Food-borne diseases, including diseases caused by *Campylobacter*, are a major public health problem. *Campylobacter* is the leading cause of bacterial food-borne gastroenteritis in the United States and around the world (1, 13, 36). There are over 18 million cases of food-borne illness caused by *Campylobacter* spp. in the United States, costing over 18 billion dollars a year, which is more than the cost for any other known agent, including *Salmonella* (36). Bacterial contamination of poultry products by *Campylobacter jejuni* is a major food safety issue (1, 13). Furthermore, *C. jejuni* has been associated with the neuropathological disease Guillain-Barré syndrome (GBS) (16, 32, 42). In recent years, some attempts have been undertaken to develop an effective vaccine against *Campylobacter*, with modest success. Baqar and coworkers were successful at stimulating an immune response against *Campylobacter* in primates by using heat-labile enterotoxin of *Escherichia coli* as an oral adjuvant for killed whole-cell *Campylobacter* (2). Since *C. jejuni* is the most common pathogen associated with the development of GBS, there is concern about the use of whole-cell vaccines for humans, as it is possible that such vaccines could induce the syndrome (12, 22, 25, 38). In the case of GBS, there appears to be an immune response against lipooligosaccharides on *C. jejuni* that cross-reacts with and causes an autoimmune-mediated response against human nerve cell gangliosides affecting the peripheral nervous system (32). This concern increases if multiple strains are combined in order to generate broad cross-serotype-specific whole-cell *Campylobacter* vaccines for use in humans (32).

An alternate approach is to utilize specific *Campylobacter* proteins in a recombinant subunit vaccine to elicit protection against multiple *Campylobacter* serotypes. There are several proteins in the literature that may make excellent candidates for peptide subunit vaccines expressed from a bacterial vector. One protein appearing as an immunodominant antigen and a candidate for a vaccine is flagellin. The *Campylobacter* flagellin has regions that are highly conserved and thus are potential candidates for vaccine development. Immunization with purified flagellin has been shown to elicit an immune response in chickens, with reductions in gut colonization of *Campylobacter* (44). It appears that the majority of epitopes for flagellin are not surface expressed (33), so epitope selection must be made with extreme care. An additional potential problem with flagellin is that these molecules apparently have variable glycosylation, leading to strain-specific immune responses for some epitopes (26).

In addition to flagellin, several other membrane proteins appear to be recognized in humans following exposure to *Campylobacter* (12). Many of the epitopes for potential vaccine candidates, such as PEB1, PEB3 (31), and Omp18, were discovered to be highly immunogenic by use of sera from human infections (6). Other potential vaccine candidates, such as CjaA, CjaC, and CjaD, were identified using *Campylobacter coli*-specific rabbit antiserum (29). An outer membrane 18-kDa protein (Omp18) has been identified as a major immunogenic protein in human patients following campylobacteriosis (6). Rabbit serum against Omp18 reacts with all *Campylobacter* serotypes. There are several proteins in the literature that may make excellent candidates for peptide subunit vaccines expressed from a bacterial vector. One protein appearing as an immunodominant antigen and a candidate for a vaccine is flagellin. The *Campylobacter* flagellin has regions that are highly conserved and thus are potential candidates for vaccine development. Immunization with purified flagellin has been shown to elicit an immune response in chickens, with reductions in gut colonization of *Campylobacter* (44). It appears that the majority of epitopes for flagellin are not surface expressed (33), so epitope selection must be made with extreme care. An additional potential problem with flagellin is that these molecules apparently have variable glycosylation, leading to strain-specific immune responses for some epitopes (26).

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screening process, investigators identified other immunodominant proteins in addition to CjaD, namely, CjaA (Cj0982c) and CjaC. All three are conserved in 30 strains of C. jejuni and C. coli (30). These immunodominant proteins were then expressed from Salmonella vectors (45). Chickens immunized with a Salmonella vector expressing CjaA showed a strong immune response. Vaccination of chickens with the Salmonella vector expressing CjaA decreased the number of colonizing C. jejuni bacteria by log from 3 to 12 days following challenge with 2 × 10^9 bacteria (45). In a very recent work evaluating a live-attenuated Salmonella vaccine with CjaA, Buckley and coworkers found that with oral vaccination there was a significant (1.4-log10) reduction of C. jejuni at 56 days post-primary vaccination following infection challenge (5). Also, using recombiant CjaA postchallenge, they found a significant reduction of C. jejuni postchallenge.

Prokhorova and coworkers utilized proteomics to identify several potential vaccine candidates (34). Eight proteins were selected and expressed in an E. coli system, and the expressed proteins were purified and used to immunize mice. The mice had high antibody titers and were then challenged with C. jejuni. Two of the mice immunized with candidate polypeptides cleared a C. jejuni challenge faster than controls. More recently, this group published information about two additional candidate proteins that were tested in a mouse challenge model (37). Mice were immunized and boosted with either an ACE83 or ACE393 (Cj0420) candidate protein and then orally challenged with C. jejuni. Both peptide groups had a dramatic decrease in fecal shedding compared to nonimmunized mice. The C. jejuni Cj0420 gene encodes the peptide ACE393, a probable periplasmic protein which may prove to be an excellent vaccine candidate (37).

Very recent work in our laboratories showed that a site-specific insertion of sequence for a foreign peptide epitope in a candidate aroA- and htrA-attenuated Salmonella vector provided rapid and sustained seroconversion against the peptide when the immune-enhancing CD154 ligand was coexpressed (10, 24). CD154 (CD40 ligand [CD40L]), a member of the tumor necrosis factor (TNF) ligand family, is expressed primarily on the surfaces of activated T cells and plays several key roles in the regulation of cellular immune responses (46). Studies have demonstrated that intracellular communications via the CD40-CD154 pathway can upregulate costimulatory molecules, activate antigen-presenting cells (APCs), and influence T-cell-mediated effector functions (15, 27). The CD40-binding regions of CD154 have been identified (41), and this information was recently used in our laboratories to engineer a bacterium that displays these CD40-binding regions on its surface, resulting in an enhanced immune response against a copresented foreign peptide sequence (10, 24). The focus of the current project was to engineer an attenuated Salmonella enterica serovar Enteritidis phage type 13A strain to express antigenic peptide epitopes of C. jejuni at the cell surface and to evaluate the effectiveness of this recombiant as a vaccine candidate.

**Materials and Methods**

Attenuation of Salmonella vaccine candidate strains. S. Enteritidis was attenuated by introducing defined, irreversible deletion mutations in the arao and/or htra gene of the S. Enteritidis genome as previously described (21). Briefly, the target gene sequence in the bacterial genome of S. Enteritidis was replaced with the kanamycin resistance (Kmr) gene sequence. This was performed by using a combination of 3 PCR products to create a single insert and by electroporation of this product into electrocompetent Salmonella cells containing the pKD46 plasmid. The resulting cell mixture was plated on LB agar plates supplemented with Km to select for positive clones containing a Kmr gene. The Kmr gene was inserted into the genomic region containing the gene of interest (arao or htra) by flanking the Kmr gene with sequences homologous to the gene of interest. Once Kmr mutants were obtained, the deletion mutations were confirmed by PCR and DNA sequencing (data not shown). All Kmr genes were removed before epitope insertion was started.

**Construction of recombiant vaccine candidates.** We selected the following three potential peptide vaccine candidates from the literature, as described in the introduction: Cj0113 (Omp18/CjaD), Cj0982c (CjaA), and Cj0420 (ACE393). The selection of DNA sequences for insertion into our expression system was based on the antigenic potential predicted using the Network Protein Sequence Analysis program (9), using published sequences for Cj0113 (GSVITVEGNCD ESWGTDYNOA), Cj0420 (KVIDLDAEIGVKQGKDGEK), and Cj0982c (KVLAGVAPKDSNTISVEDLKDGLTKLNNGTDTAD) found in the EMBL and NCBI databases. All inserts additionally contained a sequence for CD154 (WMTTYSAPTS).

**Recombinant S. Enteritidis strains containing stable integrated DNA sequences.** Cj0113, Cj0420, or Cj0982c were constructed using the method of Cox et al. (10). Briefly, an I-SceI enzyme site along with a Kmr gene was introduced into loop 9 of the lamB gene by design of a PCR product which had the I-SceI enzyme site and the Kmr gene flanked by approximately 200 to 300 bp of DNA on each side (homologous to the up- and downstream regions of loop 9). The PCR product was electroporated into electrocompetent S. Enteritidis cells containing electrocompetent Saltamella cells containing the pKD46 plasmid and the resulting cell mixture was plated on LB agar plates supplemented with Km to select for positive clones now containing a Kmr gene. After the I-SceI/Kmr mutation was performed in loop 9, this region was replaced by a codon-optimized (7) foreign epitope DNA sequence (10). This second 35-PCR product produced the foreign epitope insert flanked by loop 9 up- and downstream regions, and the resulting PCR product was electroporated into electrocompetent S. Enteritidis 13A cells containing the I-SceI/Kmr mutation described above. Plasmid pBC-I-SceI was also electroporated into the cells along with the insert, as the plasmid produces the I-SceI enzyme, which recognizes and cleaves a sequence creating a gap at the I-SceI enzyme site in the loop 9 region of the LamB gene where the foreign epitope sequences inserted into the S. Enteritidis 13A genome (23). The plasmid also carries with it a chloramphenicol resistance (Cmr) gene, as the inserts that will replace the Kmr gene must have a new selection marker to counterselect against the previous I-SceI/Kmr mutation. After electroporation, cells were plated on LB agar plates containing 25 μg/ml Cm for the selection of positive mutants.

**Confirmation of recombiant inserts.** Once positive mutations/insertions were suspected, PCR and DNA sequencing were performed to confirm that the insertion sequences were present and correct (data not shown).

**Challenge with Campylobacter jejuni.** Three wild-type isolates (WCLCJ1 to -3) of C. jejuni were grown individually to log phase, combined, serially diluted, and spread plated for conventional culture enumeration as previously described (17). These were diluted to approximately 10^7 to 10^8 CFU/ml for challenge by oral gavage, using spectrophotometric density and comparison to a previously generated standard curve. The empirically determined number of CFU administered is reported for each experiment involving challenge (see below).

**Vaccination study 1.** In the first immunization study, day-hatch broiler chicks (Cobb-500) were obtained from a local commercial hatchery and randomly assigned to one of four treatment groups (50 birds per group): saline only (negative control) or one of three vaccine candidate groups (Cj0113, Cj0420, and Cj0982c). Each treatment group was housed in an individual floor pen on fresh pine litter and provided water and feed ad libitum. On the day of hatch, all chicks in each treatment group were inoculated via oral gavage with 0.25 ml of either saline or a suspension containing approximately 10^8 CFU/ml of the appropriate vaccine treatment. On day 21 posthatch, all birds in each treatment group were challenged with a mixture of three C. jejuni isolates via oral gavage (0.25 ml) with a suspension containing 1 × 10^7 CFU/ml (17). On days 3, 11, 21, and 32 posthatch, 10 birds (days 3 and 11) or 15 birds (days 21 and 32) from each treatment group were euthanized in accordance with an Institutional Animal Care and Use Committee-approved protocol, and their livers, spleens, and cecal tonsils were removed aseptically for the determination of organ invasion (liver and spleen), colonization (cecal tonsils), and clearance of the Salmonella vaccine vector strains as previously described (19). The S. Enteritidis strains used for the constructs were resistant to novobiocin (NO) and Km and were selected for
resistance to nalidixic acid (NA). The birds were humanely killed by CO₂ inhalation, and livers and spleens (collected together) and cecal tonsils (collected separately) were removed aseptically. Tissues were enrobed in tetrathionate broth overnight (37°C) (19). Following enrichment, each sample was streaked for isolation on brilliant green agar plates containing 25 μg/mL of NO and 50 μg/mL or 20 μg/mL of NA. The plates were incubated at 37°C for 24 h and examined for the presence or absence of antibiotic-resistant Salmonella. Also, on days 21 and 32 posthatch, ileum sections were removed and processed for use in quantitative real-time PCR (qPCR) to enumerate C. jejuni, and on day 32, the mucus was removed from a separate ileum sample, diluted (1:5 wt/vol) in saline, and used to test for secretory immunoglobulin A (sIgA). In addition, blood samples were collected from the wing veins of 10 birds per treatment group, and sera (obtained from overnight clotting) were used to determine IgG antibody responses on days 21 and 32 postvaccination.

**Vaccination study 2.** In experiment 2, day-old hatch broiler chicks were obtained from a local commercial hatchery and randomly assigned to one of two treatment groups: saline only (vehicle control) or Salmonella vaccine candidate Cj0113 (n = 55/group). Each treatment group was housed in an individual floor pen on fresh pine litter and provided water and feed ad libitum. On the day of hatch, all chicks in each treatment group were inoculated via oral gavage with 0.25 mL of either saline or a suspension containing approximately 10⁸ CFU/mL of C. jejuni, and on days 3, 11, 21, and 32 posthatch, chicks from each treatment group were euthanized, and their livers, spleens, and cecal tonsils were removed aseptically for the determination of organ invasion, colonization, and clearance of the Salmonella vaccine strains. Also, on days 21 and 32 posthatch, ileum sections were removed and processed for use in qPCR. In addition, blood samples were collected from 10 birds per treatment group, and the sera were used to determine antibody responses on days 21 and 32 posthatch.

**Vaccination study 3.** A third experiment was similar to vaccination experiment 2 (described above), except for the addition of a third group of chicks receiving S. Enteritidis 13A araC htrA without the Campylobacter epitope as a control for oral vaccination with the vector itself. All sample collections were the same as those for vaccination study 2, except that on day 32 posthatch an additional section of ileum was used to harvest the mucosal layer for sIgA determination as in experiment 1.

**Measurement of Campylobacter-specific antibody response.** Sera collected from birds in all three vaccination studies were used in an enzyme-linked immunosorbent assay (ELISA) to determine relative antibody responses (35, 40). Briefly, individual wells of a 96-well plate were coated with the three wild-type isolates of C. jejuni used for the challenge (10⁵ CFU/well). Antigen adhesion was allowed to proceed overnight (4°C), and the plates were then washed and blocked with Superblock (Thermo Scientific) for 1 h at room temperature. Plates were then incubated (2 h) with a 1:50 dilution of the previously collected sera. The plates were rinsed again, followed by incubation with a peroxidase-labeled anti-chicken IgG secondary antibody (Jackson ImmunoResearch) for an additional hour. After subsequent rinsing, the plates were developed using a peroxidase substrate kit (BD OptEIA; Fisher Scientific), and after the addition of sulfuric acid, the absorbance was read using a spectrophotometer (450 nm). Each plate contained a positive-control well and a negative-control well, which was a pooled sample from vaccinated chicks and preimmune chicken serum, respectively, replaced the sera from the treatment groups. The absorbances obtained for the positive-control, negative-control, and experimental samples were used to calculate sample-to-positive-control ratios (S/P ratios) (4, 11), using the following equation:

\[
\text{S/P ratio} = \frac{\text{sample absorbance}}{\text{positive control absorbance}}
\]

For the determination of sIgA, the mucosal layer of a section of the ileum 2.5 cm from the cecal tonsil was collected, diluted (1:5 wt/vol) with saline, and frozen (−20°C) immediately until analysis. Just prior to use, the samples were vortexed and then centrifuged (1,000 × g for 15 min), and the supernatant was used for slgA determination. The ELISA method used for detection of slgA was similar to the assay described above for serum immunoglobulin, except that goat anti-chicken IgG conjugated with horseradish peroxidase (GenTex) was used in place of the anti-chicken IgG antibody conjugate.

**DNA isolation and quantitative PCR for C. jejuni.** DNA extraction from ileal samples was achieved using a QIAamp DNA stool minikit (Qiagen). The manufacturer’s recommendations were modified slightly in the following ways: ileal contents were removed to include the mucosal layer and diluted 1:5 wt/vol with ice-cold phosphate-buffered saline (PBS) plus 0.05% Tween 20, and 1 mL of the slurry was added to 1 mL of the included ASL buffer in a 2.0-mL microcentrifuge tube, mixed, and heated (70°C for 5 min). Subsequently, the manufacturer’s recommendations were followed to the last step, when the DNA was eluted into a final volume of 50 μL.

**Quantitative determination of C. jejuni.** Quantitative determination of C. jejuni was accomplished using a previously published method, with slight modifications (28, 39). The assay was optimized for use on an MX3005P instrument (Agilent Technology) and with Brilliant II QPCR master mix (Agilent Technologies). All other mixture components, primers, probes, and cycling conditions remained as published.

A standard curve (see the supplemental material) was prepared using a culture of pooled isolates (n = 3) of C. jejuni serially 10-fold diluted and added to a constant background of ileal content. Total DNA isolation was quantified by qPCR as described above and correlated with enumeration by standard microbiological methods.

**Statistical analysis.** Data were analyzed using analysis of variance with pairwise comparison, using post hoc Tukey-Kramer analysis, to determine the differences between groups and controls, using JMP statistical software (SAS Institute Inc.). P values of <0.05 were considered significant.

## RESULTS

Three experiments were performed using Salmonella vectors expressing multiple copies of selected linear peptide epitopes of Campylobacter on the cell surface. The first experiment had three potential linear Campylobacter candidate sequences selected from the literature expressed on separate Salmonella vectors. Subsequent experiments utilized only the Cj0113-expressing vector and controls.

**Immune response following vaccination with linear Campylobacter peptide-expressing Salmonella vectors.** In experiment 1, we observed significant levels of colonization by the three candidate vectored vaccines within the cecal tonsils by day 3 postvaccination, as well as significant invasion of the internal organs by the Cj0113-expressing vector at the same time point (Table 1). However, by day 11 postvaccination, there was a decline in the amount of colonization of all three vectors, and by day 21 postvaccination, the vectors had been cleared completely from the cecal tonsils as well as the internal organs (Table 1). We observed a similar trend in liver/spleen invasion
in our follow-up vaccination study (experiment 2) using vector-expressed Cj0113 as a vaccine candidate (Table 1).

Serum samples collected for each experiment on days 21 and 32 postvaccination were used to determine the levels of C. jejuni-specific IgG antibodies. In the first experiment, all three vaccine candidates (Cj0420, Cj0113, and Cj0982) caused significantly higher IgG antibody levels at both time points than those for the group which received saline only (Fig. 1). Also in the first experiment, the group vaccinated with Cj0113 showed significantly higher IgG antibody titers than the groups vaccinated with Cj0420 and Cj0982 (Fig. 1). An ELISA was also used to determine mucosal sIgA antibodies specific for Campylobacter. Vaccination with the Cj0113 vector caused a significant increase in the level of sIgA compared to those for the saline group and the two groups receiving either Cj0420 or Cj0982 (Fig. 2). The results from the second and third studies, in which only Cj0113 was used as a vaccine candidate, showed results similar to those for experiment 1, with vaccinated birds having significantly higher levels of antigen-specific IgG and sIgA antibodies to C. jejuni than birds receiving saline only (data for experiment 3 are shown in Fig. 3; data for experiment 2 were similar and are not shown). Also, in the third experiment, the antibody levels for the backbone strain (SE13) were not different from those for saline controls (Fig. 3).

Campylobacter sp. challenge in broilers vaccinated with linear Campylobacter peptide epitopes expressed on Salmonella vectors. Chickens were challenged with C. jejuni (mixture of three field isolates) on day 21 postvaccination. Ileal mucosal samples were obtained on days 21 and 32 postvaccination (days 0 and 11 postchallenge) and used for DNA sample preparation to enumerate C. jejuni within the gut, as described above. There was a high degree of correlation (>99%) of the levels of C. jejuni obtained using conventional microbiological enumeration techniques versus qPCR (see the supplemental material). Prior to challenge with C. jejuni on day 21, samples from 15 birds tested negative for C. jejuni in the ileum (data not shown). Vaccination with vector candidates Cj0420 and Cj0982 caused approximately 1-log and 2-log reductions (P < 0.05), respectively, in the levels of C. jejuni present in the ileal samples. Using the Cj0113 vaccine candidate, there was a marked, 4.8-log reduction (P < 0.05) of C. jejuni in the ileum compared to the level for the control birds (Fig. 4).

In experiment 2, a repeat of the primary vaccination study was done with only the vaccine candidate expressing Cj0113. In this study, qPCR data revealed an approximately 4-log reduction, to undetectable levels, of C. jejuni in Cj0113-expressing Salmonella vector-vaccinated birds compared to the birds receiving saline only. Additionally, in experiment 3, vaccination with the Cj0113 peptide-expressing vector caused an approxi-
The Salmonella nivation of Schrotz-King and coworkers (37) were able to decrease colo-

protein ACE393 from an

lobacter numbers following challenge. Using the expressed pro-

humoral response, yet it did not significantly decrease

Campy-

following challenge with

P/H11021 are significantly different (\(P < 0.05\)). Groups with different lowercase letters are significantly different (\(P < 0.05\)).

approximately 4-log reduction, to below detectable levels, of C. jejuni compared with the levels with saline or the Salmonella parent strain (13A), which contained no epitope insert (Fig. 5).

**DISCUSSION**

We have developed an *aroA* and *htrA* deletion Salmonella mutant to use as a vector for vaccine development (10). Insertion of three potential Campylobacter peptide epitopes of Cj0113, Cj0982c, and Cj0420 into this Salmonella vector produced strong IgG and sIgA immune responses to *Campylobacter*, with the vector persisting for only a brief period. In addition to strong and consistent seroconversion with the candidate vaccines, administration of a single dose of these candidates caused marked and significant decreases in *Campylobacter* colonization with two of these candidates (Cj0982c and Cj0113). Consistent with the results of Wyszynska and coworkers (45), one of the candidates (Cj0982c) was found to significantly decrease *Campylobacter* levels following challenge in broiler chicks. A recent review article indicated that when the Cj0113 gene was inserted into a *Salmonella* plasmid vector and then used as a vaccine in chickens, the result was an increased humoral immune response (14). In this work, we have shown that the use of small immunodominant peptide epitopes of Cj0113 inserted into the genomic DNA of the *Salmonella* vector (Fig. 1 to 3) gave a strong immune response and decreased *Campylobacter* levels to below detectable levels following challenge with C. jejuni (Fig. 4 and 5).

The vector candidate Cj0420 (ACE393) elicited a good IgG humoral response, yet it did not significantly decrease *Campylobacter* numbers following challenge. Using the expressed protein ACE393 from an *E. coli* recombinant expression vector, Schrotz-King and coworkers (37) were able to decrease colonization of *Campylobacter* in mice. The lack of protection with the *Salmonella* vector expressing Cj0420 in our system may be due to the selection of a single short-chain epitope that was less effective than the purified expressed protein of Schrotz-King et al. (37). The Cj0420 vector may elicit an immune response to the inserted linear epitope which may not prove to be immunoprotective against a direct challenge. However, both conformational and linear epitopes can provide protection against challenge. But as in the case of the major outer membrane protein (MOMP) on *C. jejuni*, protection was elicited primarily through conformational epitopes (8, 20, 47).

Cj0113 is the outer membrane protein Omp18, which was found to be an immunogenic protein found in all *Campylobacter* species tested (6, 30). The Cj0113 protein (Omp18/CjaD) has sequence similarities to the peptidoglycan-associated lipoprotein (Pal) of *E. coli* (6, 14). The Pal protein is part of a Tol-Pal protein system that is important for membrane integrity (14). Pal (Omp18 or Cj0113) is also a pathogen-associated molecular pattern signal that interacts with Toll-like receptors that modulate the immune system (14). Cj0113 (Omp18) appears to be essential for survival and therefore may be a prime candidate for vaccine development (14).

Vaccination with Cj0113 (Omp18) expressed on the cell surface of our *Salmonella* vector decreased *C. jejuni* colonization to undetectable levels following challenge of chickens. While the humoral immune response was strong for all three vaccine candidates, the Cj0113-expressing *Salmonella*-vectored vaccine had the highest levels of sIgA response in the ileum and showed the greatest protection following challenge. A possible explanation for the highly successful protection conferred by Cj0113 could be related to the high levels of antigen-specific sIgA that we observed in these experiments. Cj0113 has been shown to be a major immunogenic protein, with convalescent-phase patient sera reacting with an 18-kDa protein identified as Cj0113 (6).

While the mechanism of action was not explored in the present studies, vaccination using selected *Campylobacter* epitopes elicited a strong humoral immune response and pro-
tection against challenge through day 32. This protection against challenge may have involved complement-mediated killing of the bacteria (3), opsonization with subsequent phagocytosis (43), and sIgA binding to Campylobacter to enhance excretion (18). Further investigations will be required to more fully elucidate the mechanisms involved in the marked protection afforded by vaccination with attenuated Salmonella-vec
tored Cj0113 and, to a lesser extent, Cj0982c.

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