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# Engineering Bacterial Vectors for Delivery of Genes and Proteins to Antigen-Presenting Cells

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**Abstract:** Bacterial vectors offer a biological route to gene and protein delivery with this article featuring delivery to antigen-presenting cells (APCs). Primarily in the context of immune stimulation against infectious disease or cancer, the goal of bacterially mediated delivery is to overcome the hurdles to effective macromolecule delivery. This review will present several bacterial vectors as macromolecule (protein or gene) delivery devices with both innate and acquirable (or engineered) biological features to facilitate delivery to APCs. The review will also present topics related to large-scale manufacture, storage, and distribution that must be considered if the bacterial delivery devices are ever to be used in a global market.

**Keywords:** Gene and protein delivery; *Salmonella*; *Escherichia coli*; *Shigella*; *Listeria*; antigen-presenting cells

## 1. Introduction

Bacterial vectors represent an alternative approach to mammalian cell gene and protein delivery. However, the concept is very similar to efforts using viral or polymeric carriers (as highlighted in other articles within this issue) with the bacterial cell substituted as the protein or gene delivery device. This article will focus on candidate bacterial delivery systems, their inherent or modifiable properties that influence cellular delivery potency, vector manufacturing and distribution, and the specific application of antigen-presenting cell (APC) delivery for immune modulation.

To begin, it is important to establish the role of the bacterial cell in gene and protein delivery. In this review, the bacterial cell is viewed as a delivery device, one that carries innate and adjustable properties for improving delivery. For example, though bacterial vectors may have natural properties that aid in APC targeting, uptake, and intracellular delivery, several experimental tools (primarily driven by molecular biology) exist to further influence the antigen delivery process. These tools provide precision and flexibility when one is trying to engineer biological vectors for improved gene and protein delivery and offer a powerful biological engineering approach to improving bacterial vectors during immune-modulating delivery efforts.

The ability to influence the delivery process becomes important at several stages of protein or gene delivery. The bacterial cell offers opportunities to aid or improve the final delivery effort by addressing the individual steps along the way to final intracellular localization. The first issue is one of protein or gene packaging. How can the bacterial cell concentrate and package a macromolecule for cellular delivery? Second, the cargo and delivery device must enter the mammalian cell. How can the relatively large bacterial delivery device overcome this obstacle? Once inside the cell, the bacterial delivery device must release its cargo for intracellular delivery and trafficking to the correct final location, typically either the cellular cytosol or nucleus. Are there ways to overcome intracellular delivery barriers through biological engineering of the bacterial carrier? Each of these steps will be addressed for the specific bacterial vectors described below.

It is also important to specify the physical, chemical, and biological properties that natively influence bacterially mediated gene and protein delivery. First, the bacterial cell is typically confined to spherical or cylindrical shapes between 1 and 5 $\mu$m. In one regard, this size limits mammalian cell

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delivery applications to antigen-presenting cells or other professional phagocytic cells; alternatively, the size of a typical bacterial vector then innately targets these same phagocytic cells, aiding efforts in antigen protein or gene delivery.[1] As one might expect, bacterial cells naturally possess biological components that activate APCs. Gram-negative cells contain lipopolysaccharide (LPS), whereas Gram-positive cells possess lipoteichoic acid. These and other features (CpG motifs[2−4] and peptidoglycan[5,6]) naturally influence bacterial uptake and activation by APCs. Such properties have the potential then to act as natural adjuvants that beneficially improve APC uptake and the overall immune response.[7−9] In addition, the simple growth properties and storage options associated with bacteria offer new opportunities for vector manufacture, allotment, and storage. Finally, the ever-expanding molecular toolbox associated with bacterial organisms opens the possibility of rapidly improving protein and gene delivery through molecular engineering efforts aimed at improving intracellular delivery of bacterial cargo.

The biological plasticity of bacterial vectors will be highlighted throughout this article as well as their manipulation for the improvement of gene and protein delivery, at both the cellular and manufacturing scales. Also highlighted will be the range of bacterial hosts available, their historical development as delivery vectors, and the advantages each provides. Overall, the goal of the article will be to examine the types, tools, and potential of bacterial delivery for immune-modulation applications.

## 2. Target Cells: APCs

The bacterial vectors described seek to stimulate a robust immune response by targeting APCs. In so doing, there is an implicit goal of influencing antigen delivery and immune response through vector engineering. Vector engineering will take place in vitro; however, the benefits of this engineering will generally occur in vivo. Toward this end, the delivery route for bacterial vectors will be explained from administration to the body to interactions with APCs.

Before direct in vivo APC interaction, the bacterial device must be delivered to the body. Introduction of the bacterial vectors must take into account the biological nature of the vector and the likelihood of initiating contact between the vectors and APCs. In addition, safety precautions must be considered when a foreign biological organism is added to the body. The foreign and/or pathogenic nature of the bacterial cell can trigger potentially deadly immune reactions. Pathogenic bacteria naturally possess endotoxins, exotoxins, or both that can lead to maladies ranging from mild cases of food poisoning (and associated vomiting, diarrhea, and fever) to potentially deadly septic shock. While exotoxin activity [derived from toxins such as shiga toxin (*Shigella dysenteriae*), cholera toxin (*Vibrio cholera*), and anthrax toxin (*Bacillus anthracis*)] can be avoided by choosing strains lacking the particular toxin, endotoxins are often intrinsic components of the bacterial cell. As mentioned, key endotoxins include Gram-negative LPS and Gram-positive lipoteichoic acid. These and other bacterial cell components, when present at sufficiently high concentrations, set off a series of humoral and cellular immune responses. The humoral response includes recognition by antibodies, complement, and acute-phase proteins.[10] In addition, macrophage and dendritic cells, perhaps aided by the humoral recognition, will also converge upon and consume the bacterial cells.

The bacterial delivery vector will then naturally elicit an immune response. The natural immune reaction can be beneficial as the bacteria will have a built-in adjuvant effect.[11] The key is to match the correct (and safe) dosage, vector, and adjuvant response with the desired immune reaction. Typically, a bacterial infection will innately lead to APC cytokine (TNF-α, IL-1, IL-6) release[12] and attract other APCs

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and polymorphonuclear leukocytes to the site of infection.[13] Surrounding tissue is at risk during the digestion of the foreign bacteria as APCs and polymorphonuclear leucocytes also release microcidal agents (lysozyme, bacterialcidal/permeability-increasing protein, acid hydrolases, nitric oxide, and oxygen free radicals) that can damage nearby endothelial cells.[14−17] Potential untoward side effects (resulting from cytokine release) might include fever, capillary leakage, and myocardial depression, and, at the extreme, multiple-organ dysfunction, shock, and death. However, again, tempering these extreme reactions while delivering a specific antigen is the goal of bacterially mediated immunomodulation.

Concerns over adverse reactions to the bacterial vectors can be lessened through modifications made to the bacteria or the form of administration. At one extreme, the bacteria can be killed prior to in vivo addition. *Escherichia coli* cells killed with paraformaldehyde fixation were still very potent at offering antitumor protection.[18] Other methods for limiting bacterial toxicity include genetic manipulations to attenuate the bacterial vectors. Examples of such manipulations will be detailed below according to the specific bacterial vectors profiled. Still, another alternative is to load the bacterial vectors ex vivo.[18−20] Here, APCs such as dendritic cells are harvested, exposed to the bacterial vectors carrying the antigen of choice, and then reintroduced in vivo to elicit a response to the antigen. Since numerous studies have shown the in vitro capability of bacterial vectors to prime APCs,[21−25] ex vivo loading is a logical answer to concerns over in vivo bacterial administration. Drawbacks include the invasive and tedious nature of harvesting dendritic cells for antigen loading and subsequent reintroduction to the body.[26] However, the method appears to require only small numbers of antigen-loaded dendritic cells to be effective and would appear to overcome immune tolerance precipitated by certain diseases (such as cancer).[27,28]

Recently, other attempts to combat undesired side effects involve the use of bacterial minicells for DNA and protein delivery. Minicells are produced due to premature septation by most bacterial species and are typically 100−400 nm achromosomal vesicles that do not divide. Prior efforts have used minicells to effectively deliver both protein and DNA-encoded antigens to APCs. Though more work has to be done to generate and purify the minicells, they offer a relatively new (and potentially safer) approach to APC antigen delivery.[29]

However, many studies focus on the in vivo administration of bacterial vectors, and in this context, the location of vector administration should maximize the chance of APC encounter. Primary locations include mucosal surfaces, under the skin, and other areas likely to be faced with foreign antigen entry.[1] The most typical delivery routes include oral,[9,30−32] intranasal,[33] intramuscular,[34,35] subcutaneous,[34] intraperito-


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neal,[34,36,37] and intravenous.[36] Of these delivery routes, oral administration is common and offers the advantages of simple, noninvasive administration and, therefore, better potential patient compliance.

Once the vector is properly introduced into the body, the next desired step is APC interaction. Before describing specific interactions between bacteria and APCs, we will present a broad overview of APC−bacterial interactions with an emphasis on bacterial delivery of protein or genetic antigens.

A foreign bacterial cell has the potential to elicit either a humoral (antibody) or cell-mediated immune response. The difference often comes down to the bacterial "dosage", delivery route, and bacterial type. While a distinct possibility is the generation of antibodies against the bacterial carrier, the real objective is to generate antibodies against the protein or genetic antigen carried by the bacterial cell, if a humoral response is the primary objective. Alternatively, the objective may be to generate and influence the cell-mediated response to the delivered antigen.

APCs such as B cells, macrophages, and dendritic cells are considered professional APCs and stand on the border between innate and adaptive immunity. In particular, macrophage and dendritic cells patrol sections of the body prone to bacterial invasion (skin, mucous membranes, and GI tract). Once the cells recognize a bacterial cell, often triggered by interactions between key features (i.e., LPS, peptidoglycan, and lipoteichoic acid) of the bacteria and surface receptors on APCs, the bacteria are engulfed by phagocytosis or endocytosis. These essential features of pathogens are recognized by transmembrane pattern recognition receptor (PRR) proteins on the surface of APCs called toll-like receptors (TLRs). TLR-4 elicits an immune response upon recognition of LPS in Gram-negative bacteria, whereas components of Gram-positive bacteria are recognized by TLR-2. Furthermore, the engagement of TLRs strengthens the phagocytic ability of APCs as well as activating macrophages and dendritic cells, resulting in stronger antigen presentation to subsequent immune cells. APC activation by TLRs is commonly done through a complex signal transduction pathway, initiated when microbial components bind to the extracellular portion of TLRs.[38] Parenthetically, bacterial cells such as *Yersinia*, *Listeria*, *Salmonella*, and *Shigella* employ invasion or secretory systems to induce uptake by nonphagocytic cells such as epithelial cells.[39]

Once inside the cell, the bacteria and the internal protein or encoded antigen are processed as depicted in Figure 1. Two separate presentation routes exist for the delivered antigens, leading to production of antibodies by B cells or the activation of T-cells programmed to locate and kill any other cells that present the delivered antigen.

Given this background, an underlying theme of APC delivery is to influence the type of antigen presentation (and hence the type of immune response) as well as the overall antigen presentation efficacy. Bacterial vectors are in a good position to influence antigen presentation through the delivery of proteins (exogenous antigens) or DNA (leading to endogenous antigens). The end goal is a directed immune assault on the bacterially delivered antigens as a means of attacking the current presence of such antigens in the body or to act as a prophylactic method of preempting antigen introduction (i.e., vaccination). What's more, the bacterial cell itself offers opportunities to improve upon delivery using biological engineering to influence everything from immune response to the number and type of antigens delivered. Other papers in this issue will be dedicated to specific applications, but two of the most obvious are for cancer and infectious disease.

## 3. Bacterial Choices for Delivery

Detailed below are bacterial choices for gene and protein delivery. Early efforts provide a historical backdrop for continued efforts with new bacterial vectors. Each vector will be profiled with emphasis placed on innate delivery advantages and use in APC delivery.

**A. First Reports.** The first acknowledged transfer of genetic material from bacterial to mammalian cells involved the transfer of plasmid-encoded simian virus 40 DNA from *E. coli* to CV-1 monkey cells.[40] The work was groundbreaking but yielded low gene transfer success rates at 1 per $10^7$

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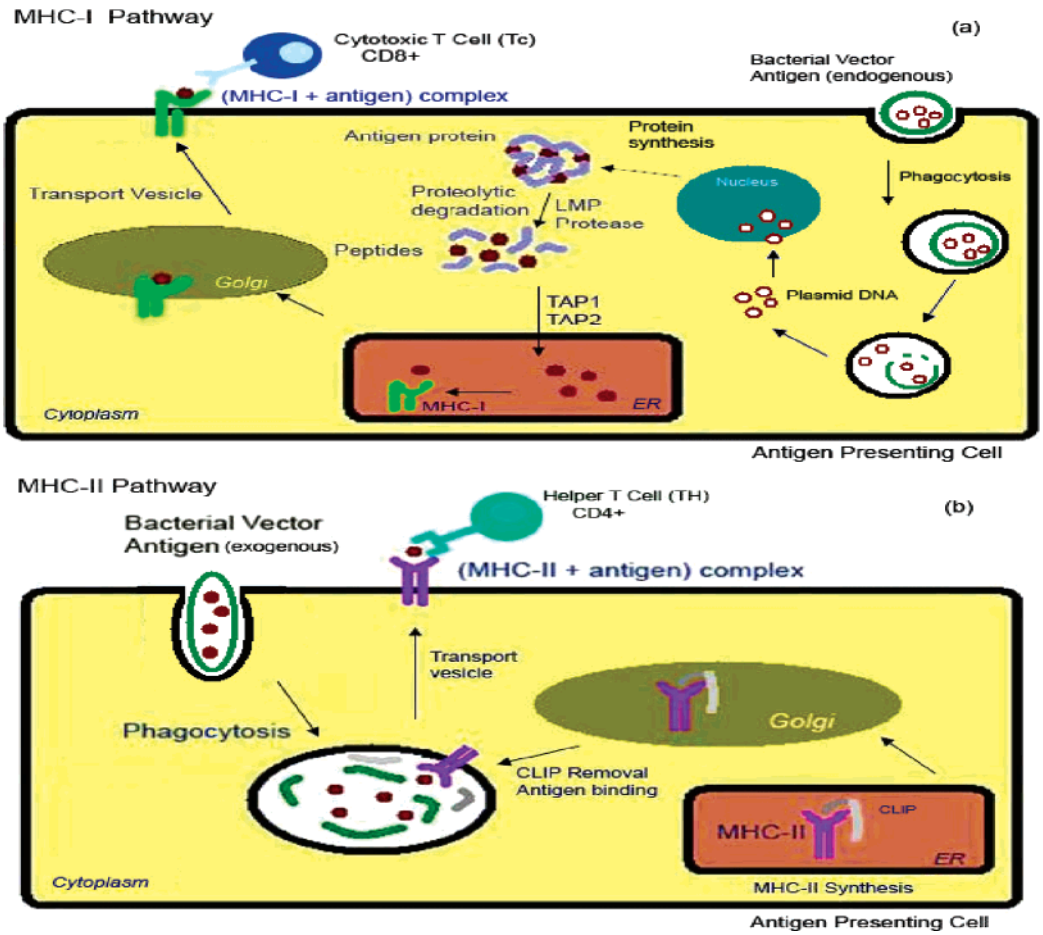


***Figure 1.*** Processing of bacterial vectors by APCs. (a) Encoded antigens are delivered for nuclear antigen production (termed endogenous antigens) and are recognized and presented via major histocompatibility complex I (MHC-I) to illicit a cytotoxic T-cell ($T_c$, CD8+) response. (b) In a second pathway, processing of exogenous protein antigens delivered by bacterial vectors and destined for MHC-II presentation for helper T-cell ($T_H$, CD4+) activation.

***Table 1.*** Common Bacterial Delivery Vectors

| bacteria | morphology/size[a] | Gram-positive or Gram-negative | key natural delivery features[b] |
|---|---|---|---|
| *E. coli* | rod-shaped/1.1−1.5 $\mu$m × 2−6 $\mu$m | Gram-negative | LPS which triggers uptake by APCs |
| *Salmonella* | rod-shaped/0.7−1.5 $\mu$m × 2−5 $\mu$m | Gram-negative | LPS; APC uptake and phagosomal survival |
| *Shigella* | rod-shaped/0.4−0.6 $\mu$m × 1−3 $\mu$m | Gram-negative | LPS; APC uptake and phagosomal escape to cytosol |
| *Listeria* | rod-shaped (rounded ends that can approach coccoidal shape)/0.4−0.5 $\mu$m × 0.5−2 $\mu$m | Gram-positive | phagosomal escape to cytosol |

[a] Size data and other general features taken from *Bergey's Manual of Determinative Bacteriology*, 9th ed.; Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., Williams, S. T., Hensyl, W. R., Eds.; Williams & Wilkins: Philadelphia, 1994. [b] This does not imply that these are the only features needed for APC delivery, but those presented represent key physical or biological features associated with the given bacterial vectors.

monkey cells. Still the work introduced the notion, a first bacterial vector (*E. coli*), and a baseline for future improvement.

The success also introduces the first bacterial vector to be profiled, *E. coli*. Table 1 highlights certain physical and biological features of *E. coli*. The bacterium is perhaps most famous for being the workhorse of biotechnology, a feature closely linked to the cell's rapid growth rate and simple growth requirements. These same features have prompted a comprehensive analysis of the organism.41 Since the initial efforts with gene transfer to mammalian cells, numerous molecular biology tools for optimizing the cell further for gene and protein delivery have been developed (with specific case examples presented in a subsequent section).

The use of *E. coli* cells for delivery of genes and proteins to APCs is not as well-developed as several other bacterial

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types to be presented. The primary reason is that most nonpathogenic *E. coli* lines lack dedicated mechanisms of cell entry (most likely leading to the relatively low transfection efficiency observed in early reports). However, several cases have been reported for nonpathogenic *E. coli* cells used as delivery vectors. For each case cited, experiments confirmed successful delivery to macrophage or dendritic cells.[18,24,25,35,42−44] However, as will be detailed later, many more efforts have focused on conferring recombinant properties upon *E. coli* to enhance cellular entry and delivery.

**B. Common Bacterial Options.** Though *E. coli* represented the first successful bacterial-to-mammalian delivery vector, several other candidates emerged thereafter. One of the common links between these other vectors is their pathogenic nature. Many pathogens have an evolutionary advantage with regard to cellular entry and intracellular survival. These traits are advantageous during bacterially mediated delivery schemes. However, we must account for the pathogenic nature if the vectors are to be used safely in a therapeutic setting.

**i. *Salmonella*.** *Salmonella* are perhaps the most widely used bacteria for antigen delivery applications (Table 1). In large part, this stems from their pathogen capability for preferentially gaining entrance to APCs and surviving within intracellular phagosomes. *Salmonella* strains hold the additional benefit of being readily modified genetically, which has been used to weaken their native pathogen effects and influence subsequent steps in the cellular delivery process.

*Salmonella* are capable of triggering cellular uptake that especially targets APCs.[22,45,46] *Salmonella* strains release a set of invasion proteins post-contact with host APC cells; the invasion proteins help facilitate the uptake of the bacteria.[47,48] Once inside the phagosome, the bacteria evade cellular mechanisms of intracellular defense (by limiting or delaying exposure to NADPH oxidase and lysosome fusion[49,50]), replicate, and thrive as pathogens safe from extracellular antibiotic therapy. These qualities innately aid protein and gene delivery mediated by the bacterium. However, the bacteria are often modified to attenuate pathogenicity and/or improve cytosolic antigen delivery.[51−53] Modified strains include *phoBQ*, *purB*, *aroA*, *purI*, and *msbB* mutants with the *aroA*, *purI*, and *purB* mutations leading to defects in aromatic amino acid and purine biosynthesis and the *phoBQ* and *msbB* mutations causing improved lysosomal fusion and altered LPS biosynthesis, respectively. The strategy has proven to be effective for the cytosolic delivery of protein and genetic antigens.[9,22,31,54−59] However, though

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successfully used, the attenuated strains must not be so compromised that an immune response is limited altogether.[51]

Modified *Salmonella* strains have proven to be effective in several clinical examples.[60−64] Numerous studies have shown the effectiveness of *Salmonella* carriers able to elicit humoral and cytotoxic T-cell responses against infectious diseases and cancer.[9,55,65−74] In addition, these clinical successes have spurred products and companies. AVANT Immunotherapeutics Inc., Berna Biotech, and Microsciences Ltd. have produced *Salmonella*-based products.[72,75−77]

**ii. *Shigella*.** Like *Salmonella*, *Shigella* bacteria thrive as delivery vehicles because of their pathogen properties. *Shigella* also release invasion proteins that spark cellular uptake by mammalian cells.[47,48] Unlike *Salmonella*, however, *Shigella* are adept at breaking free of the phagosome and making their way to the cytosol for replication. Hence, the bacteria have built-in mechanisms for overcoming the first few cellular barriers to intracellular cytosolic antigen delivery and, because of this, are particularly adept at generating cellular immune responses.

However, again, native *Shigella* pose the danger of pathogenicity. To address this, several attenuated mutants have been used to temper pathogenic properties and boost therapeutic ones. Mutations in cell wall biosynthesis (*dap*-, *asd*), LPS formation (*rfbF*), amino acid synthesis (*aroA*), cell spreading (*iscA*), guanine biosynthesis (*guaBA*), and enterotoxins (*sen*, *set*) have produced attenuated *Shigella* strains used in therapeutic settings.[33,78−91] These include applications in both


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gene and protein delivery for infectious disease and cancer applications.

**iii. *Listeria*.** *Listeria* is the third commonly used bacterial pathogen for antigen delivery purposes. The bacteria share features with *Shigella*, including the ability to escape the phagosome and spread from cell to cell.[92] One notable difference is the Gram-positive nature of *Listeria* and the subsequent lack of LPS. A key to the *Listeria* cell's escape from the phagosome and entrance into the cytosol is the production of listeriolysin O, a pore-forming protein that disrupts the phagosomal membrane.[93,94] Another advantage of *Listeria* is the mild apoptotic effects the cell has on APCs.[24] *Shigella* and *Salmonella* induce APC apoptosis,[95] and though this may not limit them as antigen delivery vectors, the milder effects elicited by *Listeria* may give this vector an advantage.

As with other pathogenic bacteria, *Listeria* must be attenuated for therapeutic use. Attenuating mutations include *actA* (actin nucleator), *prfA* (positive regulatory factor), *plcB* (phospholipase B), *dal* (alanine racemase), *dat* (D-amino acid aminotransferase), *inlB* (nonphagocytic cell invasion), and *aroA/B/E* (aromatic amino acid synthesis).[23,96] Such strains have proven to be effective in both infectious disease and cancer antigen delivery applications.[97−112] Perhaps because of the organism's advanced cytosolic delivery capabilities,


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*Listeria* has been able to elicit strong CD8+ T-cell responses.[98,109,113,114]

**iv. Others.** Besides the major examples that are highlighted, other bacterial systems have also been used for antigen delivery. These systems include *V. cholera*,[115−117] *Yersinia pseudotuberculosis*,[118] *Yersinia enterocolitica*,[119−121] *Bordatella bronchiseptica* and *pertussis*,[122] *Mycobacterium bovis* (Bacille Calmette-Guerin, the oldest and most widely used bacterial vaccine),[123,124] *Streptococcus gordonii*,[125] *Lactococcus* and *Lactobacillus*,[126,127] *Brucella abortus*,[128] *Erysipelothrix rhusiopathiae*,[129] and *Bacillus* strains.[130]

## 4. Efforts Directed toward Understanding and Improving Delivery: Cellular Engineering

The bacteria noted above (in particular, *Shigella*, *Salmonella*, and *Listeria*) all have natural antigen delivery properties, primarily because of their pathogenic nature. Attenuating the pathogen properties provides the balance between strong antigen delivery and therapeutic application (as opposed to a negative effect). In a sense, attenuating pathogenic antigen delivery bacteria provides improvement by subtraction. The


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second method of improvement involves the recombinant addition of antigen delivery capabilities.

Perhaps the best-studied case is that of listeriolysin O (LLO). Listeriolysin O is found in *Listeria monocytogenes* and is one of the causative agents in the intracellular survival of this pathogen.[131] It was quickly recognized that listeriolysin could play an important role in directed efforts of cellular delivery.[132,133]

Listeriolysin O works by weakening phagosomal membranes, thereby allowing *L. monocytogenes* to escape the phagosome and enter and propagate within the mammalian cytoplasm. The protein forms pores in phagosomal membranes and is optimally active at phagosomal pH values (∼5.5).[134] Hence, LLO provides the mechanism for phagosomal escape and bacterial entrance and delivery to the cytosol.

Another protein that has aided efforts directed at bacterially mediated gene and protein delivery is the invasin protein from *Y. pseudotuberculosis*.[135] Invasin allows *Y. pseudotuberculosis* to gain entrance to mammalian cells during pathogenesis. The invasin protein was first isolated by Isberg and Falkow and appears to function by binding to cell surface integrins which then signal cytoskeletal rearrangement and phagocytosis.[136] Other pathogenic bacteria candidates for bacterial delivery vectors also possess mechanisms of cell entry. For example, *Listeria* produces surface-associated proteins internalin A and B (InlA/B), which help facilitate nonprofessional phagocytic cellular uptake.[137,138] In the case of *Salmonella* and *Shigella*, SipB/C and IpaB/C are the invasion proteins facilitating cell entry through the type III secretory system (TTSS).[39,139,140]

As mentioned, besides revealing basic mechanisms for cell entry and phagosomal escape, the action of invasin and listeriolysin has been used to manipulate bacterial gene and protein delivery.[18,24,42,43,141−143] One of the first reports of this was the work of Grillot-Courvalin and co-workers.[144] In this

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context, the invasin and lysteriolysin proteins were engineered within *E. coli* to facilitate the entry and gene delivery to several mammalian cell lines. The importance of this work was the first steps toward engineering and improving bacterially medicated delivery with hosts that are not natively pathogenic.

Subsequently, several efforts have used recombinant strains (primarily *E. coli*) for APC gene and protein delivery.[18,24,42] With APCs, the need for invasin does not appear to be crucial for intracellular delivery of bacterial carriers. However, LLO can greatly enhance intracellular bacterial delivery.[18,42]

Besides the recombinant addition of genes meant to aid antigen delivery, the antigens themselves are also typically introduced using recombinant DNA technology. As such, both the newly introduced antigens and the mechanisms (listeriolysin O or invasin) for aiding antigen delivery can be influenced by the molecular biology techniques used to introduce them.

First, molecular biology techniques can influence recombinant gene dosage. Plasmids are commonly used to introduce the antigen, the delivery-enhancing additional genes, or both. The plasmids themselves can be maintained in the bacteria at either high or low copy numbers (from 1 to 700 copies).[145,146] Further, the recombinant genes may also be localized to the chromosome of the bacteria, stably placing the recombinant gene as a low-copy number replicate. These efforts provide one degree of freedom in influencing antigen delivery, and such efforts will influence the final level of recombinant protein produced either in the bacteria or in the targeted APC, with intuition suggesting that more of the recombinant molecule will lead to better antigen presentation and immune response. However, this is not always the case. Excess lysteriolysin O appears to damage APCs during use in antigen delivery.[18,147] In the cited work, LLO toxicity was mitigated by killing the bacterial vectors prior to administration. Another possible approach is to vary or tune the LLO gene copy number within the cell prior to or during administration. Hence, alternative gene dosage strategies provide a readily accessible tool in the goal of optimizing antigen delivery.

Like the gene copy number, promoter and control elements will also influence final protein levels. Several promoter and control options exist and are in large part dictated by the expression system and organism that are used. Several well-established promoter systems exist for *E. coli*, including the T5, T7, tac, trc, $P_{BAD}$, and lacUV5 systems.[148] Because of the similarities between the two organisms, the same promoters could most likely be adapted to *Salmonella* strains. Promoter systems for *Lysteria* include *actA*,[23,48,58] *inlC*, and *tet*,[23] and promoters used for *Shigella* include T7[78,86] and ompC.[84−86,88] If gene expression is designed to commence after delivery to the APC nucleus, the promoter of choice is usually the powerful cytomegalovirus (CMV) immediate-early promoter which calls for strong levels of mammalian cell-driven gene expression.[9,33] Constitutive or inducible control over gene expression can also influence overall antigen presence and delivery. Constitutive systems automatically provide a steady level of recombinant proteins, yet inducible systems, especially those coupled to strong promoters, can rapidly boost antigen or recombinant protein levels. Promoter/control systems (such as *actA*) have also been developed to call for gene expression in the cytosol of APCs, providing a unique way to induce gene expression in the context of gene delivery.[23,149] Table 2 summarizes common promoters and plasmids (and associated copy numbers) used in recombinant engineering of bacterial vectors.

Another issue potentially affecting antigen delivery is the spatial location of the antigen within the bacterial carrier. In a Gram-negative carrier, the final protein antigen may reside in the cytosol, the periplasmic space, or the outer cell wall or be openly secreted.[58,150] Gram-positive systems would have similar options minus the periplasmic space location. This concept was tested by compartmentalizing antigen epitopes to the *E. coli* cell surface, periplasmic space, or cytoplasm, with results placing less emphasis on location and more emphasis on total antigen as being a determinant for successful antigen processing.[44] Still, other studies have shown the benefits of antigens secreted from bacterial vectors.[22,112,151,152] Specific secretion systems used for recombinant antigen delivery include the type I and III

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***Table 2.*** Plasmids and Promoters Used for Common Bacterial Delivery Vectors

| *E. coli* | *Salmonella* | *Shigella* | *Listeria* |
|---|---|---|---|
| | Replicon/ Copy Number | | |
| pUC/500−700 | pUC/500−700 | pUC/500−700 | pUC/500−700 |
| pMB1/15−20 | p15A/18−22 | p15A/18−22 | p15A/18−22 |
| | pMB1/15−20 | ColE1/15−20 | ColE1/15−20 |
| | pBR/15−20 | | pMB1/15−20 |
| | pSC101/∼5 | | |
| | Promoters | | |
| araBAD | CMV | CMV | actA |
| $\beta$-actin | dmsA | lpp | CMV |
| CMV | htpG | nir15 | hly |
| crl | htrA | ompC | lac |
| lacz | irgA | trc | p60 |
| SP6 | nirB | T7 | SV40 |
| SV40 | nir15 | | T7 |
| tet | pagC | | |
| T7 | Pr | | |
| | Ps | | |
| | SV40 | | |
| | tac | | |
| | trc | | |
| | T7 | | |
| | References | | |
| 18, 24, 34, 39−41, 45 | 9, 22, 25, 30, 43, 45, 48, 50, 52, 56, 62, 63, 65, 66, 70, 71, 74 | 32, 78−88 | 23, 45, 55, 93, 97, 100−103, 105, 111 |

systems.[152−154] While the type I system simply transports a given protein from the bacterial cytoplasm to the surrounding environment, the type III system provides a portal for facilitating transport of the protein from the bacterial to the eukaryotic cytoplasm. The common type I system employed is the α-hemolysin machinery from *E. coli*, whereas the type III systems from *Salmonella* and *Shigella* offer the ability to transport proteins directly to the cytoplasm of nonphagocytic cells as an initial means of bacterial cellular entry. Though phagocytic APCs have a natural propensity for bacterial uptake, the type III systems have been employed for the improved cytosolic delivery of protein antigens. Compartmentalization can also be applied to spore-forming bacteria (i.e., *B. subtilis*) with a recombinant gene fused to a spore surface proteins (CotB and CotC) for antigen surface display.[155,156] The ability to control antigen location in the bacterial vector serves as another variable for improving overall antigen delivery.

## 5. Future Obstacles and Opportunities

**A. Efforts at Bacterial Vector Design.** As mentioned, a key advantage that bacterial vectors offer is techniques in genetic and metabolic engineering for improving antigen delivery. First, a range of gene localization and expression techniques that influence antigen copy number and location exist. As more and more antigens are identified for various infectious diseases or cancer, bacterial vectors will offer a shuttle for introducing the newly identified antigens to the immune system. Furthermore, it should be possible to coordinately express more then one antigen at a time (i.e., a panel of disease specific antigens) to mount a complete immune response against a particular disease in one particular administration. As an example of complete antigen challenge and the use of specialty bacterial plasmids, the complete murine cytomegalovirus (MCMV) genome was cloned into a bacterial artificial chromosome (BAC, for the stable, low-copy number maintenance of large DNA constructs) plasmid. The MCMV BAC was then introduced into a *Salmonella* vector for vaccination against MCMV challenge.[34]

The uses of listeriolysin and invasin are fine examples of biological solutions to cell entry and intracellular release of bacterial cargo and highlight efforts to introduce recombinant biological properties for improving bacterially derived delivery. However, those are only two example proteins engineered to improve bacterial antigen delivery. The likelihood of additional proteins available to further aid delivery efforts seems plausible. For example, there may be undiscovered mechanisms associated with *Listeria*, *Salmonella*, *Shigella*, or other bacteria adept at entering mammalian cells

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that could be transferred to *E. coli* or other recombinant systems to improve delivery further.

One barrier most likely still hindering gene delivery is the intracellular trafficking of gene cargo to the nucleus. Current systems might be able to efficiently enter the cell cytoplasm and release genetic cargo, but the use of designed mechanisms for facilitating nuclear transport has not been investigated thoroughly. Still, options for incorporating nuclear localization mechanisms from other systems, with viruses a likely one, for improving gene delivery may exist.[157−159]

**B. Overcoming Administration Problems.** Several undesirable outcomes are possible when using bacterial vectors. As mentioned, there is an intrinsic danger when administering bacterial organisms to the body. Besides sepsis, other undesired outcomes include arthritis or urethritis.[51] Problems can be minimized through vector choice (attenuated or nonpathogenic bacteria), dosage limit, delivery location, and ex vivo delivery. In addition, another option available to those adversely affected is treatment with antibiotics. Another concern is the use of commensal organisms that may readily colonize mucosal locations and, through prolonged presentation, cause immune tolerance toward the antigen meant to be delivered for immune system stimulation.[160]

**C. Meeting the Challenges of Vector Demand.** Finally, it is important to look beyond the molecular and cellular aspects that drive bacterially mediated gene and protein delivery and also focus on the vector manufacturing schemes that will ultimately determine the large-scale distribution of a given bacterially based therapy. Here, bacterial vectors offer several advantages. Simple growth requirements and conditions offer cost-effective methods of large-scale, environmentally friendly production strategies. For example, organisms like *E. coli* and *B. subtilis* can be cultured to high cell densities using fed-batch bioreactors (initially developed for recombinant protein production) that provide additional nutrients over time to overcome growth limitations and allow impressive cell density measurements.[161−163] Through a combination of process adjustments meant to provide continuous nutrients and prevent the buildup of stationary-phase growth inhibitors, cell densities have steadily expanded over time from bioreactors capable of supporting 54 g dry cell weight (dcw)/L to 190 g dcw/L with a theoretical maximum of 350 g dcw/L.[164,165] In essence, the operation makes a modest-sized reactor behave like a significantly larger operation. Thus, the speed and unique scalability of fed-batch bioreactor systems offer a distinct advantage for bacterial vector manufacture, especially if a rapid and sizable production effort is needed. The approach could help ease concerns over vector supply and the costs needed for production.[166]

At the same time, the bacterial vectors are easily manufactured with an intact antigen. Bacterial/antigen purification is then quite simple since filtration or centrifugation can be used to collect the final bacterial vectors. The smaller bioreactors used in fed-batch operations also reduce the operating and capital costs before, during, and after the bioreactor unit operation. This includes the size of equipment needed for vector collection, making the overall process more economically sound. In addition, like other bioreactor operations, the process is generally considered to be environmentally friendly with no concerns over harmful solvents or operating conditions (such as high temperature or pressure).

Formulation and storage are additional points of consideration for bacterial vectors. Once harvested, the cells may be divided into appropriate dose sizes and preserved (with samples stored at 4 °C for short-term storage or −80 °C for longer-term storage). A perhaps minor but necessary consideration is whether the bacteria are sensitive to sunlight as certain bacterial vectors lose potency with exposure.[167] While small individual aliquots would ease the burden of massive storage space, the need for lowered temperatures would still present an energy cost when manufacturing the vectors. However, especially in developing countries, successful cold storage has proven to be important in maintaining vector potency and immunization success.[168−170]

As an alternative approach, the cells may be lyophilized

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and stored dry at room temperature. Lyophilization is another common industrial unit operation that could be directed toward bacterial vector manufacture in this scenario. A key question is the efficacy of the vectors before and after lyophilization. From one standpoint, if therapeutic potency is maintained, lyophilization would provide a physical form of attenuation (if not outright cell death) that limits the unwanted problems potentially caused by live bacterial administration. On the other hand, lyophilization may damage the cell and its macromolecular contents needed for efficacious immunomodulation, a problem enhanced by the necessary sample freezing step.[171]

Excipients that weaken the damaging effects of cold storage and lyophilization are available. Glycerol is a common, and safe, cryopreservant used for −80 °C storage of bacterial stocks. However, studies suggest glycerol does not provide the same level of protection during lyophilization (in addition to being difficult to remove during freeze-drying). Here, disaccharides such as trehalose and sucrose have shown promise in the preservation of lyophilized bacterial samples,[172] with the presence of such sugars providing a physical encasing that slows biochemical reactions and replaces the hydrogen bonding capabilities of water during the drying process.[173,174] In *E. coli*, natural trehalose biosynthesis can be induced through osmotic pressure (elevated concentrations of salt); the lyophilized strains then naturally exhibit better storage and reconstitution.[175,176] Alternatively, recombinant efforts have introduced foreign proteins also capable of improving *E. coli*'s response to drying.[177]

Finally, spore-forming bacterial vectors (i.e., *B. subtilis*) offer a long-term and cost-effective storage option.[178,179] Here, vegetative cells are induced to form spores by limiting a carbon, nitrogen, or phosphorus source.[180,181] And like non-spore-forming bacteria, the spores can be collected through simple centrifugation or filtration unit operations. However, because the spores have formed specifically to ensure long-term survival of the bacteria, they can then be safely stored immediately after collection. Coupled with antigen surface location, spores then provide a ready-made antigen delivery device.

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