Rational design of *Salmonella* recombinant vaccines

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**Abstract**

*Salmonella enterica* is an important pathogen of animals and humans causing a variety of infectious diseases. The large number of cases of typhoid fever due to *S. enterica* serovar Typhi infections gives rise to the continuous need for improved vaccines against this life-threatening infection. However, *S. enterica* is also an interesting organism to act as a live attenuated carrier for the presentation of recombinant heterologous antigens. Comprehensive experimental studies have been performed and a detailed knowledge of the molecular mechanisms of important virulence factors is available. This allows the rationale design of improved *Salmonella* carrier strains and the development of novel strategies for the expression and presentation of recombinant antigens. Here, we review recent advances in generation of live attenuated *Salmonella* vaccines and discuss criteria for expression strategies of heterologous antigens by *Salmonella* carrier strains.

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**Introduction**

Diseases caused by *Salmonella enterica* are a continuously threat to human health. Depending on the serovar of *S. enterica* causing the infection, highly different disease outcomes can be observed. Infections with *S. enterica* serovar Typhi (S. Typhi) can lead to typhoid fever, a life-threatening systemic infection. While typhoid fever in industrialized nations is mainly a disease imported by travelers, typhoid fever is still endemic in many developing countries with an estimated disease burden of 16–33 million cases and 500,000–600,000 deaths annually (according to WHO). Infections with non-typhoidal serovars of *S. enterica*, predominantly *S. Enteritidis* and *S. Typhimurium* are even more frequent and occur in both developing and industrialized nations. These infections are associated with gastrointestinal inflammation and diarrhea and are usually self-limiting without the need for antibiotic treatment. However, systemic infections by non-typhoidal *Salmonella* can occur and the increasing frequency of strains with multiple resistances against currently used antibiotics caused problems for the treatment. *Salmonella* is transmitted by contaminated food or water. While the only host for *S. Typhi* is man, gastrointestinal infections by *S. enterica* are typical zoonotic infections from reservoirs in all important livestock animals.

Given the high disease burden of typhoid fever, there is an obvious need of efficient vaccines for protection against *S. Typhi* infections. In order to combat *Salmonella* gastroenteritis, the vaccination of livestock animals appears to be more suitable than vaccination of humans. In addition to vaccination against infections...
with Salmonella, there is another area of vaccination where Salmonella is of interest. Live attenuated S. Typhi and S. enterica serovar Typhimurium (S. Typhimurium) are among the most promising candidates for the engineering of live recombinant mucosal vaccines (reviewed in Mollenkopf et al., 2001). For many important infectious diseases such as malaria or HIV, efficient vaccines are urgently needed. One strategy to develop new vaccines is the use of live attenuated bacteria as carriers for the presentation of heterologous antigens. Salmonella strains are of particular interest since these strains can be administered orally, i.e. by the natural route of infection, and may induce mucosal as well as systemic immune responses. Both humoral and cellular immune responses can be primed by this form of application. Furthermore, convenient methods for the genetic manipulation of Salmonella are available, and one can express single or multiple heterologous antigens from other bacteria or from viruses or parasites, allowing to create a single recombinant vaccine for simultaneous protection against S. Typhi and other pathogens. However, rational vaccine design requires numerous issues regarding safety and efficacy to be addressed. More than 20 years of experience with a licensed live attenuated Salmonella vaccine, S. Typhi Ty21a (Vivotif®, Crucell/Berna biotech) are available and indicate that this strain is safe in mass vaccination against typhoid fever (Levine et al., 1990). The development of new Salmonella vaccines for recombinant vaccination is also facilitated by the important knowledge that has accumulated over the last 10 years on the pathogenesis of Salmonella and on the immune response against this pathogen. S. Typhi is restricted to human hosts, but infection of susceptible mice with S. Typhimurium leads to a lethal disease which resembles in many aspects human typhoid fever. By means of this animal model, major Salmonella virulence factors have been identified and characterized in detail, especially regarding the interaction with the host and its immune system. The following is a review of the recent advances in the generation of new attenuated typhoid vaccines and in the optimization of these strains for recombinant vaccination.

Currently available vaccines against typhoid fever

At present, two internationally licensed vaccines are available for protection against typhoid fever. The first vaccine, Typhim Vi® (Pasteur Merieux), is composed of purified Vi-antigen from S. Typhi. The Vi-antigen is a polysaccharide capsule only present in S. enterica serovars Typhi, Paratyphi C and Dublin. The vaccine is administered intramuscularly in one single dose and provides 55–75% protection against typhoid fever. As with many other pure polysaccharide vaccines, no booster effect is observed by repeated injections, indicating the lack of immunological memory (Keitel et al., 1994). In addition, this vaccine is not effective in infants less than 2 years old (Plotkin and Bouveret-Le Cam, 1995). The use of the second licensed typhoid vaccine Vivoti® is also restricted to adults and children over 6 years of age (Murphy et al., 1991). This vaccine consists of the attenuated live S. Typhi Ty21a strain and was generated by chemical mutagenesis of the parental virulent S. Typhi Ty2. The Ty21a strain was initially isolated and characterized as a GalE mutant (Germanier and Furer, 1975). Subsequently, it has been shown that Ty21a is also unable to produce the Vi polysaccharide and about 30 further mutations were characterized in this strain (Germanier and Furer, 1983). While the reasons for the strong attenuation of Ty21a are still not fully understood, the presence of multiple mutations rendered this strain very stable and safe, two essential requirements for an efficient live vaccine. This oral vaccine is well tolerated and is available in enteric-coated capsule or liquid formulation. For either formulation, three doses taken 2 days apart on an empty stomach are required to induce protection. The protection observed after the last boost is more than 70% over 7 years of follow-up for the liquid formulation, whereas the corresponding protective efficacy for the enteric-coated capsule is around 60% (Levine et al., 1999). However, both currently applied vaccines have two major flaws: their short-termed and incomplete protection, which makes frequent vaccination campaigns necessary, as well as their lack of efficacy in young children.

Development of new live attenuated Salmonella strains

Having in mind the safety data of Ty21a, the design of genetically defined new live oral Salmonella vaccines appears to be a promising issue. Such vaccines are easy to administer, target mucosal and systemic immunity, and can be used as carrier strains expressing heterologous antigens to protect against other infectious diseases, in addition to typhoid fever. The major asset of S. Typhi Ty21a, i.e. the high safety due to the presence of multiple mutations, is also a main drawback of this strain. Its low immunogenicity might be a consequence of over-attenuation. Most currently used live vaccines are the result of a non-directed mutagenesis process and have many non-characterized defects. However, the present requirements for licensing new vaccines are rather rigid and call for strains that are well defined and carry characterized mutations.
Antibiotic resistance genes widely used to select new mutants should not be present in the genome of the final strain used for vaccination. Finally, the strain should be highly immunogenic as well as sufficiently attenuated in order to be safe for application in immuno-compromised individuals. Because of the considerable advances in genetic manipulation and molecular biology, the two first requirements are minor issues in the development of new Salmonella-based vaccines. However, finding the right balance between attenuation and immunogenicity is still a major challenge. To achieve this goal, different kinds of mutations can be introduced. As for all prototrophic bacteria, Salmonella could be attenuated by targeting its main metabolic pathways. In addition, during co-evolution with its host, this pathogen acquired specific virulence factors and became a facultative intracellular bacterium. Being able to survive in rather different environments, Salmonella has evolved efficient mechanisms to sense environmental factors and cues of the host organism in order to regulate the expression of genes required for adaptation. From the dissection of all these mechanisms, many targets have already been identified to attenuate Salmonella and many combinations are possible. Nevertheless, the recent observations on the interferences of Salmonella virulence factors in the priming of the adaptive immune system will probably have the major impact in the rational design of more immunogenic recombinant Salmonella vaccines.

Using the current knowledge about the physiology and virulence mechanisms of Salmonella, around ten different attenuated strains have been rationally designed and are currently under evaluation (Levine et al., 2001; Pasetti et al., 2003). The preliminary results in humans were promising, but these approaches need optimization for successful use in large vaccination campaigns (Pasetti et al., 2003).

The recent detailed characterization of the host–pathogen interactions, as well as the description of mechanisms used by Salmonella to escape both innate and adaptive immunity should lead to further rational design of well-defined strains that are more immunogenic and optimized for recombinant vaccination.

**Metabolic pathways and auxotrophic strains**

Most of the Salmonella-based vaccines currently used in experimental work and early clinical trials are auxotrophic strains. For example, mutants in metabolic pathways have been engineered by deleting essential genes for the biosynthesis of purine (pur), aromatic amino acids (aro) or guanidine (gua). Although many of these mutants were attenuated and highly immunogenic in mice, many of the strains did not succeed in clinical trials due to adverse reactions in vaccinees or lack of immunogenicity. Strains with a mutation in the purA gene are the most widely studied of purine biosynthetic pathway mutants. However, these strains have been shown to be poorly immunogenic even in combination with aroA mutation during clinical studies (O’Callaghan et al., 1988, 1990; Sigwart et al., 1989). In contrast, an aroA aroD double mutant (strain PBCC211) derived from the CDC10-80 strain or aroC aroD CVD906 and CVD908 derived from ISP1820 or Ty2, respectively, were immunogenic at a single dose. However, these strains caused fever and/or other adverse reactions such as vaccinaemia in some volunteers vaccinated with high doses (Tacket et al., 1992; Dilts et al., 2000; Hone et al., 1992). Some of these strains have been further attenuated by deletion of the htrA gene which encodes a periplasmic serine protease required for Salmonella survival in macrophages (Tacket et al., 1997b; Dilts et al., 2000b). One of these strains, CVD908-htrA, is a promising vaccine strain which is currently in further clinical trials. It is well tolerated in volunteers even at high doses and no case of vaccinaemia could be detected. More recently, the phase II study reveals high immunogenicity of this vaccine candidate. Vaccinated volunteers developed not only a good humoral immune response, but CVD908-htrA also primed cellular immune responses in some of the vaccinees (Tacket et al., 2000b). Other Salmonella strains have been constructed by deleting guaBA (Wang et al., 2001) or by targeting DNA recombination and repair (rec) (Buchmeier et al., 1993). Good safety and immunogenicity profiles were observed in the mouse model for typhoid fever. However, phase I clinical trials have to reveal the value of these strains as potential alternative candidates.

**Facultative intracellular lifestyle and virulence factors of Salmonella**

During evolution, Salmonella acquired many virulence factors that enabled this pathogen to gain access to new niches in a host during the complex pathogenesis. Many of the virulence factors are encoded on large genetic elements, termed Salmonella pathogenicity islands (SPIs), that often show distinct base composition of the DNA and are absent in non-pathogenic relatives of Salmonella (Hensel, 2004). Depending on the S. enterica serovar analyzed, up to 12 SPIs have been identified. Two of these SPIs encode type III secretion systems (T3SSs) for the translocation of effector proteins into eukaryotic host cells. The SPI1-encoded T3SS (SPI1-T3SS) is involved in the invasion of non-phagocytic cells by Salmonella and the elicitation of intestinal inflammation. After passage of the mucosal barrier, Salmonella spreads to deeper tissue where it encounters macrophages and dendritic cells (Salcedo et al., 2001). By means of the SPI2-T3SS and specific secreted effectors, Salmonella does not only escape...
NADPH oxidase- and inducible nitric oxide synthase (iNOS)-dependent killing (Chakravortty et al., 2002; Vazquez-Torres et al., 2000), but is able to actively modify its intracellular habitat, termed Salmonella-containing vacuole or SCV (Kuhle and Hensel, 2004; Abrahams and Hensel, 2006). This modification appears to be important for the intracellular replication of Salmonella in eukaryotic cells.

In murine models, SPI1-deficient strains of S. Typhimurium are only slightly attenuated in systemic virulence, thus SPI1 has not been considered for generating attenuating mutations. In contrast, SPI2 mutant strains were identified by virtue of their strong attenuation of systemic virulence in the mouse model (Hensel et al., 1995). This observation led to the use of mutations in SPI2 as vaccines against typhoid fever and a new vaccine called M01ZH09 was recently designed (Khan et al., 2003). This Ty2 aroC strain with a defined mutation in the SPI2 genessaV seems to be well tolerated and highly immunogenic. This promising vaccine candidate (Kirkpatrick et al., 2005, 2006) is currently evaluated in large-scale clinical trials.

Besides the attenuation of systemic virulence, the control of gastrointestinal symptoms caused by the vaccine is an important issue. Many promising candidate strains failed in clinical trials due to causing intestinal inflammation and diarrheic disorders in the vaccinees. Until recently, gastrointestinal inflammation by S. enterica could only be evaluated in models with large animals, but a murine model now allows a broader study of the molecular mechanisms underlying gastroenteritis (Hapfelmeier and Hardt, 2005). Translocation of effector proteins by the SPI1-T3SS is not only required for host cell invasion, but has also been shown to elicit gastroenteritis (Wallis and Galyov, 2000). Thus, in combination with mutations attenuating virulence, mutations in the SPI1-T3SS or specific effectors may be useful in order to reduce gastrointestinal complications.

Regulatory systems

By means of different regulatory systems, Salmonella tightly controls gene expression patterns in response to the different environments encountered. Two vaccine strains Ty800 and γ4073, both derived from Ty2 but carrying mutations in distinct regulatory systems, have already proven their potential in different clinical trials (Hohmann et al., 1996; Tacket et al., 1997a). Ty800 was generated by deletions in the phoP/phoQ regulon, which controls bacterial survival in macrophages by regulating expression and repression of many virulence genes and other regulatory systems, such as SsrAB, which controls SPI2 expression. In case of γ4073, a global regulatory system has been targeted by mutations in genes encoding adenylate cyclase (cya) and cAMP receptor protein (crp), as well as in the virulence gene cdt involved in bacterial dissemination from the gut to deeper organs. Further global regulatory systems that have been proposed as interesting targets to develop new candidate vaccines are the alternative sigma factors RpoS and RhoE (Coyne et al., 1996; Humphreys et al., 1999) and RfaH, an anti-terminator required for the expression of large operons such as those involved in LPS biosynthesis pathways (Nagy et al., 2006).

Another global regulatory mechanism was recently targeted for the generation of attenuated live Salmonella strains. Inactivation of the DNA adenine-methylase (Dam) resulted in attenuation, probably by the inability of the resulting strain to control the expression of virulence genes and stress response genes (Heithoff et al., 1999). The use of dam mutant strains of S. Typhimurium as live vaccines was evaluated and Heithoff et al. (2001) observed that cross-protection was elicited against infections with other S. enterica serovars.

Controlling bacterial interference with adaptive immunity

A weak and delayed T cell response was observed in human typhoid fever and in animal models of typhoid fever that suggests the presence of immunosuppressive effects associated with Salmonella infection (Dupont et al., 1971; Mittrucker and Kaufmann, 2000). One explanation for these observations was the immunosuppression induced by nitric oxide (NO), a radical that is abundantly produced by macrophages to kill microbial pathogens (Al-Ramadi et al., 1992). Further mechanisms have been described more recently (Matsui et al., 1998; van der Velden et al., 2005; Qimron et al., 2004; Tobar et al., 2006; Cheminay et al., 2005; Link et al., 2006). Salmonella is able to directly inhibit T cell proliferation via a contact-independent interference mediated by the Salmonella-derived T cell inhibitor (STI) (Matsui et al., 1998), as well as via a contact-dependent effect mediated by a so far unknown virulence factor (van der Velden et al., 2005). The genes yejA and yejE were recently shown to encode virulence factors affecting CD8-positive T cell immune responses (Qimron et al., 2004). However, these factors do not directly target CD87 T cells, but inhibit the major histocompatibility complex class I (MHC-I) presentation pathway in macrophages. Recently, dendritic cells (DC) were characterized as an early and important target for Salmonella. Compared to macrophages and neutrophils, DC are professional antigen-presenting cells (APC) and are the major sentinels present in the skin and mucosal surfaces. Bacteria which pass through the M-cells of Peyer’s patches are taken up by underlying DC (Hopkins et al., 2000). In addition, Salmonella
can be sampled by DC directly from the intestinal lumen (Rescigno et al., 2001). In contrast to macrophages which permit bacterial proliferation, Salmonella in DC do not replicate, but persist by an unknown mechanism (Niedergang et al., 2000) as a static, non-dividing population which resides inside a specific compartment (Jantsch et al., 2003). Although the SPI2-T3SS does not appear to have a role for replication of Salmonella in DC, this virulence system influences the biogenesis of the SCV in DC (Jantsch et al., 2003) and interferes with MHC-II as well as MHC-I antigen presentation pathways (Cheminay et al., 2005; Tobar et al., 2006). The regulatory system PhoPQ (Wick et al., 1995), likely via an influence on the SsrAB regulon, as well as the SPI5-encoded effector SopB (Link et al., 2006) also exhibit immunosuppressive activities on APC. In addition, viable S. Typhimurium induces iNOS expression in DC, which leads to the production of NO in the microenvironment of the immunological synapse between DC and T cells. The virulence mechanism involved is still unknown, but it is absent in non-pathogenic S. bongori and dependent on bacterial protein synthesis (Cheminay et al., 2005). Further work is required to characterize the unknown factors interfering in the priming of the adaptive immune response. For the rationale design of improved carrier strains, the inactivation of genes that enable Salmonella to interfere with adaptive immunity should be a logical step to improve the efficacy of the vaccine.

In addition, it is important to characterize the immune responses induced by candidate vaccine strains in detail. It has been shown that S. Typhimurium strains carrying independent mutations resulting in similar levels of attenuation could stimulate quite distinct immune responses (Raupach et al., 2003). It was also possible to modulate the immune response against a heterologous antigen using two recombinant Salmonella vaccines differing only by their distinct mutation within the SPI2 locus (Medina et al., 1999). This kind of results is not surprising since SPI2 and probably also other virulence factors interfere with cytokine signaling pathways (Uchiya et al., 2004; Uchiya and Nikai, 2005; Lee et al., 2000). A systematic and comparative analysis of both cytokine profile and immunoglobulin expression pattern in response to various attenuated strains may be useful to rationally design recombinant vaccines against...

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**Fig. 1.** Strategies for heterologous antigen expression by Salmonella. (A) For optimal expression of heterologous antigens within the vaccinated host, an in vivo-inducible promoter ($P_{vi}$) can be used. Of particular interest are intracellularly induced promoters. The fusion of the heterologous antigen to secretion or translocation signals allows the presentation on the bacterial surface, or the translocation of the fusion protein into the cytoplasm of the host cell, ideally an antigen-presenting cell. (B) Generation of marker-less carrier strains with chromosomal integrations of expression cassettes for heterologous antigens. Expression cassettes are generated as described in (A) and contain a selectable marker (e.g. kanamycin resistance, $aph$) flanked by FLP recombinase target sites (black triangles). For chromosomal integration, linear targeting constructs are generated by PCR using primers with flanks (open boxes) complementary to the chromosomal target gene of the carrier strain. After electroporation, Red-mediated recombination results in the replacement of a chromosomal gene by the targeting construct. If the chromosomal gene encodes an important metabolic or virulence function, the integration also results in attenuation. The resistance marker can be removed by subsequent FLP-mediated recombination.
diseases for which a targeted immuno-modulation is required.

Technologies for heterologous antigen expression by Salmonella carrier strains

The rational design of efficient carrier strains and expression systems can be guided by the detailed knowledge of the pathogenesis of Salmonella infections, the molecular understanding of virulence factors and the gene regulation in Salmonella. For expression of heterologous antigens by Salmonella carrier strains, so-called expression cassettes are frequently used (Fig. 1A). Expression cassettes are assembled in a modular fashion with a promoter and one or several genes encoding the heterologous antigens. Additional elements may be present such as optimized ribosome-binding sites, transcriptional terminators or coding sequence of bacterial genes for the generation of fusions to the heterologous antigens (transgenes). Expression cassettes may be integrated into the bacterial chromosome or be present on episomal elements, usually plasmids. However, strategies for stabilization of episomal elements are required to prevent loss of the transgene within the vaccinee.

For the rational design of new vector candidates with increased protective capacities, systems for stabilization have to be combined with a regulated and compartmentalized expression of the heterologous antigen.

Transgene stabilization

Frequently, recombinant plasmids are used for the expression of heterologous antigens. For the selection and maintenance of plasmids during propagation of the vaccine during experimental evaluation, resistances to antibiotics are frequently used. A characteristic feature of the various plasmid vectors is the copy number that can range from single copy to high copy numbers (>200 copies per cell). Although multiple copies are present, many plasmids are unstable in absence of selective pressure after vaccination, and the loss of these plasmids is a parameter restricting the efficacy of live recombinant vaccines (Dunstan et al., 2003). Due to the growing pressure to restrict the release of genetically modified organisms containing antibiotic resistances and to optimize stability, various alternatives have been developed. In the plasmid-based balanced lethal systems, a chromosomal mutation leading to auxotrophy causing lethal effects on the carrier strain is complemented by the corresponding gene present on the plasmid, leading to its maintenance (Curtiss et al., 1990; Galan et al., 1990; Morona et al., 1991). Stabilization of plasmids was also achieved by insertion of hok sok parA, or crs rsd rrnB genes that encode an optimized toxin-antitoxin system and a site-specific recombination system associated to a gene transcriptional terminator, respectively (Galen et al., 1999; Stephens et al., 2006).

In contrast to the presence on episomal elements, the integration of expression cassettes into the chromosome usually alleviates the need for stabilization. However, compared to generation of recombinant plasmids, chromosomal integration of expression cassettes often requires extensive genetic manipulation. A recently described method based on ‘recombineering’ using phage λ Red recombinase (Husseiny and Hensel, 2005b) may significantly accelerate the generation of carrier strains with chromosomal expression cassettes (Fig. 1B). This technique allows a rapid and gene-specific insertion of an expression cassette, for example in loci encoding important metabolic functions or virulence traits. Thus an attenuating mutation and stable integration of the expression cassette can be performed in a single step. In addition, the removal of antibiotic resistance markers can easily be performed. Red-based recombineering also offers the possibility of multiple insertions of two or more different expression cassettes, thus the generation of multivalent carrier strains should be possible (Husseiny and Hensel, 2006). The performance of such strains in experimental vaccination, however, has to be evaluated.

Controlling antigen expression

The expression level, as well as the spatially and temporally controlled expression of heterologous antigens is another parameter that can now be addressed in a rational manner. The lack of immunogenicity of the first generation of live recombinant vaccines based on Ty21a is likely to be as consequence of the high attenuation by multiple mutations of this strain. Rather disappointing was the inefficacy of new prototype carriers using more immunogenic strains such CVD908, CVD908-htrA, γ4073 or Ty800. These strains were expressing Plasmodium falciparum circumsporozoite protein, the tetanus toxin fragment C, Hepatitis B virus core-pre-S protein, or Helicobacter pylori urease, respectively, from plasmids or after chromosomal integration of expression cassettes (Tacket et al., 1997b, 2000a; Gonzalez et al., 1994; Nardelli-Haefliger et al., 1996; DiPetrillo et al., 1999). One reason for these results could be the suboptimal expression level of the heterologous antigen.

Chromosomal integration of expression cassettes improves the stability of the transgene expression, but the low level of expression from a single gene copy compared to multiple copies on plasmids may be a serious limitation. In contrast, plasmid-based expression of the transgene can lead to an over-attenuation of the
carrier and by consequence a lack of immunogenicity if expression is not properly controlled (Knodler et al., 2005; Bumann, 2001). In addition, the level of antigen produced is influencing directly the quality of the immune response induced (Bumann, 2001; Covone et al., 1998), an optimal and controlled expression is required. The construction of tandem repeats of heterologous antigen on a plasmid is a possible response against suboptimal expression (Chabalgoity et al., 1996; Khan et al., 1994; Doggett et al., 1993), but a more promising approach is the use of in vivo-inducible promoters. Such promoters limit the expression of the heterologous antigen to a specific host environment, ideally an intracellular location within APC, and reduce the interference with bacterial viability and colonization capacities that is sometimes associated with use of constitutive promoters (Bumann, 2001). In comparison to constitutive promoters, in vivo-inducible promoters such as nirB, dnaS, pagC, npc, dph, phoF, ompC, htrA, groE, katG, sseA, or sseG lead to better immune responses against the heterologous antigen in the animal model (Marshall et al., 2000; Husein and Hensel, 2005a; Hohmann et al., 1995; Orr et al., 2001; Oxer et al., 1991; Bullifent et al., 2000; Everest et al., 1995). One study also shows that qualitative modulation of the immune response can be observed (Medina et al., 2000). In parallel to large screening approaches for identification of more effective in vivo inducible promoters (for example (Rollenhagen et al., 2004)), this approach can be optimized by the construction of dual promoter systems as recently shown with T7-nirB (Salam et al., 2006). The efficacy of such in vivo-inducible promoters has yet to be determined in clinical evaluations for protection in humans.

**Antigen localization**

Expression of the heterologous antigen in the bacterial cytoplasm can lead to humoral and cellular immune responses (Brown et al., 1987). However, mechanisms to display the antigen in the periplasm or on the bacterial surface, the secretion of the antigen into the Salmonella-containing vacuole or the translocation into the host cytoplasm may influence the strength and the type of immune response induced (Hess et al., 1996; Kang and Curtiss, 2003). The fusion of a heterologous antigen to MalE was used for delivery into the bacterial periplasm (O’Callaghan et al., 1990). For display on the bacterial surface, various technologies are now available. The antigen can be fused to outer membrane proteins such as OmpA (Pistor and Hobom, 1990), Lpp OmpA (Burnett et al., 2000), LamB (O’Callaghan et al., 1990; Charbit et al., 1993) or fimbriae (Chen and Schifferli, 2000). Surface display can also be achieved by using autotransporter systems such as AIDA from Escherichia coli (Kramer et al., 2003; Rizos et al., 2003; Pistor and Hobom, 1990) or MisL (Ruiz-Perez et al., 2002; Ruiz-Olvera et al., 2003). Such approaches for display may elicit mainly humoral immune responses. In contrast, secretion in the phagosomal environment mediated by the hemolysin (Hly) secretion system enhances CD4 and CD8 T cells responses (Hess et al., 1996). Enhanced cytotoxic T cell responses can be observed upon direct delivery of the antigen into the cytoplasm of the host cell. This can be mediated via the formation of pores in the phagosomal membrane by Salmonella carriers expressing a listeriolysin-Hly fusion (Hess et al., 1996) or through an SPI1-T3SS-dependent translocation (Rübsmann et al., 1998). However, a recent clinical trial did not indicate superior immune responses for the latter approach (Kotton et al., 2006). In contrast to the SPI1-T3SS which is expressed in the intestinal lumen and mediates invasion by extracellular bacteria, the SPI2-encoded T3SS is expressed during the intracellular phase of Salmonella pathogenesis. Recent approaches used the SPI2-T3SS for the translation of heterologous antigens by Salmonella carrier strains. Strategies using the SPI2-T3SS indicate efficient antigen delivery in APC (Panthel et al., 2005; Hussein et al., 2007) and this system is a promising candidate for the induction of T cell responses (see Fig. 1A for a model).

**Perspectives**

Combining the different strategies described above, one should be able to rationally design specific Salmonella-based recombinant vaccines against many kinds of infectious diseases and to direct the immune responses to the type required for protection. The value of most of the approaches to generate improved vaccines still has to be confirmed by clinical trials. Further efforts are required to characterize the immune-suppressive mechanisms of Salmonella, an issue that will contribute to the understanding of pathogenesis as well as to improved vaccines. In addition, although many ethical questions will be associated with environmental spread of genetically modified organisms encoding human cytokines or chemokines, such approaches could also be beneficial to enhance and modulate the immune response, and should be considered to optimize Salmonella-based recombinant vaccines (Dunstan et al., 1996; Al-Ramadi et al., 2001; Liew, 1994; Whittle et al., 1997; Xu et al., 1997).

Due to the comprehensive knowledge on pathogenesis, gene regulation and virulence mechanisms, together with long experience in using Salmonella vaccines in human vaccination and in various experimental systems, we consider Salmonella to be one of the most promising organisms for generation of live attenuated vaccines.
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References


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