Chapter 2
Live-Attenuated Bacterial Vectors for Delivery of Mucosal Vaccines, DNA Vaccines, and Cancer Immunotherapy

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Contents

Abstract Vaccines save millions of lives each year from various life-threatening infectious diseases, and there are more than 20 vaccines currently licensed for human use worldwide. Moreover, in recent decades immunotherapy has become the mainstream therapy, which highlights the tremendous potential of immune response mediators, including vaccines for prevention and treatment of various forms of cancer. However, despite the tremendous advances in microbiology and immunology, there are several vaccine preventable diseases which still lack effective vaccines. Classically, weakened forms (attenuated) of pathogenic microbes were used as vaccines. Although the attenuated microbes induce effective immune response, a significant risk of reversion to pathogenic forms remains. While in the twenty-first

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century, with the advent of genetic engineering, microbes can be tailored with desired properties.

In this review, I have focused on the use of genetically modified bacteria for the delivery of vaccine antigens. More specifically, the live-attenuated bacteria, derived from pathogenic bacteria, possess many features that make them highly suitable vectors for the delivery of vaccine antigens. Bacteria can theoretically express any heterologous gene or can deliver mammalian expression vectors harboring vaccine antigens (DNA vaccines). These properties of live-attenuated microbes are being harnessed to make vaccines against several infectious and noninfectious diseases. In this regard, I have described the desired features of live-attenuated bacterial vectors and the mechanisms of immune responses manifested by live-attenuated bacterial vectors. Interestingly anaerobic bacteria are naturally attracted to tumors, which make them suitable vehicles to deliver tumor-associated antigens thus I have discussed important studies investigating the role of bacterial vectors in immunotherapy. Finally, I have provided important discussion on novel approaches for improvement and tailoring of live-attenuated bacterial vectors for the generation of desired immune responses.

2.1 Introduction

Vaccines provide protection against numerous life-threatening infectious diseases, by activating the adaptive immunity against specific pathogen-derived antigens. Since the introduction of active immunization, several vaccines have been licensed for human use. These include some subunit vaccines, which are preferred for their superior safety profile. However, their success is limited by their poor immunogenicity, as multiple booster immunizations and adjuvants are required to achieve an adequate level of protective immunity. Moreover, a subunit vaccine is only applicable for pathogens where a well-defined protective antigen has been discovered. Subunit vaccines are also limited in their ability to induce cell-mediated immunity. In contrast, the live-attenuated/live-inactivated vaccines exhibit superior immunogenicity and induce humoral as well as cell-mediated immunity. Although attenuated viruses and bacteria are both utilized as vaccine vectors, this review will focus only on attenuated bacterial vaccine vectors. Bacteria harbor natural adjuvants in the form of pathogen-associated molecular patterns (PAMPs) (Fig. 2.1). PAMPs, which are recognized by components of the innate immune system including Toll-like receptors (TLRs), facilitate the release of pro-inflammatory mediators and recruitment of antigen-presenting cells (Fig. 2.2). Furthermore, even after attenuation, a limited degree of proliferation and dissemination capacity is retained in the attenuated pathogens. Overall, these factors contribute to the superior immunogenicity of live-attenuated bacteria, which consequently elicit a robust and durable immunity against the cognate antigens.

With the advent of molecular biology and genetics, it is feasible to effectively excise or insert desired genes into bacteria. Bacterial vectors can be engineered to

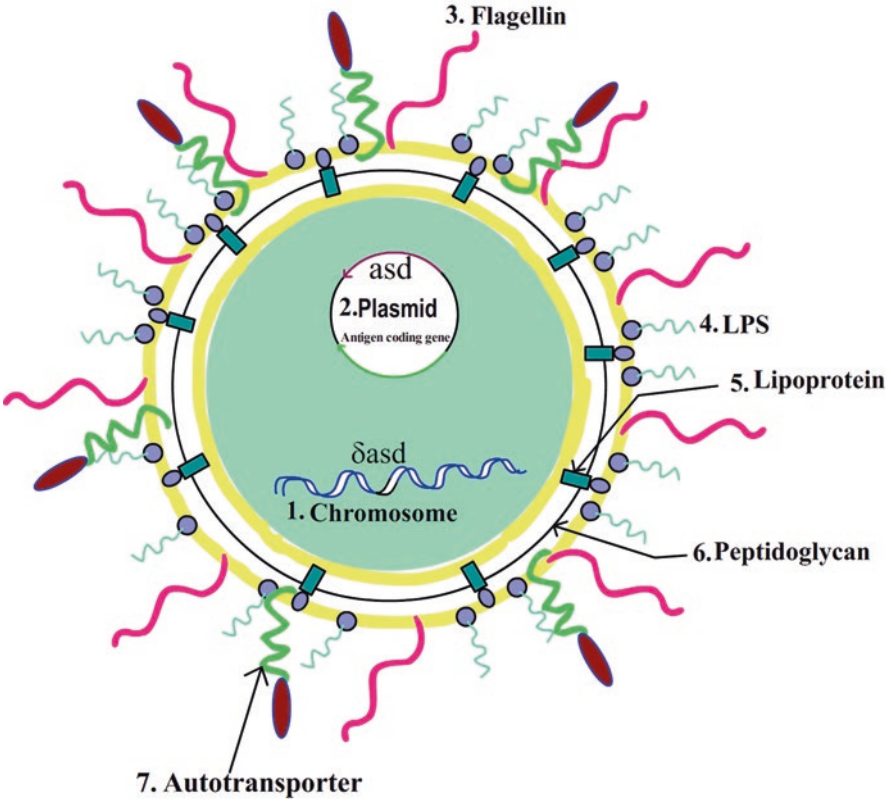

Fig. 2.1 Essential components of live-attenuated bacterial vectors: To construct live-attenuated bacterial vectors, the pathogenicity of bacteria is attenuated by creating mutations in various virulence genes (*1*). The *asd* mutation in the chromosome (*1*) is complemented with a functional copy of *asd* gene, inserted into the plasmid (*2*); this feature ensures antibiotic-free maintenance of plasmids. The plasmid (*2*) also carries genes encoding antigenic proteins. Various pathogen-associated molecular patterns including flagellin (*3*), lipopolysaccharide (*4*), lipoprotein (*5*), and peptidoglycan (*6*) facilitate the interaction with and signal the activation of antigen-presenting cells, while the additional appendages like autotransporters (*7*) facilitate surface display of antigens

express and deliver heterologous proteins, such as antigens or therapeutic proteins, in mammalian hosts. Moreover, by genetic manipulation, bacterial vectors can be engineered with properties including reduced virulence, high immunogenicity, properties which are desirable in a vaccine vector. Thus far, a variety of live-attenuated bacterial vectors including *Mycobacterium bovis* strain *Bacillus* Calmette-Guérin (BCG), *Salmonella* spp., *Listeria monocytogenes* (*Lm*), *Vibrio cholerae*, *Escherichia coli*, and *Shigella* spp. have been utilized for the delivery of heterologous proteins into mammalian hosts as vaccine antigens or therapeutic proteins. Such bacteria are called, live-attenuated bacterial vectors (LABVs).

Vaccines elicit distinct immune responses depending on the route of immunization. Mucosal immunization induces strong systemic as well as mucosal immune

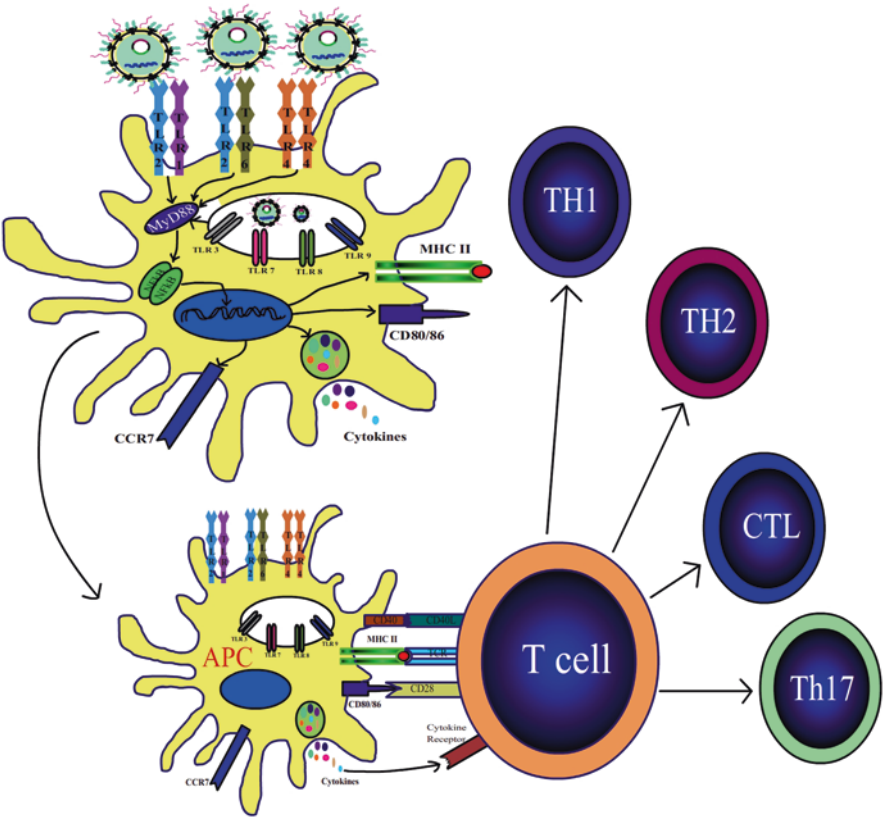

Fig. 2.2 Activation of antigen-presenting cells by live-attenuated bacterial vectors leads to adaptive immune response: Various pathogen-associated molecular patterns present in the live-attenuated bacterial vectors interact with Toll-like receptors expressed on the surface or in endosomal membranes. The signaling initiated by this interaction leads to the activation of antigen-presenting cells. Activated antigen-presenting cells express costimulatory molecules CD80, CD86, and CD40 as well as enhance expression of MHC-II. Costimulatory molecules are required to deliver the essential second signal for T-cell activation, while the first signal is received via TCR-MHC-peptide interaction. Importantly, CCR7 expressed by activated APCs help migration to draining lymph node. Moreover, the type of cytokines directs the fate of T-cell polarization to Th1, Th2, or TH17. Cytosolic delivery of antigens gives rise to CTL response

response, whereas parenteral immunization induces potent systemic but a poor mucosal immune response. Since birth, mucosal surfaces of the human body are constantly challenged with agents of the external environment that are either completely harmless (food ingredients and nonpathogenic microbes) or pathogenic (pathogenic microbes). Thus, in order to restrict pathogenic insults at mucosal surfaces, mucosa-associated lymphoid tissues (MALT) are organized. In fact, MALT constitutes the largest immune system of the human body. The oral route is the most favored route for mucosal immunization over other mucosal routes including nasal,

vaginal, and rectal. Upon oral administration, antigens travel through the gastrointestinal tract and reach the mucosal inductive sites called Peyer's patches. Peyer's patches are lined with specialized epithelial cells called M cells which serve as a point of entry into the lamina propria. In the lamina propria, dendritic cells take up the antigens and migrate to the draining lymph nodes where they present the antigens to T cells. A specialized feature of dendritic cells from Peyer's patches and mesenteric lymph nodes is that they induce gut-homing receptors α4/β7 and CCR9 on T and B cells. This feature is not found in the dendritic cells from cervical lymph nodes and spleen. Thereby, T and B cells primed at the mucosal sites are destined to migrate to mucosal tissues (Pasetti et al. 2011) (Fig. 2.3). Live-attenuated microbes exhibit superior ability to deliver vaccine antigens to the mucosal immune system, as many of them are derived from natural mucosal pathogens, including *Salmonella* spp., *Lm*, *E. coli*, *V. cholerae*, and *Shigella* spp.

This review explores the current knowledge about the LABV application in the delivery of vaccine antigens (to the mucosal immune system), DNA vaccine, and immunotherapy. Mechanism of immune responses elicited by LABV-based vaccines, the recent advances, and future perspectives have been discussed.

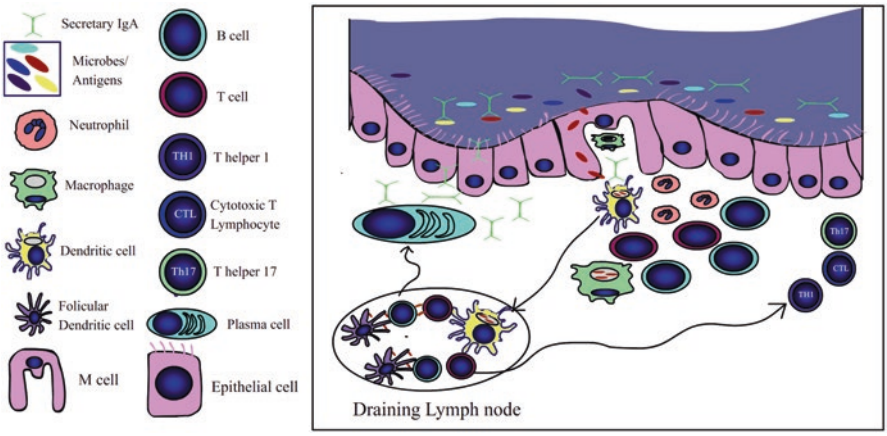

Fig. 2.3 Mucosal immune response elicited by live-attenuated bacterial vectors: Mucosal inductive sites including gut-associated lymphoid tissues and nasal-associated lymphoid tissues facilitate sampling of antigens through M cells. M cells allow the passage of bacteria through the mucosal epithelium, where they are taken up by antigen-presenting cells including DCs and macrophages. APCs undergo activation upon interaction with live-attenuated bacterial vectors, and the associated antigens are presented to T cells in the lymphoid follicles or the draining lymph nodes. Activated T cells help B cells differentiate into IgA-producing plasma cells. The secreted IgA provide effective protection against mucosal pathogens. The T and B cells primed at mucosal sites migrate back to mucosal sites where they perform their effector functions

2.2 Desired Features of Live-Attenuated Bacterial Vectors

Nonpathogenic commensals including the lactic acid bacteria and *Bacillus subtilis* as well as the attenuated versions of the pathogenic bacteria including *Salmonella* spp., *E. coli, Shigella* spp., *Lm*, and *V. cholerae* have been utilized as LABVs. While the commensal microbes are generally regarded as safe or food grade, the virulence of the pathogenic microbes needs to be significantly attenuated before they can be considered safe to deliver vaccine antigens into humans (Fig. 2.1).

Generally, plasmids are employed for the expression of heterologous antigens in LABVs. Plasmids can be easily manipulated in *E. coli* (a universal tool for genetic engineering) and subsequently introduced into the desired bacterial strains. Shuttle vectors (plasmids) carry genetic elements for replication in *E. coli* and promoter elements for gene expression in other bacterial or mammalian cells. Thus, mammalian expression vectors can be first manipulated and propagated in *E. coli* and then delivered via LABVs to mammalian cells, where the desired antigens are expressed (Fig. 2.1).

2.2.1 Attenuation

In early days, attenuation of the pathogenic microbes was achieved by in vitro cultivation for several generations, followed by the evaluation of virulence in successive generations. The classic example is BCG, where Albert Calmette and Camille Guerin, by culturing a virulent strain of *M. bovis* for more than 230 serial passages in vitro (between 1908 and 1921), generated the attenuated strain BCG. BCG is still the only vaccine available for prevention against tuberculosis. Subsequent genetic analysis revealed that BCG lack multiple virulence factors associated with *M. bovis* (Zheng et al. 2015). Similarly, the search for vaccines against typhoid fever led to the generation of attenuated live vaccine strains of *Salmonella*. *Salmonella enterica* serotype Typhi (ST) strain Ty21a (ST-Ty21a) was generated by chemical mutagenesis of wild-type ST strain Ty2. The ST-Ty21a is considerably attenuated which is now licensed for humans use as an oral vaccine (Wang et al. 2001). However, the strain exhibits low immunogenicity, as 3–4 doses are required to achieve adequate levels of protection. With the advances in microbial genetics and genetic engineering techniques, it has now become routine to identify and inactivate virulence genes. Interestingly, various auxotrophic mutant *Salmonella* strains, which lack the ability to synthesize aromatic amino acids, were found to be avirulent (Hoiseth and Stocker 1981). It is known that in *Salmonella*, the gene products of *aroA*, *aroC*, and *aroD* are required for the biosynthesis of aromatic amino acids, as well as several essential vitamins. Hoiseth and Stocker note that these factors are not found in mammalian hosts in sufficient amount; thus *Salmonella* aroA mutants cannot proliferate in mammalian hosts (Hoiseth and Stocker 1981). Harnessing this knowledge, various *Salmonella enterica* serovar Typhi (*ST*) or Typhimurium (*STm*) have been created,

with mutations in *aroA* (Dalla Pozza et al. 1998; Roberts et al. 2000; Arnold et al. 2004), *aroC* (Khan et al. 2003; Capozzo et al. 2004), *aroD* (Capozzo et al. 2004; Sevil Domènech et al. 2008), or *aroAD* (Strugnell et al. 1992; Roberts et al. 2000). Notably, the ST strain CVD908 which carries *aroC* and *aroD* mutations exhibits residual virulence in humans (Wang et al. 2001), while other investigators have also targeted genes in nucleotide biosynthesis pathways for creating attenuated *Salmonella*. Wang et al. engineered a *Salmonella* strain with a mutation in *gua*BA operon, which interferes in the guanine nucleotide biosynthesis. The resultant strain, called CVD 915, exhibits safety profile comparable to that of the typhoid vaccine strain ST-Ty21a. Importantly, ST-Ty21a, CVD-908-*htrA* (harboring mutations at *aroC*, *aroD*, and *htrA*), and CVD 915 all exhibit a high level of immunogenicity (Wang et al. 2001). Not surprisingly, *ST* strains with *gua*BA mutations have been widely utilized as LABV (Pasetti et al. 1999, 2000; Wang et al. 2001). Another approach of attenuation of *Salmonella* is to introduce mutations in *cya* (adenylate cyclase) and *crp* (cyclic AMP) receptor genes. These proteins are transcriptional regulators of many important genes. Although cAMP is found in mammalian cells, their concentrations in gastrointestinal tissues are below the requirement of *Salmonella*. Thus *cya* and *crp* mutants show reduced virulence (Tacket et al. 1992; Chen and Schifferli 2003; Wyszyńska et al. 2004; Ferreira Oliveira et al. 2012). Mutations in the two-component regulatory system PhoP/PhoQ, which controls more than 40 virulence genes involved in resistance to antimicrobial peptides, nutrient scavenging, and lipid A modifications, significantly decrease *Salmonella* virulence (Raupach and Kaufmann 2001). *Salmonella* strains with PhoP/PhoQ mutations have been used in a number of studies as LABV (Angelakopoulos and Hohmann 2000; Kotton et al. 2006; Galen et al. 2009; Wang et al. 2013). *RpoS* is an alternate sigma factor that regulates resistance under stress induced during gastrointestinal infection such as pH, nutrient starvation, change in osmolarity, and temperature. ST-Ty21a contains multiple mutations including *rpoS* (Wang et al. 2001). *SsaV* is a component of *Salmonella* type III secretion system, which is required for secretion of SPI-2 genes (essential for growth in macrophages). The ST strain ZH9 which contains *aroC* and *ssaV* mutations is highly attenuated and immunogenic in humans (Hindle et al. 2002). Tacket et al. generated mutations in *htrA* gene, which encodes a heat shock protein. The resultant strain was avirulent because of reduced ability to survive and replicate in host tissues (Tacket et al. 2000). *HtrA* mutant *Salmonella* strains have been used in multiple studies as LABVs (Galen et al. 1999; Roberts et al. 2000; Pasetti et al. 2002; Capozzo et al. 2004; Fraillery et al. 2007).

Similarly, for attenuation of *Lm*, multiple virulence factors have been targeted. *ActA* which encodes for a surface protein required for actin polymerization in host cells and helps in intracellular migration has been a prominent target for *Lm* attenuation. Together with the mutation in internalin B (*inlB*), the *actA* mutation renders *Lm* unable to infect hepatocytes; thus these strains are highly attenuated (Brockstedt et al. 2004). Phospholipase-C B (PlcB) is required for efficient escape from phagosomal vacuoles. *PlcB* mutants are thus attenuated due to defect in escape from secondary vacuoles (Peters et al. 2003; Starks et al. 2004; Stevens et al. 2004; Johnson et al. 2011; Jia et al. 2012; Liang et al. 2014). Cell wall biosynthesis genes

specifically *dal* (alanine racemase) and *dat* (D-amino acid aminotransferase) have been mutated in several attenuated *Lm* vaccine vectors (Friedman et al. 2000; Verch et al. 2004; Jiang et al. 2007; Im et al. 2013). The double mutant of *Lm* requires D-alanine for cell wall biosynthesis and is highly attenuated. Recently McLaughlin et al. demonstrated that deletion of *Lm* fur-regulated virulence factor A (*frvA*) results in attenuation in murine models of infection, due to the inability of iron homeostasis (McLaughlin et al. 2013).

Attenuated strains of *Shigella* are also being used as LABV. Noriega et al. (Noriega et al. 1996) generated *gua*BA mutant of *Shigella* CVD1204, which is highly attenuated in animals and is widely used as LABV. Other approaches of mutations include SC602, with deletions on *ics*A (mediate intra- and intercellular spread) and *iucA* (aerobactin); this strain is highly attenuated and immunogenic (Ranallo et al. 2005).

2.2.2 Plasmid Maintenance

Introduction of heterologous genes into bacterial vectors is facilitated by plasmids. Plasmids are extrachromosomal circular DNA, which are introduced into bacteria by a process called transformation. Generally, bacteria maintain the plasmids utilizing antibiotic resistance mechanism. During in vitro growth, antibiotic selection pressure ensures stable plasmid maintenance; however, in the in vivo conditions, the lack of antibiotic selection pressure plasmid-less bacteria outgrows plasmid-bearing bacteria. Moreover, the use of antibiotic markers are also discouraged, due to the risk of horizontal gene transfer to other microbes with pathogenic potential (Lin et al. 2015; Mignon et al. 2015). Novel antibiotic-free approaches of plasmid maintenance have been devised to mitigate these concerns. One such approach, known as the balanced lethal system, utilizes mutation in an essential gene in the bacterial chromosome, while the plasmid carries the functional copy of the same gene, thereby ensuring its maintenance by the bacteria (Fig. 2.1). Galen et al. generated a balanced lethal system for *STm* based on mutation in *asd* gene. *Asd* encodes aspartate semialdehyde dehydrogenase, an enzyme required in the biosynthesis pathway of DAP (diaminopimelic acid), which is an essential component of bacterial cell wall. DAP is needed for growth and maintenance of *asd* mutants. A copy of *asd* gene is inserted into the plasmid; thus *asd*-deficient bacteria are forced to maintain the plasmid in order to survive in DAP-deprived conditions, such as in the mammalian tissues. The resultant *Salmonella typhimurium* (*STm*)-based balanced lethal system exhibits high degree of plasmid stability. This system also exhibits stable expression of the associated heterologous genes (Galán et al. 1990). Balanced lethal system has been most widely used in various LABVs including *ST* (Tacket et al. 1997), *STm* (Kang et al. 2002), and *S. flexneri* (Zheng et al. 2005). Similarly, thymidine auxotrophy has also been utilized in *ST* (Bumann et al. 2010), *STm* (Mignon et al. 2015), and lactic acid bacteria (Bermúdez-Humarán et al. 2011) for balanced lethal system approach of plasmid maintenance. Glutamine auxotroph *V. cholerae* complemented with *glnA* gene is another example of the balanced lethal system utilized for antibiotic-free plasmid maintenance (Ryan et al. 2000).

2.3 Immune Mechanisms of Vaccines Delivered by Live-Attenuated Bacterial Vectors

Various mucosal pathogens and nonpathogenic food grade microbes have been extensively utilized for LABV development. *Salmonella* infect via orogastric route and enter the intestinal lamina propria by transcytosis via M cells, which are present in the mucosal inductive sites (Peyer's patches). In the lamina propria, *Salmonella* is taken up by various phagocytes including neutrophils, macrophages, and dendritic cells. The infected phagocytes then carry *Salmonella* to various organs including the liver and spleen via blood or to the mesenteric lymph nodes via lymph. The virulence factors, clustered in *Salmonella* pathogenicity islands (SPI-1 and SPI-2), facilitate invasion, survival, and proliferation in the intracellular spaces of macrophages (Pham and McSorley 2015). *Salmonella* possess a variety of pathogen-associated molecular patterns (PAMPs) including lipoprotein, lipopolysaccharide (LPS), flagellin (FliC), and CpG. These PAMPs are recognized by host pattern recognition receptors (PRRs) including TLR 2(1/6) (lipoproteins), TLR4 (LPS), TLR 5 (FliC), or TLR9 (CpG). Activation of these PRRs leads to the expression and secretion of cytokines such as TNFα, IL1β, IL6, IL8, IL12, IL-18, and IL-23 (Broz et al. 2012). These pro-inflammatory factors recruit neutrophils, macrophages, and dendritic cells. On the other hand, upon interaction with *Salmonella* LPS and flagellin, dendritic cells increase the expression of CCR7, CD80, CD86, and CD40. These mature dendritic cells with enhanced capability to process and present antigens can migrate to T-cell areas and initiate adaptive immune responses to cognate antigens. Studies suggest that *Salmonella* induces humoral as well as $CD4^+$-, $CD8^+$-, and Th17-dependent cell-mediated immune responses (Pham and McSorley 2015). Heterologous antigens carried by *Salmonella* elicit serum IgG (Frey et al. 2013), mucosal IgA (Allen et al. 2000; Ferreira Oliveira et al. 2012; Pei et al. 2015; Lalsiamthara and Lee 2017), $CD4^+$ (Ramirez et al. 2009; Ashraf et al. 2011), and $CD8^+$ T cells (Luria-Perez et al. 2007; Sevil Domènech et al. 2008; Berchtold et al. 2009). Due to the versatile immune response elicited by *Salmonella*, *ST*- and *STm*-based LABVs have been utilized to develop vaccines against numerous viral, bacterial (extracellular and intracellular), and parasitic pathogens. Importantly, it is well known that neutralizing antibodies and CTL responses confer adequate protection against viral pathogens; thus *ST*- and *STm*-based LABVs are capable of eliciting adequate antiviral immune responses. Antigens from viral pathogens including H1N1, H5N1, HIV, and SARS virus, when delivered by *ST* or *STm*, elicit antigen-specific antibody response (Karpenko et al. 2004; Luo et al. 2007; Pei et al. 2015; Hajam and Lee 2017). On the other hand, CTL response is generated against dengue virus (NS3) and HIV antigens (Karpenko et al. 2004; Luria-Perez et al. 2007). LABVs based on *ST* and *STm* elicit serum IgG, mucosal IgA, $CD4^+$ T-cell, and $CD8^+$ T-cell responses against a variety of bacterial pathogens including *B. anthracis* (Galen et al. 2010), *B. pertussis* (Dalla Pozza et al. 1998), *E. coli* (Ferreira Oliveira et al. 2012), *Helicobacter pylori* (Angelakopoulos and Hohmann 2000), *L. monocytogenes* (Igwe et al. 2002), *Pseudomonas aeruginosa* (Bumann et al. 2010), *Streptococcus pneumoniae* (Shi et al. 2010), and *Yersinia pestis* (Branger et al. 2010). While antigen-specific Th1

responses are generated against some parasitic pathogens including *Leishmania mexicana* (González et al. 1998), *Schistosoma japonicum* (Chen et al. 2011), and *Taenia solium* (Ding et al. 2013), mucosal IgG and IgA are generated against *Giardia lamblia* (Abdul-Wahid and Faubert 2007) and *Cryptosporidium parvum* (Benitez et al. 2009) by *ST*- and *STm*-based LABVs carrying related antigens.

Besides *Salmonella*, *Listeria* is the most extensively studied bacteria as LABV. Similar to *Salmonella*, *Listeria* infection begins with orogastric infection. *Lm* moves across gastrointestinal epithelial barrier by first attaching to and invading epithelial cells. Adhesion and internalization require *Lm* protein Ami and internalin A (inlA), respectively. Once in the lamina propria *Lm* rapidly spreads systemically. *Lm* primarily targets liver with the help of fibronectin binding protein (FbpA). FbpA recognizes fibronectin on the surface of hepatocytes, and at this point another molecule, called internalin (inlB), facilitates *Lm* internalization. On the other hand, phagocytes specifically macrophages and monocytes recognize lipoteichoic acid via scavenger receptors. After the phagocytosis *Lm* escapes phagocytic vesicles by synergistic activities of listeriolysin O (LLO) and two phospholipase C (PlcA and PlcB). Another virulence factor is ActA, which has actin polymerization activity and helps *Lm* migrate from cell to cell (Liang et al. 2014). *Lm* expresses various TLR agonists including peptidoglycan, flagellin, and bacterial DNA, which induces pro-inflammatory cytokines including TNFα, IFNγ, IL1β, and IL12. *Lm*-induced cell death results in secretion of IL6, which helps in recruitment of neutrophils. IL12 helps in induction of IFNγ by NK cells and $CD8^+$ T cells. *Lm* can also induce type-I IFNs (IFNαand IFNβ), which is desirable as antiviral immunity. Evidences suggest that *Lm* induces both $CD4^+$ and $CD8^+$ T-cell-mediated immune response (Zenewicz and Shen 2007; Liang et al. 2014). *Lm*-carrying viral antigens such as HIV/SIV-gag (Frankel et al. 1995; Friedman et al. 2000; Im et al. 2013), HPV-E7 (Jia et al. 2012), and LCMV-NP118-126 (Tvinnereim et al. 2002) induces CTL immune response. *Lm*-based LABV also induces neutralizing antibody against HIV-gp160 (Lakhashe et al. 2011). However, there are limited reports on *Lm* as LABV against bacterial and parasitic pathogens. In one study, *Lm*-carrying *Coxiella burnetii* antigen T4SS (epitopes) induced $CD8^+$ T-cell immune response (Xiong et al. 2017). In another study *Lm*-carrying *Francisella tularensis* antigen IglC induced IFNγ producing $CD4^+$ and $CD8^+$ T-cell-mediated immune response.

BCG, an attenuated *M. bovis*, has also exhibited potential as LABV. Following immunization, BCG interacts with phagocytes such as macrophage, dendritic cells, and neutrophils. Various PRRs of macrophages involved in interaction with BCG include CR3, TLR2 (1/6) and TLR-4. However, dendritic cells utilize a different set of phagocytic receptors including CR3, CR4, DC-SIGN (CD209), and DEC 205. Infected dendritic cells upregulate expression of MHC-II and costimulatory markers CD80, CD86, CD40, and CD54 which are involved in activation of adaptive immune response (Moliva et al. 2017). BCG is known to induce humoral as well as T-cell-mediated immune response (Abomoelak et al. 1999). The T-cell responses induced by BCG include polyfunctional $CD4^+$ T cells that secret TNF, IL-2, and IFNγ (Moliva et al. 2017). BCG expressing IL12 and two *M. tuberculosis* (*Mtb*) antigens (secreting antigen Ag85B and culture filtrate antigen CFP10) induce antigen-specific Th1-type immune response including IFNγ-producing cells and

IgG2a (Chen et al. 2017). Antigen-specific humoral immune response is induced by BCG expressing a hepatitis-B surface antigen (Rezende et al. 2005). BCG-induced $CD8^+$ T cells also secrete IFNγ (Moliva et al. 2017). In a mouse model, BCG carrying *Mtb* antigen (Ag85B) exhibit antigen-specific Th17 immune response (Hatano et al. 2016). Pertussis toxin subunit S1 expressed by BCG induces IFNγ producing $CD4^+$ T cells which completely protects against lethal *Bordetella pertussis* challenge (Nascimento et al. 2008). Furthermore, in a mouse model of an intracellular pathogen *Lm,* BCG expressing *Lm* antigen p60 induced $CD4^+$ and $CD8^+$ T-cell-dependent protection (Grode et al. 2002). Studies have demonstrated that BCG induces long-lived mycobacteria-specific memory B cells. Moreover, following BCG immunization, hosts secrete robust *Mtb*-specific serum antibodies including IgG isotypes IgG1, IgG2, and IgG3. However, it is not known if specific mucosal IgA is induced by BCG (Moliva et al. 2017).

S. flexneri infection occurs through orogastric route. Once in the colon, *S. flexneri* crosses epithelial layer through highly endocytic M cells. *S. flexneri* then adhere to and infect colonic epithelium through the basolateral surface. Colonic epithelial cells engulf *S. flexneri* by macropinocytosis, and through the activity of IpaB and IpaC, they are released from macropinocytic vacuoles to the cytosol (Mellouk and Enninga 2016). *S. flexneri* invasion causes activation of innate immune system and release of a variety of cytokines including IL-1, TNF-α, IL6, TGF-β, and IL-8 (Fernandez and Sansonetti 2003; Jennison and Verma 2004). Though *Shigella* has the capacity of cell-to-cell translocation, its infection is limited to lamina propria of the intestine, and it doesn't migrate to other organs (Maurelli and Sansonetti 1988). In the lamina propria *S. flexneri* is phagocytosed by macrophages and dendritic cells. Infected macrophages undergo apoptosis, which leads to the release of pro-inflammatory cytokine IL1, IL18, and IFNγ (Fernandez and Sansonetti 2003). *S flexneri* induces both systemic and mucosal antibody response including IgM, IgG, and secretory IgA (Jennison and Verma 2004). *S. flexneri 2a* with *gua*BA mutation (CVD 1204) has limited invasiveness, and proliferative capacity. Attenuated *S. flexneri* expressing ETEC antigens CFA-I, LTB, CS2, CS3, and CS4 induce antigen-specific serum IgG and mucosal IgA (Koprowski et al. 2000; Barry et al. 2003; Strain et al. 2003; Ranallo et al. 2005; Zheng et al. 2005).

Food grade bacteria including *B. subtilis* and *Lactobacillus lactis* are considered important candidates for LABV, due to their superior safety profile. Upon oral administration, *B. subtilis* spores can safely transit through the stomach, germinate, and proliferate in the upper intestine and finally undergo re-sporulation in the colon (Cutting et al. 2009). Nevertheless, the mechanism of immune response in response to *B. subtilis* delivered antigens is not fully understood. Antigens delivered by *B. subtilis* have been shown to induce humoral as well as Th1-mediated immune response (Cutting et al. 2009). *B. subtilis* has been used as LABV for various bacterial and parasitic pathogens including pathogenic *E. coli*, *H. pylori*, *Mtb*, *Clonorchis sinensis*, and *S. japonicum. B. subtilis* induces systemic IgG (Amuguni and Tzipori 2012; Zhou et al. 2015), mucosal IgA (Amuguni and Tzipori 2012; Zhou et al. 2015), and Th1/Th17 (Sibley et al. 2014; Stasilojc et al. 2015) immune response against cognate antigens. The lactic acid bacteria are among the microbes, which occur physiologically in animal digestive tracts and like other natural microflora

through their metabolites and interaction with macrophages can stimulate cytokine production. Peptidoglycan of the lactic acid bacteria induces secretion of IL1, IL6, and TNF, by monocytes (Bermúdez-Humarán et al. 2011; Szatraj et al. 2017). Unlike attenuated strains of otherwise pathogenic microbes used as LABV, *B. subtilis* and *L. lactis* do not invade through the gut mucosa and serve mainly as protein (antigen) factories, which supply vaccine antigens to gut-associated lymphoid tissue (GALT). Protective antigens of pathogenic viruses H1N1 (HA) and H5N1 (HA) expressed by *L. lactis* induce mucosal antibodies. *L. lactis* expressing bacterial antigens *Campylobacter jejuni* (cjAD) (Kobierecka et al. 2016), *Clostridium difficile* (TcdA) (Yang et al. 2013), *Clostridium perfringens* (epsilon toxoid) (Alimolaei et al. 2016), *H. pylori* (omp22, HpaA, cag12, and UreaseB) (Kim et al. 2006; Gu et al. 2009; Zhang et al. 2016b), and *V. cholerae* (WZM) (Zamri et al. 2012) also induce mucosal antibodies.

2.4 Delivery of DNA Vaccines by Live-Attenuated Bacterial Vectors

In preclinical models, DNA vaccines have proven to confer protective immunity against a variety of infectious agents including HIV, herpes simplex virus (HSV), *Plasmodium* spp., and *Mtb* (Schoen et al. 2004). An attractive feature of DNA vaccine is that it can induce humoral as well as cell-mediated immune response. While antibodies alone can protect against many pathogens and toxins, cell-mediated immunity is required for protection against intracellular pathogens and cancer. The DNA vaccines in the form of eukaryotic expression plasmids are delivered either by intramuscular injection of naked DNA, intradermal bombardment using DNA coated on gold particles with help of a gene gun, or electroporation following needle injection. However, most of these methods induce only moderate levels of protection in animal models and fail to show efficacy in clinical trials (Schoen et al. 2004). In recent years many bacterial vectors have been utilized to deliver plasmids into the host cells (Schoen et al. 2004). As many attenuated strains are being developed for delivery of vaccine antigens, similar strains can also be utilized to deliver plasmids as DNA vaccines. Attenuated strains of gut pathogens including *ST*, *STm*, or *L. monocytogenes* are of particular importance, as they colonize and infect mucosal epithelial cells.

As discussed above, *Listeria* infection begins at gastrointestinal tract, and after invasion through intestinal mucosa, *Listeria* migrate through blood vessels and lymph to other organs. *Listeria* can infect a wide array of cell types including intestinal epithelial cells, hepatocytes, dendritic cells, and macrophages. *Listeria* escape phagocytic vesicles and multiply in cytosol where they release the plasmids. Listeriolysin O helps *Listeria* lyse and escapes the phagosomal vacuoles (Liang et al. 2014). Miki et al. engineered a self-destructing *Lm*-based vaccine delivering a eukaryotic expression plasmid encoding *Mtb* antigens Ag85a/Ag85b and MPB/MPT51. The vaccine induced protective immune response against *Mtb* in a mouse model (Miki et al. 2004).

Salmonella also infects via gastrointestinal tract, and after crossing epithelial barrier through M cells, *Salmonella* is taken up by macrophages (Pham and McSorley 2015). *Salmonella* has the capability of surviving and replicating in phagocytic vacuoles (Pham and McSorley 2015). However, through unknown mechanisms, they can release plasmid DNA into the cytosol (Schoen et al. 2004). *Salmonella* strains expressing listeriolysin O have been shown to escape the phagosome vesicles to the cytosol, thus making gene transfer by *Salmonella* more efficient (Schoen et al. 2004). HIV-1 T-cell epitopes in the form of eukaryotic expression plasmid delivered by attenuated *STm* induced CTL as well as antibody immune response (Karpenko et al. 2004). Another study targeting an *S. pneumoniae* protective antigen PsaA and PspA delivered by *STm* induced mucosal IgA against both antigens. Thus immunized mice were protected against nasopharyngeal colonization by *S. pneumoniae* (Zhang et al. 2011). Pathogenic parasites *Trichinella spiralis* and *Trypanosoma cruzi* have also been targeted for *STm*-mediated DNA vaccination. Yang et al. constructed a DNA vaccine against *T. spiralis* using antigen Ts87 and *STm* as the delivery vehicle. Mice immunized orally with this vaccine induced antigen-specific mucosal IgA which correlated with protection against *T. spiralis* larval challenge. *Salmonella*-delivered *T. spiralis* DNA vaccine induced a Th1-/Th2-type immunity and IL5, IL6, and IL10 cytokines (Yang et al. 2010). In another study, Matos et al. using *STm* delivered *T. cruzi* antigens (Tc-52) into mice via the oral route. Immunized mice elicited specific antibodies with higher IgG2a/IgG1 ratio, suggesting a Th1 bias. The vaccinated group also induced strong cell-mediated immunity and mucosal IgA (Matos et al. 2014).

Most bacteria used as DNA delivery vehicles were designed to disintegrate after infecting host cells. If the bacterial DNA vaccine vectors are destroyed in the phagolysosomes, before reaching the cell cytoplasm, it will lead to inefficient delivery of the plasmid. To circumvent this problem various approaches have been devised. One such approach takes advantage of phage lysin to disintegrate ΔaroA-*Lm* after reaching host cell cytosol. The inclusion of phage lysin significantly improved bactofection (bacteria-mediated delivery of plasmid DNA into mammalian cells) efficiency in phagocytic as well as non-phagocytic cells (Pilgrim et al. 2003). Recently, Kong et al. developed a universal DNA vaccine delivery platform, which includes several modalities for enhanced delivery and immune response to cognate antigens. The attenuated *STm* includes the capability to escape the phagosomal compartment to the cytosol of the host cells, before phagolysosomal degradation (Kong et al. 2012). SifA proteins direct *Salmonella*-induced filament formation when *Salmonella* is contained in the endosomal vacuoles, and the deletion of *sif*A gene results in the release of *Salmonella* into the cytosol. Hence, mutation of *sif*A gene in *Salmonella* plasmid carriers allowed successful transfer of plasmid DNA into the cytosol of the host cells (Kong et al. 2012). Kong et al. also incorporated elements that guide the plasmid into the nucleus. Transcription factors such as NF-κB and AP2 bind to plasmids carrying NF-κB and AP2 binding sequences and transport them to the nucleus where the desired antigens are transcribed (Kong et al. 2012). *Salmonella* induces apoptosis/pyroptosis in infected cells that diminishes the overall transfection efficiency. Deletion of *tlpA* and *sseL* genes significantly reduces apoptosis in

host cells (Kong et al. 2012). Moreover, *Salmonella* degradation is delayed due to the regulated expression of the *Salmonella* lysis program. This allows a limited number of replication and invasiveness, thereby ensuring optimal delivery of plasmids. An influenza antigen (HA) delivered by this platform induced enhanced HA-specific IgG, which correlated with protection against influenza virus challenge (Kong et al. 2012).

2.5 Immunotherapy Against Cancer Using Live-Attenuated Bacterial Vectors

A nineteenth-century physician, William B. Coley, for the first time observed regression of malignant tumor in one of his patients after a bacterial infection. Coley went on to develop the first bacterial therapy against cancer using killed gram-positive bacteria streptococci and a gram-negative bacteria *Serratia marcescens*. This mixture called "Coley's toxins" when injected into patients suffering from various forms of cancer resulted in partial to complete regression. In cases of soft tissue sarcoma, long-term disease-free survival was achieved in approximately 50% of the patients. Nevertheless, despite the remarkable success of "Coley's toxins," with the advent of chemotherapy and radiotherapy, this line of investigation was prematurely abandoned (Bickels et al. 2002). However, in recent years this approach is regaining attention. In fact, BCG is currently being used as immunotherapy for bladder cancer and exhibits superiority over epirubicin and IFNα2b, mitomycin, and epirubicin alone (Fuge et al. 2015). Since the first report of BCG's use in cancer treatment in 1936, preclinical and clinical investigations of BCG have also been reported for other forms of cancer. Mice preimmunized with BCG exhibited slower tumor growth compared to control (Zheng et al. 2015). Morton et al. reported complete regression of tumor lesions in melanoma patients, upon intralesional injection of BCG in 684 out of 754 lesions. Similarly, survival benefits against cancer were also reported in other clinical trials. See Zheng et al. for a detailed review on application of BCG in cancer therapy (Zheng et al. 2015).

Bacteria, specifically anaerobes, exhibit natural tropism toward solid tumors. This phenomenon, although poorly understood, is theorized that certain characteristics of tumor microenvironment facilitate this phenomenon. The deeper pockets of tumors, which are devoid of new blood vessels, are poorly oxygenated and show limited accessibility to chemotherapeutic drugs (Lee 2012; Lin et al. 2015). Forbes et al. demonstrated that *STm* accumulate at a rate of 2000-fold more compared to other organs including the liver, spleen, lung, heart, and skin (Forbes et al. 2003). Using an in vitro model, Kasinkas and Forbes demonstrated that *STm* exhibits chemotaxis. Depending on the availability of specific receptors (tsr, tar, and trg), *STm* were differentially attracted to corresponding chemoattractants expressed in the tumor microenvironment (serine, aspartate, and ribose/glucose), while the wild-type strains accumulate around necrotic zones inside tumors (Kasinskas and Forbes 2007). Moreover, various immunosuppressive mechanisms manifested by the tumor microenvironment also support the proliferation of microbes (Lin et al. 2015).

Distinct tumor-homing property of microbes, including *Lm* and *Salmonella*, has been harnessed to deliver various tumor therapeutic modalities, including therapeutic vaccine antigens, DNA vaccines, and anticancer drugs. Various tumor-associated antigens (TAAs) have been targeted for therapeutic vaccines using LABV as delivery vehicles. PSA (prostate-specific antigen) is secreted by prostate epithelial cells and is overexpressed in malignant prostate cells. Attenuated *Lm* expressing PSA (*Lm*-LLO-PSA) antigen was tested as therapeutic vaccine in mouse tumor models expressing human PSA. Immunization with *Lm*-LLO-PSA completely regressed tumors in five out of eight mice and induced PSA-specific cellular immune response. Immunization of *Lm*-LLO-PSA significantly increased infiltration of PSA-specific $CD8^+$ T cells in tumors and decrease in CD4/CD25/$FoxP3^+$ T_{reg} cells (Wallecha et al. 2009). HER2/neu is overexpressed in about 25–30% of breast cancers and is a potential target for immunotherapy. Shahabi et al. engineered an *Lm*-based vaccine incorporating HER2/neu as antigen (ADXS31-1642). ADXS31-164 elicited HER2-specific $CD8^+$ T cells. The vaccine caused a significant delay in the formation of mammary tumors, and 50% of mice were tumor-free till 45 weeks of the experiment, whereas all sham-treated mice developed tumors and succumbed to the disease. This vaccine also resulted in significant increase in tumor-infiltrating $CD8^+$ T cells and a decrease in the intratumoral $FoxP3^+T_{reg}$cells (Shahabi et al. 2011). *P. aeruginosa* can also deliver heterologous antigens using its type III secretion system. In an experimental model of B-cell melanoma expressing ovalbumin (OVA), Chauchet et al. demonstrated antitumor efficacy of *P. aeruginosa*-based vaccine expressing OVA. *P. aeruginosa* induced a long-lasting and polyfunctional $CD8^+$ T-cell immune response against the cognate antigen, wherein antigen-specific $CD8^+$ T cells expressed IFNγ, TNFα, and IL2 simultaneously. These $CD8^+$ T cells also showed enhanced tumor infiltration property and a greater ratio between effector versus regulatory T cells (Chauchet et al. 2016). Recently Mei et al. utilized a composite approach of DNA vaccine and bacterial surface expression to achieve $CD8^+$ and $CD4^+$ T-cell-mediated immunity targeted to a tumor-associated antigen. The *Salmonella*-based vaccine included AIDA-I autotransporter-Melan A (a murine melanoma antigen) fusion protein and a DNA vaccine element encoding two murine melanoma epitopes (Mei et al. 2017).

2.6 Novel Technologies for Tailored and Enhanced Immune Response

2.6.1 Control of Gene Expression: Use of Plasmid Copy Number and In Vivo Promoters

High levels of antigen synthesis by multicopy plasmids exert metabolic burden to LABV, which results in hyperattenuation, low colonization, loss of viability, and most importantly poor immunogenicity. Various strategies have been adapted to circumvent this problem including the use of low-copy plasmid, use of in vivo

inducible promoters (IVIP), and use of arabinose-inducible promoters (Loessner et al. 2007). Among the first promoters introduced in LABV is $P_{nir}B$, which is activated under anaerobic conditions. $P_{pag}C$ and $P_{ssa}G$ are macrophage-inducible promoters from *Salmonella*. Dunstan directly compared the immunogenicity of antigens upon expression of antigens regulated by $P_{nir}B$ and $P_{pag}C$ and found significantly higher antibody response with $P_{pag}C$ compared to $P_{nir}B$ (Dunstan et al. 1999). Arnold et al. achieved differential antigen expression in vivo using in vivo inducible promoters $P_{pag}C$, comprising variable ribosomal binding site (RBS). By this approach, strains with a high level of expression of heterologous protein exhibited low level of colonization, while a moderate amount of expression resulted in a significantly improved infection rate in mesenteric lymph nodes. A very low level of in vivo inducible antigen expression resulted in unhampered infectivity compared to the parent strain. Immunogenicity was dependent on the rate of infection, as well as the level of antigen expression. Notably, the best immune response was achieved with moderate level of antigen expression and infectivity, while high antigen-expressing strain resulted in little to no immune response. On the other hand, a moderate level of immune response was generated with high infectivity and low antigen expression (Arnold et al. 2004). Wang et al. developed a regulated delayed antigen synthesis system, consisting of *LacI* repressor to repress transcription from P_{trc} during in vitro cultivation. The arabinose-regulated promoter P_{BAD} drives LacI expression in vitro in medium supplemented with arabinose. Upon immunization and lack of external arabinose supplementation, P_{trc} is derepressed, leading to the synthesis of antigens. The regulated delayed antigen synthesis system induced equivalent levels of antibody and protection to that of $P_{pag}C$-controlled antigen synthesis and better than that of $P_{ssa}G$-controlled antigen synthesis (Wang et al. 2011).

2.6.2 Acid Resistance

Upon oral immunization, LABV must withstand acidic environment of the stomach for successful colonization. Enteric pathogens including *E. coli*, *L. monocytogenes*, *Shigella* spp., and *L. lactis* can tolerate extreme acidic pH (below pH 2.5) because they possess the most potent acid resistance (AR) system known as GDAR (glutamate-dependent acid resistance) pathway. Attenuated strains of *ST* and *STm* have limited acid tolerance and exhibit moderate immunogenicity (Dharmasena et al. 2016a). By engineering AR components from *Shigella* spp., Dharmasena et al. significantly (10^5-fold) enhanced acid tolerance of attenuated *ST*-Ty21a (Dharmasena et al. 2016a).

2.6.3 Detoxification of Lipopolysaccharide

LPS plays important role in survival and infectivity of bacteria. However, it is also involved in toxicity to the host. Various attempts at the use of LPS O- antigen mutants of *STm* resulted in poor attachment and intestinal invasion and survival

following oral immunization. By regulated expression of LPS O- antigen components such that they are expressed in vitro and at the time of immunization, but soon after colonization their synthesis is stopped, it is expected to achieve maximal infectivity and minimal toxicity (Wang et al. 2013). Kong et al. engineered a *Salmonella* strain where LPS O- antigen synthesis genes *rfc* and *rfaH* are kept under the control of the promoter *araC*-P_{BAD}, which is tightly regulated by arabinose. This strain is highly attenuated nevertheless exhibits superior immunogenicity (Kong et al. 2009, 2010). Another approach of detoxification of *Salmonella* LPS included removal of 1-phosphate group from lipid A of LPS. Kong et al. introduced an inner membrane phosphatase LpxE from *F. tularensis*, which can selectively remove the 1-phosphate group from *Salmonella* lipid A. The resultant LPS had reduced toxicity while preserved adjuvant activity(Kong et al. 2011). See Wang et al. for a detailed review on LPS modifications in *Salmonella*-based LABV (Wang et al. 2013).

2.6.4 Optimization of Virulence: Control of Safety and Immunogenicity

Many methods employed for attenuation, although make the LABV strains less pathogenic and safe to administer at high doses, it often renders them poorly immunogenic due to their inability to circumvent physicochemical defense of the host. Moreover, inability of penetration through mucosal barrier also makes them poorly immunogenic. To circumvent this problem, Curtiss et al. generated a regulated delayed attenuation system (RDAS), which retains full virulence till the passage through gastrointestinal tract and infection of epithelial cells. In the modified RDAS strains, *Salmonella* virulence genes *fur*, PhoP/Q, *rpoS*, and *crp* are expressed under the control of *araC*-P_{BAD} promoter. Arabinose concentration in human tissues is very less. Thus, in vitro these strains express all the virulence genes in medium supplemented with arabinose, whereas in vivo under the arabinose deprivation, many virulence genes are suppressed, resulting in attenuation of *Salmonella*. This approach results in high immunogenicity combined with tolerance at high doses (Curtiss 3rd et al. 2009).

2.6.5 Cytosolic Delivery of Antigen

In order to evoke $CD8^+$ T-cell (CTL) response, antigens need to be delivered into the cytoplasm of host cell. Various approaches are in use to accomplish the cytosolic delivery of antigens including the use of a type III secretion system that can directly deliver vaccine antigens into the host cell cytoplasm and use of a-hemolysin (HlyA) secretion system of *E. coli* which is fully active in *Salmonella* (Gentschev et al. 1996). On the other hand, escape from endocytic vacuoles is also a feasible approach. Unlike *Lm*, *ST* and *STm* do not reach cytoplasm of infected cells and elicit $CD4^+$ T-cell response more effectively compared to $CD8^+$ T-cell response to cognate

antigens. Chen et al. used secretion signal of a type III secretion system *Salmonella* outer protein E (SopE) and HlyA (secretion signal) to deliver *S. japonicum* antigen Sj23-LHD-GST. The *Salmonella* vaccine constructs carrying Sj23 LHD-GST fused to HlyA (secretion signal) or SopE effectively expressed and delivered antigens into cytoplasm of murine macrophages in vitro. This vaccine construct induced Sj23-LHD-GST-specific Th1 type response and protected against *S. japonicum* infection (Chen et al. 2011). Gentschev et al. reported that two Listerial antigens delivered by *STm* using HlyA (secretion signal) generated protection against *Listeria* infection (Gentschev et al. 1996). Simultaneous delivery of two Listerial antigens (LLO and p60) by *STm* using *Yersinia* outer protein E (YopE) as a carrier molecule for *Salmonella* type III secretion system developed LLO- and p60-specific T cells and protection against murine listeriosis (Igwe et al. 2002). SopE-mediated delivery of Listerial antigen p60 generated $CD8^+$ T-cell-mediated protection against *Listeria* infection (Berchtold et al. 2009).

2.7 Conclusion

What makes bacteria an excellent vaccine delivery vehicle is their natural ability to induce potent and long-lasting immune response. LABVs possess the capacity to induce humoral as well as cell-mediated immune response. While the humoral immune response includes serum IgG and mucosal IgG and IgA, the cell-mediated immunity is characterized by Th1-, Th2-, and Th17-type CD4+ T cells and CD8+ CTLs. IgA and IL17 have been specifically implicated in mucosal protection against various mucosal pathogens. The cell-mediated immunity is required for intracellular pathogens. It should be noted that subunit vaccines have a poor capacity to evoke mucosal as well as cell-mediated immunity. LABVs have also shown the capacity to overcome immunosuppressive nature of various forms of tumors. These characteristic of LABVs, together with their tumor-tropic capacity, makes them a highly suitable vector for cancer immunotherapeutic vaccines. In the past two decades, tremendous progress has been made regarding LABV-mediated delivery of vaccine antigens for prevention of a variety of viral, bacterial, and parasitic diseases. Recent advances have further improved the safety and immunogenicity profile of several LABV platforms. The new-generation LABVs can withstand harsh physicochemical conditions of gastrointestinal tract, exhibit regulated attenuation, regulated antigen expression, and targeted antigen delivery. LABVs have exhibited effectiveness in various preclinical and preliminary clinical trials (Table 2.1). However, a limited number of clinical trials have been conducted to date using LABVs, due to potential safety concerns. Further optimization would result in a versatile, safe, and highly immunogenic vaccine delivery platforms.

Table 2.1 Examples of vaccines delivered by live-attenuated bacterial vectors

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
Viral pathogens					
BCG		Hepatitis B	Surface antigens	Antibodies	Rezende et al. (2005)
BCG		HIV	SIV-Gag and CD8+ T-cell epitopes	CD 8+ T cell	Venkataswamy et al. (2014) and Mahant et al. (2017)
BCG		HIV and SIV	gp120, Gag	T cells	Hart et al. (2015)
L. lactis		HPV-16-E7	LL-E7	Th1 immune response	Almeida et al. (2016)
L. lactis		H1N1	HA	IgA, Antibodies	Joan et al. (2016)
L. lactis		H5N1	HA	IgA	Bobek et al. (2010)
Lm	Δ*act*A/	H1N1	NP	Th1	Johnson et al. (2011)
	Δ*plc*Band Δ*act*A/				
	Δ*inl*B				
Lm	Δ*dal*, Δ*dat*	HIV	Gag, gp160	CD8+, CTL, nAb	Frankel et al. (1995), Friedman et al. (2000), Rayevskaya and Frankel (2001), Rayevskaya et al. (2002), Jiang et al. (2007) and Lakhashe et al. (2011)
Lm	Δ*act*A, Δ*plc*B	HPV17	E7	CTL	Jia et al. (2012)
Lm	Δ*act*A	LCMV	NP118-126	CD8+ T cells	Tvinnereim et al. (2002)
Lm	Δ*dal*, Δ*dat*	SIV	Gag	CD8+, Cellular immune response	Sciaranghella et al. (2011) and Im et al. (2013)
STm	Δ*aro*A	Dengue virus	NS3-MisL	CTL	Luria-Perez et al. (2007)
STm	Δ*cpx*R, Δ*lon*, Δ*asd* and Δ*wba*P	H1N1	HA and M2e	IgG1 and IgG2a and Th1 cell response	Hajam and Lee (2017)
STm	Δ*aro*A	H5N1	HA, NA, NP	IgG and mucosal IgA and gamma-producing T cells	Ashraf et al. (2011) and Pei et al. (2015)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*aro*C	HIV-1	10- E8, Gag	Antibody, CTL	Karpenko et al. (2004), Chin'ombe and Ruhanya (2013), Li et al. (2016)
STm	Δ*aro*A	Measles virus	B-cell and T-cell epitopes	IgG	Spreng et al. (2000)
ST	Δ*pil*S	SARS	Nucleocapsid protein	IgG2a and IgA	Luo et al. (2007)
STm	Δ*aro*A	TGEV	N gene, C and A epitopes	IgG	Chen and Schifferli (2003, 2007) and Zhang et al. (2016a)
Bacterial pathogens					
BCG		*B. pertussis*	Pertussis toxin Subunit S1	Th1	Nascimento et al. (2008, 2009)
BCG		*Lm*	p60 Ag	CD4 and CD8 T cell	Grode et al. (2002)
BCG		*Mtb*	Ag 85B	IL 17A T cells	Hatano et al. (2016)
BCG		*B. pertussis*, tetanus, *Mtb*	Pertussis-tetanus toxin fusion	Humoral and cellular	Abomoelak et al. (1999)
BCG		*S. pneumoniae*	PspA	IL-17A and IFNg	Goulart et al. (2017)
B. subtilis		ETEC	CfaB	Sera and mucosal Ab	Amuguni and Tzipori (2012)
B. subtilis		*H. pylori*	Urease B	IgG, IgA, Th1/Th17	Stasilojc et al. (2015) and Zhou et al. (2015)
B. subtilis		*Mtb*	MPT64	Th1	Sibley et al. (2014)
B. subtilis		Tetanus	TT C fragment	IgG, IgA	Amuguni et al. (2011)
E. coli	Δ*intimin*, Δ*stx*1 and Δ*stx*2	ETEC	CFA-I, LThK63	IgG and IgA	Byrd and Boedeker (2013)
E. coli	Δ*ler*	EHEC	Stx1B	Antibody	Zhu et al. (2006)
L. casei		*C. perfringens*	Epsilon	IgG, IgA	Alimolaei et al. (2016)
L. lactis		*C. jejuni*	cjAD	Antibody	Kobierecka et al. (2016)
L. lactis		*C. difficile*	TETC-TcdA	Antibodies	Yang et al. (2013)
L. lactis		*H. pylori*	Omp22 or HpaA, cag12, urease B	Antibody	Gu et al. (2009), Kim et al. (2009), Li et al. (2014), and Zhang et al. (2016b)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
L. lactis		*L. monocytogenes*	Listeriolysin O	CD8+ T cells	Bahey-El-Din et al. (2008)
L. lactis		*S. pyogenes*	M protein (CRR)	Mucosal IgA	Mannam et al. (2004) and Mannam et al. (2004)
L. lactis		*V. cholera*	Wzm	IgG and IgA	Zamri et al. (2012)
Lm	Δ*act*A, Δ*inl*B	*C. burnetii*	T4SS (Epitopes)	CD8+ T cells	Xiong et al. (2017)
ST	Δ*aro*C, Δ*aro*D, and Δ*htr*A	*B. anthracis*	PA83, PAd4	IgG, Ifng	Galen et al. (2004, 2010)
STm	Δ*aro*A	*B. pertussis*	Pertussis toxins S1, S2, S3, S4, and S5	IgG	Dalla Pozza et al. (1998)
STm	Δ*lon*, Δ*cpx*R	*B. abortus*	BCSP31, Omp3b, and SOD	IgG and sIgA	Kim et al. (2016) and Lalsiamthara and Lee (2017)
STm	Δ*aro*C, Δ*aro*D and Δ*htr*A	EHEC O157:H7	Intimin, CFA-I, CS3, STx2	Mucosal IgG and IgA	Girón et al. (1995), Rojas et al. (2010) and Ferreira Oliveira et al. (2012)
STm	ΔPhoP/Q	*H. pylori*	Urease B, hpaA, adhesin AB, babA2/ ureI, CagA, and VacA	Antibodies	Angelakopoulos and Hohmann (2000), Bai et al. (2004), Xu et al. (2005) and Liu et al. (2011)
STm	Δ*aro*A/ Δ*spt*P	*L. monocytogenes*	Listeriolysin and p60	CD8+ T cell, IFNg	Igwe et al. (2002), Sevil Domènech et al. (2008) and Berchtold et al. (2009)
STm	ΔaroA	*Mtb*	ESAT6-Ag85B	T cell, IFNg	Wang et al. (2009)
STm	ΔaroC, Δ*aro*D, and Δ*htr*A	*P. aeruginosa*	OprF/OprI, LPS	Antibodies and TH1, mucosal IgG, and IgA	Arnold et al. (2004), Digiandomenico et al. (2004) and Bumann et al. (2010)
STm	Δ*cya* and Δ*crp*	*P. gingivalis*	HagB	IgG and IgA and mucosal IgA	Isoda et al. (2007) and Pathangey et al. (2009)
STm	Δ*gal*E	*S. dysenteriae*	LPS	Serum antibody	Dharmasena et al. (2016b)
STm	Δ*gal*E	*S. sonnei*	LPS	Serum antibody	Dharmasena et al. (2013)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*crp* and Δ*asd*A	*S. pneumoniae*	PspA, PspC	Th1/Th2, IgG, and IgA	Kang et al. (2002), Xin et al. (2009), Shi et al. (2010), Wang et al. (2010, 2011), Kong et al. (2011) and Frey et al. (2013)
ST	Δ*aro*C, Δ*aro*D, and Δ*htr*A	Tetanus	TetC	IgG, IgA	Dunstan et al. (1999), Allen et al. (2000), Orr et al. (2001) and Capozzo et al. (2004)
STm	Δ*aro*A	*Y. enterocolitica*	HSP-60	T cell, IFNg	Kramer et al. (2003)
STm	ΔPhoP/Q	*Y. pestis*	F1, V, YadC, YadBC, PsaA, LcrV, Psn, and HmuR	IgG, CD4, and CD8+ T cells	Ramirez et al. (2009), Branger et al. (2010), Torres-Escobar et al. (2010), Sizemore et al. (2012), Sun et al. (2014) and Galen et al. (2015)
S. flexneri	Δ*gua*BA	ETEC	CFA-I and LTB, CS2, CS3, CS4, and CFA/A	Serum IgG and mucosal IgA	Koprowski et al. (2000), Barry et al. (2003), Ranallo et al. (2005) and Zheng et al. (2005)
V. cholerae	Δ*ctx*	*C. difficile*	TcdA	IgG	Ryan et al. (1997)
V. cholerae	Δ*att*RS1	*E. coli*	LT	Serum IgG and mucosal IgA	Ryan et al. (1999)
V. cholerae	ΔCTA	*H. pylori*	HpaA	IgG	Tobias et al. (2017)
V. cholerae	Naturally attenuated	Tetanus toxin, *B. pertussis*	TetC, BP-TCF	IgG	Chen et al. (1998)
Parasitic pathogens					
B. subtilis		*C. sinensis*	Enolase	Mucosal IgG and IgA	Yu et al. (2015)
B. subtilis		*S. japonicum*	GST protein	Mucosal IgG and IgA	Li et al. (2009)
E. coli	Δ*ler*	Malaria	NANP	Antibody	Zhu et al. (2006)
Lm	Δ*act*A	*L. major*	LJM11, p36, and LACK	Th1	Soussi et al. (2002), Saklani-Jusforgues et al. (2003) and Abi Abdallah et al. (2014)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*pmi*, Δ*fur* and Δ*crp*	*G. lamblia*	α1-Giardin, CWP2	Th1/Th2, mucosal IgG, and IgA	Abdul-Wahid and Faubert (2007) and Jenikova et al. (2011)
STm	Δ*aro*C, Δ*aro*D, and Δ*htr*A	*C. parvum*	Cp23, CP15, and Cp40	Antibody	Benitez et al. (2009) and Roche et al. (2013)
STm	Δ*aro*A	*E. histolytica*	Gal/Gal-NAC lectin	Antibody	Mann et al. (1997)
ST	Δ*aro*C and Δ*aro*D	*L. mexicana*	gp63	Th1 type immunity	González et al. (1998)
STm	Δ*pur*l and Δ*msb*B	*S. japonicum*	Sj23LHD-GST	Th1 type immunity	Chen et al. (2011)
STm	Δ*crp* and Δ*cya*	*T. solium*	TSOL18	CD4 and CD8 T cells	Ding et al. (2013)

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