</tbody>
</table>

### Table 3. Evaluation of virulence and efficacy of isogenic deletion mutants of S. enterica serovar Enteritidis in mice

<table>
<thead>
<tr>
<th>S. enterica serovar Enteritidis 76Sa88 strain</th>
<th>Immunization</th>
<th>Challenge</th>
<th>Disease symptoms</th>
<th>Disease symptoms</th>
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<tr>
<td>Wild type</td>
<td>Dose (CFU/mouse)</td>
<td>No. of survivors/ total (no. of days until death)</td>
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<td>No. of survivors/ total (no. of days until death)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe symptoms from day 5 onward</td>
<td></td>
<td>Severe symptoms from day 5 onward</td>
</tr>
<tr>
<td>ΔfliC</td>
<td>1.4 × 10^8</td>
<td>0/3 (6, 8, 8) Severe symptoms from day 4 onward</td>
<td></td>
<td>Severe symptoms from day 5 onward</td>
</tr>
<tr>
<td>ΔguaB</td>
<td>7.6 × 10^8</td>
<td>5/5 Mild symptoms from day 11 until day 18</td>
<td>2/5 (8, 8, 19)</td>
<td>None</td>
</tr>
<tr>
<td>ΔguaB ΔfliC</td>
<td>1.2 × 10^8</td>
<td>5/5 Reduced activity from day 11 until day 13</td>
<td>5/5</td>
<td>None</td>
</tr>
<tr>
<td>None (no infection)</td>
<td>4/4 None</td>
<td>0/4 (8, 8, 9)</td>
<td>0/4 (8, 8, 9) No symptoms from day 5 onward</td>
<td></td>
</tr>
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</table>
days following initial oral immunization of BALB/c mice with approximately 10^8 CFU per mouse of, respectively, the *S. enterica* serovar Enteritidis ΔguaB and *S. enterica* serovar Enteritidis ΔguaB ΔfliC 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 ΔguaB and mice immunized with *S. enterica* serovar Enteritidis ΔguaB ΔfliC 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 ΔguaB and ΔfliC single mutants and the ΔguaB ΔfliC 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 ΔguaB ΔfliC mutant is safe in 1-day-old chicks.** Ten 1-day-old chicks were inoculated intratracheally with the *S. enterica* serovar Enteritidis ΔguaB mutant strain (group 1) at 1.3 × 10^6 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 ΔguaB mutant strain is not safe in chickens when administered at 1.3 × 10^6 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 ΔguaB ΔfliC 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 ΔguaB ΔfliC 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 ΔguaB ΔfliC 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 ΔguaB ΔfliC mutant protects chickens against infection of the internal organs by *S. enterica* serovar Enteritidis.** Immunization of chicks with *S. enterica* serovar Enteritidis ΔguaB and *S. enterica* serovar Enteritidis ΔguaB ΔfliC 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 ΔguaB and *S. enterica* serovar Enteritidis ΔguaB ΔfliC 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::miniTn5lacZ2 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 ΔguaB mutant could be a limiting factor for gene expression, resulting in decreased
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, 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 (IL-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 ΔguaB vaccine strain was investigated. Deletion of the *fliC* gene in the *S. enterica* serovar Enteritidis ΔguaB 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 ΔfliC 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 up-regulation 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 ΔguaB and ΔfliC single mutants and, in particular, the ΔguaB ΔfliC 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 adhesion 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 ΔguaB ΔfliC mutant was more attenuated than the ΔguaB mutant in chickens, resulting in a safe vaccine strain. The vaccine candidates *S. enterica* serovar Enteritidis ΔguaB and *S. enterica* serovar Enteritidis ΔguaB ΔfliC 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 feces 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 ΔguAB ΔflfC 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 their animal hosts, and can be distinguished from the S. enterica serovar Enteritidis ΔguAB ΔflfC vaccine strain by PCR (replacement of the guAB and flfC 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 ΔguAB ΔflfC 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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