Bacterial Vectors for Delivering Gene and Anticancer Therapies

Engineered bacteria deliver genes or proteins into specific cells to treat tumors or genetic diseases, or trigger immune responses

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Genes can transfer from bacteria into a broad range of recipient cells, including phylogenetically remote bacteria, yeast, and plants. In addition, after incubation with phagocytic or non-phagocytic cells, bacteria can transfer functional genes into mammalian cells. Bacteria of several genera, including Shigella, Salmonella, and Listeria, that transfer DNA to cells can also invade such cells. This property, shared by a large array of gram-positive and gram-negative bacteria, depends on interactions between specific bacterial ligands and host-cell receptors.

We have studied this gene transfer mainly in nonpathogenic but invasive strains of Escherichia coli that are deficient in cell-wall synthesis. During this gene-transfer process, called bactofection or suicidal invasion, the bacteria lyse after they enter a host cell and release their plasmids, which are then transferred from the host cytoplasm to the nucleus where some of them are expressed (Fig. 1). This process can stimulate humoral and cellular immune responses.

Harnessing this bacterial suicidal invasion process to introduce genes into eukaryotic cells may prove medically valuable for gene therapy and treating cancers. The inexpensive production, diverse natural and modified tropism profiles, and large and diverse DNA-packaging capacity of bacteria, coupled with their immunological tolerance in target organs and relative ease of control in the case of adverse events, make bacterial delivery an attractive alternative to consider for gene delivery to the gastrointestinal, respiratory, and urogenital tracts.

Summary

- During bactofection, bacteria lyse after entering a host cell, releasing their genes, which transfer from the host cytoplasm to the nucleus where they are expressed.
- Harnessing bactofection provides a means for introducing genes into mammalian cells that may prove medically valuable for gene therapy, treating cancers, and vaccination.
- Several types of bacteria, including Escherichia coli, Shigella, Salmonella, and Listeria, are being developed as vectors for transferring genes into mammalian cells.
- Bacterial vectors also can be paired with RNA interference, potentially providing a steady supply of siRNAs to sustain therapeutic benefits.
- Bacterial vectors are being evaluated in several early-stage clinical trials in which they are used to target cancers, to deliver siRNA to patients with familial adenomatous polyposis, and to deliver interleukin-10 to patients with ulcerative colitis.

Initial Findings

Because Shigella flexneri readily enters intestinal epithelial cells and escapes from endocytic vesicles, we and other research groups began using these bacteria as a means for transferring plasmids directly into cells. In those experiments, we used a diaminopimelate auxotroph mutant (dap') of Shigella or a dap' invasive strain of E.

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coli, which lyse when they enter mammalian cells because they cannot synthesize their cell wall. These bacterial vectors harbor plasmid pCMVβ, which synthesizes β-galactosidase under the control of a eukaryotic promoter. Typically, 48 h after these suicidal bacteria have invaded mammalian cell lines, 1–2% of the cells express transfected β-galactosidase. Moreover, after these bacterial strains are introduced intranasally into mice, both humoral and cellular anti-β-galactosidase responses are detected, indicating that in vivo delivery of plasmid DNA by Shigella stimulates immune responses to plasmid-encoded antigen.

Bacteria deliver DNA (or proteins) into mammalian cells by a two-step process: entry into the cell vacuole followed by escape of the bacteria or their plasmid DNA from the vacuole to the cytosol. For instance, Yersinia pseudotuberculosis depends on invasin, encoded by the 3.2-kb inv locus, to enter nonphagocytic cells expressing β1-integrins; its presence is sufficient to allow E. coli to invade such cells (Fig. 2). Listeriolysin O (LLO) from Listeria monocytogenes, encoded by the 1.5-kb hly gene, enables Listeria to escape the entry vesicle.

We introduced the inv and hly genes cloned in the non-self-transferable, low-copy-number plasmid pGB2 into the dap auxotroph strain of E. coli along with a plasmid that directs synthesis of the green fluorescent protein (GFP) in mammalian cells but not in bacteria. When these invasive bacteria were incubated with HeLa, CHO, and COS-1 cells, up to 20% of them became GFP-positive within 2 days. Within 1–3 h, the bacteria localize to the cell phagolysosomes, according to confocal immunofluorescence microscopy (Fig. 3). After 72 h, the intracellular bacteria are all dead, and their chromosomal DNA can no longer be detected after 20 days.

Other Bacterial Vectors

Other intracellular bacteria also can transfer genes into mammalian cells. For instance, attenuated strains of S. typhimurium can transfer plasmids to phagocytic cells even though the bacteria remain within phagocytic vacuoles.

![Schematic representation of bacteria-cell DNA delivery process.](image-url)
Grillot-Courvalin: Piano and Culinary Interests but Science Dominates

Catherine Grillot-Courvalin decided years ago to move from clinical medicine to basic research. A pediatric immunologist, she found that treating—and sometimes losing—young children took too big an emotional toll. Also, she believed she could do more for them through research. “I was a resident in pediatrics and mother of a young child. It was sometimes difficult,” she says. “I felt that to do research would be a more efficient way to help these children.”

Grillot-Courvalin, 63, switched to microbiology and became a pioneer in the field of bacterial-mediated gene delivery. In 1998, her group described the delivery of plasmid DNA via engineered cells of Escherichia coli into a variety of mammalian cells. She and her collaborators as well as other researchers continue to develop bacteria-based technology into an efficient means for transferring DNA into recipient cells as a means of delivering therapeutic or immunogenic proteins.

Grillot-Courvalin considers bacterial vectors “a great new approach,” noting that it also promises to be relatively inexpensive. The good news is that an economical and efficient delivery system could make a big difference in countries where medical resources are limited. The approach has many potential applications, including gene therapy and delivery of other treatments, as well as DNA-based vaccines.

Grillot-Courvalin is an associate professor at the CNRS in France, the French National Center for Scientific Research, which is “the equivalent of the National Institutes of Health,” she says. She works at the Institut Pasteur in Paris, often collaborating with her husband Patrice Courvalin, who first proposed designing a bacterial delivery system. “It started with an idea of Patrice that I put to work” she says. The two met during her infectious diseases residency at the hospital of the Institut Pasteur. “He had been a resident there and was working on antibiotic resistance mechanisms in one of the Institut Pasteur laboratories,” she says. “It was my first year of residency, and a doctor colleague suggested I ask him for advice. His help has been indeed precious, and was the beginning of a collaboration which is still ongoing 35 years later.” They married two years later and soon moved to the University of Wisconsin, Madison, where they did postdoctoral research.

“French people did not often go abroad for a postdoctoral stay at that time, and certainly not in Wisconsin and certainly not for three years,” she says. “Being, apparently, the first French citizens on the campus, we were treated in a very special way. Since there were high expectations for French cuisine, I started to become a very good cook, and was able to start a French bakery class. I also became a competent gardener, able to grow, for example, leeks that were difficult to find at that time.”

She enjoyed their time in Wisconsin, although “I remember in May there was still snow,” she says. “For us, May is the time of flowers. The first winter was striking because of its length, and the short days—but then we got used to it.”

Born near Alsace in Kehl, Germany, Grillot-Courvalin says that she traveled with her family as a child because of her father’s military service. “It gave me the opportunity to live as a child in various places, for example, Algeria, in the charming village of Cherchell, which was an important military base, and then in Paris,” she says. “I was ready to continue to travel around the world, and a research career is great for this purpose.”

One of four children—the others chose careers in pharmacy, engineering, and dentistry—she credits her mother for encouraging all of them to put education before everything else. “She was a very bright woman, but could not go to school,” she says. “My grandmother forced her to quit school to learn cooking and sewing. She was really disappointed not to continue her studies, so she convinced us to be good in school and to achieve.”

Grillot-Courvalin received her medical degree from the Medical School in Paris in 1972 and a master’s degree in immunology from the Pasteur Institute in 1978. In 1980, she earned a master’s degree in human biology from the Medical School, and a Ph.D. in 1989 from the Faculty of Sciences, in Paris. She received a diploma in “Prospective and Evaluation of Research and Technology,” from the Centre National des Arts et Métiers, in Paris, in 1999.

She and her husband have two grown sons, ages 33 and 30. The older one lives in Paris and is practicing law. The younger son, a banker, lives in New York, and is married with two young daughters. Grillot-Courvalin and her husband make no effort to bar science from their home, although she squeezes time to bar indulge other interests, including playing classical piano, cooking, and gardening. “We do talk about science when we are home although we also discuss other things.”

Marlene Cimons
Marlene Cimons lives and writes in Bethesda, Md.
Oral administration of recombinant *Salmonella* carrying antigen-encoding eukaryotic expression plasmids to mice stimulates a strong immune response. This experimental vaccine approach is being actively developed, with the focus on improving plasmid stability and further attenuating the vector.

Other types of bacteria are being developed as vectors for carrying antigens—that is, as potential vaccines. For example, *Listeria monocytogenes*, a gram-positive species, invades a wide range of mammalian cell types. Once internalized, these bacteria rapidly escape from the primary vacuole into the cytosol to replicate. One engineered attenuated strain self-destructs in the cell cytosol when it produces a phage lysin under control of the *actA* promoter, which is preferentially activated in the host cytosol. This delivery strategy proves efficient at transferring plasmids into cells of epithelial and endothelial origin. This experimental vaccine approach is being actively modified to improve safety as well as the efficiency of delivery of antigen-encoding DNA and RNA.

Lactococci also are being used to deliver proteins or DNA molecules to eukaryotic cells. For instance, food-grade *Lactococcus lactis* can deliver eukaryotic expression plasmids to mammalian cells in vitro. Further, *L. lactis* administered orally to mice can transduce intestinal epithelia to produce β-lactoglobulin. In turn, β-lactoglobulin stimulates a transitory Th1 immune response, modulating the Th2 response against milk-protein allergens. Engineering *L. lactis* bacteria to produce either internalin A from *L. monocytogenes* or fibronectin-protein A from *Staphylococcus aureus* improved the efficiency of their plasmid delivery.

**E. coli Vectors: Further Development**

We are continuing to develop nonpathogenic but invasive dap auxotroph derivatives of *E. coli* K12 BM2710. For instance, some carry the *inv* and *hly* genes integrated in the chromosome. By inactivating the *msbB* gene to modify the LPS, we produced a strain with reduced proinflammatory activity. These vectors can invade and transfer functional DNA or heterologous proteins to epithelial cells in vitro as efficiently as our first-generation bacterial vectors. Moreover, they do not carry plasmids and harbor no antibiotic resistance markers.

These bacterial vectors can deliver DNA into immortalized airway epithelial cells from healthy individuals or those with cystic fibrosis, or into primary bronchial epithelial cells grown as an explant-outgrowth cell culture. Approximately 2% of the immortalized cells express GFP from transferred DNA encoding that protein. However, only a few primary cells were transfected, and they were poorly differentiated, a finding that is consistent with β1-integrins being present only on these cells (Fig. 2). Subsequently, the *E. coli*-containing phagosomes mature into phagolysosomes, according to confocal and electron microscopy (Fig. 3). These experiments demonstrate for the first time that recombinant bacteria can transfer genes into primary airway epithelial cells, provided that they can invade those cells.

Alternatively, after being adminis-
tered intranasally, bacterial vectors move to the pulmonary alveoli of recipient mice. Although they transfer *lux* genes into such cells, they do so with an efficiency that is lower than that of lipid-mediated transfer and further modifications of the bacterial vectors are needed for efficient lung delivery.

**E. coli Vectors: Potential Applications**

Bacterial vectors can transfer large genomic DNA constructs, including human artificial chromosomes. Successful gene therapy requires persistent, tissue-specific expression of particular transgenes and, therefore, delivery of large DNA molecules containing additional regulatory and promoter elements. Such transgenes can be stabilized either by integrating into host chromosomes or by carrying elements that allow them to replicate and faithfully segregate during mitosis. These large segments of genomic DNA can be difficult to transfer because they have to be intact in a low copy number, ideally one or two per cell, for physiologically relevant expression.

These conditions are difficult to fulfill by lipofection. However, with the invasive *E. coli* vector, we could deliver alpha satellite DNA cloned into a P1-based artificial chromosome into HT-1080 cells. The construct carries a 250-kb bacterial artificial chromosome (BAC), including all 26 exons of factor VIII, the introns, and more than 40 kb of upstream and 20 kb of downstream DNA. We can also deliver a BAC covering the whole genomic area of the CFTR gene by using an invasive *E. coli* in which the CFTR BAC is stably propagated. Thus, bacteria provide a means for transferring large genomic DNA constructs that can assemble as functional chromatin.

Bacterial vectors also can be paired with RNA interference, a conserved posttranscriptional gene silencing mechanism in which small interfering RNA (siRNA) molecules degrade specific target mRNAs. Because synthetic siRNAs decrease target gene expression only transiently, a steady supply of siRNAs is needed to sustain therapeutic benefit. Bacteria prove to be an effective and safe tool for delivering siRNA to cells, efficiently silencing specific genes in vitro and in vivo.

For instance, *E. coli* cells carrying a transkingdom RNA interference plasmid (TRIP) produce siRNA, invade target cells, release siRNA into the cell cytoplasm, and activate the RNAi pathway to induce gene silencing. The TRIP plasmid carries the *inv* and *hly* genes and also a small hairpin RNA (shRNA) expression cassette, which drives expression of shRNA under the control of the T7 RNA polymerase promoter and terminator. The shRNA, which is released when the bacteria die inside the host cell, is then processed intracellularly by the host Dicer enzyme into siRNAs.

One TRIP plasmid carries a siRNA that is directed against the human colon cancer oncogene *CTNNB1*, which overproduces catenin β-1. When this plasmid was transferred into *E. coli* BL21DE3 and then introduced into SW480, a human colon cancer cell line, it lowered β-catenin levels by down-regulating *CTNNB1*. When orally administered, these bacterial strains also lowered β-catenin in the intestinal epithelium of mice. On the basis of such experiments, TRIP is being developed as a potential therapy for familial adenomatous polyposis with applications in colon cancer prevention.
Other engineered invasive bacteria are being used to deliver to the nucleus plasmid expression systems for gene-specific shRNAs. This approach is believed to induce a more sustained silencing since recipient cells continue to produce siRNA as long as the plasmid is maintained in the nucleus.

**Perspective**

For gene therapy, a key limiting step is delivering specific genes to specific cell types. However, receptor-ligand interactions at the cell surface provide a potentially useful, cell-specific way to bring DNA molecules into the cytoplasm. Early efforts involving bacterial gene transfer focused on antigen-presenting cells.

However, attention is shifting to vectors that deliver functional DNA into epithelial or other differentiated cells and tissues, taking advantage of the capacity of bacterial pathogens to target specific cell types and to gain access to intracellular compartments. Those pathogens can be rendered safe for this use by making them auxotrophs, as in the case of attenuated *Salmonella* diaminopimelate auxotrophs whose cell wall synthesis capacity is impaired. But one can also use auxotrophy to target bacterial vectors to specific tissues. For example, polyauxotrophic mutants of an invasive *Salmonella typhimurium*, defective in purine and multiple amino acid biosynthesis, invade nutrient-rich tumor cells in mice much more effectively than other nutrient-poor tissues, enabling us to deliver the gene for a prodrug convertase to those tumors. Similarly, one could exploit anaerobic conditions that are common within tumors.

Once bacterial vectors enter a targeted cell, the transgene may be expressed via the host cell or directly by the bacteria. Cytosolic endonucleases can degrade plasmid DNA delivered by bacterial vectors in the cytosol, limiting nucleus entry. Alternatively, bacteria-mediated transgene or RNAi expression can bypass cytosolic and nuclear barriers. Moreover, by enclosing plasmids, bacterial vectors can evade host defenses against exogenous DNA, potentially enabling long-term expression of the transgene. In any case, since DNA cargoes for gene therapy are normally bacteria-derived plasmids, the bacterial delivery system thus combines production and delivery of the transgene or its product. Moreover, and as opposed to viral vectors, bacteria can accommodate large constructs and several genes for gene therapy.

To date, bacterial vectors are being developed and evaluated mainly for cancer gene therapy and for use as DNA-based vaccines. Although they are also being developed to treat genetic diseases such as cystic fibrosis, transgene expression levels so far are low compared to other delivery methods. Indeed, the nuclear envelope remains a key barrier to all nonviral gene delivery systems. It is estimated that only about 1 to 10% of unmodified plasmids can be detected in the nuclear fraction of recipient cells. Efforts to improve nuclear targeting and import of plasmids will depend on designing better plasmids and improving our understanding of peptides that bind to and mediate nuclear import of DNA. Another potential advantage of bacterial vectors is the possible “hijacking” of their protein synthesis machinery to produce proteins favoring DNA transfer to the host cell nucleus.

The toxicity of bacteria varies among species and strains. In gene transfer clinical trials involving direct intratumoral or intravenous injections of attenuated *Salmonella*, patients experienced no significant side effects at low doses of $7.6 \times 10^7$ CFU over the course of 4 days for intratumoral injections and $10^8$ CFU/m$^2$ of body surface area for single intravenous injections. Although some patients experienced bacteremia-related symptoms at higher doses, those symptoms resolved with antibiotic treatment. Oral administration of *E. coli* for gene delivery to the intestinal epithelium in mice is well tolerated, and immune tolerance exists for many gut-associated bacteria; this also holds true for *L. lactis*. Therefore, engineered bacterial flora could prove a safe vector for organs, such as the gut, upper respiratory tract, and vagina. If the attenuated bacteria were to spread beyond the target organ, antibiotics could be used to control the infection.

We know of at least four phase I clinical trials in which bacterial vectors are used to target various cancers, including to deliver tumor antigens via *S. typhimurium*, cytotoxic genes via *L. monocytogenes*, and to deliver siRNA against β-catenin orally via *E. coli* in familial adenomatous polyposis. In addition, there is a phase II trial in which *L. lactis* is a vector for delivering interleukin-10 to patients with ulcerative colitis.
SUGGESTED READING