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Chapter 64

Antigen Delivery System II: Development of Live Attenuated Bacterial Vectors

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Chapter Outline

INTRODUCTION

This chapter will focus on manuscripts published during the past 10 years (from 2005–2013) that focus on design and properties of recombinant attenuated bacterial vaccine (RABV) delivery of antigens and DNA vaccine vectors. These vaccine delivery systems were developed for administration on or through mucosal surfaces to prevent infection by bacterial, viral, and parasite pathogens that cause disease

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on or invade through mucosal surfaces. I have therefore not included reference to use of these vector systems to confer protection against insect-borne pathogens or in cases not using a mucosal route of immunization or not measuring any mucosal antibody or cellular immune responses. Readers are referred to the chapter in the previous edition of this book (Curtiss, 2005), as well as to recent reviews (Curtiss, 2002; Cheminay and Hensel, 2008; Van Huynegem et al., 2009; Galen et al., 2009; Hegazy and Hensel, 2012; Simon et al., 2011; Bahey-El-Din and Gahan, 2011; Berlec et al., 2012; Bermúdez-Humarán et al., 2011; Mohamadzadeh, 2010; Wells, 2011) for discussion of prior work and for additional perspectives and information. Other reviews also address means for vaccines to induce mucosal as well as systemic and cellular immunities based on mucosal routes of immunization (Pasetti et al., 2011; Anjuere et al., 2012; Kim et al., 2012a; Kong et al., 2013). In regard to *Salmonella* vectored vaccines, there is a critical need for Peyer's patches (PP) to induce a mucosal immune response with production of secretory IgA (SIgA) after oral immunization (Hashizume et al., 2008). Whether PP are needed to induce mucosal S-IgA responses when the recombinant attenuated *Salmonella* vaccine (RASV) is administered via other mucosal routes or by parenteral immunization has so far not been examined. However, Seo et al. (2010) have used eye drop immunization with an RASV to induce mucosal antibodies assayed in vaginal secretions and also induce protection to challenge with a wild-type virulent *Salmonella enterica* serovar Typhimurium strain. Previous work (Hopkins et al., 1995) had demonstrated that delivery of an RASV strain by diverse mucosal routes induced antibodies in distal mucosal sites, as well as in serum, except for rectal immunization, which only resulted in mucosal responses in the intestinal tract. In contrast, Kantele et al. (1998) observed that rectal immunization of human volunteers with antityphoid vaccine strain Ty21a induced better mucosal immune responses in nasal and rectal secretions and tears than did oral administration, which induced better mucosal responses in saliva and vaginal secretions. Both routes were equivalent in inducing mucosal responses in intestinal secretions and in serum. Other studies (Srinivasan et al., 1995) confirmed Hopkin's results in mice, finding antibody responses in secretions and in serum following oral, intranasal, and intravaginal immunization, and also observed this to be true for intraperitoneal immunization. This may be a unique attribute of RASVs since *Salmonella* vaccines do colonize the gut-associated lymphoid tissue (GALT) in mice following intraperitoneal immunization. This is also possibly true for other invasive vaccine vectors with the potential to colonize diverse internal effector lymphoid tissues after parenteral inoculation. It is apparent that more studies comparing mice and humans and routes of immunization are needed. It is now evident from the increasing number of studies using noninvasive lactic acid bacteria as antigen delivery vectors that invasion is not necessary to induce mucosal responses. In these regards, it would be valuable to have comparative studies with different bacterial vector systems using the same protective antigens, strain of mice, and immunological evaluations, including challenge with the same pathogen strain. It would be particularly important in these studies to evaluate duration of immunity and protection and the induction of memory responses.

ATTENUATED BACTERIAL ANTIGEN DELIVERY SYSTEMS

Bacterial Vector Components of RABV Antigen Delivery Systems

Introduction

The most widely studied bacterial genera for delivery of recombinant protective antigens and DNA vaccine vectors are *Salmonella* as an invasive vector and *Lactobacillus* as a noninvasive vector. *Edwardsiella, Listeria, Mycobacterium, Shigella,* and *Yersinia* are other invasive bacteria used while *Clostridium, Escherichia coli*, and *Vibrio* are used as noninvasive vaccine vectors. In reviewing the literature on RABVs, one must remember that not all parental isolates used in various studies are the same; while an attenuated derivative of one strain can induce protection against itself and other strains, the reciprocal is not necessarily so (Zhang et al., 1997). In other words, different strains of the same species also have differences in the degrees to which they are able to induce protective immunity. Also, the choice of outbred versus specific inbred strains of mice and the antigen studied can significantly impact the results in induction of immune responses and protective immunity (Sbrogio-Almeida et al., 2004; Simon et al., 2011).

Impact of Route of Immunization

The objective of mucosally delivered vaccines is to induce mucosal antibody and cellular immune responses at portals of entry of bacterial, viral, fungal, and parasite pathogens. Although immunization via any route will induce mucosal responses at other mucosal sites, there is often a much better response, at least a short-term response, in the mucosal site used for primary immunization. Unfortunately, the impact of route of immunization on duration of response and memory has not been adequately studied. Although the oral route of immunization is most often used for invasive *Salmonella* vectored vaccines, especially for *S. enterica* serotype Typhimurium (hereafter *S*. Typhimurium) in mice and other animals, an increasing number of studies most often when evaluating human adapted *S. enterica* serotypes Typhi and Paratyphi A (hereafter *S*. Typhi and *S*. Paratyphi A)

in mice have used intranasal (i.n.) immunization (see Pickett et al., 2000; Galen et al., 2010). In the increasing number of studies using *Lactobacillus*-vectored vaccines, the i.n. route has now almost become the norm. While I fully subscribe to investigating vaccines in ways to most readily assess what works and what works best, I must remind readers and investigators that i.n. administered vaccines are unlikely to ever be approved for human use unless means to preclude access of bacteria and their products administered by the i.n. route to the brain are discovered and implemented. This problem has been clearly demonstrated for wild-type *S*. Typhimurium and *S*. Typhi, as well as their attenuated derivatives, which after i.n. inoculation traverse along the olfactory nerve through the cribriform plate of the ethmoid bone to the olfactory bulb and then to the brain (Bollen et al., 2008). Eliminating the SPI-1 *inv* genes did not block this brain access. In this regard, other vaccines administered i.n. have been taken off the market, and it is well known that *S*. Typhi infection in children can result in meningitis (Henderson, 1948). Even oral infection of mice with wild-type strains can result in neurological symptoms associated with brain colonization (Wickham et al., 2007). It therefore might be worth a more complete investigation of using the sublingual route of immunization (Cuburu et al., 2007). While these issues are particularly relevant when using live invasive bacterial strains as vaccine vectors, problems might also be encountered when using noninvasive bacterial vectors. This is particularly true in that a preponderance of recent studies with lactic acid bacterial vectors has observed much better efficacy in using i.n. immunization compared to oral delivery.

Desired Attributes

Safety

An important feature of highly immunogenic live bacterial vaccine vectors is that they recruit innate immune responses that are usually accompanied by an inflammatory response. However, too much of a good thing is not always acceptable. Thus, using invasive bacterial vectors can possibly lead to too much inflammation. Those using *S*. Typhimurium in mice may never realize this since mice do not get gastroenteritis from *S*. Typhimurium infections. Thus, safety and evaluation of the degree of excessive inflammatory responses associated with invasive RABVs should probably be evaluated using rabbit ileal loop assays or calves. Bollen et al. (2008), using rabbit ileal loops, found that inclusion of *sopB* and/or *msbB* mutations in *S*. Typhimurium decreased excessive fluid secretion and inflammatory responses. More recently, Kong et al., (2011b) genetically modified *S*. Typhimurium to synthesize mostly monophosphoryl lipid A, which is the nontoxic adjuvant form of lipid A, and showed that this strain was not excessively inflammatory in rabbit ileal loop studies. Vaccine strains with this modification were highly immunogenic. These considerations are likely important for *S*. Typhi and *S*. Paratyphi A vectored vaccines in humans since mild diarrhea has been observed in some subjects in some trials (Tacket et al., 1997).

Much of the early focus in developing RASVs for humans was in selecting attenuation strategies that would be safe and efficacious in healthy young adults (18- to 40-year-olds), because of FDA guidelines. Fortunately, these were not well codified when Germanier and Furer (1975) described the attenuated *S*. Typhi Ty21a vaccine and clinical trial success soon led to its testing for safety and efficacy in young children in Chile (Black et al., 1990). In the early 2000s, there was a global effort to identify the major Grand Challenges in Global Health and after much deliberation, 14 such Grand Challenges were selected. The first three were to develop (1) safe, efficacious vaccines for newborns, (2) vaccines to be delivered needle-free, and (3) vaccines to be thermostable. We thus spent several years in evaluating various combinations of attenuating mutations to render *S*. Typhimurium totally safe for newborn mice while retaining immunogenicity. One such strain, *S*. Typhimurium χ9558, was found to be totally safe at doses in excess of 10^8 CFU in newborn mice (Gunn et al., 2010) when the LD_{50} for the wild-type parent was 10. This strain, mostly constructed by Bronwyn Gunn with help from others, was unable to colonize the brain after i.n. immunization (Bollen et al., 2008) was immunogenic in adult (Li et al., 2009b), infant (Shi et al., 2010a), malnourished (O. Wijburg et al., unpublished), and immunocompromised (O. Wijburg et al., unpublished) mice. *S*. Typhi vaccine strains with the same mutations as χ9558 have been evaluated and found to be safe in human volunteers (Frey et al., 2013). Periaswamy et al. (2012) screened for secondary mutations in an *S*. Typhimurium strain with an *ssaV* attenuating mutation and identified some that conferred safety in immunocompromised mice and yet retained immunogenicity. Vishwakarma et al. (2012) reported that a Δ*ssaV fur* double mutant was safe and immunogenic in some strains of immunocompromised mice. However, the involvement of Fur in upregulating *hilD* expression and thus in enhancing SPI-1 invasion functions (Teixido et al., 2011) would suggest that inclusion of a *fur* mutation in a vaccine strain would lessen invasion and thus immunogenicity. Peters et al. (2010) introduced a *trxA* deletion to eliminate thioredoxin and reduce inflammation and splenomegaly in i.v. immunized mice. While attenuated, the mutant strain still induced protection to the *Salmonella* challenge, even though numbers of CD4 and CD8 T cells and B lymphocytes in the spleen were reduced.

Absence of Antibiotic Resistance

Since gene cloning and early recombinant DNA manipulations very much relied on plasmid vectors and antibiotic resistance markers to select recombinant clones

(Cohen et al., 1973), it follows that much of the work in developing live recombinant bacterial vaccines also used plasmid vectors with antibiotic-resistance markers for selection. However, in the late 1980s, scientists at the USDA and the FDA let it be known that antibiotic resistance would not be a good attribute to have in live bacterial vaccines. The use of balanced-lethal vector-host systems (Nakayama et al., 1988; Galan et al., 1990) and the *hok-sok* plasmid addiction system (Galen et al., 1999) to eliminate the use of plasmids or strains with antibiotic resistance were reviewed previously (Curtiss, 2005). As a new system, Garmory et al. (2005) described construction of an operator-repressor titration (ORT) system for maintenance of plasmid vectors in a host strain in vivo, in the absence of either drug resistance or other selective markers. The system involves insertion of the *lacI* (or $lacI^q$ sequence in opposite orientation to P_{tac} (or P_{trc} or P_{lac})) with the *lacO* sequence intact to drive expression of an adjacent-essential gene such as *dapD*. Growth of this strain was dependent on addition of IPTG. However, introducing a plasmid-encoding, protective antigen with the *lacO* sequence titrated LacI and enabled the strain to grow in the absence of IPTG. Loss of the plasmid caused the vaccine cell to undergo a diaminopimelic acid (DAP)-less death and lyse. Garmory et al. (2005) reported that this construction delivering the *Yersinia pestis* (*Y. pestis*) F1 antigen was very efficient and induced complete protection to the *Y. pestis* challenge after a single oral immunization. Galen et al. (2010) developed an additional novel means for stable maintenance of plasmid vectors in the absence of antibiotic resistance. They deleted the gene encoding a single-stranded DNA binding protein, essential for DNA metabolism, and placed the gene on antigen-specifying plasmids. Introducing the lethal mutation into the chromosome of vaccine strains requires use of a maintenance plasmid to be replaced by the antigen-specifying plasmid for final RASV construction. Xin et al. (2012) describe an additional balanced-lethal plasmid maintenance system relating to the requirement of D-alanine for peptidoglycan synthesis.

Host Specificity

S. Typhi is specifically restricted to humans (and chimps) and therefore most research directed toward developing *S.* Typhi vectored vaccines for humans work out the details using *S*. Typhimurium constructions to evaluate in mice. However, recombinant attenuated *S.* Typhi vaccines can be evaluated for the ability to induce mucosal and systemic antibody responses and cellular immunity to the delivered protective antigen by using i.n. immunization (Londono-Arcila et al., 2002; Morton et al., 2004; Galen et al., 2004). In this regard, it is now possible to modify *S*. Typhi strains to be more infectious for mice to enable a more complete evaluation of the safety and immunogenicity of recombinant attenuated *S*. Typhi vaccines (Spano and Galan, 2012).

Lactic acid bacteria are being increasingly investigated as antigen delivery vectors. Since these bacteria are noninvasive and unknown to cause disease, they are generally regarded as safe (GRAS). *Lactococcus lactis* and *Lactobacillus casei* do not persist or establish as commensals in orally immunized animal hosts since they grow optimally at 25–30 °C, whereas those species that grow at 37 °C (*Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus helveticus,* and *Lactobacillus delbrueckii*) persist longer in the immunized animal host and have the potential to establish as commensals. This raises an issue of induction of tolerance that may also depend on which animal host and the particular strain of *Lactobacillus* being used.

In regard to possible induction of oral tolerance, either to the bacterial vector used to deliver protective antigens or, more importantly, to the delivered protective antigens, there have been no well-designed studies to establish the magnitude of these problems, if any, whether in mice or in humans. Although there have been many studies demonstrating induction of tolerance by administration of purified antigens, with and without various adjuvants, to different mucosal surfaces (see Mestecky et al., 2007), we need a better understanding of these potential problems with regard to bacterial vectored vaccines. It seems that commensal bacteria become tolerated, whereas pathogens are not, and this may predict a better long-term outcome of using invasive bacteria as antigen delivery vectors rather than those derived from commensal bacterial strains. This speculation requires investigation.

Precise Genetic Manipulation

Kang et al. (2002a) developed a suicide vector approach to generate precise deletions, with or without insertions, that makes use of counterselection using kanamycin or chloramphenicol resistance for initial selection and sucrose sensitivity due to the presence of the *sacB* gene for selection in the reverse direction. Once created, the mutation can be quickly moved from one strain to another using P22-mediated transduction. Husseiny and Hensel (2005, 2008) have described an adaptation of the λ*red*-based system, described by Datsenko and Wanner (2000) for *E. coli* to enable repetitive deletions and insertions (often of expression cassettes) in the *S.* Typhimurium chromosome. The system also only uses antibiotic resistance genes transiently with the final constructs being antibiotic-sensitive. One potential complication is the accumulation of multiple FRT scars (FLP recombinase recognition target of 82–85 nt) in place of each deletion that could lead to potential genetic instability. Some regulatory scientists dislike these, as well as antibiotic resistance in live vaccine strains. Sun et al. (2008) therefore developed a strategy analogous to that proposed by Husseiny and Hensel (2005) for both *Salmonella* and *Yersinia* that generates

multiple scarless genetic modifications. The suicide vector, coupled with the P22 transduction method of Kang et al., (2002a), also avoids the problem with DNA scars. Kwon et al. (2007) has also used fusion of a unique 18-bp sequence to a sequence encoding antibiotic resistance to insert in place of a chromosomal deleted sequence and then use the homing nuclease I-SceI (Kang et al., 2004) that recognizes this unique 18-bp sequence for excision to generate scarless allele deletions and replacements.

Elimination of Means by Which the Host Bacterium Suppresses, Modulates, or Diverts Induction of Protective Immunity

Most bacterial pathogens have multiple means to be successful as pathogens and do so by escaping recognition by the innate immune system or in suppressing, modulating, or diverting induction of acquired immunity. Success in developing a bacterial vaccine vector system is therefore dependent on identifying and then inactivating these means. The SopB protein of *S.* Typhimurium is an effector secreted by the SPI-1 T3SS that prevents induction of apoptosis (pyroptosis), which is advantageous for *Salmonella* rather than the host (Knodler et al., 2005). Deletion of the *sopB* gene in a vaccine strain lessens induction of fluid secretion in the intestinal tract (Gunn et al., 2010), without decreasing invasion and also enhances induction of mucosal immune responses (Li et al., 2008). Jones et al. (2008) report that the *S.* Typhimurium *avrA* gene acts to subvert innate and apoptotic signaling pathways. Further studies (Wu et al., 2012) revealed, however, that *S*. Typhimurium with a Δ*avrA* mutation caused more extensive intestinal inflammation, led to increased apoptosis of epithelial cells, and achieved higher titers in internal organs, including the spleen. St. John and Abraham (2009) reported that *S*. Typhimurium LPS via a TLR4-mediated process downregulate production of the homeostatic chemokines CCL21 and CXCL13, which impact functional organization of the draining lymph nodes. It is likely that the modification of *S.* Typhimurium lipid A to the 1-dephosphorylated form (Kong et al., 2011a) eliminates this problem. Kullas et al. (2012) reported that *S.* Typhimurium produces an L-asparaginase II enzyme encoded by the *ansB* gene that enhances virulence by inhibiting T cell responses.

S. Typhi and presumably some strains of *S*. Dublin produce the Vi capsular antigen that is immunosuppressive (An et al., 2012) by blocking invasion and being antiphagocytic. Thus many *S*. Typhi RASVs have complete or partial deletion of the *tvi* operon (Shi et al., 2010b). It remains to be seen whether mutations or other genetic alterations that lessen induction of immune suppression by *S.* Typhimurium in mice will be applicable to *S.* Typhi in humans. On the other hand, the Vi antigen is a protective antigen and is used as a conjugate vaccine to prevent *S.* Typhi infection (Kossaczka et al., 1999). Wahid et al. (2007) have therefore generated an effective live *S*. Typhi vaccine, CVD 909, that constitutively synthesizes the Vi antigen.

As already mentioned, it is possible that use of those *Lactobacillus* species that can establish a commensal relationship with the vaccinated host may induce tolerance, and this needs to be investigated. If this occurs, vaccine vectors will benefit from being altered to preclude induction of tolerance so as to permit repeat use as a vector.

Consequences of Attenuating Mutations on Vaccine Attributes

For years, the majority of papers and reviews on live bacterial vaccines have noted the importance of ensuring complete attenuation with induction of no disease symptoms while maintaining high immunogenicity. I finally realized that this was improbable, if not impossible. We thus devised a number of strategies so that the vaccine strain at the time of administration would be as nearly like the wild-type pathogen as possible, in being able to withstand the host defenses and stresses encountered in colonizing targeted effector lymphoid tissues. The first strategy was to design strains with regulated delayed attenuation. We have achieved this in three ways. The first (Curtiss et al., 2007) was to use *Salmonella* vaccine strains with mutations such as *pmi* or *galE*, or deletion-insertion mutations, such as ΔP_{rfc}::TT a*raC* P_{BAD} *rfc* (Kong et al., 2010) and ΔP_{rfaH}::TT *araC* P_{BAD} *rfaH* (Kong et al., 2009). These strains would synthesize lipopolysaccharide (LPS) O-antigen and/or outer core when supplied with a sugar (mannose, galactose, and/or arabinose) during growth and cease such synthesis in vivo where that sugar would be unavailable such that a rough phenotype would be manifested after some six to eight cell divisions in vivo (Curtiss et al., 2007). The second was to delete promoters for genes essential for virulence and pathogenicity and replace them with the *araC* P_{BAD} regulatory cassette. So that expression of these genes would be dependent on the presence of arabinose during vaccine strain growth and would cease to be expressed in vivo due to the absence of arabinose (Curtiss et al., 2009). In these cases, the imposition of attenuation is gradual since the gene product is diluted by half at each cell division in vivo. The third strategy is regulated delayed lysis of the vaccine strain in vivo, a system designed for release of a bolus of protective antigen (Kong et al., 2008) or a DNA vaccine vector (Kong et al., 2012b), which provides total biological containment with no vaccine persistence in vivo and no survival if the vaccine strain is shed in feces.

Many of the problems with the presence of attenuating mutations at the time of immunization was due to a lessened ability of the vaccine strain to contend with host defenses between the time of entrance into the body and achieving a reasonable titer of bacterial vaccine cells in effector lymphoid tissues. The strategies of regulated delayed

attenuation noted above were designed to contend with these problems. I suspect that later efforts will be to further improve means by which the vaccine strain survives these stresses to enhance induction of protective immune responses.

Another problem with some means of attenuation is reduction in ability of the vaccine strain to attach to, invade into, or colonize in lymphoid tissues. It is well known that the structure of LPS very much influences attachment to intestinal epithelium due to reduced ability to traverse mucin, which thus negatively impacts immunogenicity. Kong et al. (2011a) have made a series of defined mutations to better define this problem. Based on the results of these studies, it is important to have wild-type levels of LPS present on vaccine cells at the time of immunizing an animal host. The presence of *rpoS* mutations in *S*. Typhimurium diminishes the strain's ability to colonize the GALT after oral immunization (Coynault et al., 1996; Nickerson and Curtiss, 1997) and thus decreases immunogenicity, a potential problem with derivatives of *S*. Typhi Ty2, which also possess a *rpoS* mutation (Robbe-Saule and Norel, 1999).

Lastly, some means of attenuation can eliminate or reduce production or display of pathogen associated molecular patterns (PAMPs) that interact with toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domains (NODs) to initiate innate immune responses. Such impairments are likely to reduce immunogenicity. Whether such problems exist can often be evaluated using cell lines such as HEK cells each endowed with responsiveness to a given PAMP due to presence of a single TLR or NOD.

Consequences of Synthesis of Protective Antigens

It has been recognized since the 1980s that synthesis of a protective antigen can sometimes be toxic to the vaccine cell to reduce growth and efficiency at colonizing effector lymphoid tissues. Thus selective deletion of portions of genes encoding protein sequences with multiple cysteine residues or encoding hydrophobic domains was often helpful. As an alternate strategy, using promoters activated in vivo such as P_{nirB} (Chatfield et al., 1992) and P_{pagC} (Hohmann et al., 1995) were even more helpful and universally usable. More recently, we developed a universal means for regulated delayed synthesis in vivo of the recombinant protective antigen (Wang et al., 2010a). In this system, plasmid-encoded antigen genes are controlled by the strong promoter P_{trc}, which during growth of the vaccine strain is repressed by LacI produced in response to growth of the vaccine culture in the presence of arabinose due to an *araC* P_{BAD} *lacI* cassette.

In the 1990s, it was recognized that expression of genes encoding a recombinant antigen often contributed to attenuation. Since loss of antigen synthesis capability would restore virulence, it was well recognized that recombinant antigen synthesis could not be relied on as a basis for attenuation. However, Yang et al. (2012) reported that overexpression of antigen CFA/1 synthesis was a basis for attenuation, making cells more permeable and thus more susceptible to host destruction. They termed this attenuating gene expression (AGE). For the reason stated above, this would be unacceptable for a human vaccine, although it might be acceptable for a vaccine to be used in an agriculturally important animal.

Means to Enhance Entry and Facilitate Colonization of Mucosa-Associated Lymphoid Tissue (MALT) and Internal Lymphoid Tissues

Based on the report by Teixido et al. (2011) that Fur activates expression of *hilD* and thus SPI-1 genes to increase invasion, it follows that overexpression of *fur* at the time of mucosal immunization could increase colonization of MALT. We have observed this to be so using the regulated delayed attenuation associated with *fur* gene expression (Curtiss et al., 2009). Thus *S*. Typhimurium vaccine strains with the ΔP_{fur33}::TT *araC* P_{BAD} *fur* construction are more invasive and better colonizers of PP than strains with the ΔP_{fur81}::TT *araC* P_{BAD} *fur* construction that synthesizes less Fur when grown in medium with arabinose. It should be noted that this strategy might not be ideal if the vaccine strain is designed to overexpress Fur-regulated genes for iron acquisition proteins due to problems of iron toxicity. An additional means to increase invasiveness was reported by Kong et al. (2012b), who replaced the SPI-1 *hilA* promoter with P_{trc} lacking the *lacO* sequence. Wild-type strains with this ΔP_{hilA}::$P_{trc\Delta lacO}$ *hilA* construction are more invasive, colonize all lymphoid tissues to higher levels, and have a lower LD_{50} than the wild-type parent. This enhanced virulence is not observed, however, in vaccine strains with other attenuating mutations, although the increased invasiveness and tissue-colonizing abilities are retained (Kong et al., 2012b).

Generation of Particulate Antigens to Enhance Immunogenicity

Particulate antigens are often very immunogenic, possibly because of their persistence after immunization. Although there is some debate as to whether HPV16 L1 capsomers form pentamers or complete capsids after synthesis in attenuated *Salmonella* strains, Baud et al. (2004) found them to be highly immunogenic. Ameiss et al. (2010) made fusions of the exposed M2e region of influenza virus to the Woodchuck hepatitis core (WHc) to enable formation of core particles within the attenuated *S*. Typhimurium vaccine strain composed of 180 subunits each with the M2e

sequence spliced into the spike domain. Higher titers of both mucosal IgA and serum IgG responses were induced when using an *S*. Typhimurium antigen delivery strain exhibiting regulated delayed lysis in vivo (Kong et al., 2008) than a strain that did not undergo programmed lysis. Mice immunized with the lysing RASV exhibited 65% protection to challenge with 10^4 $TCID_{50}$s of WSN influenza virus administered intranasally. The use of other virus antigens, such as HBV cores that form highly immunogenic particulate antigens, were discussed in the chapter in the preceding edition of this book (Curtiss, 2005).

Cytokine Delivery and Adjuvant Effects

As we learn more about RABV constructs and the interactions with immunized hosts, it becomes worthwhile to investigate how coexpression of sequences encoding cytokines, chemokines, and other immune modulators might improve the immune responses to be induced. Such efforts were initiated in the late 1990s with production of IL-2 by an attenuated *S*. Typhimurium strain (Saltzman et al., 1997) as part of an antitumor therapy strategy. One very interesting application of this technology was the delivery of human IL-10 (as a treatment for inflammatory bowel disease) by chromosomal insertion of the sequence encoding IL-10 to replace the *thyA* gene of *L. lactis* (Steidler et al., 2003). Another interesting application was the design of an attenuated *S*. Typhimurium synthesizing swine IL-18 and IFN-α used as an adjuvant to enhance Th-1 biased protection against the pseudorabies virus to be coadministered with the PrV vaccine (Kim et al., 2012b). Other studies with RABVs delivering cytokines are listed in other sections of this chapter. Although some results reported are encouraging, more detailed and well-controlled studies will be needed to better delineate how expression of these cofactors would enhance immune responses and protection, especially in regard to mucosal immune responses and protection against pathogens infecting via a mucosal surface. I should note, however, that Yoon et al. (2011) discovered that engineering a commercial DNA vaccine vector to specify IL-12 synthesis to be delivered by wild-type *S*. Typhimurium UK-1 (low doses) resulted in attenuation of the *Salmonella*. Although Yoon et al. (2011) suggest this as a means to attenuate virulent pathogens and transform into vaccines, I would argue that this, like over-synthesis of a recombinant antigen, is not a safe, acceptable, or reliable means for RABV attenuation, since loss in ability to synthesize IL-12 would restore virulence.

Future Needs and Objectives

Although progress in improving RABV delivery of protective antigens and DNA vaccines has been impressive, there is still much to learn. Thus, we could benefit by knowing how to design strains that would target a specific tissue, cell type, and/or cell compartment, or to direct the antigen for more specific delivery for Class I and/or Class II presentation. We still do not fully understand how to achieve safe, persistent production of antigens, or understand the means/mechanism by which the immunized host kills and lyses bacterial vaccine cells to release and process protective antigens. Although much work has focused on delivery of protective protein antigens, means to deliver protective carbohydrate antigens are mostly unstudied. Means to enhance production of outer membrane vesicles (OMVs) that are highly immunogenic would be valuable. In the arena of immunology, we need to understand how to better induce long-term memory responses for mucosal immunity, as opposed to short-term local mucosal immune responses. In terms of safety and efficacy, very few studies have focused on immune responses to RABVs in aged individuals.

Plasmid Vector Components for RABV Antigen Delivery Systems

Introduction

Although plasmid-specified synthesis of protective antigens is generally the norm for RABV systems, chromosomal integration is still sometimes used either when low amounts of protective antigen are needed to stimulate cellular immunity or the antigen in high amounts is toxic to the bacterial cell, so as to decrease growth rate and lessen the ability to colonize effector lymphoid tissues. Since gene expression levels vary greatly depending on site and direction of chromosomal insertion, one must empirically determine the optimal location. Proximity to the origin of chromosome replication might be better if higher levels of antigen synthesis are desired. Also of importance is the selection and design with modification of the promoter region and the increase in translation efficiency by inclusion of double lysine AAA codons immediately after the start codon (Stenstrom et al., 2001).

In regard to plasmids for specifying recombinant protective antigens, certain features were discussed in the preceding section on host bacteria. Thus, although use of antibiotic resistance markers on plasmid vectors is a convenience, stability of such constructs cannot be assured in vivo and regulatory agencies frown upon the inclusion of antibiotic resistance in any live bacterial vaccine for ultimate clinical or commercial use. Means for regulated delayed synthesis of recombinant protective antigens in vivo were also discussed above as a means to achieve higher levels of RABV colonization of effector lymphoid tissues.

Attributes of Plasmid Vectors

Levels for synthesis of recombinant protective antigens can be influenced by choice of plasmid vectors with different copy numbers. This sometimes requires modifications in the level of synthesis of the selective marker, since

excessive synthesis levels can cause hyperattenuation and reduce the fitness of the vaccine strain (Kang et al., 2002b; Xin et al., 2012). Thus, elimination or alteration of promoter and/or Shine-Dalgarno sequences on high copy number plasmids is often necessary. One way to enhance the level of antigen synthesis when inducing antibody responses is to use a runaway plasmid replication vector that is maintained at low copy number during growth of the strain. Then in vivo, a release from repression leads to plasmid amplification and high levels of protective antigen synthesis (Torres-Escobar et al., 2010a). It should be noted that this system also provides complete biological containment since the runaway plasmid replication seems to not be reversible, so that no viable vaccine cells can be recovered after mucosal immunization.

In addition to the balanced-lethal vector-host systems, a number of investigators are using complementation of deletion mutations that, while not lethal in vivo, do constrain the survivability and/or persistence of vaccine strains. Use of Δ*thyA*, Δ*glnA*, Δ*purB*, and Δ*aroA, C, and D* chromosomal deletion mutations have been used with the plasmid vector possessing the complementing wild-type allele. As with the balanced-lethal systems overexpression of the complementing genes on high copy number vectors can also result in hyperattenuation with less efficacious induction of protective immunity. Sizemore et al. (2012) compared delivery of *Y. pestis* F1 and LcrV antigens by *S.* Typhimurium strains with a Δ*phoPQ* mutation as a function of plasmid copy number and selective marker. In this case, pBR *ori* vectors were superior to pUC *ori* vectors and the Asd$^+$ balanced-lethal plasmid addiction system yielded higher antibody titers than using PurB$^+$ and GlnA$^+$ complemented systems.

As described above, expression of genes encoded on plasmid vectors is often accomplished by using promoters that permit constitutive synthesis of the protective antigen. However, this most often interferes with the ability of the RABV to efficiently colonize effector lymphoid tissues and thus reduces immunogenicity. Because of this, most constructions now make use of either in vivo-activated promoters or regulated delayed in vivo expression of genes encoding protective antigens.

Protective Antigen Delivery by Secretion

One of the first means investigated for secretion of protective antigens was use of the T1SS associated with hemolysin secretion in *E. coli* (see Curtiss, 2005). Although still used and investigated, it is inferior to other means for delivery of protective antigens out of the RABV cell. The next system described was use of the T3SS (Rüssmann, 1998) to both secrete antigens out of the cell but, more importantly, to translocate them into the cytosol of host cells where they are processed in the proteasome for class I presentation to augment induction of CD8-dependent CTL responses. Most of the initial work used the SPI-1 T3SS effectors to fuse to antigens with T cell epitopes (see Curtiss, 2005). More recently, Husseiny et al. (2007) have expanded such antigen delivery by characterizing use of the SPI-2 T3SS and associated effectors for antigen delivery. In this regard, the effector SseF seemed to work the best. Xu et al. (2010) also evaluated a number of SPI-2 in vivo-activated promoters for T3SS delivery of the model antigen OVA. As will be noted in later sections, many of the mucosally administered RABVs stimulate not only antibody production at mucosal sites but also cellular immunities. In fact, there is a significant need to better characterize these induced cellular immunities.

Since early work with RABVs often did not result in inducing high antibody titers to synthesized protective antigens compared to the high titers of antibodies induced to RABV antigens, Kang et al. (2002b) investigate use of the β-lactamase signal sequence to deliver the *Streptococcus pneumoniae* PspA protective antigen out of the cytosol into the periplasm. In this study, antibody titers to the PspA antigen were as high as the antibody titers to the *Salmonella* LPS and outer membrane proteins (OMPs) and it was found that some 15–20% of the PspA antigen was in the supernatant fluid. In a comparative study, the use of this β-lactamase T2SS yielded antibody titers to PspA that were about 100 times the titers when PspA was retained in the interior of the RASV strain (Kang and Curtiss, 2003). Since C-terminal sequences in β-lactamase also enhance its secretion (Koshland and Botstein, 1980), Branger et al. (2007) found that inclusion of both the N-terminal and C-terminal signal sequences of β-lactamase led to better secretion and higher antibody titers to the *Y. pestis* LcrV antigen. Xin et al. (2008) have investigated use of several other T2SS signal peptides, but the consensus to date is that the β-lactamase fusions are most efficient in the export of protective antigens.

Protective Antigen Delivery by Lysis of RABV

Achieving lysis of RABVs in vivo has generally made use of blocking synthesis of unique peptidoglycan constituents, due to deletion mutations in genes for their synthesis or by regulating synthesis of enzymes for peptidoglycan synthesis to cause a regulated delayed cessation in synthesis of peptidoglycan. In either case, cell wall-less death occurs with release of any protective antigens present in RABV cells at the time of their lysis. Grangette et al. (2004) generated alanine racemase-deficient *L. lactis* and *L. plantarum* strains and found that both were superior in inducing tetanus antitoxin C antibodies but with the *L. plantarum* strain being superior, especially in inducing antitoxin IgA responses in intestinal secretions. Thus, lysis to release synthesized antigen is a superior means of antigen delivery, especially to induce mucosal immunity. Zhao et al. (2006) and Jiang et al. (2007) used a D-alanine requiring

Listeria monocytogenes strain to deliver the HIV-1-Gag antigen, and observed with one strain induction in mice of protective mucosal antibody and cellular responses as measured by challenge with a Gag-encoding recombinant vaccinia virus vector. Significantly, induction of high numbers of Gag-specific $CD8^+$ T cells was observed in MALTs following oral vaccination.

Kong et al. (2008) designed an RASV strain with regulated delayed lysis in vivo by using the arabinose-dependent *araC* P_{BAD} regulation for expression of the *asdA* and *murA* genes on a specially designed regulated delayed lysis plasmid with deregulated synthesis of antisense mRNA for the *murA* and *asdA* genes to completely prevent their expression. The chromosomal *murA* gene expression was also under *araC* P_{BAD} regulation. The strain possessed a mutation to block synthesis of colanic acid that is able to protect cells undergoing cell wall-less death and a *relA* mutation to also ensure complete lysis by uncoupling cell growth from a dependence on protein synthesis. This system has been used to obtain better immune responses to protective antigens than achieved with nonlysis RASV strains (Ameiss et al., 2010; Ashraf et al., 2011; Juarez-Rodriguez et al., 2012a, 2012b). Juarez-Rodriguez et al. (2012b) encoded two different protective antigens (a fusion of two antigens), with one delivered by the T2SS and the other by the T3SS on a regulated delayed lysis plasmid such that the antigens would be secreted by either of two means continuously prior to cell lysis when a bolus of synthesized antigens was released.

As discussed below, lysis of gram-negative RABVs will release LPS with the lipid A endotoxin to possibly cause excessive inflammation or even sepsis. There are several means to alleviate this problem by modification of lipid A (Gunn et al., 2010; Kong et al., 2011b, 2011c; 2012a).

Vectors that Result in Surface Display of Synthesized Protective Antigens

Kramer et al. (2003) encoded the autotransporter AIDA-I (causes diffuse adhesion of *E. coli* to intestinal epithelial cells) into a $ThyA^+$ plasmid vector for maintenance in an attenuated *thyA S.* Typhimurium vaccine strain. Fusion of several antigens to AIDA-I resulted in surface localization. This was confirmed by Rizos et al. (2003), using *Heliocobacter pylori* UreA epitopes fused to AIDA-1 for surface display and presentation. Breau et al. (2012) encoded the autotransporter HgbA in the pTetC*nirB* vector for use in *S.* Typhimurium vaccine strains to enable surface antigen display.

Isoda et al. (2007) made a C-terminal fusion of the *Porphyomonas gingivalis* hemagglutinin (HagB) to a Lpp-OmpA fusion for display on the surface of attenuated *S.* Typhimurium. Synthesis of this fusion protein was toxic, necessitating a modification of P_{trc} to lead to decreased antigen synthesis. The construct induced much higher serum antibody (IgG and IgA) and higher vaginal IgA than did an isogenic vaccine strain retaining HagB in the cytoplasm. Further work by this group (Pathangey et al., 2009) demonstrated that the level of protective antigen synthesized altered the fitness of the RASV strain, but the higher the level of synthesis, while correlated with less fitness, resulted in more rapid induction of especially mucosal immune responses.

Kim et al. (2008) constructed a vector for use in *L. lactis* that enables fusions to domains of a surface protease that target fusion proteins to the cell surface. Other vectors for surface localization of protective antigens on *L. lactis* (Raha et al., 2005), *L. casei* (Hou et al., 2007), *L. acidophilus* (Chu et al., 2005; Moeini et al., 2011; Kajikawa et al., 2012), and *L. plantarum* (del Rio et al., 2010a) have been described. In most all cases when comparisons were made, the surface display of antigens led to higher antibody titers to the delivered antigen than when the antigen was synthesized and retained in the cytoplasm.

Although there is much evidence that secretion or surface display of protective antigens on RABV strains yields better immune responses and protective immunity (Kang and Curtiss, 2003; see above), this is not always true and Sizemore et al. (2012) found that much higher antibody titers against *Y. pestis* F1 and LcrV antigens were induced when they were retained in the cytoplasm of the attenuated Δ*phoPQ S.* Typhimurium strain rather than when they were secreted.

Multiple Vectors Encoding Protective Antigens and Use of rec Mutations

Inclusion of multiple plasmids encoding multiple protective antigens (Xin et al., 2012) or complex plasmids encoding multiple antigens as an operon (Juarez-Rodriguez et al., 2012a, 2012b) or of multiple genes independently regulated (Zhang et al., 2009), often results in genetic instability due to both inter- and intraplasmidic recombination. In *S.* Typhimurium, these activities are encoded by the *recF* and *recJ* genes that can be deleted to enhance plasmid stability (Zhang et al., 2011). The genes and the enzymes they encode are not the same or even present in all *Salmonella* serotypes. A *recA* mutation can also be included, but this results in total attenuation of most pathogens and has the potential to reduce immunogenicity. In contrast, *recF* and *recJ* mutations do not alter virulence or colonizing ability of *S.* Typhimurium (Zhang et al., 2011).

RECOMBINANT ATTENUATED BACTERIAL VACCINE ANTIGEN DELIVERY SYSTEMS

Introduction

Many studies have been conducted during the past 10 or so years to explore means for constructing and delivering various RABV strains, most often to stimulate all three

branches of the immune system (mucosal, systemic, and cellular) but with a focus on inducing protective immunity to those pathogens that colonize on or pass through a mucosal surface to result in disease. While there has been much progress and development of new systems, it does not appear that many vaccines have reached the market place. There has also been a rapid expansion in the number of journals publishing findings in this field of mucosally administered vaccines. In reviewing this recent literature, I found many deficiencies and would not have recommended the publication of many papers without addition of specific information and probable conduct of additional experiments, including controls. I add this as a cautionary word to note that reference and citation of published work does not serve as an endorsement of its accuracy. Although many of the new systems described offer promising results, issues of safety and efficacy are not often addressed adequately. In regard to induction of mucosal immune responses, some of the more exciting observations pertain to the induction of cellular immune responses in mucosal tissues. This raises a major question that has yet to be addressed in any meaningful way. Namely, what is the duration in production of mucosal antibody responses following immunization, as well as for the duration of induced cellular immunity? More specifically, are memory responses induced, and what are the immunological bases for such memory? In these regards, the turnover and replacement of mucosal tissues is much more rapid than other internal tissues.

Table 1 includes a listing of recently described RABVs listing the infectious disease addressed, the protective antigens delivered, the vector system used, and the reference. Table 1 is also subdivided to list all the invasive bacterial vectors and then the noninvasive bacterial vectors. *Salmonella*-based vaccines continue to predominate, but there has been a significant increase in investigations of noninvasive commensal and noncommensal lactic acid bacterial vectors. A more substantial presentation and discussion of the key findings follows.

INVASIVE BACTERIAL VECTOR DELIVERY SYSTEMS

Edwardsiella

Mu et al. (2011) evaluated many iron-regulated promoters in *E. tarda,* and found that P_{dps} and P_{yncE} gave highest transcription of the sequence encoding the protective antigen glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the fish pathogen *Aeromonas hydrophlia.* Plasmid vectors with antibiotic-resistance markers were introduced into various attenuated *E. tarda* strains and the recombinants used for bath vaccination of turbot (*Scophtalmus maximus*). Protective immunity to intramuscular (i.m.) challenge with both *E. tarda* and *A. hydrophilia* was observed. Mucosal immunity was not evaluated. Santander et al. (2010) and Choi and Kim (2011) developed balanced-lethal recombinant vector systems for antigen delivery in *E. ictaluri* and *E. tarda*, respectively, by deleting the *asdA* gene for aspartate semialdehyde dehydrogenase that imposes an obligate requirement for DAP. In both cases, this enables the development of recombinant vaccines that do not display undesirable resistance to antibiotics. Santander et al. (2010) also reported that both *E. tarda* and *E. Ictaluri* possess an inactive *asdB* gene that is present in an active form in most gram-positive bacteria and also in *Vibrio* species, which have both *asdA* and *asdB*. For attenuation, both *crp* (Santander et al., 2011) and *fur* (Santander et al., 2012) mutations render *E. ictaluri* attenuated and immunogenic for catfish (*Ictalurus punctatus*). Wang et al. (2010b) have described a T3SS encoded in part by genes in the *ese* operon, which is essential for *E. tarda* virulence, with the EseD protein, used to induce protective immunity to an *E. tarda* challenge in turbot.

Listeria

Stevens et al. (2004) orally immunized cats with an *L. monocytogenes* strain with feline immunodeficiency virus (FIV) genes encoding Gag chromosomally integrated and Env on a DNA vaccine vector. Significant S-IgA titers were detected in vaginal secretions in three of five immunized cats and in all five cats after challenge with FIV. The immunization also significantly reduced FIV titers following vaginal challenge. In a follow-up study, Stevens et al. (2005) employed the same system to show that cats previously immunized with an *L. monocytogenes* vector control could still be productively orally immunized with the recombinant strain delivering Gag and a DNA vaccine encoding Env to induce S-IgA antibodies in vaginal secretions, saliva, and feces.

Zhao et al. (2006) used a D-alanine requiring an *L. monocytogenes* strain to deliver the HIV-1-Gag antigen, and observed with one strain induction in mice of protective mucosal antibody and cellular responses as measured by challenge with a Gag-encoding recombinant vaccinia virus vector. The observation of induction of high numbers of Gag-specific $CD8^+$ T cells in MALTs following oral vaccination was significant. Jiang et al. (2007) also used a D-alanine requiring an *L. monocytogenes* strain to deliver the HIV-Gag antigen chromosomally encoded by insertion into the *sepA* gene into orally vaccinated rhesus monkeys. Doses of 10^{12} CFU were orally administered in whipped cream at three or two intervals, with the group only getting two oral doses at weeks zero and six, and receiving an intramuscular dose of 10^{12} CFU on week 10. D-Alanine was administered i.v. 15 min before and 2.5 h after vaccination. Mucosal immunity was detectable in vaginal and intestinal

TABLE 1 Recombinant Attenuated Bacterial Vaccine Antigen Delivery Examples Mucosal Immune Responses or Protection

Pathogen	Protective Antigen(s)	Vector	References
Invasive Bacterial Vectors			
Edwardsiella Vectored			
Aeromonas hydrophilia	GAPDH*	*E. tarda*	Mu et al. (2011)
Listeria Vectored			
Bacillus anthracis	PA	*L.m.** Δ*actA prfA**	Qiu et al. (2011)
FIV*	Gag, Env	*L.m.*	Stevens et al. (2004)
HIV*	Gag, Env	*L.m.* D-ala⁻	Zhao et al. (2006)
HIV	Gag	*L.m.* D-ala⁻	Jiang et al. (2007)
Mycobacterium Vectored			
Eimeria tenella	Rhomboid protein	*M.b.** BCG	Wang et al. (2009)
EPEC*	Intimin, BfpA	*M. smegmatis*	Vasconcellos et al. (2012)
	Intimin, BfpA	*M.b.* BCG	Vasconcellos et al. (2012)
HIV	Env	*M. smegmatis*	Yu et al. (2006)
HIV	Env V3J1 peptide	*M.b.* BCG	Kawahara et al. (2002)
SIV	Gag, Env, Nef	*M.b.* BCG	Mederle et al. (2003)
Salmonella Vectored			
a. Murine Hosts			
B. anthracis	PA	Typhi	Galen et al. (2004)
B. anthracis	PA	Typhi	Baillie et al. (2008)
EHEC*	Stx2ΔAB (nontoxic)	Typhimurium	Rojas et al. (2010)
EHEC	EspA, intimin, Stx2B	Typhimurium	Gu et al. (2011)
EHEC	Intimin	Typhimurium	Oliveira et al. (2012)
ETEC*, EPEC	K88, K99, FasA, F41, intimin	Typhimurium	Hur and Lee (2011b)
Heliocobacter pylori	UreA, UreB	Typhi	Londono-Arcila et al. (2002)
H. pylori	AIDA-UreA epitopes	Typhimurium	Rizos et al. (2003)
H. pylori	UreB	Typhimurium	Liu et al. (2005)
H. pylori	UreB	Typhimurium	Yang et al. (2005)
H. pylori	UreA, UreB	Typhimurium	Becher et al. (2010)
H. pylori	BapA2, UreI	Typhimurium	Liu et al. (2011a)
H. pylori	CagA-VacA-UreB fusion	Typhimurium	Liu et al. (2011b)
Mycobacterium avium paraTB	Ag85A, Ag85B, SOD, Ag74F	Typhimurium	Chandra et al. (2012)
M. tuberculosis	ESAT-6, Ag85A	Typhimurium	Hall et al. (2009)
M. tuberculosis	ESAT-6	Typhimurium	Jong et al. (2012)
M. tuberculosis	ESAT-6, CFP-10	Typhimurium	Juarez-Rodriguez et al. (2012a)
M. tuberculosis	ESAT-6, CFP-10, Ag85A	Typhimurium	Juarez-Rodriguez et al. (2012b)
Mycoplasma hyopneumoniae	Adh, NrdF	Typhimurium	Matic et al. (2009)
Porphyomonas gingivalis	HagB	Typhimurium	Isoda et al. (2007)

Continued

TABLE 1 Recombinant Attenuated Bacterial Vaccine Antigen Delivery Examples Mucosal Immune Responses or Protection—cont'd

Pathogen	Protective Antigen(s)	Vector	References
P. gingivalis	HagB	Typhimurium	Pathangey et al. (2009)
Proteus mirabilis	TetC MrpA fimbriae	Typhimurium	Scavone et al. (2011)
Pseudomonas aeruginosa	OprF, OprI	Typhimurium	Arnold et al. (2004)
P. aeruginosa	LPS O11	Typhimurium	Digiandomenico et al. (2004)
P. aeruginosa	LPS O11	Typhimurium	Digiandomenico et al. (2007)
Streptococcus mutans	Saliva-binding protein	Typhimurium	Salam et al. (2010)
S. mutans	Saliva-binding protein	Typhimurium	Xu et al. (2011a)
Streptococcus pneumoniae	PspA, PspC	Typhimurium	Xin et al. (2008)
S. pneumoniae	PspA	Typhimurium	Li et al. (2008)
S. pneumoniae	PspA	Typhimurium	Li et al. (2009b)
S. pneumoniae	PspA fusions	Typhimurium	Xin et al. (2009)
S. pneumoniae	PspA	Typhimurium	Shi et al. (2010a)
S. pneumoniae	PspA	Typhi	Shi et al. (2010b)
S. pneumoniae	PspA	Typhi	Shi et al. (2013)
S. pneumoniae	PspA	Typhimurium	Wang et al. (2010a)
S. pneumoniae	PsaA	Typhimurium	Wang et al. (2010b)
S. pneumoniae	PspA	Typhimurium	Wang et al. (2011)
S. pneumoniae	PspA	Typhimurium	Kong et al. (2011b)
S. pneumoniae	PspA	Typhimurium	Kong et al. (2011c)
S. pneumoniae	PspA, PspC	Typhimurium	Xin et al. (2012)
S. pneumoniae	PspA	Typhimurium	Kong et al. (2012b)
Yersinia pestis	LcrV	Typhimurium	Garmory et al. (2003)
Y. pestis	F1	Typhi	Morton et al. (2004)
Y. pestis	F1	Typhimurium	Garmory et al. (2005)
Y. pestis	F1, LcrV	Typhimurium	Liu et al. (2007)
Y. pestis	LcrV	Typhimurium	Branger et al. (2009)
Y. pestis	LcrV	Typhimurium	Torres-Escobar et al. (2010a)
Y. pestis	PsaA	Typhimurium	Torres-Escobar et al. (2010b)
Y. pestis	Psn, HmuR, LcrV	Typhimurium	Branger et al. (2010)
Y. pestis	FI, LcrV, F1-LcrV fusion	Typhimurium	Sizemore et al. (2012)
Cryptosporidium parvum	Cp23, Cp40	Typhimurium	Benitez et al. (2009)
Giardia lamblia	α1-Giardin, α-enolase	Typhimurium	Jenikova et al. (2011)
Trypanosoma cruzi	Cruzipain	Typhimurium	Schnapp et al. (2002)
Enterovirus 71	VP1	Typhimurium	Chiu et al. (2006)
HIV	Gag	Typhimurium	Tsunetsugu-Yokota et al. (2007)
HIV	Gag	Typhimurium	Chin'ombe et al. (2009)
HPV*16	L1	Typhimurium	Baud et al. (2004)

Continued

TABLE 1 Recombinant Attenuated Bacterial Vaccine Antigen Delivery Examples Mucosal Immune Responses or Protection—cont'd

Pathogen	Protective Antigen(s)	Vector	References
HPV16	L1	Typhimurium	Echchannaoui et al. (2008)
Influenza virus	M2e fused to WH core	Typhimurium	Ameiss et al. (2010)
PRRSV*	ORF7 protein	Typhimurium	Han et al. (2011)
Pseudorabies virus	PrV vaccine coadministered	Typhimurium	Kim et al. (2012b)
SARS* virus	PilV-SARS N gene fusion	Typhi	Luo et al. (2007)
TGEV*	987P-spike (S) epitopes	Typhimurium	Chen and Schifferli (2003)
TGEV	MisL-S epitope fusions	Typhimurium	Chen and Schifferli (2007)
b. Human Hosts			
H. pylori	UreA, UreB	Typhi	Metzger et al. (2004)
P. aeruginosa	OprF, OprI	Typhi	Bumann et al. (2010)
c. Avian Hosts			
Campylobacter jejuni	TetC-CjaA, TetC-Pep1A	Typhimurium	Buckley et al. (2010)
C. jejuni	Dps	Typhimurium	Theoret et al. (2012)
Clostridium perfringens	C-terminal α-toxin	Typhimurium	Zekarias et al. (2008)
C. perfringens	FBA, PFOR, AgX	Typhimurium	Kulkarni et al. (2008)
C. perfringens	α-Toxin B-cell epitopes, AgX	Typhimurium	Kulkarni et al. (2010)
Influenza virus	HA	Typhimurium	Liljebjelke et al. (2010)
d. Porcine Hosts			
ETEC	K88, K99, FasA, F41	Typhimurium	Hur and Lee (2012a)
ETEC	K88, K99, FedA, FedF	Typhimurium	Hur and Lee (2012b)
Shigella *Vectored*			
HPV	L1	*S.f.*2a sh42	Yang et al. (2005)
HPV	L1	*S.f.*2a Δ*virG*	Li et al. (2009a)
Noninvasive Bacterial Vectors			
Escherichia coli Vectored			
ETEC	F4 and F18	LT⁻ ETEC	Kovšca Janjatović et al. (2010)
EPEC Δ*ler*	NANP & Stx1B	rEPEC	Zhu et al. (2006)
Influenza virus	HA 110-120	Nissle 1917	Westendorf et al. (2005)
Lactobacillus Vectored			
a. Murine Hosts			
B. anthracis	PA	*L. acidophilus*	Mohamadzadeh et al. (2009)
Borrelia burgdorferi	OspA	*L. plantarum*	del Rio et al. (2008)
Brucella abortus	Cu, Zn SOD	*L. lactis*	Saez et al. (2012)
Clostridium tetani	Tetanus toxin C	*L. lactus*	Grangette et al. (2002)
C. tetani	Tetanus toxin C	*L. plantarum*	Grangette et al. (2002)
C. tetani	Tetanus toxin C	*L. plantarum*	Reveneau et al. (2002)
ETEC	GFP-ST-LTB	*L. reuteri*	Wu and Chung (2007)

Continued

TABLE 1 Recombinant Attenuated Bacterial Vaccine Antigen Delivery Examples Mucosal Immune Responses or Protection—cont'd

Pathogen	Protective Antigen(s)	Vector	References
ETEC	F41	*L. casei*	Liu et al. (2009)
ETEC	K99	*L. casei*	Wei et al. (2010)
ETEC	K88, K99	*L. casei*	Wen et al. (2012)
ETEC	K99	*L. acidophilus*	Chu et al. (2005)
Helicobacter felis	Urease B	*L. plantarum*	Corthesy et al. (2005)
S. Enteritidis	FliC	*L. casei*	Kajikawa et al. (2007)
S. pneumoniae	PsaA, PspA	*L. casei*	Oliveira et al. (2003)
S. pneumoniae	PsaA	4 LAB species	Oliveira et al. (2003)
S. pneumoniae	PspA	*L. casei*	Campos et al. (2008)
S. pneumoniae	PppA	*L. lactis*	Medina et al. (2008)
S. pneumoniae	PspA, PspC	*L. casei*	Ferreira et al. (2009)
Y. pestis	LcrV	*L. plantarum*	del Rio et al. (2010b)
Cryptosporidium	P23	*L. casei*	Geriletu et al. (2011)
HIV	Gag, *S.*T. FliC	*L. acidophilus*	Kajikawa et al. (2012)
HPV16	E7	*L. lactis*	Cortes-Perez et al. (2007)
		L. plantarum	
HPV16	E6, E7	*L. casei*	Adachi et al. (2010)
HPV16	E7	*L. casei*	Taguchi et al. (2012)
HPV17	L2	*L. casei*	Yoon et al. (2012)
PEDV*	Nucleocapsid (N) protein	*L. casei*	Hou et al. (2007)
PEDV	N & spike (partial) proteins	*L. casei*	Liu et al. (2012)
SARS	S protein segments	*L. casei*	Lee et al. (2006)
TGEV	N-terminal S	*L. casei*	Ho et al. (2005)
b. Piscine Hosts			
IPNV* of trout	VP2	*L. casei*	Min et al. (2012)
c. Porcine Hosts			
CSFV* & porcine parvovirus	CTL 290 & VP2	*L. casei*	Xu et al. (2011a)
Porcine parvovirus	VP2	*L. casei*	Xu and Li (2007)
Porcine rotavirus	VP4, VP4-LTB	*L. casei*	Qiao et al. (2009)
d. Avian Hosts			
Avian H5N1 influenza virus	HA	*L. delbrueckii*	Wang et al. (2012b)
CAV* of chickens	VP1, VP2	*L. acidophilus*	Moeini et al. (2011)

*GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *L.m.*, *Listeria monocytogenes*; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; *M.b.*, *Mycobacterium bovis*; EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; ETEC, enterotoxigenic *eromonaseromonas*; HPV, human papilloma virus; PRRSV, porcine reproductive and respiratory syndrome virus; SARS, severe acute respiratory syndrome; TGEV, transmissible gastroenteritis virus; PEDV, porcine epidemic diarrhea virus; IPNV, infectious pancreatic necrosis virus; CSFV, classic swine fever virus; CAV, chicken anemia virus.

secretions for only short durations in the monkeys getting the intramuscular boost.

Qiu et al. (2011) used an attenuated *L. monocytogenes* Δ*actA* mutant with an altered *prfA** gene (Yan et al., 2008) to cause constitutive expression of *prf* and *prf*-regulated genes and a 100-fold increased secretion of recombinant antigens in vivo to deliver HIV-Gag or *Bacillus anthracis* PA by i.n. immunization of mice. This route of immunization was superior to parenteral routes of immunization in inducing mucosal, as well as cellular immune responses in the pulmonary tract, but was not as efficacious as i.v. vaccination in stimulating systemic cellular immune responses.

Mycobacterium

Yu et al. (2006) parenterally immunized mice with a recombinant *M. smegmatis* strain secreting HIV-1 Env and in prime-boost regimens induced very significant levels of cellular immunity against HIV in the lungs. More recently, Sweeney et al. (2011) have described a much-improved *M. smegmatis* vector to protect against *M. tuberculosis* infection, which can be used as a vector to deliver protective antigens from other pathogens. Vasconcellos et al. (2012) constructed both *M. smegmatis* and BCG strains delivering the EPEC intimin and BfpA, a bundle-forming pilus, antigens. These constructs were used for oral immunization of mice that developed both serum IgG and IgA, as well as fecal IgA against intimin and BfpA. Adhesion of the virulent EPEC strain to Hep-2 cells was blocked by either the induced antiintimin or anti-BfpA IgA or IgG antibodies. Jensen et al. (2012) describe a replication-deficient, recombinant attenuated *M. tuberculosis* H37Rv strain mc^{2}6435 (Ranganathan et al., 2009) with Δ*leuCD* Δ*panCD* Δ*secA2* mutations and with plasmid-specified SIV Gag delivered orally with and without i.d. boosts that was totally safe in SIV-infected infant macaques. This is important since the licensed BCG vaccine causes disseminated disease in HIV infected children.

Mycobacterium BCG

Manuscripts not using mucosal delivery or not measuring mucosal immunity (antibody or cellular) of recombinant BCG vaccines are not cited or discussed. It should be noted, however, that Dennehy and Williamson (2005) carefully reviewed many publications indicating the ability of BCG-based vaccines to elicit strong mucosal immune responses in mice and guinea pigs when administered by oral, i.n., intragastric, and rectal routes, but not when administered parenterally.

Kawahara et al. (2002) constructed a recombinant BCG synthesizing the HIV-1 Env V3J1 peptide and orally immunized guinea pigs that developed both intestinal intraepithelial T cell lymphocytes, as well as antigen-specific T cells in systemic compartments. Memory was fully detectable 1.5 years after the oral immunization. Mederle et al. (2003) used a mixture of three recombinant BCG vaccines, each delivering SIV Gag, Env or Nef. The rBCG-SIV^3 vaccine mixture was initially used to intradermally vaccinate five sites on the backs of macaques. This was followed 16 weeks later by either three intragastric vaccinations or three intrarectal vaccinations. Both regimens resulted in significant anti-SIV IgA in rectal lavage fluids.

Wang et al. (2009) compared two recombinant BCG strains with a plasmid-specified and chromosomal-integrated rhomboid protease gene from the coccidiosis-causing *Eimeria tenella* for intranasal vaccination of chickens. Immunized chickens displayed increased CD4 and serum antibody titers and, importantly, after oral challenge with oocysts showed significant reductions in intestinal lesion scores, weight loss, and shedding of oocysts in feces.

For additional consideration and evaluation of local and mucosal immune responses induced by *Mycobacteria tuberculosis* and other mycobacteria, see the chapter by J. Ivanyi in this book.

Salmonella

Salmonella-derived RABVs remain the most extensively developed and studied. However, in view of the significant mucosal cellular immune responses induced by both *Listeria*- and *Mycobacterium*-vectored vaccines, it is unfortunate that more effort has not been focused in studying induction of mucosal cellular immune responses by *Salmonella*-vectored vaccines. Digiandomenico et al. (2004) used an *aroA S.* Typhimurium strain to induce mucosal and systemic immune responses to a *P. aeruginosa* LPS O11 antigen after oral immunization, but did not observe significant protective immunity. A later study (Digiandomenico et al., 2007) using i.n. immunization of mice did, however, induce significant protective immunity. Thus, the route of immunization can impact the degree of success in conferring protective immunity. These results mandate the need to develop attenuation strategies that preclude *Salmonella* delivered by the i.n. route from traversing to the brain (Bollen et al., 2008). The alternative might be to investigate the use of sublingual immunization (Cuburu et al., 2007).

RASV strains have most often been used to deliver recombinant protective protein antigens often specified by sequences with codons optimized for high-level expression in *Salmonella*. In rare cases, the coding sequences have been modified to remove stem-loop structures in mRNA so as to reduce nuclease attack and increase mRNA half-life. In this regard, Becher et al. (2010) used a recombinant *aroA S.* Typhimurium strain, synthesizing the

Helicobacter pylori UreA and B, to orally immunize mice to demonstrate induction of increased populations of $CD4^+$ T cells and neutrophils in the gastric mucosa and a reduction in $CD4^+$ Treg cells, which collectively led to reduced titers of *H. pylori* after the challenge. It is thus evident that RASVs are able to induce protective mucosal immune responses in the stomach compartment of the gastrointestinal tract. Induction of protective immune responses in the urinary tract was reported by Scavone et al. (2011), who used a TetC fusion to a *P. mirabilis* mannose-resistant fimbriae subunit (MrpA) to induce an immune response that significantly reduced titers of *P. mirabilis* in bladders and kidneys of i.n. immunized mice. As described in earlier sections and listed in Table 1, we have extensively modified RASV strains to enhance safety and immunogenicity to deliver multiple protective pneumococcal antigens to mice (see Curtiss et al., 2010; Wang et al., 2012a). Our objective has been to develop a vaccine to be orally administered to newborns that would protect against any and all *S. pneumoniae* serotypes that might be encountered anywhere in the world. Many of these improved strategies have also been used to improve the abilities of RASVs to induce protective immunity to both pneumonic and bubonic plague (Table 1). While RASVs have been extensively used to develop vaccines for diarrheal diseases, they also have been effective against cariogenic and periodontal pathogens, at least in rodents (see Table 1).

RASVs have begun to be used in developing vaccines against parasite infections in cases where the parasite resides in proximity to a mucosal surface or invades through such a surface. This is particularly important in developing RASVs to protect against intestinal protozoan parasites, such as *Giardia lamblia* (Jenikova et al., 2011). Although RASVs have also been used as vaccine vectors for insect-born parasites, those examples have been ignored for this chapter since there is no evidence that mucosal immunity would be of any importance in conferring protection.

Use of RASVs to deliver viral antigens has given mixed results. When viral protective protein antigens require glycosylation, the antibodies induced are generally nonneutralizing and nonprotective. Some viruses without glycosylated capsid proteins can sometimes assemble into capsomere structures or even into virus-like particles (VLPs) that are highly immunogenic and antibody responses that usually confer protective immunity. This has worked well for human papilloma virus (HPV) L1 (Baud et al., 2004) and for the assembly of a woodchuck hepatitis core antigen with influenza M2e fusions (Ameiss et al., 2010). On the other hand, delivering nucleocapsid antigens is often effective in inducing protective cellular immunity, and it is unfortunate that examination of cellular immunity in mucosal tissues was not often evaluated since the mucosal route of immunization was invariably the optimal means to induce the immune responses measured.

Chiu et al. (2006) used an RASV to deliver VP1 of enterovirus 71 (EV 71) to female mice 1 week prior to mating. This is a picornavirus with capsids that are not glycosylated. No information was provided whether capsid proteins assembled in *Salmonella* to form VLPs. Newborn mice were partially protected from a low-dose lethal challenge but not to a high-dose lethal challenge with EV 71. All unimmunized or vector-immunized infant mice succumbed to either dose.

Tsunetsugu-Yokota et al. (2007) constructed an RASV using the *aroA S.* Typhimurium SL7207 to deliver human immunodeficiency virus (HIV) Gag synthesized from a codon-optimized sequence on a pUC *ori* vector for high-level gene expression in *Salmonella*. Oral and i.n. immunization led to low levels of mucosal IgA in nasal and intestinal secretions and to serum IgG antibodies. Cytotoxic T lymphocytes (CTLs) were also induced in mucosal surface tissues, as well as in internal lymphoid tissues. The issue of the inability of *Salmonella* to glycosylate Gag was not addressed. Therefore, it is highly dubious that any of the antibody responses induced have any significance or potential for conferring protection against infection in contrast to inducing CTLs, which might confer protection.

Luo et al. (2007) inserted the severe acute respiratory syndrome (SARS) virus N gene into the chromosomal *pilV* gene in a *galE S.* Typhi strain such that synthesis of the N antigen was under the control of the type IV pilus operon. The RASV was administered by the i.n., i.p., oral, and i.v. routes with the i.n. route inducing the highest mucosal IgA and serum IgG anti-N antibody titers and also the highest CTL response (in the spleen), as evaluated by the killing of a SARS N-expressing tumor cell line.

The report of Liljebjelke et al. (2010) showing protection of chickens to an influenza challenge after being immunized by an RASV strain synthesizing HA that was nonglycosylated and largely insoluble is difficult to understand. No data on whether antibodies were neutralizing were provided. Similar experiments done over 20 years ago, in collaboration with a major influenza research group with RASV producing HA protein, failed to induce neutralizing antibodies and failed to show specific immune protection to an influenza challenge. We therefore discontinued research on delivery of antigens that would require glycosylation since it would not be likely that the antibodies induced would be effective in conferring protective immunity.

Echchannaoui et al. (2008) used an RASV delivering HPV16 L1 for the intravaginal immunization of mice. The vaccine was somewhat inflammatory but induced mucosal and systemic antibody responses, and protected mice against proliferation of HPV16-induced tumors. The results

with intravaginal immunization were essentially the same as observed earlier for oral immunization (Baud et al., 2004).

Newly Identified Genetic Functions Required for Virulence or Immunogenicity

Many means to attenuate *Salmonella* while retaining immunogenicity were described in previous reviews listed in the Introduction. YqiC is a newly identified protein with high α-helical content and a coiled-coil structure that permits trimerization to display fusogenic activity with membranes (Carrica et al., 2011). Its absence leads to attenuation, even though it retains the ability to invade into and replicate in epithelial cells and macrophages. This gene and its gene product certainly deserve further study in regard to effects on immunogenicity.

Hur and Lee (2011a) used an *S*. Typhimurium Δ*lon* Δ*cpxR* Δ*asdA* strain to secrete LTB fused to a β-lactamase SS on an AsdA$^+$ vector to demonstrate a significant adjuvant effect in increasing serum and especially mucosa antibody responses over levels induced by a strain not releasing LTB. The adjuvant strain also exhibited biological containment features with failure to detect viable bacteria at any time after oral immunization. In a study to develop a mixture of vaccines delivering secreted K88, K99, FasA, and F41 pilins plus intimin, Hur and Lee (2011b) also failed to detect viable vaccine cells in excreted feces at any time. They did not examine extent and duration of vaccine cells in visceral tissues. In subsequent studies, they demonstrated complete protection in pigs from diarrheal disease caused by a mixture of ETEC challenge strains causing severe disease in control unvaccinated pigs (Hur and Lee, 2011a, 2011b). The vaccines displayed the same containment features as observed in mice. Attenuation by the *lon* and *cpxR* mutations has not been adequately investigated relative to enhancing antigen delivery and effects of Th1 versus Th2 responses and, importantly, in inducing mucosal antibody and cellular immune responses.

The influence in altering synthesis or modification of *Salmonella* PAMPs that interact with various TLRs and NOD factors has not been well studied in terms of enhancing desired mucosal antibody and cellular immune responses. Gat et al. (2011) observed that *S*. Paratyphi A strains with intact flagella induced much better immune responses after i.n. immunization of mice than strains that produced FliC flagellin without assembly into flagella. However, Rojas et al. (2010) compared *S*. Typhimurium RASV strains delivering a nontoxic Stx2 with and without flagella, and observed no differences in mucosal or systemic antibody titers induced.

As a general feature in using RASVs to stimulate protective immune responses, Griffin and McSorley (2011) found that generation of *Salmonella*-specific Th1 cells requires sustained antigen stimulation for a minimum of 2 weeks. They used interruption of the immunization process by antibiotic therapy to regulate duration of vaccine strain persistence in mice. This methodology could have numerous applications in achieving a better understanding of induction of different immune responses by RABVs.

New Developments in Antigen Delivery and Display

Galen et al. (2004) have developed use of the *S*. Typhi cytolysin A hemolysin as a means to export protective antigens out of the vaccine cells into the supernatant. This was demonstrated using the *B. anthracis* PA antigen induction of a significantly higher level of immunity after i.n. immunization of mice than observed when the PA was not secreted. Baillie et al. (2008) compared delivery of the *B. anthracis* PA antigen using the HlyA hemolysin and ClyA export system from the licensed *S*. Typhi Ty21a vaccine using i.n. immunization of mice. Delivery of PA via the ClyA system was superior, and the investigators found that the recombinant Ty21a vaccine was very effective in priming a significant immune response to PA administered parenterally at a later time.

Chen and Schifferli (2003, 2007) compared delivery of TGEV spike epitopes as fusion to a subunit of the P987 fimbriae versus using the MisL autotransporter by several attenuated *S*. Typhimurium strains. Although antibody titers to the S epitopes were higher for the MisL display system, the neutralizing antibody titers were higher for the P987 fimbrial presentation system. Thus, the presentation must have influenced conformation since the MisL fusion construct was synthesized at a 2.5 times higher level than the P987 fusion construct.

Autotransporters (T5SS) have been investigated for some time as a means to export and present antigens on the cell surface (see Curtiss, 2005). All systems have a transmembrane β-barrel and an external domain that may or may not be cleaved for release by a protease such as OmpT. One can preclude this release by deleting some of the basic amino acids in the hinge region. The Hbp autotransporter studied by Jong et al. (2012) for secretion and display of heterologous antigens in attenuated *S*. Typhimurium such as ESAT-6 from *M. tuberculosis* only differs by two amino acids from the autotransporter Tsh that was the first autotransporter identified in the *Enterobacteriaceae* (Provence and Curtiss, 1994). Both Hbp and Tsh bind heme and digest hemoglobin, thus contributing to septicemia caused by ExPEC strains.

Repeat Use of Recombinant Vectors

A concern with any bacterial-vectored vaccine is whether the individual to be immunized has already been exposed

to the pathogen or one closely related to the one from which the vaccine vector is derived. In this case, the individual might not respond and elicit a significant immune response against a delivered protective recombinant antigen. There is also the issue of tolerance to the bacterial antigen delivery vector, a potential problem as discussed above, when using commensal bacterial strains as antigen-delivery vectors. Metzger et al. (2004) evaluated this question using human volunteers and *S*. Typhi Ty21a with and without delivery of plasmid-encoded *H. pylori* UreA and B subunits. In these studies, prior immunization and existing antibodies to *Salmonella* LPS did not alter the frequency (56%) of individuals showing cellular immune responses to the UreA and B subunits. Attridge and Vindurampulle (2005) also reviewed this issue and therefore examined whether mice primed by oral immunization with *S*. Stanley, a serotype naturally attenuated for mice that transiently colonizes PP but not the liver and spleen, would lessen immune responses to the K88 pilus antigen when delivered later by a recombinant *S*. Stanley strain. In all cases, even with priming by i.n. and i.p. routes, immune responses to K88 based on antibody production were muted after immunization of the primed mice with the recombinant vaccine.

In anticipation of potential problems in repeat use of the same vaccine vector for multiple vaccines to protect against diverse pathogens, we (see Curtiss et al., 2010) commenced to modify *Salmonella* antigen-delivery vectors to eliminate or reduce synthesis of immunodominant and serotype-specific surface antigens in vivo to hopefully diminish induction of antibody responses to them. In evaluating such strains of *S*. Typhimurium (Shi et al., 2010a) and *S*. Typhi (Shi et al., 2013), it was observed that prior immunization of potential mothers 2 weeks before breeding gave rise to pups that mounted after immunization with the identical RASV at 7 days of age higher levels of immune responses and higher levels of protective immunity than did pups from nonimmunized mothers. In other studies not yet complete, prior immunization with an RASV delivering one heterologous protective antigen had no adverse effect on the induction of an immune response by the same RASV vector delivering a different heterologous protective antigen.

Role of TLRs in Host Response

Salam et al. (2010) used TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice immunized by the i.n. route with an RASV delivering a nonsecreted portion of an *S. mutans* saliva-binding protein (SBP). TLR2 signaling enhanced serum immune responses to SBP, whereas the response to *Salmonella* LPS benefitted from signaling via TLR4. However, mucosal IgA response to SBP was TLR2-, TLR4-, and MyD88-dependent while the mucosal IgA response to *Salmonella* was TLR4- and MyD88-dependent. Park et al. (2008) found that MyD88 signaling is not essential for inducing PspA-specific B-cell responses leading to mucosal and serum antibodies, but is essential for inducing protective immunity to an *S. pneumoniae* challenge.

Shigella

Li et al. (2009a) describe construction of a recombinant attenuated *S. flexneri* 2a Δ*virG* strain expressing the HPV L1 protein that was used for intraocular vaccination of guinea pigs. Vaccination protected the guinea pigs from *Shigella*-induced keratoconjunctivitis, and led to significant induction of L1-specific IgA antibody secreting cells in spleen and lymph nodes. Suzuki et al. (2006) describe a potentially much improved *S. flexneri* 2a vaccine vector by eliminating the means by which *Shigella* invades epithelial cells that result in excessive inflammation (by deleting the *ipaB* gene) and substituting use of the *Yersinia pseudotuberculosis inv* gene, which promotes invasion through the M cells of the bronchus-associated lymphoid tissue (BALT) and GALT. Mice vaccinated i.n. with this vaccine strain produced high titers of serum IgA and IgG antibodies to LPS, low titers of inflammatory cytokines, and were protected from an i.n. *S. flexneri* 2a challenge.

Yersinia

Although suitable attenuated derivatives of *Y. pseudotuberculosis* and *Y. enterocolitica* have been generated and validated as safe orally administered vaccines, they have been used in a recombinant mode most often using their T3SS to effectively deliver viral and parasite antigens to stimulate protective CD8 and CD4 immunities with little or no attention as to whether they would induce significant mucosal immune responses. This is unfortunate since the systems developed might be very proficient at inducing cellular immunities in mucosal compartments, such as the lungs, as well as inducing production of mucosal IgA responses. Their invasiveness might also engender induction of long-term protective mucosal immune responses.

NONINVASIVE MUCOSAL COLONIZING BACTERIAL DELIVERY SYSTEMS

Clostridium

A nonattenuated *C. perfringens* engineered to synthesize a plasmid-specified HIV-1 Gag or p24 protein was used to orally immunize mice in combination with HIV-1 VLPs and with oral adjuvants cholera toxin (CT) or mutant heat-labile toxin (mLT) or CpG to induce vaginal and serum antibody responses and CD4 and CD8 T cell responses against the HIV antigens (Pegu et al., 2011). Since the immune responses were barely significant when using the

recombinant *C. perfringens* strain alone and were significantly elevated when VLPs were administered either before or after the recombinant *C. perfringens* strain, it is unclear what the role of the noninvasive bacterial host was in inducing the reported results. However, Chen et al. (2004) had previously observed that fusion proteins produced by *C. perfringens* were present in dendritic cells (DCs) in PP following the oral immunization of mice. It was also not clear whether the strain used by Pegu et al. (2011) produced enterotoxin or α-toxin that could have facilitated antigen presentation by the VLPs or the means by which the HIV antigens were released from the bacterial cells to be taken up by DCs or the GALT.

Escherichia

Westendorf et al. (2005) describe the use of the probiotic *E. coli* strain Nissle 1917 as a vector to deliver HA epitopes in the influenza virus. Harms et al. (2009) genetically modified the nonpathogenic *E. coli* DH5α to express genetic sequences encoding invasin from *Y. pseudotuberculosis* and listeriolysin O from *L. monocytogenes* to exhibit invasiveness and intracellular release of antigen. They used green fluorescent protein (GFP) as a model antigen and demonstrated induction of a predominant Th1 response with production of CTLs. The ultimate aim was to deliver antigens from *Brucella*. They did not examine induction of mucosal immunity. Zhu et al. (2006) used a Δ*ler* mutant of an EPEC strain causing diarrheal disease in rabbits and used it to deliver a plasmodium circumsporozoite NANP antigen and the Stx1B toxin subunit by employing the *E. coli* hemolysin export system and the MisL autotransporter of *Salmonella*. Biliary IgA and serum IgG were induced against the antigens. More protective antigen was delivered to the cell surface and secreted by the hemolysis system and, after oral administration, this construct also induced some protection to challenge with a virulent RDEC strain.

Helicobacter

Marshall and Schoep (2007) review efforts, so far unsuccessful, to develop an attenuated *H. pylori* antigen delivery strategy to induce protective immunity to other pathogens.

Lactobacillus

Lactobacillus and *Lactococcus* strains are administered orally, often by gastric intubation, in relatively large amounts such as ~10 mg wet weight or 1 mg dry weight or 10^9 to 5×10^{10} CFU per mouse given 3–5 days in a row during weeks one, two, and four with a boost or every two or three weeks. Thus, the total dose probably approximates 10^{10} to 10^{12} bacteria for the entire oral immunization regimen. Many recent studies have used i.n. immunization with lower doses, often with better results. These bacteria that grow optimally at lower than mammalian and avian body temperatures (*L. lactis* and *L. casei*) do not persist or establish as commensals in orally immunized animal hosts, whereas those species that grow at 37 °C (*L. plantarum, L. acidophilus, L. helveticus*) persist longer. An issue not addressed concerns the use of bacterial vaccine vectors that are selected from strains with the potential to be commensals and thus induce immunological tolerance. The high repetitive doses needed to induce desired immune responses would seem to favor this outcome.

Grangette et al. (2002) compared plasmid encoded synthesis and delivery of tetanus toxin fragment C by *L. lactis* that cannot persist and colonize the intestinal tract of mice and *L. plantarum* that can. The recombinant *L. plantarum* was best and the UV-irradiated construct also was inferior to the live viable strain. Mice were immunized intragastrically with 10^9 CFU on three consecutive days with two or three repeat immunizations three weeks apart. Conclusions are based on induced serum IgG levels and neutralization of tetanus toxin. Reveneau et al. (2002) compared three *L. plantarum* strains that either retained tetanus toxin fragment C in the cytoplasm, secreted it, or displayed it as cell-surface exposed. The three recombinant vaccine strains were then administered to mice by intragastric, i.n., and s.c. routes. All routes induced significant antitoxin antibodies with the strain with cell surface localizing fragment C requiring the lowest dose to induce antibodies but with the highest antibody titers observed for the strain having cytoplasmic localization of the antigen. Grangette et al. (2004) generated alanine racemase deficient *L. lactis* and *L. plantarum* strains and found that both were superior in inducing antitoxin C antibodies but with the *L. plantarum* strain being superior, especially in inducing antitoxin IgA responses in intestinal secretions. Thus, lysis to release synthesized antigen is a superior means of antigen delivery, especially to induce mucosal immunity.

Adachi et al. (2010) introduced plasmid encoding a mutated form of the HPV-16 E7 protein to render it nontumorogenic while retaining immunogenicity and the K6 protein into a sugar-apple isolated *L. casei* strain that was then grown at 30 °C with antibiotic selection and attenuated with heat. The cells were washed several times with distilled water, dried to a powder, and stored at 4 °C. The dried powders were suspended in phosphate-buffered saline (PBS) and administered in 200 μl by an intragastric tube to mice that had been fasted for 3 h. Mice were immunized 5 days a week during weeks one, two, four and eight. This vaccination induced significant proportions of $CD4^+$ and $CD8^+$ integrin $\alpha_4\beta_7^+$ cells in the intestinal mucosal lymphocytes and these cells exhibited CTL activities. Taguchi et al. (2012) extended these studies to demonstrate that coadministration

of two different Japanese herbal medicines with the recombinant heat-inactivated *L. casei* strain enhanced the mucosal cellular immune responses to E7. Yoon et al. (2012) chose to deliver the HPV-16 L2 amino acids 1 to 224, which induces cross-reactive antibodies that would be prophylactic to other HPV types. The poly-γ-glutamate synthetase A protein of *B. subtilis* was used to achieve surface display of the L2 sequence on *L. casei* that was repetitively used to immunize mice by intragastric lavage. Serum IgG and vaginal IgG and IgA were induced, and using bovine pseudovirions, they demonstrated neutralizing activity against the HPV pseudovirions of the types-18, -45, and -58. Sera from bovine papilloma virus (BPV)-16 L1 immunized mice showed no such neutralizing activity. Cortes-Perez et al. (2007) compared delivery of plasmid-specified HPV16 E7 from *L. lactis* and *L. plantarum*, using i.n. and intragastric immunization. The i.n. route was superior in terms of inducing both serum IgG and mucosal IgA and in therapeutically reducing growth of HPV16-induced tumors. Also, the *L. plantarum* strain outperformed the *L. lactis* strain.

Oliveira et al. (2006) initially introduced *S. pneumoniae* PsaA encoding sequences into *L. lactis, L. casei, L. plantarum*, and *L. helveticus*, but replaced its signal sequence and lipidation site during fusion to the *usp45* SS. The recombinant *L. lactis* was least able to colonize after i.n. vaccination and elicited the lowest levels of anti-PsaA antibodies, compared to the other strains. The *Lactobacillus* species were thus superior in colonization and induction of immune responses with the *L. plantarum* and *L. helveticus* strains capable of growing at 37 °C, giving the best antibody results. These results are in accord with the findings of Grangette et al. (2004) discussed above and it is thus surprising that the Oliveira group in subsequent work, as well as other groups, continued to focus on use of *L. casei* strains. It should be noted that Wang et al. (2010b) found that only full-length PsaA with its signal sequence and lipidation signal was able to induce an immunity to block pneumococcal colonization in the upper respiratory tract of mice, whereas delivery of all truncated forms of PsaA were unable to induce such protection. Campos et al. (2008) (in the Oliveira group) thus expressed constitutively an *S. pneumoniae* D39 sequence encoding the α-helical domain and proline-rich region of the *pspA* gene lacking a signal sequence from a plasmid vector in *L. casei* and administered to mice in two i.n. doses of 10^9 CFU. Serum IgG responses were induced and antibodies were able to coat the surface of a number of PspA Clade 1 and 2 strains. A modest level of protective immunity to a low-dose i.p. challenge with *S. pneumoniae* was observed. The recombinant *L. casei* persisted in the nasal mucosa for up to 6 days, but induction of mucosal immune responses was not monitored. This work has been expanded to also deliver the α-helical domain of the *pspC* gene (Ferreira et al., 2009), but i.n. immunization with six doses of 10^9 CFU of the *L. casei* strain delivering PspC did not result in protective immunity. Hernani Mde et al. (2011) extended this work to demonstrate that i.n. immunization with the recombinant *L. casei* strain delivering PspC did reduce intranasal colonization by *S. pneumoniae*, and that challenge resulted in a detectable intranasal IgA response. As described above, Xin et al. (2008, 2009, 2012) observed significant protective immunity to several *S. pneumoniae* strains with different challenge routes with T2SS delivery by attenuated *Salmonella* vaccines of PspA and PspC alone, together, and as fusions to represent the diversity of epitopes in the α-helical domains and proline-rich regions of diverse pneumococcal serotypes.

Medina et al. (2008) delivered the *S. pneumoniae* PppA surface protein by exporting it with the signal sequence of a cell wall protein to the cell surface of *L. lactis*. Nisin-induced cultures were used for i.n. immunization that led to induction of serum (mostly IgG) and bronchial alveolar lavage fluid (mostly IgA) anti-PppA antibodies. Mice were protected against a lethal i.p. challenge with the *S. pneumoniae* strain from which the *pppA* gene had been cloned.

Wu and Chung (2007) used *L. rueteri* to deliver a secreted green fluorescent protein-*E. coli* heat-stable toxin-*E. coli* heat-labile toxin subunit B (GFP-ST-LTB) fusion that possessed GM ganglioside binding ability for repetitive oral immunization of mice to induce fecal mucosal IgA and serum IgG and protect the mice from an oral challenge with the ST- and LT-producing ETEC strain ATCC35401. They used the nisin-inducible expression-secretion vector pNIES. Liu et al. (2009) used a plasmid vector encoding the poly-γ-glutamate synthetase A protein of *B. subtilis* (Lee et al., 2006; Hou et al., 2007) to enable cell surface display of the ETEC F41 fimbrial antigen on *L. casei* cells. Repetitive oral immunization led to high titers of mucosal IgA and serum IgG with a Th2 skewing. Vaccinated mice challenged with a lethal dose of the $F41^+$ ETEC strain cleared the infection from the intestinal tract and survived. Wei et al. (2010) also used the poly-γ-glutamate synthetase A anchoring system to display K99 pilin on the surface of *L. casei* cells. Oral and i.n. immunization of mice induced mucosal IgA and serum IgG anti-K99 antibodies, and mice immunized by either route displayed 80% protection to an oral challenge with the ETEC strain C83912. Further studies, including induction of protective immunity, with this surface display of pilin subunits and antigen delivery by *L. casei* for the K88 and K99 fimbrial antigens has been reported by Wen et al. (2012). Chu et al. (2005) constructed a fusion of the gene for K99 to the S-layer gene of *L. acidophilus* to be expressed from a plasmid vector under control of the constitutive S-layer protein promoter. The recombinant *L. acidophilus*, unlike *L. casei* and *L. lactis*, grows at 37 °C and was used to colonize pigs after oral administration. K99 antigen was found on the cell surface and in the supernatant and the recombinant adhered to the intestinal brush border and competitively displaced

ETEC strains with K99 fimbriae. Induction of immune responses was not reported.

Corthesy et al. (2005) used an *L. plantarum alr* mutant to deliver a plasmid-specified urease B subunit from *H. felis* to mice using intragastric immunization of 10^9 CFU 3 days in a row, repeated two additional times 3 weeks apart. Antiurease serum IgA and IgG were induced and *H. felis* titers were significantly reduced in immunized mice.

del Rio et al. (2008) expressed synthesis of the *Borrelia burgdorferi* OspA gene without any codon optimization on a plasmid vector in *L. plantarum* that was grown, harvested, frozen, and later recovered to administer 4×10^{10} CFU by oral gavage given twice daily for 8 days to mice. Colonization was transient since bacteria were not recovered 48 h after the last inoculation. Anti-OspA fecal IgA and serum IgG were induced and the mice were 100% protected from a tick-transmitted *B. burgdorferi* infection. In a subsequent paper (del Rio et al., 2010b), the effect of lipidation of OspA was carefully analyzed to reveal a Th1-inducing bias when the nonlipidated OspA is delivered by the *L. plantarum* vaccine vector. del Rio et al. (2010b) used the *L. plantarum* vaccine vector to deliver the *Y. pestis* LcrV antigen with and without fusion to the *B. burgdorferi* OspA protein to enable LcrV to be cell-surface localized. Mucosal IgA and serum IgG were induced, but no evidence for protective immunity to *Y. pestis*-caused pneumonic and bubonic plague and to *Y. pseudotuberculosis* and *Y. entercolitica* was provided, as has been achieved with attenuated *Salmonella*-vectored vaccines (Branger et al., 2007, 2009; 2010).

Geriletu et al. (2011) used *L. casei* to deliver the P23 surface protein of *Cryptosporidium parvum* to orally immunized mice. Fasted mice receiving 10^{10} CFU for three consecutive days each week developed fecal anti-P23 IgA and serum IgG responses. No information on challenge studies was reported.

Ho et al. (2005) constitutively synthesized the N-terminal sequence of the protective TGEV coronavirus glycosylated spike protein that was secreted to the surface of the recombinant *L. casei* strain. This construct at a dose of 2×10^9 CFU was administered daily for 3 days and repeated twice at 2-week intervals. This particular strain seemed to persist at higher titers in various intestinal compartments than strains used by others and induced good fecal IgA and serum IgG titers. Most importantly, both inhibited transmissible gastroenteritis virus (TGEV) plaque formation on monolayers of swine testicular cells. We had earlier delivered TGEV S protein from an attenuated *S.* Typhimurium vaccine (Smerdou et al., 1996a) to orally immunized mice to induce mucosal IgA and serum IgG responses that were not virus-neutralizing (unpublished). An improved strategy was employed to fuse a DNA sequence encoding multimers of a 25 amino acid protective S-gene epitope to the C-terminal end of LT-B with its signal sequence intact (Smerdou et al., 1996b). This vaccine strain used to orally immunize rabbits did induce both intestinal IgA and serum IgG at titers sufficient to neutralize TGEV. Although the inability of bacteria to glycosylate viral proteins has often precluded inducing protective immune responses, the results of Ho et al. (2005) would suggest that secretion of such antigens by the recombinant vaccine vector is more likely to yield some protective immunity.

Lee et al. (2006) developed a protective antigen surface display system using the poly-γ-glutamate synthetase A protein of *Bacillus subtilis* encoded by the *pgsA* gene to enable presentation of SARS spike (S) protein amino acids 2 to 114 (segment A) and 264 to 596 (segment B) by *L. casei* administered repetitively intragastrically and i.n. to induce high titers of intestinal and lung mucosal IgA and serum IgG. Oral immunization yielded higher titers of both serum and intestinal antibodies able to neutralize SARS pseudoviruses than did i.n. immunization. The studies also identified three new neutralizing epitopes.

Hou et al. (2007) also used the poly-γ-glutamate synthetase A protein of *B. subtilis* to anchor the nucleocapsid protein of porcine epidemic diarrheal virus (PEDV) to the surface of recombinant *L. casei*. Repetitive oral (20 doses) or i.n. (eight doses) immunizations of mice yielded induction of serum IgG and lung IgA. Sows were immunized by the addition of heat-killed recombinant *L. casei* to feed and resulted in high IgG and IgA titers in colostrum, which were able to neutralize the virus to prevent plaque formation. The surface display attribute of this vaccine was likely the basis for success. Liu et al. (2012) used plasmid vectors with the cell surface display *pgsA* gene from *B. subtilis* and with a signal sequence to cause export of PEDV S and N proteins out of *L. casei* cells. Mice were repetitively immunized orally. Mucosal IgA antibodies were measured in nasal washes, ophthalmic fluid, intestinal washes, and vaginal fluids. Highest titers were induced by the strains delivering both S and N antigens as surface displayed antigens. Serum IgG responses were able to neutralize the virus as shown by reduction in plaque formation.

Kajikawa et al. (2007) constructed a recombinant *L. casei* strain with surface-exposed FliC from *S.* Enteritidis to repetitively (nine doses) intragastrically immunize mice with 5×10^{10} CFU. Although flagellin-specific IgA and IgG responses were not observed, mice produced FliC-induced INFγ and were protected from an *S.* Enteritidis lethal challenge. The results thus suggested that protection was due to induction of cellular immunity. In a follow-up study, Kajikawa and Igimi (2010) fused the *S.* Enteritidis SipC to the C-terminal end of surface-localized FliC, and now observed induction of antibodies to both SipC and FliC with a predominant Th1 response. Kajikawa et al. (2012) surface-displayed HIV Gag and *S.* Typhimurium FliC on a recombinant *L. acidophilus* strain by fusions to the signal sequence and anchor motif of a mucus-binding

protein. The FliC interacted with TLR5 on HEK cells to trigger synthesis of NFκB. Repetitive intragastric immunization led to serum IgG and mucosal IgA with much higher titers, especially of mucosal IgA, induced by the strain also displaying FliC.

Mohamadzadeh et al. (2009) describe the construction of a surface-exposed antigen delivery system with specific targeting via DC-binding peptides to DCs residing in the intestinal mucosa and in peripheral tissues. In this study, the plasmid sequence encoding the *B. anthracis* PA was engineered for secretion and DC targeting by C-terminal fusion to a 12 amino acid DC-targeting or nontargeting peptide. The plasmid-containing *L. acidophilus* strains were fully evaluated in mice after repetitive gastric intubations, and the strain with the DC-targeting peptide induced PA neutralizing antibodies, intestinal IgA, and, importantly, protection against an i.p. challenge with *B. anthracis* Sterne. This work is also fully described in a review (Mohamadzadeh, 2010).

Xu and Li (2007) intragastrically immunized mice with an *L. casei* strain delivering porcine parvovirus VP2 to induce mucosal IgA and serum IgG capable of neutralizing the porcine parvovirus. A subsequent study (Xu et al., 2011b) also included a strong CTL epitope 290 from classic swine fever virus (CSFV) in addition to the VP2 antigen. Importantly, both mucosal and systemic $CD8^+$ CTL responses were induced against CSFV.

Raha et al. (2005) used C-terminal sequences with one of three 44 amino acid repeats from the *N*-acetylmuraminidase of *L. lactis* that specifically cause tight cell wall binding of peptide sequences fused to the repeat. Such constructs were made using N-terminal sequences of the VP1 protein from enterovirus 71 with the protein products synthesized in *E. coli* and used to bind to *L. lactis* cells. Tests on stability during immunization were not reported.

Saez et al. (2012) used a secretion vector to deliver the periplasmic superoxide dismutase with Cu and Zn cofactors (Cu, Zn SOD) of *B. abortus* from *L. lactis* used to orally immunize mice. SOD-specific IgA was induced in nasal and bronchoalveolar lavage fluids and SOD-specific IgG in serum. Immunized mice caused reductions in *B. abortus* titers after the challenge and antigen-specific T cell proliferation was equal 2 weeks and 6 months after completion of the immunization regimen. Thus memory was induced.

Wang et al. (2012b) reported the induction of mucosal IgA and serum IgG against HA of the avian H5N1 influenza virus delivered by a recombinant *L. delbrueckii* strain orally administered to chickens. No data on virus neutralization were given nor was mention made about the glycosylation of HA. Nevertheless, many of the studies using *Lactobacillus* antigen delivery strategies, especially with secretion, surface display, or release by lysis, gave promising results in inducing mucosal immunity and protection to challenge.

Vibrio

Ludi et al. (2008) endowed the attenuated *V. cholerae* CVD103-HgR vaccine with ability to synthesize and assemble ETEC colonization antigens CFA/1 CS3 and CS6 as specified by plasmids conferring antibiotic resistance. CFA/1 and CS3 fimbriae were visualized on the cell surface by electron microscopy and negative staining. Tobias et al. (2008) also constructed in a non-CT producing *V. cholerae* strain ability to synthesize ETEC CFA/1 specified by a plasmid with a $thyA^+$ gene to complement a chromosomal *thyA* mutation, thus yielding an antibiotic-sensitive vaccine strain. Only formalin-killed preparations were used for oral immunization. Roland et al. (2007) constructed a derivative of the attenuated *V. cholerae* vaccine strain Peru-15 to oversynthesize and secrete CT-B from a balanced-lethal plasmid construct yielding an antibiotic-sensitive recombinant vaccine strain. Intranasal immunization of mice and oral immunization of rabbits resulted in anti-CTB-B titers some 30 times those induced by the parental Peru-15 strain and serum antibodies neutralized CT. Mucosal IgA titers were not determined.

Xiao et al. (2011) used the attenuated *Vibrio anguillarum* strain MVAV6203 (virulence plasmid cured and Δ*aroC*) to evaluate various plasmid replicons resulting in selection of plasmid pUTat with the replicon from pAT153 (derived from pBR322) as being most stable in vitro and in vivo. The GAPDH gene from *E. tarda* was cloned into the plasmid vector that did specify antibiotic resistance transformed into the vaccine strain, which was evaluated by intracoelomic (i.c.) immunization. Although partial protection to challenge with *E. tarda* in turbot was observed, use of mucosal immunization was not investigated.

RECOMBINANT ATTENUATED BACTERIAL DNA VACCINE DELIVERY SYSTEMS

Introduction

Results with delivery by attenuated bacteria of protective antigen synthesis capability encoded on DNA vaccine vectors can often be misleading. The problem is compounded by use of high copy number pUC *ori* replicons. Most commercially available DNA vaccine vectors make use of the cytomegalovirus (CMV) promoter to drive expression of an inserted DNA sequence in the eukaryotic immunized host and a selective marker sequence to be expressed in the bacterial host. It is often not recognized that expression of genes cloned on high copy number vectors do not need a promoter (Kang et al., 2002b); thus, one can get expression of the sequence to be expressed under control of the CMV promoter in the immunized animal host in the bacterial strain prior to or during immunization. To further complicate matters, bacterial RNA polymerase can transcribe

sequences thousands of bp from a promoter, such as the one associated with the selective marker. Thus, unless one has strong transcription terminators on either side of the eukaryotic expression cassette or bacterial synthesized antisense mRNA to preclude expression of the CMV promoter-associated coding sequence (Kong et al., 2012b), one is likely to have presynthesized protective antigens present in the RABV before immunization. When delivery of a DNA vaccine is desirable so that the encoded product can be glycosylated in the cells of the immunized host, we often will have a mixture of antigen with some synthesized in the bacterial vaccine cell being nonglycosylated. This can result in a mixed immune response rather than one of high specificity. These problems can be addressed by careful design of the DNA vaccine vector with careful use of transcription terminators, stop codons, and by direction of transcription of various coding sequences.

Using an attenuated bacterial vector to deliver a DNA vaccine has a potential benefit when the vector strain is administered at a mucosal portal of entry. This is because there is a likely induction of mucosal antibody and cellular immune responses. Unfortunately, such mucosal responses have not been rigorously quantified and shown to only be due to delivery of the DNA vaccine and not to delivery of preformed antigens as discussed above. Deciding to use a DNA vaccine delivery system is very much dependent on the pathogen type that the vaccine is designed to protect against. Since most bacteria do not glycosylate their gene products (there are exceptions), the delivery of bacterial genes encoding protective antigens is not a wise choice, unless it is shown that the gene product synthesized in bacteria and in suitable eukaryotic cells are the same with no posttranslational modifications in the eukaryotic cell that would lead to induction of a nonprotective immune response. Elsewhere in this book, Raska and Turanek discuss the induction of mucosal immunity by DNA vaccines administered as naked DNA plasmids.

Many of the papers describing construction and attributes of DNA vaccines did not focus on their delivery by recombinant attenuated bacterial strains. Nevertheless, a number of reviews (Montgomery and Prather, 2006; Brandsma, 2006; Williams et al., 2009; Carnes et al., 2010) provide useful guidance, although they do not address the unique problems associated with their delivery by RABV vectors. Thus, one important issue concerns elimination of sequences in the DNA vaccine vector subject to nuclease attack in either the bacterial delivery strain or the immunized host. Also of critical importance is inclusion of enhancers and nuclear targeting sequences to improve expression of the DNA vaccine encoded sequence.

In addition, there are other features of the bacterial delivery vector that are also of critical importance. It will thus be best to release the DNA vaccine by lysis of the RABV, preferably in the host cell cytosol, and to eliminate bacterial nucleases by inclusion of appropriate mutations. Since many bacteria induce apoptosis/pyroptosis in eukaryotic cells in which they invade, and since these events would decrease the ability of the nucleus to transcribe the DNA vaccine encode sequences, one must lessen the ability of the vaccine vector to induce apoptosis/pyroptosis. One can also alter the display of PAMPs to recruit desired innate immune responses. In this regard, Li et al. (2007a) described constructing a DNA vaccine vector encoding short 40 bp and long 750 bp hairpin RNA molecules to exert adjuvant effects. The short-stem molecule did not enhance apoptosis or reporter gene expression but did enhance induction of antigen-specific cellular immune responses. On the other hand, the long-stem RNA increased apoptosis but did not enhance cellular immunity. The question arises as to where in the cell does dsRNA act to enhance recruitment of innate immunity to result in the desired cellular immunities? Another potentially useful attribute has been described by Carvalho et al. (2010), in which DNA vaccine vectors specify fusions to target encoded antigens to extracellular spaces, lysosomes, and/or the endoplasmic reticulum.

Another issue, often ignored in delivery of DNA vaccines relative to their efficacy in inducing protective immunity, is the ability of the DNA vaccine to direct synthesis of the protective antigen. In addition to the need to use bacterial vectors that either do not induce or induce diminished or delayed apoptosis/pyroptosis in the cells in which the DNA vaccine is delivered (as discussed above), there is a need to deliver the DNA vaccines to tissues with cells able to synthesize the protective antigen and to modify it correctly by posttranslational modification. Raska et al. (2008) have demonstrated that delivery of a DNA vaccine encoding the HIV gp120 antigen induced maximal humoral immune responses when the DNA vaccine was delivered i.v. in a manner to enhance delivery to the liver. They also noted the superior ability of the liver to carry out appropriate glycosylation of the gp120 antigen, which also contributed to induction of neutralizing antibodies. In this regard, DNA vaccines delivered by invasive bacterial strains, such as *Salmonella* and *Listeria,* that effectively colonize the liver and spleen most likely will be better for DNA vaccine delivery than noninvasive bacterial strains. Such comparisons need to be made as do studies to relate the amount of antigen synthesized to relative induction of humoral immune responses versus CD4- and CD8-dependent cellular immunities (Table 2).

BACTERIAL GENERA BEING DEVELOPED FOR DNA VACCINE DELIVERY

Edwardsiella

Choi and Kim (2011) constructed a Δ*alr* Δ*asd* auxotroph of *E. tarda* to deliver an Asd$^+$ DNA vaccine with no antibiotic-resistance genes to deliver GFP as a model antigen

TABLE 2 Recombinant Attenuated Bacterial DNA Vaccine Delivery Systems

Pathogen	Protective Antigen(s)	Host	Reference
Edwardsiella tarda Vectored			
Hemorrhagic septicemia virus		Olive flounder	Choi et al. (2012b)
Streptococcus iniae	Sia10	Japanese flounder	Sun et al. (2012b)
Salmonella Vectored			
H. pylori	UreB, IL12	Mouse	Xu et al. (2007)
M. tuberculosis	Mpt64	Mouse	Huang et al. (2010)
Toxoplama gondii	Sag1, Sag2, CTA_2B	Mouse	Cong et al. (2005)
Eimeria tenella	C-term EtMIC4	Chicken	Du and Wang (2005)
SARS	spike protein	Mouse	Woo et al. (2005)
Infectious bursal disease virus (IBDV)	VP2-4-3 polyprotein	Chicken	Li et al. (2006)
White spot syndrome virus	Env. Prot VP28	Crayfish	Ning et al. (2009)
TGEV	spike protein	Mouse	Yang et al. (2009)
Infectious bronchitis virus (IBV)	S1 & N proteins	Chickens	Jiao et al. (2011)
Avian reovirus	Env. Proteins σB & σC	Chickens	Wan et al. (2012)
Duck enteritis virus	UL24 & LTB-UL24	Ducklings	Yu et al. (2012)
Influenza virus	HA	Mouse	Kong et al. (2012b)
Shigella flexneri 2a Vectored			
Influenza virus	HA	Mouse	Vecino et al. (2004)
Lactobacillus Vectored			
Foot & mouth disease virus (FMDV)	VP1	*L. acidophilus*	Li et al. (2007b)

with a million-fold attenuation due to lysis as a result of the absence of D-alanine in vivo. Subsequent work (Choi and Kim, 2012) demonstrated the presence of GFP activity in liver, spleen, and kidney after either oral or intracoelomic (i.c.) vaccination of olive flounders to induce good antibody titers against *E. tarda* and GFP. Choi et al. (2012a) improved the balanced-lethal Asd^+ DNA vaccine vector by replacing the CMV promoter with the marine medaka (*Oryzias dancena*) β-actin promoter and found higher titers of neutralizing antibodies against the viral hemorrhagic septicemia virus and also observed higher survival after challenge of flounders with the virus. They did not indicate whether antibodies were in the mucous layer covering the fish, which would indicate induction of mucosal antibodies.

Sun et al. (2012b) have used a rifampicin-passaged, rifampicin-resistant attenuated *E. tarda* strain (Sun et al., 2010) to deliver a DNA vaccine vector encoding a protective antigen Sia10 (Sun et al., 2012a) from the fish pathogen *Streptococcus iniae* to vaccinate Japanese flounder (*Paralichthys olivaceus*) orally and by bath immersion to induce partial immunity to challenge with either virulent *E. tarda* or *S. iniae*.

Salmonella

Huang et al. (2010) designed a high-copy number DNA vaccine vector using the operator-repressor titration (ORT) system (Garmory et al., 2005) that maintains plasmids stably in the absence of antibiotic-resistance markers and selection. The DNA vaccine encoded the *M. tuberculosis* MptA antigen for synthesis in vivo under control of the CMV promoter. The RASV delivering the DNA vaccine was delivered both intragastrically (one dose) and i.n. (three doses) prior to an aerosol challenge with *M. tuberculosis*. BCG was given s.c. to a group of mice as a positive control. Although the vaccine decreased bacterial loads in lungs and spleens, the BCG vaccination was much more effective. Mucosal immune responses to the MptA antigen were not monitored. No mention was made as to whether the MptA was glycosylated in vivo.

Ning et al. (2009) made a derivative of pcDNA3.1 encoding an envelope protein VP28 from the White Spot Syndrome Virus (WSSV), which kills crayfish, to be delivered by an *S.* Typhimurium with *dam* and *phoP* mutations. The *S.* Typhimurium was fed on food to crayfish

with no adverse effects. Therefore, crayfish were orally immunized with the *S.* Typhimurium with the recombinant DNA vaccine vector. The vaccine strain persisted in tissues for up to 7 days and induced significant protective immunity to crayfish injected with WSSV at a dose that killed 100% of the controls. The nature or basis of the protective immunity was not discussed. I suspect that since the ORT system is designed such that loss leads to DAP-less death, it is possible that some VP28 antigen synthesized by the *Salmonella* could be released to act as the immunogen.

Jiao et al. (2011) used a pVAX vector to encode infectious bronchitis virus (IBV) S1 and N gene products to be orally and nasally delivered to chickens by the *aroA S.* Typhimurium strain SL7207. Mucosal and systemic antibody responses were induced and immunized chickens demonstrated protective immunity to challenge with IBV. The results were the same for both routes of immunization.

Yang et al. (2009) specified the glycosylated spike protein of TGEV on pVAX to be delivered by the *aroA S.* Typhimurium SL7207 strain. Oral immunization of mice induced mucosal and humoral anti-TGEV antibodies, and the highest dose of vaccine given (10^9 CFU) gave the highest antibody titers.

Wan et al. (2012) constructed pVAX DNA vaccine vectors encoding two avian reovirus envelope proteins σB and σC, capable of inducing neutralizing antibodies, and introduced them into the *aroA S.* Typhimurium strain SL7207. Chickens were orally immunized with three doses of 10^9 CFU at 2-week intervals. Serum IgG and intestinal IgA were induced and chickens immunized to receive both DNA vaccines encoding the two Env proteins were best protected with 87.5% of birds showing no signs of infection 5 days after the challenge.

Yu et al. (2012) constructed a pVAX DNA vaccine derivative encoding the UL24 core gene from the duck enteritis virus (DEV), an α-herpes virus, and delivered it to orally immunized ducklings using the *aroA S.* Typhimurium strain SL7207. Mucosal antibodies were induced in bile and intestinal secretions, as well as DEV neutralizing antibodies in serum. Ducklings receiving the highest dose of 10^{11} CFU demonstrated the greatest protection of 40% to a DEV challenge with 1000 LD_{50}s.

Kong et al. (2012b) used an attenuated *S.* Typhimurium strain with regulated delayed in vivo lysis that was engineered to be hyperinvasive, to escape from the endosome (SCV) to lyse in the cytosol. This caused reduced induction of apoptosis/pyroptosis to enhance transcription of the DNA vaccine delivered efficiently to the nucleus by inclusion of nuclear targeting sequences. The DNA vaccine vector was also improved to remove DNA vaccine vector sequences susceptible to digestion with host nucleases (that were also minimized) by including an *endA* mutation in the *Salmonella* to eliminate a potent endonuclease. The DNA vaccine also had strong transcription terminator sequences on either side of the eukaryotic expression cassette to minimize synthesis of influenza HA protein in *Salmonella* prior to lysis. This vaccine induced 100% protection to mice challenged with 100 LD_{50}s of WSN influenza virus.

Shigella

Vecino et al. (2004) evaluated an *S. flexneri* 2a Δ*asdA* vaccine vector to deliver a DNA vaccine encoding influenza virus HA by i.n. vaccination of mice to induce protective immunity to an influenza virus challenge. Although anti-HA IgA was detected in bronchoalveolar lavage fluids after four immunizations, these titers were substantially increased after a lethal influenza virus respiratory challenge.

Lactobacillus

Li et al. (2007b) constructed a eukaryotic expression vector for the expression of the foot and mouth disease virus (FMDV) VP1 protein encoded on an *L. plantarum* plasmid replicon for delivery by *L. acidophilus.* Comparative studies were conducted using intragastric, i.n., i.p., and i.m. immunization. Highest titers of anti-VP1 antibodies were achieved by i.m. and i.p. immunization. Mucosal routes of immunization were not very effective.

OTHER ISSUES OF IMPORTANCE

Means to Prevent Intestinal Persistence, Colonization of Gallstones and Importance of Biofilms

RABVs have the potential to persist in vivo either in tissues or in the intestinal tract. As is well known, *S.* Typhi and *S.* Paratyphi A can persist by adhering to gallstones in the gallbladder to be later shed in feces. The genetic attributes involving biofilm formation needed for such persistence have been investigated and reviewed (see Gonzalez-Escobedo et al., 2011). We included mutations to block synthesis of thin aggregative fimbriae, colanic acid, and LPS O-antigen in vivo in RASV *S.* Typhi strains delivering pneumococcal PspA antigen as a means to block such persistence (Shi et al., 2010b). Baumler and colleagues (Kingsley et al., 2000, 2003; Weening et al., 2005) have investigated the roles of fimbrial adhesins for persistence in the intestinal tract, as well as the importance of *S.* Typhimurium surface antigens that bind to extracellular matrix proteins that also facilitate intestinal persistence. Most of the mutations contributing these features do not significantly attenuate strains, but further studies are needed since strains with these attributes are often difficult to recover from immunized animals and are not shed in feces in human volunteers.

Induction of Cytokine Storm or Sepsis by Release of Endotoxin

The need to have DNA vaccine delivery vectors lyse in vivo has been discussed above. Although gram-positive vaccine vectors such as the lactic acid bacteria do not have LPS with lipid A as an endotoxin, this is not the case with gram-negative pathogens. Gunn et al. (2010) demonstrated using rabbit ileal loops that the inclusion of Δ*msbB* and/or Δ*sopB* mutations in *S.* Typhimurium significantly reduced intestinal inflammation and fluid secretion. Kong et al. (2011c) genetically modified *S.* Typhimurium by inclusion of several deletion mutations (Δ*pagL,* Δ*pagP* and Δ*lpxR*) and insertion of a codon-optimized *lpxE* gene from *Francisella tularensis* to produce 1-dephosphorylated lipid A, which is nontoxic but has strong adjuvant activity. These mutations will be particularly useful in strains with regulated delayed lysis in vivo (Kong et al., 2008, 2012b), as well as in strains undergoing lysis by other means as discussed above, since in all cases endotoxin will be released during lysis and could result in a cytokine storm or sepsis.

Biological Containment Features of Recombinant Attenuated Bacterial Antigen and DNA Vaccine Delivery Systems

Since live bacterial vaccines have the potential to survive after exit from an immunized individual and are also invariably genetically modified, it is appropriate to consider how they might be modified to ensure their nonsurvival in the environment. Although some success of the live polio vaccine was contributed by excretion of the vaccine virus to contaminate waterways leading to the involuntary immunization of many, it is now established that it is unethical to vaccinate individuals who did not elect to be immunized and give their informed consent. There is also an economic basis for biological containment and nonsurvival of vaccine strains in the environment. This is most relevant for vaccines used to control infections in agriculturally important animals where commercial development is dependent on repeat sales of the vaccines.

Kong et al. (2008, 2012b) described two regulated delayed lysis in vivo systems, one for delivery of protective antigens synthesized by the *Salmonella* vaccine strain and the other for delivery of a DNA vaccine for expression of the genes encoding the protective antigens within the nucleus of the cells in the immunized animal host. In the former case, the synthesized antigens can be delivered by a combination of using antigen secretion by either or both T2SS and T3SS, as well as by lysis (Juarez-Rodriguez et al., 2012b). These strains are unable to persist in vivo and fail to survive if excreted.

Kim et al. (2011) constructed a conditional lethal *S.* Typhimurium strain using an *araC* P_{BAD} *asd* fusion using *araC* P_{BAD} from *S.* Typhimurium SL1344 and *asd* from pYA3332. Strains with this genetic construction are analogous to the strains with ΔP_{murA}::TT *araC* P_{BAD} *murA* constructed by Kong et al. (2008). In both cases, strains will commence to lyse after several cell divisions, but will not persist as long or achieve as high titers in effector lymphoid tissues as the recombinant regulated delayed lysis strains constructed by Kong et al. (2008, 2012b). Both constructions would provide complete biological containment.

Grangette et al. (2004) used *alr* mutants of *Lactobacillus plantarum* and *L. lactis* and, like the Δ*asdA Shigella* strains (Sizemore et al., 1995; Vecino et al., 2004), will commence to undergo lysis during vaccination even when D-alanine or DAP are supplied during the process. Both types of mutations confer complete biological containment.

Lee (2010) described multiple means for attenuating and providing biocontainment to *Lactobacillus* strains, and noted that the genetically modified microorganisms (GMM) are no longer generally recognized as safe (GRAS). He also stresses that GMM vaccines cannot express antibiotic resistance attributes. He discusses *alr* mutants and then pyrimidine-requiring mutants and noted that *thyA* mutants are bacteriocidal in the absence of thymidine or thymine, whereas *pyr* mutants are bacteriostatic in the absence of pyrimidines. Furthermore, a *thyA* mutant of *L. lactis* was stably maintained in pigs. This was shown by Steidler et al. (2003), who recognized that the *thyA* mutation with human Il-10 insertion might be replaced by a *thyA*$^+$ gene from some other bacterium. They were mainly concerned with survival/persistence of the *thyA* vaccine strain after excretion, such that thymine-less death under these conditions would be sufficient. This is interesting, since *thyA* mutants of *S.* Typhimurium are not attenuated (Curtiss and Kelly, 1987). Of course, *L. lactis* does not need additional attenuation. It is therefore not clear whether a *thyA* mutation would be needed to attenuate or even provide biological containment in the intestinal tract, which contains substantial quantities of digested DNA components.

Guan et al. (2011) fused a Fur regulated promoter P_{vluB} to the ΦX174 *E* gene introducing transmembrane holes and tested efficacy in a laboratory *E. coli* strain. In animals, it is hard to know whether the iron concentration would repress or derepress the E gene. Thus, it might be well to couple the E gene with the ΔP_{fur}::TT *araC* P_{BAD} *fur* construction (Curtiss et al., 2009). Whether this system as described provided complete biological containment was not established.

Wang et al. (2013) deleted the genes for the twin arginine secretion pathway (Δ*tatABCD*) to render *E. tarda* salt-sensitive (also more sensitive to other stresses) and thus confer containment for use in a marine environment. However, the deletion was not sufficiently attenuating such that other means would be required in an *E. tarda* recombinant vaccine. No tests were done to see if this strategy contributed to biological containment.

Biotechnological and Synthetic Biology Considerations in Improving Vaccine Efficacy

When expressing genetic information from heterologous organisms, there is a need to screen for sequences that are recognized by regulatory proteins that would interfere with gene expression or cause false internal transcription initiation. Also, expressing bacterial sequences in eukaryotes might lead to posttranslational modifications, such as glycosylation, that would lead to induction of nonprotective immune responses. The reciprocal can be true for viral, fungal, and parasite antigens synthesized in bacteria.

Kindsmuller and Wagner (2011) reviewed use of synthetic biology and computer algorithms as means to advance vaccine design and increase successes. Many researchers started using site-directed mutagenesis for codon optimization and improved promoter design over 15 years ago, and many of us have not cloned a gene for over 5 years, preferring instead to have it synthesized. Reverse vaccinology (Rappuoli, 2001) and bioinformatics analyses in general have been used for over 15 years to identify surface exposed antigens, signal secretion sequences, and protein fusion partners for antigen export and targeting. One of the perplexing discoveries in the last several years is the finding that cytoplasmic proteins, with no means yet discovered for their export from bacterial cells, can induce protective immunity. Such protective or partially protective antigens have been identified in *Mycobacterium, Clostridium,* and *Streptococcus*, possibly implying something unique in gram-positive bacterial pathogens. One possibility is release of such antigens by formation of membrane vesicles (Prados-Rosales et al., 2011; Yang et al., 2011; Thay et al., 2013) released in vivo to stimulate immune responses to these cytoplasmic proteins that must be important for virulence. I think it is interesting that engineers and computer scientists are now interested in genetic engineering, but have been obliged to rename the discipline as synthetic biology, possibly to avoid the need to master the essential knowledge of biology and genetics. We genetic engineers, however, while often progressing by applying new approaches relying on bioinformatics and computer science, have been slow to incorporate these new approaches from the engineering sciences. The problem, of course, is that many of us prefer the cerebral approach to science that requires bench work to achieve understanding, rather than to embrace mindless and costly high-throughput screening in hopes of effortless discovery of something important.

CONCLUDING THOUGHTS

There are important facets of design and development of RABVs for antigen and DNA vaccine delivery to induce mucosal antibody and cellular immune responses. Since the ultimate objective is to deliver vaccine constructs that will be safe and efficacious in protecting animals and humans against infectious disease agents, it follows that one has to contemplate conduct of relevant preclinical studies to validate vaccine performance to secure approval or funding to advance the effort and commence clinical trials. One also has to consider means for manufacture and preservation. Lastly, regulatory issues associated with using live vaccines that are genetically modified are of considerable importance.

While I have restricted the discussion to using live bacterial vectors for delivery of antigens and DNA vaccines to prevent infectious diseases, these systems are also being developed for anticancer therapies, to alter animal host physiology, and to develop contraceptive vaccines. Some applications are directed at control of zoonotic diseases, as well as in inducing infertility in rodent populations that are vectors or consume harvested foods.

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