Evaluation of *Salmonella* live vaccines with chromosomal expression cassettes for translocated fusion proteins

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**Abstract**

*Salmonella enterica* is a versatile live carrier for the presentation of recombinant vaccine antigens. Fusion proteins of a type III secretion system effector and heterologous vaccine antigens can be translocated by live attenuated *Salmonella* strains and mediate protective immunity against infections. Here we investigated the use expression cassettes for translocated fusion protein consisting of effector SseF and antigens of *Listeria monocytogenes* after stable integration into the *Salmonella* chromosome. The efficacy of chromosomal expression cassettes was compared to plasmid-based expression cassettes. Our data indicate that live *Salmonella* vaccines with chromosomal expression cassettes for translocated fusion proteins, although only present in single copy, efficiently stimulate immune responses.

1. Introduction

*Salmonella enterica* is a promising tool for the generation of live recombinant vaccines. As a gastrointestinal pathogen, attenuated *Salmonella*-based vaccines can be applied by mucosal routes and are easy to produce and administer [23]. Efficient genetic tools allow that *S. enterica* can be easily genetically modified to express heterologous antigens relevant for vaccination. Genetic manipulation allows the generation of attenuated strains that are safe for application in vaccinees as well as the introduction of heterologous antigens (recently reviewed in [2,20,26]). Important parameters for the efficacy of *Salmonella* carrier strains in presentation of heterologous antigens are the control of expression and the form of display of the antigens. Controlled high-level expression of foreign antigens can be achieved by use of *in vivo* activated and, more specifically, intracellular activated promoters [15].

For injection of virulence proteins into eukaryotic target cells, most Gram-negative bacterial pathogens use so-called type III secretion systems (T3SS) [13]. The ability of *S. enterica* to cause disease is complex and involves a multitude of virulence factors, particularly two distinct T3SS, which are important for interaction with host cells [9]. The T3SS encoded by *Salmonella* Pathogenicity Island 1 (SPI1-T3SS) is expressed by extracellular bacteria and is required for the invasion of non-phagocytic cells [6,30]. Intracellular *Salmonella* express the second T3SS encoded by *Salmonella* Pathogenicity Island 2 (SPI2-T3SS) that is required for intracellular replication and systemic pathogenesis. A large number of effector proteins are translocated by the SPI2-T3SS, among them SseF and SseJ [18]. *Salmonella* offers the application of ‘inverted pathogenicity’, i.e. the use of T3SS for a beneficial purpose [28]. Previously, we reported that expression by intracellular *Salmonella* in combination with translocation by the SPI2-T3SS was useful for the improvement of *Salmonella* live vaccine carriers [17].

A common problem is the generation of recombinant bacterial vaccine strains that allow stable expression of heterologous antigens within the vaccine even in the absence of selective pressure. Several strategies have been developed to address this requirement. The balanced lethal plasmid stabilization system is one of these strategies, in which the functional allele present on the plasmid that express the vaccine antigen complement the auxotrophy due to a mutation on the bacterial chromosome [3,7,8,22]. The insertion of DNA fragments for the expression of foreign antigens into the chromosome of the carrier strain was possible [12,32]. Furthermore, genetic switches can be utilized to shift a part of the bacterial population to antigen expression [33]. Although these approaches resulted in the construction of vaccine carrier strains, the genetic manipulations required for the construction of such strains are rather laborious and time-consuming.

Here, we introduce and evaluate a combined approach that allows (i) the stable integration of expression cassettes into the...
chromosome of S. enterica serotype Typhimurium and (ii) the translocation of fusions proteins into eukaryotic cells. These cassettes express fusion proteins for the translocation of heterologous antigens by the SPI2-T3SS. This technique also allows the simultaneous generation of deletions in the genome of Salmonella that can be used to attenuate virulence of the carrier strain. Due to the modular concept, an expression cassette consisting of translocated effector fusion can be directed to insert into various loci [14].

In this study, we evaluated the novel combination of stable chromosomal integration of expression for heterologous antigens with the use of SPI2-T3SS effector proteins for a vaccination strategy employing Salmonella as a live carrier.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. Salmonella enterica serovar Typhimurium strain NCTC 12023 (S. typhimurium) was used as wild-type strain. Bacteria were routinely cultured in LB broth and on LB agar plates. If required for the selection of recombinant strains or to maintain plasmids, carbenicillin (50 μg/ml) and/or kanamycin (50 μg/ml) were added. Synthetic chromosomal integration of expression for heterologous antigens with the use of SPI2-T3SS effector proteins for a vaccination strategy employing Salmonella as a live carrier.

Table 1

<table>
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<tr>
<th>Strain designation</th>
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<tr>
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Plasmids

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hc, high copy number; lc, low copy number; ts, temperature sensitive.

For generation of recombinant DNA molecules, all PCR reactions were performed using polymerase mixtures with proofreading activity (Expand HF kit, Roche). DNA manipulations were performed according to standard procedures and resulting plasmids were previously constructed by disruption of various loci [14] and are specified in Table 1. For the generation of a highly attenuated S. typhimurium carrier strain capable of translocating SPI2 effector proteins, a double mutant strain harboring deletions of htrA and purD was generated. This was accomplished by sequential mutagenesis using the one-step deletion approach [4,16]. Alternatively, strains were constructed using P22 transduction according to standard methods [21].

L. monocytogenes EGD Sv 1/2a was obtained from Dr. Werner Goebel (Würzburg, Germany). L. monocytogenes was cultured in brain heart infusion broth at 37 °C with aeration.

2.2. Construction of recombinant DNA

For generation of recombinant DNA molecules, all PCR reactions were performed using polymerase mixtures with proofreading activity (Expand HF kit, Roche). DNA manipulations were performed according to standard procedures and resulting plasmids were previously constructed by disruption of various loci [14] and are specified in Table 1. For the generation of a highly attenuated S. typhimurium carrier strain capable of translocating SPI2 effector proteins, a double mutant strain harboring deletions of htrA and purD was generated. This was accomplished by sequential mutagenesis using the one-step deletion approach [4,16]. Alternatively, strains were constructed using P22 transduction according to standard methods [21].
constructs were confirmed by DNA sequencing. Recombinant plasmids were introduced into *E. coli* DH5α or XL-1 Blue competent cells by electroporation. Confirmed plasmid constructs were introduced into different strains of *S. typhimurium* by electroporation.

DNA fragments were generated by PCR and the primers used are listed in Table 2. The PCR product for *L. monocytogenes* *iap* (1110 bp) was digested by EcoRI and EcorV and subcloned in plasmid p2064 digested by EcoRI/Smal to obtain plasmid p2666. The PCR product for *lisA* (1007 bp) was digested by PstI and EcoRV and inserted into p2064 digested by PstI and EcoRV to obtain plasmid p2682.

For generation of translated fusion proteins, plasmids p2803 (for synthesis of SseF-p60-HA, about 64.5 kDa) and p2810 (for synthesis of SseF-llo-HA, about 62.4 kDa) were digested by SacI and Xhol, the inserts were recovered and subcloned into SacI/Xhol-digested p2795 to obtain plasmids p3047 and p3048, respectively. For somatic expression, SacI/Xhol-digested p2666 and p2682 and inserts were cloned into SacI/Xhol-digested p2795 for generation of plasmids p3049 and p3055, respectively.

### 2.3. Analysis of synthesis of recombinant proteins

Samples obtained from *in vitro* expression experiments were separated by SDS-PAGE on 10% tricine gels and protein was transferred onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell) by semi-dry transfer as previously described [5]. Membranes were blocked with 5% skim milk in TBS-T (Tris-buffered saline containing Tween) at 4 °C overnight. Recombinant proteins were detected with monoclonal antibody against the HA tag (rat α-HA, Roche) or against the M45 tag [24]. For detection, goat α-rat horseradish peroxidase conjugate (Dianova) for detection of HA tag and goat anti-mouse IgG–HRP for detection of M45 tag were used at a dilution of 1:20,000, followed by chemiluminescent detection using the ECL system (Amersham).

### 2.4. Cell culture and infection experiments

#### 2.4.1. Bacterial infection of macrophages

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection, and maintained in DMEM containing 10% heat-inactivated FCS (Sigma) at 37 °C in an atmosphere of 5% CO₂. The cells were washed gently with phosphate-buffered saline (PBS) and removed from the flasks using a cell scraper. The cells were suspended in 24-well plates for experiments at the density of 5 × 10⁴ cells per well. For the infection of RAW264.7 cells *S. enterica* serovar Typhimurium strains were grown to stationary phase in LB with the appropriate antibiotic selection. The OD₆₀₀ of the cultures was adjusted with LB to 0.2 and the bacteria were washed once with PBS, diluted in cell culture medium and added to the cells growing in 24-well tissue culture plates at a multiplicity of infection (MOI) of about 10. The plates were centrifuged onto the cells at 500 rpm for 5 min. After infection for 25 min, the macrophages were washed three times with PBS and incubated for 1 h in cell culture medium containing 100 μg/ml gentamicin (Sigma). The medium was then replaced with fresh medium containing 10 μg/ml gentamicin for the remainder of the experiment. Analysis of protein translocation was carried out by immuno-staining 16 h after infection as stated below.

#### 2.4.2. Bacterial infection of HeLa cells

For the infection of HeLa cells, *S. typhimurium* strains were grown overnight in LB broth supplemented with the necessary antibiotic at 37 °C. The cultures were then diluted 1:30 with fresh LB broth and incubated for another 3.5 h at 37 °C with agitation to reach the late logarithmic growth phase. The OD₆₀₀ of the cultures was adjusted with LB to 0.2 and the bacteria were washed once with PBS. Bacteria were diluted in DMEM containing 10% FCS and 1% glutamine and added to the HeLa cells which are grown on glass cover slips in 24-well culture plates at MOI of about 10. The plates were centrifuged at 500 rpm for 5 min in order to synchronize infection and incubated for 25 min at 37 °C in 5% CO₂ and further processed as described for infection of macrophages.

### 2.5. Immunofluorescence

For immuno-fluorescence analysis, the cells were grown in 24-well tissue culture plates on glass cover slips. After infection and incubation for indicated time period, the cells were fixed with 3% glutaraldehyde in PBS and then washed three times with PBS. The antibodies were diluted in a blocking solution consisting of 10% saponin (Sigma) in PBS. The cover slips were incubated with various antibodies as detailed below, and were washed three times with PBS after each incubation step. The cover slips were mounted on Fluoprep (bioMerieux) and sealed with Entellan (Merck). Samples were analyzed using a laser-scanning confocal microscope (Leica TCS-NT) as described [1]. If not otherwise stated, all antibody incubations were performed for 1 h at room temperature. The following antisera and antibodies were used at the indicated dilutions: rabbit α *S. enterica* O4 antisera (diagnostic test sera, BD, 1:1000); rat α HA tag mAB (Roche, 1:300). For detection, the following conjugates (all obtained from Dianova, Hamburg) were used at the indicated dilutions: goat α rabbit Cy2-conjugate (1:1000); goat α rat Cy3-conjugate (1:1000).

### 2.6. Vaccination experiments

For vaccination, bacterial strains were grown overnight to stationary growth phase. Groups of 6–8 female BALB/c mice (6–8 weeks old) were immunized by gastric gavage with a single dose of 1 × 10¹⁰ CFU/mouse of various *S. typhimurium* carrier strains resuspended in 5% sodium bicarbonate in a volume of 250 μL, or a sub-lethal intraperitoneal (i.p.) dose of 5 × 10⁵ CFU *L. monocytogenes* 8 weeks before the challenge infection. The mice groups were challenged intravenously (i.v.) with 1 × 10⁵ CFU of log phase *L. monocytogenes* in 0.2 ml PBS. Three days after the challenge, mice were sacrificed and CFU in organs were determined by plating serial dilutions of spleen homogenates onto *Listeria* selective agar (Oxoid). Colonies were enumerated after 48 h of incubation and the log CFU per organ was calculated. Animal studies were performed in accordance with national and institutional guidelines for animal handling.
2.7. MHC pentamer staining and FACS analysis

Blood samples were collected 12 and 21 days after orogastric immunization with two successive doses of $1 \times 10^9$ CFU/mouse (0 and 14 days) of Salmonella deficient in purD/htrA strain harboring plasmid [p2810]. Blood cells were harvested, erythrocytes were lysed with ammonium chloride and cells were subsequently resuspended in RPMI complete media. Subsequently, cells were stained with R-PE labeled H-2Kd/GYKDGNEYI Pro5 pentamer (Proimmune) (Listeria-specific H-2Kd-Llo pentamers) and rat anti-mouse CD8-FITC (Proimmune). After washing, murine CD8 T cells stained with Pro5 pentamers can be analyzed by flow cytometry (FACSCalibur; BD Biosciences) and the frequency of antigen-specific T cells determined.

2.8. Statistical analysis

Statistical significance between unpaired groups and the control group was tested with Student's $t$-test for unpaired values. Statistical significance was defined as a $P$ value of < 0.05 (Prism 3).

3. Results

3.1. Integration of expression cassettes into Salmonella chromosome for translocation fusion proteins

The Red recombinase approach [4] was utilized for integration of expression cassettes into the chromosome of S. enterica serotype Typhimurium and the concomitant generation of deletions leading to the attenuation of virulence of the resulting strain [14]. By modification of this technique one can create insertions at any given point in the bacterial chromosome. Expression cassettes were constructed that contained heterologous model antigens under control of the SPI2-derived, in vivo-activated promoter $P_{sseA}$ (Fig. 1). This promoter is activated by Salmonella residing within the Salmonella-containing vacuole (SCV) of infected host cells and our previous work demonstrated that superior immune responses were induced if heterologous antigens were specifically expressed by intracellular Salmonella carrier strains [15]. The expression cassettes harbored lisa or iap for the expression of Llo or p60, respectively, as protective antigens against infections by Listeria monocytogenes. Expression cassettes allowed the in vivo-regulated somatic expression of heterologous antigens (Fig. 1A). Alternatively, gene fusions of the heterologous antigens to effector protein SseF of the SPI2-encoded type III secretion proteins generated (Fig. 1B) that should allow the translocation of fusion proteins into host cells. The cassettes were integrated into the chromosomal loci, htrA, galE or purD. The insertion of expression cassettes into these loci led to deletions that resulted in attenuation of virulence. This approach was also used to integrate two expression cassettes in independent loci in the Salmonella chromosome such as htrA and purD or htrA and galE. Targeting two distinct loci generated mutant strains with higher attenuation of virulence [16] and may allow the generation of strains that express two different heterologous antigens.

3.2. In vitro characterization of recombinant strains for chromosomal expression of translocated fusion proteins

The regulated expression of the gene fusions by S. enterica serovar Typhimurium wild-type (WT) was analyzed using previously described in vitro growth conditions [5]. Western blot

Fig. 1. Rationale for the generation of Salmonella strains with stable chromosomal insertions of expression cassettes for translocated heterologous antigens. Expression cassettes were constructed consisting of the in vivo-activated promoter $P_{sseA}$ and fragments of genes encoding heterologous antigens from L. monocytogenes (iap or lisa) without (A) or with (B) fusions to sseF encoding a translocated effector protein. Expression cassettes without a gene for a translocated effector protein (A) result in the presence of fusion proteins in the bacterial cytoplasm, while expression cassettes incorporating the effector gene (B) allow the synthesis of fusion proteins that are translocated by intracellular Salmonella. Epitope tags M45 or HA were fused to C-termini to enable standardized detection. Expression cassettes were subcloned in plasmid p2795 for the generation of template vectors for the generation of linear targeting constructs. Using Red recombinase-mediated recombination, expression cassettes were integrated into various loci of the Salmonella chromosome. Insertion of expression cassettes into target genes such as purD, htrA or galE results in mutant strains with attenuated virulence. Finally, the aph resistance gene was deleted by FLP-mediated recombination.
analyses was performed of bacterial cultures grown under non-inducing conditions (PCN medium) or inducing conditions (PCN-P medium) (Fig. 2). We observed that all analyzed strains harboring chromosomal expression cassettes showed regulated expression, since high amounts of the recombinant proteins were only observed in cultures grown in PCN-P media. There was no difference in the amount of heterologous antigen between strains harboring cassettes for the somatic expression of vaccine antigens (Fig. 2A) or sets for the expression of translocated fusion proteins (Fig. 2B and C). Similar expression characteristics in vitro were observed for strains with single or double insertions of expression cassettes (Fig. 2A and B). The data indicate that chromosomally integrated expression cassettes are functional to control the synthesis of the somatic as well as translocated heterologous antigens by Salmonella carrier strains. The effect of the culture conditions on expression of the sseF::isxA::HA cassette on a plasmid was similar to the chromosomal cassette, but higher levels of fusion protein were synthesized (Fig. 2C).

To test stability in vivo, a strain was used for oral immunization of mice in which an expression cassette with the aph gene replaced the purD locus. Analyses of feces indicated that the recovered Salmonella carrier strain was shed for up to 9 days. Plating on selective agar with or without kanamycin indicated that the recovered S. enterica serovar Typhimurium were kanamycin resistant (48.7 CFU/mg feces), supporting the stability of chromosomal integrated cassettes during vaccination. In previous studies we showed that Salmonella is able to efficiently colonize the lymphoid tissues of immunized mice [15,16]. Also we have previously demonstrated that the plasmid used in this work was stably maintained by the vaccine strains in vitro and in vivo [14,15].

We next investigated the translocation of fusion proteins by intracellular Salmonella (Fig. 3). For infection of human epithelial cells (HeLa) (Fig. 3A) or murine macrophages (RAW264.7) (Fig. 3B), double mutant strain MvP774 (ΔhtrA ΔgalE) harboring a chromosomally integrated expression cassette for translocation of an SseF::p60-HA fusion protein was used. For comparison, we used the previously described wild-type Salmonella that harbored recombinant plasmid p2643 for translocation of epitope-tagged effector SseF or plasmid p2803 for translocation of an epitope tagged SseF-p60 fusion protein [17]. As expected from previous observations, intracellular Salmonella WT translocated SseF-HA and SseF-p60-HA into both types of host cells. Translocation in epithelial cells and macrophages was also observed for strain MvP774. The subcellular localization of the translocated fusion proteins appeared to be similar to that of SseF, i.e. in association with late endosomal/lysosomal membranes rather than diffusely in the host cell cytoplasm (data not shown). As judged by the intensity of immunofluorescence signals, the amounts of fusion protein translocated by intracellular MvP774 were comparable to than those for wild-type Salmonella translocating an epitope-tagged effector protein. No translocation was observed by a strain deficient in ssaV encoding a key component of SPI2-T3SS. We also noticed that some bacteria by phagocytosed macrophages that were unable to translocate effector proteins or fusion proteins with heterologous antigens (Fig. 3B). This phenomenon was observed for epitope-tagged effector proteins as well as for translocated heterologous antigens and may reflect the different individual fate of the bacteria after uptake by host cells.

These data indicate that attenuated carrier strains with chromosomally integrated expression cassettes can be used for the translocation of heterologous vaccine antigens by intracellular Salmonella. This observation is a pre-requisite for the further use of such strains for vaccination.

3.3. Protection experiments

The efficacy of vaccination with recombinant Salmonella strains was analyzed in a vaccination model of protection against L.
monocytogenes infection. We previously reported that vaccination with recombinant Salmonella expressing Listeria antigen iap under control of an intracellular activated promoter mediates partial protection against a lethal challenge infection [15]. Also, we reported that the protection after vaccination with recombinant Salmonella expressing a translocated fusion protein consisting of SseF and Llo enhanced protection indicating a superior protection by translocation compared to somatic expression of the heterologous antigen [17].

Here, we immunized mice by oral administration of a single dose of attenuated Salmonella strains harboring different forms of expression cassettes. We compared the protection after vaccination with purD-deficient recombinant Salmonella harboring plasmids for expressing a translocated fusion protein consisting of SseF and p60 (p2803) or SseF and Llo (p2810) to Salmonella strains MvP578 and MvP580 with chromosomally integrated expression cassettes for translocated p60 or Llo, respectively (Fig. 4B). In both strains, the expression cassettes were inserted into the purD locus, leading to attenuation of the carrier strain. In a second set of experiments, carrier strains deficient in htrA were used (Fig. 4B). Compared to vaccination with carrier strains without heterologous antigens, the use of all Salmonella strains that expressed the heterologous antigens led to a significant reduction of the organ counts of Listeria, indicating a partial protection against the infection. Highest protection was obtained after vaccination by intraperitoneal injection of L. monocytogenes that resulted in a sterile immunity in many animals. Compared to vaccination with L. monocytogenes, bacterial counts in the spleen were about 2 log higher if animals were vaccinated with Salmonella strains with plasmid-based expression cassettes for Llo or p60. Higher organ burdens were detected in the cohorts vaccinated with strains with chromosomal expression cassettes and the bacterial counts in the spleen were generally higher compared to cohorts vaccinated by strains with episomal expression cassettes. However, compared to vaccination with the carrier strains vaccination with strains MvP578, MvP579 or MvP580 led to 20-, 1130- or 175-fold, respectively, reduced organ counts of Listeria.

These results show that carrier strains with chromosomal expression cassettes for heterologous antigens can be used to mediate a protective immune response after vaccination. However, in general the level of protection mediated by strains with episomal expression cassette was higher.

3.4. Antigen-specific CD8 T cell response induced by Salmonella vaccine

To determine specific cytotoxic T lymphocytes (CTLs) induction by vaccination with Salmonella, we used a Salmonella strain deficient in purD/htrA harboring plasmid (p2810). Listeria-specific H-2Kd-Llo pentamers was used, which allow longitudinal measurement of antigen-specific CTL response. Mice were divided into groups that received purD/htrA harboring (p2810) or the empty vector (as control). A booster vaccination was given on day 14. Listeriolysin-reactive CTLs were measured in peripheral blood on days 12 and 21. The data shown in Fig. 5 demonstrate that vaccination with purD/htrA (p2810) induced clearly detectable specific CTLs after single dose with subsequent increase after boost vaccinations. Therefore, oral vaccination with purD/htrA (p2810) induced antigen-specific CTLs.

4. Discussion

We have previously demonstrated that effector proteins of the SPI2-T3SS can be used to translocate vaccine antigen into antigen-presenting cells and efficiently stimulate immune responses [17]. This approach is based on the initial finding that the T3SS-mediated translocation of heterologous antigen can stimulate immune responses [29] and subsequent studies revealed that use of effector proteins of T3SS represents a powerful delivery of het-
expression cassettes were induced in a similar way by conditions thought to mimic the intracellular environment of Salmonella. The translocation of the fusion proteins expressed by episomal and chromosomal expression cassettes was detectable in epithelial cells and in macrophages. We have previously demonstrated that the expression of heterologous antigens by the cassettes was stable over many generations. We also observed that expression of chromosomally integrated cassettes containing a promoter specifically activated by intracellular Salmonella was tightly regulated [14]. A further advantage of integration of expression cassettes as described here is the option of creating attenuating mutations concomitantly with introduction of the translocating fusion protein of heterologous antigen. We could also insert expression cassettes consisting translocating fusion protein into purD, htrA, or galE of Salmonella resulting in deletion of these loci. It was shown before that mutant strains deficient in these genes are highly attenuated in virulence and represent safe and efficient vaccine carrier strains [14].

Our evaluation demonstrated that vaccines strains with chromosomal expression cassettes lead to the indication of protective immune responses against an infection with L. monocytogenes. In general the protection mediated by strains with chromosomal expression cassettes was lower than after vaccination with strains harboring expression cassettes on plasmids. This observation indicates that both forms of delivery of heterologous antigens function, but differ in their efficacy in stimulating immune responses. In further experiments, the induction of Llo-specific effector CD8 T cells after Salmonella-based immunization was demonstrated by pentamer staining. Salmonella strains that secrete Llo into the cytosol of APCs directs the listerial antigen to the MHC class I antigen-processing pathway, leading to presentation of Llo-antigen to MHC class I restricted CD8 T cells. These results demonstrate that a bacterial SPI2-T3SS can be used for heterologous antigen delivery to induce specific cytotoxic effector CD8 T cell responses resulting in an efficient protection.

One obvious limitation of chromosomal expression cassettes is the low amount of heterologous antigens (Fig. 2A and B) due to its presence in single copy, which is in contrast to high-level expression by plasmid-based systems (Fig. 2C) with multiple copies. This limitation, however, might be addressed further optimization of regulatory elements such as ribosome binding sites and the selection of stronger in vivo-activated promoters.

Another critical parameter is the selection of a suitable carrier strain for insertion of chromosomal expression cassettes. We observed different levels of protection after vaccination of htrA and purD deficient strains harboring the same expression cassettes. Thus the identification of recombinant strains with an optimal balance between attenuation of virulence and penetration of the host appears to be an important parameter.

One important feature of the integration of chromosomal expression cassettes is the possibility to introduce multiple expression cassettes for multiple antigens from one or several different infectious agents. The use of this possibility to create multivalent recombinant life carrier strains has to be evaluated by future work.

In the present work, only SseF was evaluated as a fusion partner to mediate translocation by the SPI2-T3SS. However, other effector proteins may be more efficient for an approach with a single copy chromosomal expression cassette. Currently, at least 20 SPI2-T3SS effector proteins have been described (reviewed in [11]) that may be worth further evaluation. Panthel et al. [25] investigated two SPI2 effector proteins, i.e. SspH2 and SifA. These authors observed that SspH2 as a translocation domain was sufficient to mediate translocation of fusion proteins with p60. SPI2 carrier molecule is sufficient to induce a concomitant p60-specific CD4 and CD8 T cell response in Salmonella-vaccinated mice. Moreover, T3SS-mediated antigen delivery results in an efficient priming of

![Fig. 4. Protection against Listeria infection after experimental vaccination with recombinant Salmonella carrier strains.](image)
central and effector memory CD8 T cells in spleens of these animals [26]. Although our work demonstrated that single chromosomal expression cassettes are less efficient compared to episomal expression strategies, further optimization should be possible. The benefits of this approach, namely the lack of antibiotic resistance markers, stability of strains and the possibility of creating multivalent vaccines clearly justify further work in this direction.

References