Autodisplay: Efficacious Surface Exposure of Antigenic UreA Fragments from Helicobacter pylori in Salmonella Vaccine Strains

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Live attenuated Salmonella strains expressing antigens of pathogens are promising oral vaccine candidates. There is growing evidence that the topology of expression of the foreign antigens can have a dramatic impact on the immunogenicity. We examined the potential of the AIDA-I (Escherichia coli adhesin involved in diffuse adherence) autotransporter domain to display antigenic fragments of the urease A subunit of Helicobacter pylori for the induction of a protective immune response. In the murine H. pylori model, protection is mainly mediated by CD4+ T cells, and we therefore used the AIDA-I expression system to successfully express both nearly full-length UreA and defined T-helper-cell epitopes on the surface of an attenuated Salmonella enterica serovar Typhimurium vaccine strain. Surface exposure of the large UreA fragment or of one UreA T-cell epitope mediated a significant reduction in the level of H. pylori in immunized mice after challenge infection, whereas conventional cytoplasmic expression of UreA in Salmonella had no effect. These results support the concept that surface display increases the immunogenicity of recombinant antigens expressed on oral live vaccine carriers and further demonstrate the feasibility of immunizing against H. pylori with Salmonella vaccine strains expressing CD4+ T-cell epitopes.

The approach of using live Salmonella vaccine strains to deliver recombinant antigens has been generally accepted, and to date several clinical studies have been performed in this field. The results of these studies, although promising, imply that new attenuated strains and improved antigen expression are needed to enhance the immunogenicity of Salmonella vaccine strains (14).

The localization of expressed antigens in bacterial live oral vaccines seems to be very important (20), and therefore many efforts have been made to manipulate surface-exposed proteins to display antigenic determinants (for a review see reference 13). Recently, we observed that an attenuated Salmonella vaccine strain expressing a CD4+ T-cell epitope on its surface via the autotransporter domain of AIDA-I (an adhesin involved in diffuse adherence from Escherichia coli [3]) was able to induce a specific CD4+ T-cell response (30). These findings encouraged us to investigate whether the AIDA-I expression system is able to induce protective immune responses in an animal model of infectious disease in which protection is mainly mediated by CD4+ T cells. We therefore chose the murine Helicobacter pylori infection model, because immunity against this pathogen has been reported to depend mainly on CD4+ T-helper cells (11) and we confirmed this for mice vaccinated with recombinant Salmonella which was effective in IgH+/− mice but not in major histocompatibility complex II gene-deficient mice (Aebischer, unpublished observations).

H. pylori is a gram-negative spiral bacterium that colonizes the human stomach and can cause a variety of diseases, including chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric lymphoma (23, 41, 48). Vaccination would be a cost-effective means to control this public health problem faced by one-half of the world’s population. Expression of urease subunits A and B from H. pylori in recombinant attenuated Salmonella vaccine strains induced high levels of protection against an H. pylori challenge infection in vaccinated mice (8, 16, 34), and three clinical phase I studies have already been based on this approach (2, 5, 10). Recombinant UreB has been reported to confer protective immunity against Helicobacter felis in different mouse strains (12, 38), whereas variable results have been reported for the protective effects of UreA (12, 38).

In a recent study, spleen-derived oligoclonal CD4+ T-cell lines were isolated from BALB/c mice vaccinated with attenuated Salmonella expressing urease subunits A and B from H. pylori (35). The T cells recognized urease A and could be restimulated with peptides containing predicted H-2d-restricted CD4+ T-cell epitopes (amino acids 28 to 51, 74 to 90, or 209 to 225) (35). Furthermore, adoptive transfer of these T cells into naïve mice partially protected against a H. pylori challenge.

In this study, we expressed translational fusions of a nearly full-length urease A variant or one of the three recognized urease A peptides to the C-terminal autotransporter domain of AIDA-I in attenuated Salmonella and tested these constructs for protective efficacy in the murine Helicobacter infection model.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains employed in this study are listed in Table 1. For all purposes (except preparation of frozen stocks), E. coli and Salmonella strains were grown on Luria-Bertani (LB) agar plates or in liquid medium supplemented with ampicillin (100 μg/ml) and, in the case of recombinant Salmonella, with streptomycin (90 μg/ml). Thymine (50 μg/ml) was added when required. H. pylori P76 was grown on brain heart infusion (BHI) (Difco,
TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Escherichia coli JK321</td>
<td>aei-6 fhuA23 lacY1 leu-6 mit-1 proc14 purE42 rpsL109 thi-1 trpE38 tsx-67 (ompT-pegC)</td>
<td>26</td>
</tr>
<tr>
<td>Crea238</td>
<td>S. enterica serovar</td>
<td>Creatogen AG*</td>
</tr>
<tr>
<td></td>
<td>Typhimurium SL3261 ΔureA</td>
<td></td>
</tr>
<tr>
<td>Crea294</td>
<td>S. enterica serovar</td>
<td>Creatogen AG</td>
</tr>
<tr>
<td></td>
<td>Typhimurium SL3261 ΔureA ΔhydA</td>
<td></td>
</tr>
<tr>
<td>SL3261(pYZ97)</td>
<td>S. enterica serovar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Typhimurium SL3261</td>
<td></td>
</tr>
<tr>
<td>H. pylori P76</td>
<td>Streptomyces-resistant</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>derivative of the mouse-adapted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. pylori strain P49</td>
<td></td>
</tr>
</tbody>
</table>

*a Modified as described by Hoiseth and Stocker (22).

Bacterial strains used in this study. The fusion constructs were then subcloned to obtain plasmids psdUreA27-53, BglII, and LAT183-LAT184 products, and inserted into the LAT183 and LAT184, respectively, by using pYZ97 as the template, treated with primers LAT212 and LAT198, treated with BglII and KpnI, respectively, to obtain plasmid stabilization purposes (39). A fragment encoding UreA27-53-PEYFK-CTB was amplified with primers LAT68 and LAT198 and treated with BglII and XbaI (LAT68-LAT61 product) or BglII and BamHI (LAT181-LAT182 and LAT183-LAT184 products), and inserted into the BglII site of plasmid pLAT238. The DNA fragments encoding UreA27-53, UreA27-230, and UreA74-95-PEYFK-CTB were amplified from primers LAT181 and LAT182 and treated with BglII and KpnI, respectively, to obtain plasmid pXYZ79 with primers LAT70 and MSC4 was inserted into the single BglII and Acc65I sites of the new multiple cloning site. The ureA4 fragment was transferred from this vector by digestion with Xhol and SalI to obtain plasmid pcUreA, which mediated ureA expression by the Puro promoter. The identities of the constructs were verified by dideoxy chain termination sequencing (4base lab GmbH, Reutlingen, Germany). The final plasmids are shown in Fig. 1.

**Animals.** Specific-pathogen-free female BALB/c mice that were 6 to 8 weeks old were obtained from the Bundesamt für Gesundheitlichen Verbraucherschutz (Berlin, Germany) and were kept under conditions that were in full compliance with German guidelines for animal care. All experiments were approved by the local animal welfare committee.

**Preparation of frozen stocks.** Starting from a single colony, each Salmonella vaccine strain was grown on LB agar plates overnight at 37°C. The organisms were harvested on the following day in fresh LB medium, the suspension was used to inoculate culture medium (LB medium containing 90 μg of ampicillin per ml) to obtain an optical density at 600 nm (OD600) of 0.1, and the culture was incubated overnight at 28°C and 200 rpm. The culture was harvested and resuspended in a 70% LB medium–30% glycerol mixture at an OD600 of 7 and stored at −80°C. The number of CFU per milliliter in each batch was determined by plating serial dilutions on selective LB agar plates.

**TABLE 2. Oligonucleotide used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' &gt; 3')</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM54</td>
<td>GATCTCCGGAATATTTCACGCGATCCATCGCAGCTGCGCTCAGGG</td>
<td>Linker encoding PEYFK, sense*</td>
</tr>
<tr>
<td>JM55</td>
<td>GATCTGAGACGAGGTGGACCTTGAATATTTCAGG</td>
<td>Linker encoding PEYFK, antisense*</td>
</tr>
<tr>
<td>LAT61</td>
<td>GATCGGATCCCTTTTATCCAGTCTCG 651</td>
<td>UreA27-53, antisense, BglII site</td>
</tr>
<tr>
<td>LAT68</td>
<td>GATCAGGGATCCCGCAGTATGAAATCTGATCAGCGCTTACGCGATCCATCG</td>
<td>UreA27-53, sense, BglII site</td>
</tr>
<tr>
<td>LAT70</td>
<td>GATCGAGTGCATCATATGAAATCTGATCAGCGCTTACGCGATCCATCG</td>
<td>UreA27-53, antisense, BglII site</td>
</tr>
<tr>
<td>LAT71</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA27-53, sense, BglII site</td>
</tr>
<tr>
<td>LAT75</td>
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<td>UreA27-53, antisense, BglII site</td>
</tr>
<tr>
<td>LAT81</td>
<td>GATCGAGTGCATCATATGAAATCTGATCAGCGCTTACGCGATCCATCG</td>
<td>UreA27-53, sense, BglII site</td>
</tr>
<tr>
<td>LAT82</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA27-53, antisense, BglII site</td>
</tr>
<tr>
<td>LAT83</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA27-53, sense, BglII site</td>
</tr>
<tr>
<td>LAT84</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA27-53, antisense, BglII site</td>
</tr>
<tr>
<td>LAT121</td>
<td>AAGGCCTGCTAGCTGATGAGACCCAAAATGCTTTACGCGCTTACG</td>
<td>IAIDA, antisense</td>
</tr>
<tr>
<td>LAT220</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>pEGf6, sense</td>
</tr>
<tr>
<td>MSC04</td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA, antisense, BglII site</td>
</tr>
<tr>
<td></td>
<td>GATCGGATCCCTTTGATCCGAGCTGCGCTCAGGG</td>
<td>UreA, antisense, Acc65I site</td>
</tr>
</tbody>
</table>

*See reference 36.

The BglII site is underlined.

The BglII site is underlined.

The BglII site is underlined.

The SalI site is underlined.

The Acc65I site is underlined.
Immunization experiments. Prior to oral immunization mice were left overnight without food. Salmonella stocks were thawed, diluted with a 70% LB medium-30% glycerol mixture to obtain a concentration of 1 \times 10^{10} \text{CFU/ml}, and then diluted 1:2 with 100 mM NaHCO_3 to obtain a concentration of 0.5 \times 10^{10} \text{CFU/ml}. The number of CFU per milliliter was counted after 5 days of growth under microaerophilic conditions at 37°C. Serial dilutions, and 1:1000 plating were performed to determine bacterial counts were determined after 5 days of growth under microaerophilic conditions at 37°C.

Surface exposure of AIDA-I fusion proteins. Bacteria were grown overnight on LB agar plates at 37°C and harvested in PBS the following day. The OD_{590} of the bacterial suspension was adjusted to 10.0, and surface-exposed protein domains were proteolytically cleaved off by incubation of the suspension at 37°C for 10 min with trypsin (50 \mu g/ml). Cells were washed twice in PBS with gentle centrifugation in order to remove residual trypsin and were subjected subsequently to SDS-PAGE analysis.

Preparation of outer membranes. Bacterial outer membranes were prepared as described elsewhere (31), with slight modifications. Bacteria grown overnight were harvested from agar plates and resuspended in PBS as described above. The suspension was sonicated with 30 1-s pulses at the maximum intensity by using a Branson Sonifier. Intact cells and large bacterial fragments were separated by centrifugation at 5,000 \times g for 5 min. The cleared lysate was supplemented with 1% LAURYL sarcosinate (Sigma, Deisenhofen, Germany) at a final concentration of 1% to solubilize the inner membrane. Subsequently, the outer membrane was separated from the cytoplasm, periplasm, and inner membrane by centrifugation at 20,000 \times g for 30 min at room temperature.

Statistical analysis. Statistical analysis was performed by using the GraphPad Prism program (version 3.0; GraphPad Software, San Diego, Calif.). The level of significance used was P < 0.05.

RESULTS

Construction of Salmonella vaccine strains displaying Urea fragments on the cell surface. The autotransporter domain of AIDA-I adhesin has been used in several studies to target epitopes, CTB, and \(\beta\)-lactamase to the surface of E. coli cells (29, 31, 36). In this study, we used AIDA-I to target H. pylori Urea fragments to the surface of an attenuated Salmonella vaccine strain. The Urea epitopes Urea\textsubscript{27-53}, Urea\textsubscript{4-95}, and Urea\textsubscript{209-230} were translationally fused to the N terminus of a fusion protein of the AIDA-I autotransporter domain and CTB, whereas a large Urea\textsubscript{27-238} fragment was translationally fused to the N terminus of the AIDA-I autotransporter domain without CTB but contained an HA tag sequence separating Urea\textsubscript{27-238} and AIDA-I. Plasmids psdUrea\textsubscript{27-53}, psdUrea\textsubscript{174-95}, psdUrea\textsubscript{209-230}, and psdUrea\textsubscript{27-238} are shown in Fig. 1. Intermediate constructs contained the transcriptonal fusions under control of the constitutive P\textsubscript{TK} promoter (25), which were electroporated into an araA Salmonella carrier strain for biochemical localization of the products (see below).

For in vivo studies, the thyA gene coding for thymidylate synthase was also included in the plasmid backbone of the final constructs (Fig. 1) for plasmid stabilization purposes (39). The Salmonella vaccine strain Crea\textsubscript{1294}, which contained a chromosomal deletion of the thyA gene and was derived from the araA-deficient strain Crea\textsubscript{1283} (unpublished data), was transformed with the corresponding plasmids. Complete Urea was
expressed from the $P_{\text{psyc}}$ promoter (pcUreA) as a cytoplasmic antigen localization control (Fig. 1).

Localization of AIDA-I fusion proteins in the outer membrane. Autotransporter proteins localize to the outer membrane of gram-negative bacteria. To evaluate expression and outer membrane targeting of the various UreA fusion proteins in the attenuated $S. \text{enterica}$ serovar Typhimurium aroA strains, outer membrane fractions from the Salmonella strains were analyzed by SDS-PAGE and Western blotting by using a monoclonal antibody to HA or a polyclonal antibody to cholera toxin (Fig. 2). All AIDA-I fusion proteins were detected in the outer membrane fraction. Decreased expression of the UreA$_{27-238}$–AIDA-I fusion protein compared to the expression of AIDA-I without UreA$_{27-238}$ was detected, and we also detected some degradation which might have been caused by partial proteolysis in the periplasm or at the outer membrane. This partial proteolysis most likely affected the UreA part of the fusion protein, since the degradation products were recognized by the HA antibody and remained membrane associated.

Surface localization of proteins can be investigated by trypsin treatment of intact cells, because surface-exposed protein structures that are sensitive to trypsin are cleaved off during exposure to the protease, whereas cytoplasmic, periplasmic, or protein domains embedded in the outer membrane are not affected (31, 37). This method is therefore suitable for monitoring the surface exposure of passenger domains fused to autotransporters. Physiologically intact cells, expressing the various AIDA-I fusions proteins, were subjected to trypsin digestion or were left untreated. In cells treated with trypsin UreA–AIDA-I fusion proteins were undetectable in immunoblot analyses of whole-cell lysates but were present in untreated control cells (Fig. 3). As only surface-exposed proteins were accessible to trypsin under these conditions, the results corroborated the localization data obtained by cell fractionation (Fig. 2) and further demonstrated that the vast majority of the fusion proteins were surface exposed.

Immunization with Salmonella expressing UreA$_{27-238}$–AIDA-I protects mice against an $H. \text{pylori}$ challenge. In order to analyze the vaccine potential of aroA-attenuated Salmonella expressing either cytoplasmic UreA [Crea1294(pcUreA)] or surface-exposed UreA$_{27-238}$–AIDA-I fusion protein [Crea1294(psdUreA$_{27-238}$)], protein expression was first compared in the corresponding vaccine strains. The UreA levels expressed under in vitro conditions were assessed by immunoblot analysis by using a Helicobacter-specific antiserum raised in rabbits (Fig. 4). The level of UreA expression was higher in the strain expressing UreA in the cytoplasm, Crea1294(pcUreA) (603 arbitrary staining intensity units), than in Crea1294(psdU-
For reference, UreA expression in our standard vaccine strain, SL3261 (pYZ97) expressing both UreA and UreB subunits (16), was also analyzed, and the level of UreA expression in this strain was 829 optical density units.

A second important parameter of live vaccine strains is the relative ability to colonize the host. To estimate the bacterial fitness, groups of three BALB/c mice were inoculated orally with single intragastric doses of 10^9 CFU of either Crea1294 (psdUreA27-238), Crea1294(pcUreA), the plasmidless carrier strain, or the positive control strain SL3261(pYZ97) (16). Bacterial loads were compared on day 7, when SL3261 colonization usually reached peak levels. The new UreA-expressing strains Crea1294(pcUreA) and Crea1294(psdUreA27-238) showed reduced colonization of the Peyer’s patches on day 7 postimmunization (2.2 × 10^2/100 µl and 3.9 × 10^2/100 µl CFU, respectively) compared to the colonization by the plasmidless strain (6.6 × 10^4/100 µl CFU) and the control strain, SL3261(pYZ97) (7.5 × 10^3/100 µl CFU) (Fig. 5A).

However, for the present study it was important that the new vaccine strains expressed comparable amounts of UreA and had similar colonization capabilities.

To determine vaccine efficacy, the four different strains [the carrier control, positive control SL3261(pYZ97), cytoplasmic UreA, and surface-exposed UreA strains] were orally administered to mice, and 4 weeks later the mice were challenged with 1 × 10^9 CFU of H. pylori. Three weeks after the challenge infection, mice were sacrificed, and the H. pylori burden in the stomach was determined. Vaccination with the Salmonella strain expressing UreA at the cell surface resulted in significantly reduced H. pylori colonization compared to the colonization of the carrier-immunized control group (P = 0.0089, as determined by the Student t test) (Fig. 5B). In contrast, the Salmonella strain expressing cytoplasmic UreA failed to induce a significant reduction in the H. pylori burden compared to the burden observed after administration of the carrier strain control, although the bacterial counts were lower on average.

Salmonella sp. expressing a UreA27-53 peptide on the surface partially protects against H. pylori. In a recent study, Lucas et al. identified three peptides of the UreA protein that contain T-cell epitopes recognized by protective UreA-specific CD4+ T cells (35). In the present study, we fused these peptides to AIDA-I to evaluate its potential as a T-cell epitope expression platform. All peptide fusion proteins were similarly expressed by the attenuated Salmonella aroA strain (Fig. 6), and the colonization capabilities of the constructs were comparable.

Mice were vaccinated with the three constructs or a negative control strain expressing AIDA-I without a fused UreA pep-
tide and challenged with *H. pylori* as described above. While *Salmonella* strains expressing UreA_{27-53} and UreA_{209-230} had no protective effect, the UreA_{27-53} construct induced a significant reduction in the *H. pylori* burden compared to the burden observed with the *Salmonella* negative control strain (Fig. 7) ($P = 0.03$, as determined by the Student t test).

**DISCUSSION**

Recombinant attenuated *Salmonella* strains are promising vaccine carriers for oral delivery of heterologous antigens. For example, we and other workers have previously shown that immunization with *Salmonella* strains expressing *H. pylori* ureases A and B can protect mice against a subsequent *Helicobacter* challenge infection (16).

In a number of experimental models, *Salmonella* vaccine efficacy can be enhanced by surface display or secretion of the foreign antigen. Autotransporter domains of gram-negative bacteria can be used as one such surface display system (27–29, 31, 36, 45). Autotransporters are widespread among gram-negative bacteria, and their main physiological function is to translocate virulence factors through the cell envelope to the surface (19). The fact that they are expressed as a single polypeptide chain containing all features necessary to translocate an N-terminal passenger domain to the cell surface (25) makes them attractive candidates for antigen display. Furthermore, functional B-cell epitopes (42) and T-cell epitopes (29, 30) have already been successfully expressed as autotransporter...
fusion proteins. In this study, we tested if Salmonella surface display of urease A or urease A fragments that contain T-cell epitopes (35) by using the AIDA-I autotransporter provides better anti-Helicobacter protection than an otherwise identical cytoplasmic UreA construct provides. A nearly full-length variant of UreA, as well as three different UreA peptides, could be expressed as AIDA-I fusion proteins and were displayed on the surface of attenuated Salmonella cells. The nearly full-length variant encoded on psdUreA27-238 lacks the N terminus (amino acids 1 to 26), which contains six lysine residues, to avoid potential inhibition of translocation across the inner membrane by the Sec pathway (1). Inhibition of translocation was noted when the full-length UreA was introduced into E. coli or Salmonella (Lattemann, unpublished observations).

In the murine H. pylori infection model a significant reduction in the H. pylori burden was detected in mice vaccinated with a Salmonella strain expressing either the almost complete UreA protein [Crea1294(psdUreA27-238)] or the T-cell epitope-containing peptide UreA27-53 [Crea1294(psdUreA27-53)] as an AIDA-I fusion protein. The results obtained with the latter strain also provided independent functional confirmation that there is at least one protective epitope in UreA, which has been suggested previously by Lucas and coworkers, who identified the UreA fragments mentioned above as CD4+/H11001 T-cell epitope-containing regions (35). Additionally, this study demonstrated the feasibility of immunizing against H. pylori with Salmonella vaccine strains expressing CD4+ T-cell epitopes on the cell surface. In contrast, the H. pylori burdens in mice vaccinated with a Salmonella strain conventionally expressing UreA in the cytoplasm [Crea1294(pcUreA)] were not significantly reduced compared to the burdens in mice vaccinated with the carrier strain.

CD4+ T-cell induction is directly correlated with the number of Salmonella cells colonizing the Peyer’s patches on the first few days after vaccination (4). For comparing various Salmonella vaccine constructs, it is thus important to determine the corresponding colonization capabilities. The two vaccine constructs differing in the location of the UreA fragment on the AIDA-I fusion protein were tested using an in vitro model of colonization in murine Peyer’s patches (5) and in a mouse model of in vivo colonization (6).

In the murine Peyer’s patches model, the Salmonella vaccine strain expressing the UreA27-53 peptide displayed on the surface of the bacterial cell conferred significant protection against H. pylori challenge. The results obtained with the UreA27-53 peptide-expressing Salmonella strain also provided independent functional confirmation that there is at least one protective epitope in UreA, which has been suggested previously by Lucas and coworkers, who identified the UreA fragments mentioned above as CD4+/H11001 T-cell epitope-containing regions (35). Additionally, this study demonstrated the feasibility of immunizing against H. pylori with Salmonella vaccine strains expressing CD4+ T-cell epitopes on the cell surface. In contrast, the H. pylori burdens in mice vaccinated with a Salmonella strain conventionally expressing UreA in the cytoplasm [Crea1294(pcUreA)] were not significantly reduced compared to the burdens in mice vaccinated with the carrier strain.

**FIG. 6.** Expression of UreA CD4+ T-cell epitopes by Salmonella vaccine strains. Whole-cell lysates from cells grown overnight on LB agar plates at 37°C were prepared and subjected to SDS-PAGE. (A) Coomassie brilliant blue-stained SDS-PAGE gel. (B) Immunoblot with an anti-cholera toxin antibody. Lane 1, Crea1294(psdUreA27-53); lane 2, Crea1294(psdUreA27-53); lane 3, Crea1294(psdUreA290-238); lane 4, Crea1294(pCTB-AIDA); lane 5, SL3261(pYZ97); lane 6, Crea1283.

**FIG. 7.** In vivo colonization and protective efficacy of Crea1294 expressing UreA CD4+ T-cell epitopes on the cell surface. Groups of three BALB/c mice were immunized orally with 1 x 10^9 cells of recombinant Salmonella vaccine strains. At day 7 postimmunization mice were killed, and the Peyer’s patches were harvested, homogenized, and plated on selective LB agar plates to determine Salmonella colonization (A). (B) Analysis of H. pylori burden in mice orally vaccinated with Salmonella strains expressing UreA CD4+ T-cell epitopes after challenge. The results of one representative experiment are shown (10 mice per group) (two experiments were performed). Crea1294(psdUreA27-53), displaying the UreA27-53 peptide, conferred significant protection against H. pylori compared to the protection conferred by Crea1294(pCTB-AIDA) expressing only the carrier protein. An asterisk indicates a P value of 0.03, as determined by an unpaired t test. The horizontal lines indicate means.
strains expressing cytoplasmic and surface-displayed urease A showed similar colonization levels in Peyer’s patches of immunized mice. This suggests that other factors and probably the different localizations account for the different vaccination efficacies of the two strains. Superior antigen processing of surface antigens by antigen-presenting cells and/or an altered urease A conformation might be involved, but further studies are required to test these hypotheses. Improvement of the urease A–AIDA-I fusion protein-expressing strains is, however, still needed, and this should be possible by increasing *Salmonella* fitness in vivo. Our standard laboratory vaccine strain, strain SL3261(pYZ97), colonized at least 10-fold better ever, still needed, and this should be possible by increasing *UreA*–

...are required to test these hypotheses. Improvement of the urease A–AIDA-I fusion proteins, addition of immunogenic epitopes from other antigens to the existing constructs and prime-boost regimens are potential ways to increase the efficacy of the new strains, which, as reported here, indicates that AIDA-I is an attractive candidate for the development of live vaccines against *H. pylori*.

Several other approaches have been used to develop expression systems that are alternatives to the conventional somatic expression of antigens for the induction of cellular immune responses (13, 20, 44). These include secretion of antigens into the surrounding environment (13, 20) or into the cytoplasm of the host cell (44). *Salmonella* vaccine strains endowed with the α-hemolysin secretion apparatus of *E. coli in trans* were able to secrete full-length antigens and were efficacious in several animal models (15). The type III secretion system encoded on the SPI-1 pathogenicity island of *S. enterica* serovar Typhimurium has also been successfully used in *Salmonella* live vaccine carriers to translocate major histocompatibility complex I–restricted epitopes fused to the N terminus of YopE, a secreted effector protein of *Yersinia enterocolitica*, into host cells, resulting in protective immune responses (43, 44).

Humoral immune responses have been observed after vaccination of mice with attenuated *Salmonella* strains with immunogenic determinants exposed on their surfaces by means of outer membrane proteins from *E. coli*, like OmpA (17), PhoE (24, 47), LamB (6, 18, 32), and P87 fimbriae (7), the ice-nucleating protein from *Pseudomonas aeruginosa* (33), the main flagellar component FlIC from *Salmonella* (9, 40, 40), or the *Salmonella* autotransporter MisL (42). Recently, use of *E. coli* outer membrane protein ToIC for surface display of protective listerial B- and T-cell epitopes in a *Salmonella vaccine* strain has been shown to be efficacious in vivo (46).

In summary, autotransporters facilitate effective surface display of antigenic determinants on live *Salmonella* vaccine carriers, leading to humoral (42) and cellular immune responses in vivo (29). This study demonstrated that surface display of a *Helicobacter* antigen via the AIDA-I autotransporter can induce protective immune responses.

**ACKNOWLEDGMENTS**

We thank Elke Gerland, Kirstin Hoffmann, and Kerstin Burmeister for excellent technical assistance.

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