Harnessing the Power of Microbes as Therapeutics: Bugs as Drugs
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Report on an American Academy of Microbiology Colloquium held in San Diego, CA, in April 2014

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This report is based on the deliberations of experts who gathered for two days to discuss a series of questions developed by the steering committee regarding the use of microorganisms as therapeutic agents. This report has been reviewed by the majority of participants, and every effort has been made to ensure that the information is accurate and complete. The contents reflect the views of the participants and are not intended to reflect official positions of the Academy or ASM. The Academy thanks the efforts of previous staff, Leah Gibbons and Shannon Greene, Ph.D., for their assistance on this project.

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I. Preface

In the age of modern medicine, advances across many scientific specialties, including genetics, medicinal chemistry, and molecular biology are yielding novel treatments and potential cures for an impressive array of diseases. Despite these advances, however, some diseases resist efforts to develop effective treatments against them. Cancer remains a formidable disease, with a remarkable ability to overcome many available therapies. While novel treatments improve patient life expectancies in many cases, others are prone to eventual failure. Relapse is common and deadly, particularly when failed treatments seed more aggressive and resistant forms of this disease.

Bacterial pathogens are similarly resilient in terms of becoming resistant to antibiotic treatment. Thought to be conquered decades ago with the advent of antibiotics, bacterial infections are re-emerging as a serious threat in developed countries and remain a problem in developing countries. Antibiotic resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) are increasingly common, while also gaining resistance to drugs from other potent classes of antibiotics. Researchers are trying to identify new targets for the development of novel antibiotics to enable clinicians to treat the infections being seen in their patients. Unfortunately, many new antibiotic therapies prove short-lived due to the rapid development of resistance to these agents by bacterial pathogens (1).

While cancer and bacterial infections seem like very different diseases, the challenges they pose to modern medicine are similar. Both are diseases of cellular proliferation, either of microbial pathogens or self-cells gone awry. Rapid replication and genetic mutability enable both types of cells to overcome efforts to halt their potentially deadly progress. Conventional therapies too often fail when their use selects for treatment-resistant forms of both types of disease, demanding novel tactics to combat them (2).
II. Introduction

Historically, microbes proved to be rich sources of antimicrobial and anticancer drugs. The mold *Penicillium chrysogenenum* provided the first antibiotic, namely penicillin, in 1929. Later, soil and, still more recently, marine bacteria stole the spotlight for being sources for antibiotics and anticancer agents. Now, however, researchers are beginning to develop intact microbes, including bacteria, viruses, and bacteriophage (phage), to serve as therapeutic agents.

In April 2014, the American Academy of Microbiology assembled a group of 17 experts from different scientific disciplines to consider “Bugs as Drugs,” that is, the potential for harnessing microorganisms as therapeutic products for treating human diseases. These scientists, including clinicians, veterinarians, industry experts, and representatives from regulatory agencies, brought their collective experiences in vaccine design, gene delivery, genetic engineering, and clinical research to address the prospects of using microorganisms to treat and prevent infectious diseases and cancer. For two days, they considered scientific theory and evidence, lessons from past successes and failures, current research experiences, and the role of genetic engineering in modifying microorganisms to improve their therapeutic performance as they sought to identify the best ways for moving this field forward. They concluded that, while this field of research offers great promise and is gaining momentum, it also is faced with many challenges. Thus, the immediate focus should be on overcoming those challenges while also finding practical means for moving forward.

**Advantages of Microbes over Traditional Approaches**

Colloquium attendees suggest that microbes will be best-suited for use against several types of diseases that are proving especially challenging to modern medicine, namely cancer and antibiotic-resistant bacterial infections. Not only is there a shortage of effective treatments for those diseases, but they also afflict a large percentage of the population and, in many cases, pose serious risks of mortality. Novel treatments for these diseases would substantially reduce disease burden as well as medical and social costs across the globe (1, 3). Microbes are particularly well-suited to fill this niche, with inherent capabilities to address short-falls in traditional approaches to treating these diseases.

- **Multi-Pronged Mechanisms** - Combination therapies are making it more difficult for antibiotic-resistant pathogens and drug-resistant cancers to evade multi-pronged attacks involving both conventional drugs as well as other more recently developed therapeutic products. Because the combination treatment strategy so often proves effective, the medical community continues to apply it ever more widely—for example, in the development of drug cocktails against many cancers, infections caused by viruses such as HIV and hepatitis C, and infections by antibiotic-resistant bacterial pathogens (2).

Microbes also depend on multi-pronged mechanisms for survival within different environments. Bacterial pathogens, for example, can overcome host barriers to infection, including by penetrating the skin, withstanding stomach acidity, and overcoming host-produced antimicrobial peptides such as defensins. Countering the capacity of bacterial pathogens or cancers to overcome treatment with several drugs at once by using microbes as therapeutics takes this concept a big step forward. It offers the possibility for optimizing and combining several varied and independent mechanisms to use against a particular pathogen or cancer—facing it with too many injuries to overcome or evade. Bacteria and viruses also could be combined in vaccines to prevent infections and perhaps cancers.

- **Evolution of Microbes against Resistance** - In those cases where resistance to antimicrobial drugs or chemotherapy in the case of cancer emerges, therapeutic microbes offer a novel alternative treatment strategy. To further improve on this fundamental strategy, directed evolution can be used to extend the efficacy of the microbes being developed as therapeutics, modifying them to respond to pathogens or tumors that may become resistant (See Text box 2). However, this approach will require careful monitoring for any signs that the microorganisms being used for therapy are becoming more pathogenic to the patients (or resistant to antibiotic treatment if it is needed to halt treatment).

- **Replication** - Therapeutic microbes replicate within the host at the site of disease, thereby amplifying the therapeutic dose in the environment where it is needed, while avoiding side effects from the treatment elsewhere in the body. In addition, the replication rate of the therapeutic microbe may well correlate with the burden of disease, changing the dose to reflect the amount of tumor tissue or level of pathogens within each patient during treatment. With
bacteriophages, for example, the drug-resistant bacterial pathogen is the host for the phage used for therapy, and the numbers of phages being produced will depend on the availability of the host pathogens that may be present.

- **Immune Stimulation**: Therapeutic microbes can stimulate the host immune system to attack the pathogen or tumor, enhancing the microbial mechanisms that are directed against that disease. Therapeutic microbes potentially can stimulate both innate and adaptive immune responses in a coordinated fashion, more effectively treating the disease while also generating long-term immunity to it.

- **Genetic Engineering**: Although strategies will vary depending on the disease and microbe of interest, therapeutic microbes can be modified genetically to enhance their safety and efficacy.

## III. Bacteria versus Cancer

Clinicians observed that bacterial infections can inhibit cancer growth as far back as the 1800s, noting that osteosarcoma patients with infected amputations had better outcomes than did patients who were free of infection (4, 5, 6, 7). William B. Coley was the first U.S. physician to treat cancer patients directly with *Streptococcus pyogenes*. However, his approach was highly controversial in an age without antibiotics or other means to control infections. Objections from colleagues led Coley to abandon live for heat-killed bacteria and bacterial by-products, termed “Coley’s toxins.” Although far less effective than their live counterparts, they had some beneficial impact when used for treating such cancers (8, 9).

Coley’s toxins compared to live bacteria speaks to the power of live microbes over isolated bacterial products, both for their superior ability to stimulate immune responses as well as to exert direct cytotoxic effects.

Recent advances in cancer biology and recombinant DNA technology are reawakening interest in using attenuated bacteria to treat cancer (See Text box 1). Efforts to reevaluate this approach have led to animal experiments and clinical trials showing safety as well as favorable outcomes in both canines and in humans (10, 11).

### Mechanisms of Antitumor Activity

Mechanisms of bacterial antitumor activity vary depending on the bacterial species and strain being used, but generally fall under three themes: direct cytotoxic effects on tumor cells, destruction of the vasculature that feeds those tumors, and stimulation of the immune system antitumor responses.

**Text box 1.**

**BCG (Bacillus Calmette–Guérin): A Tuberculosis Vaccine against Cancer**

BCG is a vaccine that was designed to protect against tuberculosis but is also curiously effective in treating bladder cancer (Figure 1 and 2). It consists of a live-attenuated strain of *Mycobacterium* that stimulates antitumor immune responses that lead to tumor regression and prevent relapse. Regardless of the mechanism, this example is a clear indication that live-attenuated microbes can be effective and safe cancer treatments (12).

**Figure 1.** Camille Guérin and Albert Calmette, respectively, were the developers of the BCG (nonvirulent) vaccine, an attenuated strain of *Mycobacterium bovis*. Image left retrieved from Wellcome Library, London. Image right retrieved from “1921_calmette_albert” by crootof - CC BY-NC-SA.

Each of these activities can be augmented by genetic engineering.

1. **Direct Killing Mechanisms** - Most bacteria used to treat cancers are pathogens, each one carrying a variety of virulence mechanisms capable of infecting and potentially killing host cells. These virulence mechanisms are unleashed against the tumor when bacteria contact tumor tissues, often causing tumor cell lysis. Some bacteria infect tumor cells directly, attacking them from within. Other bacteria remain extracellular, secreting toxins or enzymes that degrade tumor tissues. Some microbes attack tumor cells from both outside and inside (Table 1).

This area of research is very active. Despite extensive information about bacterial virulence mechanisms, however, much effort is needed to harness these mechanisms effectively to combat tumors (14).

The inherent cytotoxic mechanisms of bacteria include fairly recent discoveries, and some bacteria have been further engineered to produce and deliver exogenous agents. These engineered mechanisms synergize with innate mechanisms. Some examples are listed below:

- **Bacterial Toxins** - Toxin-producing pathogenic bacteria are too dangerous to use in patients without putting those toxins under the control of tumor-specific promoters and moving those promoter-controlled genes into safer bacterial host species. Some bacteria make peptides with specific antitumor activity, but they will require testing to determine whether this specificity for tumors puts enough of a limit on their toxicity to make them safe for direct use (15, 16, 17).

- **Prodrug Conversion** - Bacteria can be engineered to express enzymes that convert non-toxic prodrugs into chemotherapeutic agents that are active against tumors. A particular prodrug is converted into the toxic product only within infected tumor tissues, mitigating and sometimes altogether avoiding systemic toxicity issues and other off-target side effects (18, 19).

- **Apoptosis** - Bacteria can be engineered to induce apoptosis of cancer cells—for example, by expressing ligands that bind “death” receptors on cancer cell surfaces or other molecules such as p53 that alter intrinsic programmed cell-death pathways.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Site of Action</th>
<th>Mechanism of Toxicity</th>
<th>Impact on Tumor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria</em></td>
<td>Intracellular</td>
<td>-ROS production</td>
<td>-Tumor cell apoptosis</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>Extracellular</td>
<td>-Lipases</td>
<td>-Tissue invasion and tumor cell lysis</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Both</td>
<td>-Hyaluronidase - Type III secretion</td>
<td>-Cancer cell apoptosis and lysis - Injects effector proteins into tumor cells; can produce and/or secrete proteins by other means to kill tumor cells from within</td>
<td>(7)</td>
</tr>
</tbody>
</table>

**Figure 3.** Scanning electron micrograph of *Listeria monocytogenes*  
Image retrieved from SCIMAT/Science Source.  

**Figure 4.** Clostridium botulinum exemplifying spore formation  

**Figure 5.** Gram stain of *Salmonella typhimurium*  
Image retrieved from Michael Abbey/Science Source.
These apoptosis-inducing mechanisms are less likely than conventional therapies to generate aggressive or drug-resistant cells that underlie tumor regrowth and treatment failure (20, 21).

- **Radioactivity** - Bacteria can be used to deliver radiation directly to tumors—for example, by coupling radioisotopes to antibodies that bind to the surface of bacteria that migrate specifically to tumor tissues. This approach is particularly effective against metastases and pancreatic cancers, which are notoriously aggressive and resistant to current treatments (22) (Figure 6).

2. **Indirect Killing through Attack of Vasculature** - To meet their own high metabolic demands, tumors require new blood vessels to supply nutrients and oxygen. Novel chemotherapies block the growth of new blood vessels, a process known as angiogenesis, but have less impact on existing vessels, limiting this treatment strategy in its ability to starve tumors. However, bacterial vectors may provide a more powerful means for destroying both old and new blood vessels. For example, *Clostridium* and *Salmonella* induce apoptosis of vascular endothelial cells, destroying blood vessels in tumors while leaving normal vasculature intact (23).

Destroying the blood vessels that supply nutrients to tumors also makes them a more hospitable environment for supporting anaerobic and facultative anaerobic bacterial infections (24). Without blood vessels, tumors are less accessible to immune cells that might target the therapeutic infection, which could improve efficacy, or the cancer cells, which might undermine efficacy.

3. **Indirect Killing through Immune Activation** - Tumors are privileged sites that exclude immune cells and suppress their activation, enabling tumor cells to evade immune clearance. Infusing tumors with bacteria, however, can attract immune cells, including macrophages, neutrophils, and white blood cells to such sites (25, 26). Innate immune mechanisms trigger inflammatory responses that help to clear bacteria but which also kill host cells in the vicinity of an infection. In the case of infected tumors, non-specific inflammatory responses can damage and destroy tumor cells and the vasculature that supplies them with nutrients, reducing the chances of tumor regrowth. These innate immune mechanisms can be effective even in the absence of adaptive immune responses, which can be compromised in cancer patients. In addition, bacterial infections can sometimes elicit potent adaptive immune responses against tumor cells (26).

- **TNF-α** - Bacterial antigens such as lipopolysaccharide (LPS) induce tumor necrosis factor (TNF)-α, which has antitumor effects and makes conventional chemotherapy agents more effective, possibly by making blood vessels more permeable. However, limiting TNF-α production to tumors is critical because systemically administered TNF-α can cause deadly septic shock.

- **Myeloid-Derived Suppressor Cells (MDSCs)** - MDSCs are prevalent in cancer patients, home to tumors, and produce immunosuppressive cytokines. Bacteria that directly infect MDSCs alter their behavior. For example, *Listeria*-infected MDSCs become immunostimulatory, contributing to tumor regression (27).

- **Macrophages** - Macrophage cells of the immune system can infiltrate tumor tissues, contributing to tumor growth by promoting angiogenesis and inhibiting antitumor responses of the immune system. However, because macrophages can infiltrate tumors, they are potential vehicles for delivering therapeutic microbes to tumor sites. Some bacteria can induce inflammatory responses in macrophages, leading to antimicrobial and antitumor responses, whereas other bacteria promote an anti-inflammatory response, supporting angiogenesis and tumor growth.

- **Neutrophils** - Although neutrophils may be depleted by chemotherapy, they are heavily recruited to and activated within infected tumors, where they can destroy many types of bacteria, including *Salmonella*. The
Genetic manipulation is also required for lasting immunity. Immune responses that are mucosal, systemic, and cellular nodes, it elicits the full range of penetrates deep effector lymph this bacterial species. Recom- 
tensive research on the genetics, ensuring safety in the attenuat-
ing into patients for the sake of bacterial cells are ever introduced 
capability is reduced before the mi-
trade-offs in which colonizing 
symptoms. This approach avoids 
and unable to cause disease 
beforer becoming avirulent 
fully colonizing internal tissues 
defense mechanisms, success-
with natural barriers and host 
its availability about bacterial mechanisms of immune-
tumor causes “epitope spreading,” which helps to generate T-cells that target not only 
fighting pathogens but also tumor cells. 
Tumor-specific T-cells attack the primary 
tumor and patrol for metastases, while 
memory T-cells continue to patrol long after 
an initial treatment.

Augmenting Immune Stimulation through Genetic Engineering

Enlisting the immune system may be the key to success against primary tumors, metastases, and relapse. Bacteria are an excellent tool for this purpose, and can be genetically engineered to shape and fine-tune immune responses to maximize their antitumor effects. However, insufficient information is available about bacterial mechanisms of immune-based tumor control, or how different bacterial strains affect these responses, leaving researchers with the need to follow a trial-and-error approach. Despite this information shortfall, some promising directions have been identified:

• Cytokine Production - Bacteria can be engineered to produce cytokines that attract immune cells, stimulate immune responses, and polarize Th1/Th2 type responses. For example, some cytokines trigger monocytes/macrophages, which influence angiogenesis and also can infiltrate and survive within hypoxic tumor tissue.

• Indolamine2,3-dioxygenase I (IDO) - IDO, a mediator of immune suppression, is overexpressed by tumors and correlates with disease progression. Because IDO inhibitors are not effective on their own and lead to off-target side effects, bacteria might be used to deliver IDO-silencing shRNA to tumors, preventing over-expression of this compound. The bacterial vector might serve an additional role by attracting and activating antitumor immune cells. Suppressing IDO might make other adjuvants more effective, stimulating stronger and longer lasting immune responses. However, too much IDO suppression can lead to toxic levels of inflammation that kill adaptive immune cells, undermining lasting responses and limiting efficacy against metastases and relapse (28).

• Tumor-Specific Antigens - Bacterial vaccines can be engineered to express tumor-specific antigens, stimulating adaptive immune responses with the potential both to eradicate and prevent tumors. This approach does not require that the tumor become infected. Instead, it relies on the bacteria infecting antigen-presenting cells and on their immunogenicity as adjuvants. The multifaceted and coordinated responses elicited by such engineered microbes are substantially more powerful than single, or even multiple, adjuvant or cytokine therapies (29).

Inherent Targeting Mechanisms

Restricting therapeutic bacterial infections to remain within tumors is essential for patient safety. Off-target side effects and toxicity are major problems for current chemotherapies, many of which do not selectively target tumor tissue. In contrast, therapeutic bacteria are developed specifically to target tumors.

1. Hypoxia - Blood vessels feed only the outer rim of a tumor, leaving the inner core starved of nutrients and oxygen. This hypoxic environment is ideal for obligate anaerobic bacteria, which cannot survive in healthy oxygenated tissues but thrive in the tumor core, feeding on dying tumor cells and the nutrients they provide. Facultative anaerobes also do well under these conditions.

2. Immune Escape - The hypoxic tumor core is inhospitable to immune cells, which require oxygen for survival. The tumor also suppresses immune system components in its vicinity, preventing those components from entering the tumor while also blocking their activation. Hence, the tumor is considered an “immune-privileged” site, where bacteria can escape immune attack. Attenuated and even avirulent bacteria (and viruses) can survive within immune-privileged tumor tissue, although they are cleared by the immune system from the rest of the body. These weakened strains used for treating cancer pose less risk to the patient than would virulent strains.

3. Myeloid-Derived Suppressor Cells (MDSCs) - MDSCs can deliver bacteria to tumor tissue. Patrolling the body for invaders, MDSCs engulf bacteria and digest them. However, some bacteria, such as Listeria and Salmonella, infect
MDSCs. Once inside MDSCs, these bacteria are protected from extracellular immune responses, which could otherwise eliminate them before they reach the tumor. When MDSCs home to and infiltrate tumor tissue, bacteria escape from them to infect the tumor.

Ensuring Safety with Genetic Engineering
Genetic engineering can enhance the safety of microorganisms used for treatments in several ways, including by decreasing their toxicity, increasing susceptibility to immune clearance, and enhancing tumor targeting, to give patients the upper hand over these infective therapeutic agents. Combining these techniques further enhances safety.

1. Attenuation

Attenuation follows the same principles as are used in vaccine design to ensure that the therapeutic bacteria will not harm the patient or cause invasive disease. However, attenuation must strike a balance between safety and efficacy. Bacteria used for therapy must retain some virulence to sufficiently attack the tumor or induce antitumor immune responses. Researchers and clinicians walk a fine line between endangering patients and curing them. Erring on the side of caution, overly attenuated bacterial therapeutics have failed in clinical trials.

- **Removal of Virulence Factors**—The pathogen may be stripped of some virulence factors that injure host cells, such as toxins or secretion systems, yet others may be retained because they act against tumors. For example, C. novyi must be stripped of α-toxin for safety, but its PLC toxin is retained for efficacy (13, 31).

Some virulence factors dampen antimicrobial immune responses, allowing pathogens to evade the immune system. Deleting these factors can enhance both safety and efficacy by allowing the immune system to clear the bacteria from healthy tissue, while also enhancing tumor-targeting and increasing immune responses against infected tumor tissue.

Genes encoding virulence factors, that are upregulated during infection but perhaps not in culture, are good candidates for attenuation, as they are likely to be involved in immune evasion, tissue invasion, and colonization. In the case of RASVs, some genes are engineered to delay synthesis of their products, including antigens and toxic substances, until after they are administered to patients. Delaying their production relieves this metabolic burden when the microorganisms are being administered to the patient, and it also avoids various host responses that might reduce the ability of the microbe to colonize targeted tumor tissues.

- **Auxotrophy**—Auxotrophic mutants, those which cannot synthesize essential amino acids or nucleic acid components, cannot survive without outside sources of these building blocks. While these nutrients are present in limited supply in healthy tissue, they are plentiful in necrotic tumor tissue as byproducts of cell death, allowing auxotrophic bacteria to thrive within tumors. Thus, using auxotrophic bacteria is critical if facultative anaerobes are to be restricted to growing within tumors.

Salmonella, for example, can be attenuated by combining mutations that lead to purine and amino acid auxotrophy (32). However, purine plus amino acid auxotrophy appears to over-attenuate these bacteria, meaning they tend to lose their capacity to make tumors regress.

Auxotrophic mutants are cleared from normal tissue, even in immunodeficient mice, but target tumor tissue 1,000-fold over healthy tissues. Leucine and arginine auxotrophy, combined in a strain designated AR1, serves as a useful foundation from which further manipulations are being evaluated (33, 34). Additional means for rendering antitumor bacteria incapable of causing infectious disease in immunodeficient or immunocompromised individuals are under development.

- **LPS and Septic Shock**—One risk in using Gram-negative bacteria such as Salmonella is that lipopolysaccharides (LPS) on their outer surfaces can induce release of TNF-α in recipient individuals, resulting in potentially deadly septic shock. One approach to reduce this risk is to use msbB mutants, which do not produce the inflammatory form of these lipids, (35). Macrophages, which release TNF-α when they bind ordinary LPS, do not recognize the LPS produced by these mutants. Another approach to avoiding this risk is to use a strain of Salmonella that synthesizes mono-phosphoryl lipid A, which triggers TLR4-dependent innate immune responses without triggering septic shock (36) (Figure 7).

Only lipid A in LPS is toxic to humans. Removing the O-antigen and other parts of the LPS core by introducing various mutations leads to a variety of phenotypes—

Mutations that attenuate otherwise virulent microbial pathogens should be designed never to revert when the microbes are replicating within the patient, which could lead to invasive disease. Thus, gene deletions are preferable to amino acid changes. Attenuated mutants should be tested for reversion in culture and animal models.

LPS can cause septic shock, but it also protects bacteria from host immune responses and environmental conditions. Salmonella deficient in LPS may be too attenuated to be effective—for example, by making the outer membrane more permeable and susceptible to host complement, high acidity, and carbon dioxide, all of which these bacteria are likely to encounter in the tumor microenvironment. Indeed msbB mutants are rapidly cleared and do not colonize tumors well. However, adding a mutation in a different gene restores resistance to these environmental conditions, generating a strain that can survive in tumors without causing septic shock. This example illustrates the unpredictable challenges and fine-tuning required when optimizing strains for therapeutic use (37).
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Therefore, passaging microbes in vitro can affect efficacy in vivo. Moreover, passaging microbial strains for use as therapeutic agents may also be more powerful to use when developing microbial strains for use as therapeutic agents. Whereas individually engineered mutations can impose unintended costs on a microbial strain, directed evolution allows for multiple compensatory changes that improve the overall fitness of the microbe.

In vitro techniques focus on factors known to inhibit efficacy in vivo, and they mimic these conditions in the laboratory to force selection for a safer, more effective microbe. These factors include harsh environmental conditions, such as acidic pH that is typically encountered within the tumor microenvironment, as well as exposure to damaging host proteins and cells. For example, passaging microbial strains in fetal bovine serum selects for those microbes that can withstand exposure to host blood proteins, facilitating intravenous delivery of those selected variants. Similarly, co-culture with immune cells selects for microbes that evade immune clearance, prolonging their circulation times. However, in vitro passaging has limitations, involving factors beyond our knowledge or ability to mimic in vivo that can affect efficacy in vivo. Therefore, passaging microbes in animals and isolating those variant microbes with desired mechanisms or, alternatively, limiting cytotoxic gene expression within tumors further reduces the risk of off-target side effects from such infections.

- **Receptor Mediated:** Some bacteria have inherent affinities for specific tissue types, a trait that can be repurposed to target them or other species and strains to tissue-specific tumors. Alternatively, receptor-mediated systems can be engineered into strains. For example, a *Listeria* strain was engineered to target breast cancer by introducing into it the gene for a *S. aureus* surface protein that binds to the therapeutic antibody Herceptin, which is internalized by HER2-expressing breast cancer cells (39).

- **Targeting to Macrophage:** Because macrophages engulf microbes and shield them from immune responses or other environmental conditions, they appear to be a good choice for transporting therapeutic bacteria and oncolytic viruses to tumor tissues (39). Bacteria can be engineered to target macrophages by repurposing inherent targeting mechanisms, including phagocytic uptake, survival within the macrophage, and escape from the phagolysosome, from *Salmonella* and *Listeria*. Macrophage-targeted strains might also help to circumvent the immune-suppressive behavior of MDSCs.

- **Promoter Control:** Tumor-specific promoters can limit toxic gene expression in bacteria until after they infiltrate tumors, while promoters that respond to tumor-specific environmental conditions may also prove useful, such as

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**Figure 7.** Comparison of mono-phosphoryl lipid A and LPS structures. Image retrieved from Casella CR and Mitchell TC. 2008. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 65:323-40.

Text box 2. Directed Evolution

Even with intricate knowledge of molecular mechanisms, genetic engineering can lead to surprising results. Directed evolution procedures typically subjects microbes to conditions that select for those that survive in particular environments. This technique is exponentially faster and cheaper than genetic engineering, and may also be more powerful to use when developing microbial strains for use as therapeutic agents. Whereas individually engineered mutations can impose unintended costs on a microbial strain, directed evolution allows for multiple compensatory changes that improve the overall fitness of the microbe. Directed Evolution techniques focus on factors known to inhibit efficacy in vivo, and they mimic these conditions in the laboratory to force selection for a safer, more effective microbe. These factors include harsh environmental conditions, such as acidic pH that is typically encountered within the tumor microenvironment, as well as exposure to damaging host proteins and cells. For example, passaging microbial strains in fetal bovine serum selects for those microbes that can withstand exposure to host blood proteins, facilitating intravenous delivery of those selected variants. Similarly, co-culture with immune cells selects for microbes that evade immune clearance, prolonging their circulation times. However, in vitro passaging has limitations, involving factors beyond our knowledge or ability to mimic in vitro that can affect efficacy in vivo. Therefore, passaging microbes in animals and isolating those variant microbes with desired mechanisms or, alternatively, limiting cytotoxic gene expression within tumors further reduces the risk of off-target side effects from such infections.

- **Cell Wall:** Impairing cell wall production is another way to make bacteria vulnerable to host immune responses and to other environmental conditions that they may encounter. Moreover, like LPS-deficient bacteria, strains with defective cell walls may be cleared before they reach the tumor, and they also are unsuitable for oral delivery, which requires an intact cell wall to withstand stomach acid.

- **Motility:** Mechanisms of bacterial motility, such as flagella-based swimming, allow bacteria to spread throughout the body. Impairing these mechanisms can severely attenuate strains. There are five levels of regulation involved in expressing genes needed for fully assembled and functional flagella, and some of the regulatory genes have other functions that may be critical for virulence. However, motility can also contribute to antitumor effects, enabling bacteria to invade tumor tissue. *Listeria*, for example, exerts direct cytotoxic effects on tumor cells by propelling itself from MDSCs into neighboring tumor cells. Motile bacteria can also disperse within tumor tissue to more effectively eradicate tumors (38).

- **2. Enhanced Targeting:** Some inherent tumor-targeting mechanisms help to limit specific kinds of bacterial infections to tumor tissue. Adding targeting mechanisms or, alternatively, limiting cytotoxic gene expression within tumors further reduces the risk of off-target side effects from such infections.
the hypoxia-inducible promoter (HIP-1). Combining these approaches enhances specificity. Shiga toxin 2 expression can be controlled in Salmonella by a promoter that is tumor-specific and also responsive to acidic pH, which is characteristic of the tumor microenvironment.

Yet another approach is to use radiation-inducible promoters, thereby adding to the therapeutic effects of radiation that is being used to treat tumors. These promoters may be used to control expression of factors that are directly cytotoxic or that sensitize tumors to other treatments. For example, the cytokine TNF-α makes tumor cells more sensitive to radiation.

- **Serial Passaging**-Passaging microbial strains through tumor-bearing animals to select those that home to tumors generally leads to strains that are safer and more effective than the parent microbe. This technique can enhance targeting to specific tumor types or, possibly, can be used to identify microbial strains with broader target ranges than those of the parent (See box “Directed Evolution”). Strains might even be tailored to individual patients by passaging them through patient tumor samples ex vivo (34).

3. **Antibiotic Sensitivity**- Microbes used in therapeutic protocols should be monitored for their sensitivity to antibiotics (Figure 8) ensuring that, if a systemic infection were to occur, it could be readily treated. Pre-clinical development of such strains should avoid antibiotic selection steps that might lead inadvertently to resistance. Investigators also should not use any strains with plasmid-encoded virulence factors, including genes for toxins and antibiotic resistance that could be transferred between bacteria.

4. **Co-morbidities**- Cancer tends to strike individuals later in life, when other health problems also occur: Such co-morbidities should be taken into account when microbes being developed for therapeutic applications are being tested in animals or readied for clinical trials. For example, cancer patients who are immunocompromised are likely to fail microbe-based treatments that depend on the recipients’ immune systems responding to a particular stimulus. Moreover, some types of therapeutic microbes might prove highly invasive and thus dangerous when used in immunocompromised or elderly populations.

5. **Biologic Containment**- Efforts should be made to contain therapeutic bacteria within those patients who are being specifically treated with them, taking care not to release genetically modified organisms into the environment. Attenuating such strains generally supports other efforts to contain them biologically, leading to their inactivation and elimination by a patient’s immune system before viable therapeutic bacteria are excreted. Mutations that impair survival of such strains outside patients or engineering strains with delayed-self destruction systems are additional safeguards.

After patients are treated, steps should be taken to eliminate therapeutic microbes from recipients to prevent unintended consequences. While any remaining microbial populations may be miniscule, they are likely to persist and thus might seed subsequent infections. Moreover, persistent therapeutic microbes might be passed unintentionally to others, including healthcare workers or family members, again leading to unintended consequences. Therefore, clearing therapeutic bacteria from patients with antibiotic treatments should be considered a standard practice. However, the timing of such antibiotic treatments needs to take into account whether antitumor immune responses are fully established and tumors eradicated. The question of whether persistent microbes might be beneficial for some patients remains to be addressed.

![Figure 8. Kirby-Bauer disk diffusion susceptibility test on coagulase-negative Staphylococcus aureus grown on Mueller-Hinton agar with tetracycline (30 µg), cephalothin (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), vancomycin (30 µg), penicillin (10 µg), streptomycin (10 µg), and novobiocin (30 µg). Microorganisms used in therapeutic design include penicillin (10 µg), streptomycin (10 µg), and novobiocin (30 µg).](image)


Parameters may offer some advantages. This technique requires little or no prior knowledge of mechanisms involved, and accounts for multifaceted, interacting mechanisms that we may be unaware of or that may be difficult to simulate in culture. For example, instead of engineering complicated systems to enhance tumor targeting, bacteria can be injected into animal models for a particular kind of cancer and then isolated from tumor tissue. The strain retrieved from the tumor is expected to be better than its parent at targeting, surviving in, and multiplying within such tumors. Various serial passaging strategies could optimize microbial agents for different purposes, such as specializing them against the unique physiology of large tumors or other specific cancers (34, 41). Directed evolution can improve upon nearly any parameter, including pharmacokinetics, to optimize microbes for clinical use. For example, selecting for phages that remain in circulation vivo extends circulation times from minutes to hours. The amino acid change responsible for this improvement would have been virtually impossible to identify by other means (42).

Passaged microbial strains can be readied quickly for preclinical and clinical trials, or further modified by genetic engineering. They also provide information that can guide rational design strategies into following new directions by revealing microbial genes, proteins, and mechanisms that improve efficacy, and may also provide insights into host disease pathology. For such reasons, it makes sense to develop passaged microbial strains for use in clinical studies, while simultaneously delving into the basic biology behind them.
Meet the Players: Which Bacteria Are Best?

Bacteria with antitumor activity fall under two major categories: obligate and facultative anaerobes. Of the two obligate anaerobes are safer because they cannot survive in oxygenated healthy tissue. However, because they are excluded from the vascularized outer rim of tumors, they must somehow be endowed with the capacity to elicit strong antitumor immune responses or be used with traditional treatments to fully eradicate tumors. Further, because obligate anaerobes apparently need to be injected intratumorally (i.t), they may be precluded from use in treating patients with disseminated metastases.

In contrast, facultative anaerobes can infect hypoxic as well as oxygenated tissues, treating both necrotic and viable areas of tumors. However, they pose a greater threat of also infecting healthy tissue, and therefore must be carefully attenuated. Endowing them with auxotrophic mutations and increasing their susceptibility to immune responses are first-round attenuation strategies.

- **Obligate Anaerobes**: *Clostridium* is a genus of obligate anaerobes with potentially useful antitumor activities. *Clostridium* poses little threat to humans when its alpha/lethal toxin is removed. Moreover, it can be stably stored as spores that, when injected directly into hosts, can germinate in hypoxic tumor tissues. However, not all of the toxins produced by Clostridial species have been elucidated. Clinical trials are under way evaluating spores from *C. novyi* as a treatment against tumors (10).

- **Facultative Anaerobes**: *Salmonella typhimurium* is an example of a facultative anaerobe with antitumor activity. With extensive research to build on, many attenuated strains of *Salmonella* are available to be repurposed for evaluating their antitumor activities (5, 29).

*Salmonella* is suited for oral delivery. Whereas other bacteria pass through or are killed in the gut, *Salmonella* cells infect intestinal epithelial cells, where they induce immune responses and attract immune cells. They also infect macrophages, which can be used as delivery vehicles to target *Salmonella* to tumor tissue. Because oral delivery of *Salmonella* fosters contact with macrophages, this means for delivering them may be more effective than intravenous injection, which would expose them to circulating neutrophils that could kill them before they would reach tumor tissues.

*Salmonella* infect both intracellularly and extracellularly, delivering direct cytotoxic effects as well as inducing indirect immune responses. The hyaluronidase enzymes that *Salmonella* produce may enable them to breach fibrotic barriers that protect some tumors, including difficult-to-treat pancreatic cancers (43). This array of mechanisms helps to make *Salmonella* effective against a broad spectrum of cancers being treated in animal models, including primary and metastatic, prostate,

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Researchers avoid using commensal bacteria for therapeutic purposes for several reasons. For example, the immune system does not eliminate commensals from healthy tissue, nor are they likely to exert substantial toxic effects on tumors. While genetic engineering can overcome these drawbacks, it may be difficult to tease apart the modified therapeutic microbes from their unmodified commensal counterparts. Genetic similarities between modified and wild-type commensals also makes genetic exchange that much more likely between the two populations, leading potentially to a wide range of unintended consequences.
breast, pancreatic, colon, bone, and brain cancers. When used to treat breast tumors in nude mice, the orally delivered A1-R strain of *S. typhimurium* appears to be effective and safe (44).

*Listeria*, another facultative anaerobe being evaluated for its antitumor activity, infects MDSCs, which can be used to deliver these bacteria to tumors. Once within a tumor, *Listeria* cells escape MDSCs to infect the tumor cells, producing reactive oxygen species (ROS) and eliciting immune responses against the tumor cells. *Listeria* also alters the behavior of MDSCs to promote pro-inflammatory, antitumor immune responses. Because these mechanisms synergize and thus might damage healthy host tissues, *Listeria* used in antitumor protocols will need to be carefully attenuated (45, 46).

The highly antigenic *Listeria* enzyme, listeriolysin O (LLO), offers yet another way to harness antitumor activity from this microbe. Chimeric molecules combining LLO with tumor antigens elicit strong antitumor T-cell responses, which can drift to recognize and respond more broadly to other tumor antigens. These responses include lasting memory that protects against tumor regrowth and metastasis. *Listeria* is particularly effective against metastatic prostate, breast, and pancreatic cancers in mice.

**Advantages over Traditional Treatments**

Some cancers are particularly resistant to treatment, with 5-year survival rates of such patients under 5%, showing little improvement in treatments or patient outcomes during the past half century. In the search for novel approaches to control these aggressive diseases, therapeutic bacteria have the potential to treat otherwise incurable disease (*Figure 9*).

1. **Toxicity**- Chemotherapy is toxic not only to tumor cells but also healthy tissues, sometimes causing debilitating or life-threatening side effects. The ability of bacteria to target tumor tissue could help to reduce or potentially avoid altogether such toxic side effects. Therapeutic bacteria are safe and well-tolerated in both animals and humans, even at very high doses (10, 11).

2. **Targeting**- Selectively delivering chemotherapeutic drugs to tumors remains a major challenge, requiring identification of tumor-specific antigens and extensive development of agents to target them. However, bacteria that target tumors can meet this challenge, offering means to treat a wide array of tumors, including those that are inoperable, those that are diffusely distributed such as glioblastomas, and metastases that may be too small or too numerous to identify.

3. **Resistance**- Rapidly multiplying and genetically unstable tumor cells too readily subvert conventional chemotherapies. However, evading the overlapping and independent mechanisms that bacteria bring against tumors could prove more difficult for tumor cells to overcome.

4. **Hypoxic Core**- Chemotherapeutic agents delivered via the bloodstream reach only the vascularized outer ring of a tumor without penetrating the hypoxic and necrotic core, which drive tumor regrowth and metastasis, and are resistant to radiation. Bacteria and viruses, however, are the only agents known to penetrate this core, offering hope that they can prove more effective than current treatments against the cancer cells found there.

5. **Metastases**- Advances in cancer treatment have improved survival rates for patients with many types of cancers; however, many fail when metastases develop. Importantly, these metastases—typically aggressive, recurring, treatment-resistant, and dispersed—are the leading cause of death from cancer, particularly in cases where primary tumors are asymptomatic or otherwise difficult to detect, including pancreatic, breast, and prostate cancers.

Bacteria are particularly effective at preventing and treating metastases because they can target microscopic lesions that cannot otherwise be readily identified. Moreover, nascent metastases are more vulnerable to immune and bacterial attack. Further, by inducing immune memory, bacteria could help to establish a surveillance system that identifies and removes metastatic cells before they can take hold. In mouse models of fibrosarcoma, pancreatic, and prostate cancers, all known for their particularly deadly metastases, *Salmonella* clears metastases and primary tumors without the help of chemotherapy or radiation (47, 48). Also, *Listeria* eliminates metastases in mice with triple-negative breast cancer (49).

6. **Cancer Stem-Cells**- Cancer stem-cells, another dangerous cell type in the hypoxic tumor core, appear to be responsible for relapse and metastasis. Some chemotherapies kill less aggressive tumor cells, while leaving these more aggressive tumor stem-cells intact. Although *Salmonella* bacteria kill pancreatic cancer stem-like cells in culture, whether they do so *in vivo* is not yet known (50). Engineering bacteria to target such stem-cells would be groundbreaking.
IV. Bacteriophages against Cancer

Bacteriophages (phages), viruses that infect bacterial cells, can be genetically engineered to target cancer cells (S1, S2). Some antitumor approaches employ phages as particles to stimulate anti-cancer immune responses, while others use phages to inject cytotoxic agents into tumor cells (Figure 10 and 11).

1. Vaccination - Phages can be genetically modified to express on or several types of tumor-specific antigens that, with native immunostimulatory phage components, induce robust antitumor immune responses (S1). Because they are recognized as foreign particles and engulfed by antigen-presenting cells (APCs), phages can be used as adjuvants to amplify innate and adaptive immune responses.

There are two major types of phage-based, antitumor vaccines. In one, phages are engineered to express fusion proteins that consist partly of disease antigens and partly of immunogenic phage surface proteins (Table 2). When engulfed by APCs, the APC displays the fusion protein via MHC I and II molecules, eliciting CD4 and CD8 responses (S1). In the other, DNA-based vaccines are engineered to include disease antigens encoded within the phage genome and regulated under a eukaryotic promoter: When APCs engulf these DNA molecules, the APC cells express the disease antigen, inducing T-cell responses. These two approaches can be combined to optimize efficacy (S1).

2. Targeted Delivery - Phages can be engineered to target specific cell types through receptor-ligand interactions (S3). Because phages replicate exclusively within bacterial host cells, targeting phages to enter tumor cells is an intermediate step in a broader strategy that also requires further engineering them to deliver antitumor agents.

For the purposes of targeting phage, first a tumor specific antigen is identified to serve as a receptor—for example, a receptor that brings essential nutrients into tumor cells. This part of the strategy is based, in part, on how lambda phage invades cells of E. coli via the bacterial cell receptor for maltose (S4). Lambda and other phage display ligands on the head region or as part of their tail fibers that interact with a wide variety of bacterial receptors. However,

Text box 3. History of Bacteriophages as Therapeutic Agents

Felix d’Herelle of the Institut Pasteur first used phage therapeutically in 1919, successfully treating four patients with dysentery after ingesting it himself to test its safety. He later developed phages commercially for L’Oreal’s predecessor. In the United States, Eli Lilly and Company marketed phage products during the 1940s, providing phages that were used to treat infections caused by staphylococci, streptococci, E. coli, and other bacterial pathogens.

Phage therapies were widely abandoned following the discovery of antibiotics except in Eastern Europe and countries of the former Soviet Union. The Eliava Institute, which D’Herelle helped to establish in Georgia, and the Hirszfeld Institute in Poland were both dedicated to investigating phage therapies. Translated reports describe phage therapies for a wide range of diseases, including acute and chronic infections of the skin, eye, urinary tract, lung, and sepsis, caused by various pathogens, including Pseudomonas, Staphylococcus, Klebsiella, and E. coli. Even

Figure 10. Schematic representations of several phage-mediated vaccine delivery systems. (1) Production of virus-like particles (VLPs) by adding purified phage capsid proteins fused to antigens during assembly of phage particles in vitro. (2) Antigens are attached by chemical conjugation on preassembled phage head. (3) Antigens are displayed on phage surface by fusion of antigen genes with phage capsid protein genes. (4) Phage as a DNA delivery vehicle where antigen genes are cloned in phage genome under the control of eukaryotic promoters. (5) Phage for DNA vaccine, where phage carries antigen genes under the control of eukaryotic promoters. The phage also displays foreign proteins on its surface as fusion of phage capsid proteins. This protein targets the phage to antigen-presenting cells (APC).

Image retrieved from Adhya S et al (S1).
designing such ligands requires having a detailed understanding of receptor structures and their interactions at the molecular level.

Alternatively, phage that target tumor cells can be generated by directed evolution, which entails exposing phage with variable tail domains to tissue culture or whole animals, and then isolating phage that are found within tumor cells. This approach is potentially much faster than traditional rational design strategies (See box “Directed Evolution”).

- **Chemical Linkage**—Toxic chemotherapy agents can be linked to phage surfaces for delivery specifically to tumors, avoiding systemic exposure to those agents, thus reducing the likelihood of their causing toxic side effects.

- **Gene Delivery**—Phage can be genetically modified to inject genes encoding proteins with antitumor effects into tumor cells (53, 55). These proteins may include inhibitors of cell growth and proliferation, stimulators of apoptosis, cytotoxic proteins, enzymes that convert non-toxic precursors into toxic by-products, and immunostimulatory proteins. In addition, delivery of siRNA can block expression of endogenous proteins.

Table 2. List of phages and their virion proteins, which are used for display

<table>
<thead>
<tr>
<th>Vector phage</th>
<th>Phage virion proteins for fusion display</th>
<th>Copy number of display</th>
<th>Size of display shown</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>gpVIII</td>
<td>≈2700</td>
<td>Small peptide (6–8 amino acids)</td>
</tr>
<tr>
<td></td>
<td>gpIII</td>
<td>≈5</td>
<td>Large protein with reduced viability</td>
</tr>
<tr>
<td>T4</td>
<td>Soc</td>
<td>≈960</td>
<td>Large protein (up to 837 amino acids)</td>
</tr>
<tr>
<td></td>
<td>Hoc</td>
<td>≈160</td>
<td>Protein (up to 183 amino acids)</td>
</tr>
<tr>
<td>T7</td>
<td>10A</td>
<td>≈415</td>
<td>Protein (40–50 amino acids)</td>
</tr>
<tr>
<td></td>
<td>10B</td>
<td>≈1</td>
<td>Protein (up to 1200 amino acids)</td>
</tr>
<tr>
<td>Lambda (λ)</td>
<td>gpD</td>
<td>≈420</td>
<td>Large protein (1024 amino acids)</td>
</tr>
</tbody>
</table>

Table retrieved from Adhya S et al (51)

Figure 11. Immune activation of mammalian system with phage display antigens. (1) Particulate nature of phage activates the antigen-presenting cells (APC), which process the antigens for immune presentation. (2) Presentation of processed antigens by major histocompatibility complexes (MHC) class I molecules to CD8 T cells, which leads to T-cell activation. (3) Antigens are presented by MHC class II molecules to CD4 T cell, which in turn activates T_{H1} and T_{H2} effector cells. (4) T_{H1} cells generate cytotoxic T-cell (CTL) responses and help produce interferon-γ (IFN-γ). (5) T_{H2} cells activate B cells to make antibodies. (6) Direct activation of B cells by phage vaccines also leads to massive antibody response. T-cell receptors are denoted by TCR.

Image retrieved from Adhya S et al (51).

Reports of prophylactic approaches with phages also describe them as highly successful. In a study of more than 30,000 children in Poland, treatments with Shigella phages decreased the incidence of dysentery nearly 4-fold. Similarly, in India, prophylactic *Vibrio cholera* phages reduced deaths from this pathogen by more than 50-fold, prompting the government to require villages to use such treatments. During World War II, Russian soldiers carried phage canisters to treat battle wounds against infection.

Despite claims of clinical successes with phages, many of the Eastern European phage studies lacked rigor: some failed to run appropriate controls or to compare treated patients with those who received placebos, while other studies were poorly documented in terms of design and data analysis. Thus, further study is necessary to evaluate the validity and safety of phage-based therapies (51, 52).
that contribute to tumor growth, such as cell-cycle promoters, immunosuppressive proteins, and inducers of vascularization.

Advantages and Limitations of Phages in Cancer Treatment
The relative advantages and disadvantages of bacteria-based versus virus-based antitumor approaches need to be weighed carefully (56).

• Safety- Because phage do not infect human cells, they are inherently safe for use in humans. Both bacteria and viruses have targeting and cytotoxic mechanisms that can enhance efficacy but pose safety issues.

• Targeting- Although selecting phage to target human cells can be labor-intensive, combining phage display libraries with directed evolution can streamline this process. Typically, it will likely be necessary to manipulate bacteria or other types of viruses before they are suited for targeting specific tumors. Meanwhile, however, approaches involving antitumor vaccines do not require special targeting steps.

• Replication- Because phage cannot replicate in human cells, they pose no threat of causing infections within patients, thus avoiding many safety and related regulatory concerns, while also reducing the likelihood that tumors will develop resistance against them. Without the ability to amplify the dose in target tissue, however, phage treatments seem unlikely to eradicate every tumor cell and may not provide long-term efficacy.

• Gene Delivery- The size of phage particles limits the number and size of genes that can be engineered into their genomes. Larger viruses are more amenable to accepting higher numbers of and larger genes. Bacteria have a virtually unlimited capacity for genetic insertions, but can prove difficult to optimize for gene delivery.

V. Bacteriophages for Treating Bacterial Infections
Phages control bacterial populations in nature and are a key component of microbial ecology in the external environment as well as within our own microbiota. For more than a century, they have been used to treat various bacterial infections (See box “History of Bacteriophage”). With antibiotic resistance of growing concern, phages are being studied as an alternative means for treating drug-resistant strains of bacterial pathogens (51, 52).

A Bit about Phages: Structure and Life Cycle
A phage consists of a genome containing either RNA or DNA that is surrounded by a protein capsid. Some phages have tail fibers that interact with bacterial receptors, helping to determine host specificity. Phage tails sometimes are involved in injecting its genome into a host bacterium. In other respects, tail-host receptor interactions are largely uncharacterized, but generally limit host range to specific bacterial genera, species, or strains. Phages have specificity for their host species (Figure 12).

Once within a host bacterium, phages replicate by one of two mechanisms. Lytic phages immediately reproduce, rapidly disrupting their host cells from within and then releasing as many as 1,000 progeny per cell. Temperate, or lysogenic, phages incorporate their genomes into the genomes of their hosts to become dormant. When the bacterial cell divides, the prophage genome is replicated along with the bacterial genome.

Lytic phages are better suited than lysogenic phages for treating human bacterial infections because they rapidly and specifically kill their host bacteria while also releasing progeny phages that stimulate innate immune responses that may further help to clear remaining bacteria.

Advantages of Phages over Antibiotics
Phage therapy offers many advantages over antibiotics for treating bacterial infections. While some antibiotics merely stop bacteria from replicating, lytic phages kill the bacteria that they target. Those antibiotics that are bactericidal can take hours or days to work, whereas lytic phages typically kill bacteria within several minutes, bringing quick relief to patients and allowing the targeted bacterial pathogens little time to develop resistance. One drawback is that fast-acting phages can release a bolus of endotoxin from Gram-negative bacteria, meaning that treated patients risk developing a shock response (See Safety Concerns p 15).

• Resistance- Multiple mechanisms make phages much more difficult for bacterial pathogens to evade than single-target antibiotics. If resistance arises to a specific phage being used as an antimicrobial agent, it is possible to identify replacement phages within days to weeks, in contrast to the many years and enormous expense required to develop novel antibiotics.

• Self-Titrating Dose- Phages replicate at the site of a bacterial infection, rapidly increasing
only where they are needed. Phage replication corresponds to the level of a bacterial infection within each patient. This correspondence in dose holds true spatially and temporally as an infection grows worse or subsides. This phage property overcomes many dosing issues, suggesting that administering one-time doses of phages to a patient will be sufficient, overcoming conventional drug-compliance issues that can contribute to the development of resistance.

- **Toxicity** - In contrast to antibiotics, which damage or kill non-targeted members of the microbiota, phages are very species specific, leaving benign members of the microbiota intact and, thus, sparing patients complications that may arise when the microbiome is disrupted (See box “History of Bacteriophage”). However, phage treatments may release toxins when they disrupt targeted bacterial pathogens.

- **Biofilms** - Phages may provide an effective means for disrupting pathogens that form biofilms, many of which include a polysaccharide layer that is impenetrable to conventional antibiotics. Some types of phage produce a polysaccharide depolymerase that enables them to break through such barriers, while other phages can be engineered to produce this enzyme.

**Safety Concerns**

Phages are ubiquitous, meaning humans regularly contact, consume, and, in some cases, shed phages at rates as high as three billion plaque forming units (pfu) per day. These facts speak to the safety of phages, a claim that is further supported by a century of medical use without reports of serious side effects (See box “History of Bacteriophage”). Even so, it is prudent to proceed with caution with the development of phages for use as antimicrobial agents.

- **Septic Shock** - Some phages rapidly lyse bacteria, releasing endotoxins that can cause deadly septic shock. Though septic shock has not been observed in phage-treated patients, it is important to minimize this risk as phage antibacterial treatments are being developed. Phages can be engineered not to lyse their target bacteria by deleting the gene encoding endolysin from the phage genome. Such lysis-deficient phages still cripple their target bacteria via endonucleases that degrade the host genomes of the bacterial pathogens being targeted. Moreover, the crippled but otherwise intact bacterial cell can stimulate immune responses that will help to clear the infection (57).

- **Gene Transfer** - Some phages transfer genetic material from one bacterial cell to another, including genes encoding antibiotic resistance and toxins. However, this capacity is limited to lysogenic phages, which are incorporated into the genomes of their bacterial hosts and then excised from them, sometimes carrying host-cell genes. Lytic phages, which do not pose this threat, more rapidly and effectively kill target bacteria, making them preferable for therapeutic applications. In more general terms, phage life-cycles should be well characterized prior to their development for therapeutic use.

Lysis-deficient phages offer safety benefits but efficacy drawbacks. Because the host bacterial cell does not lyse, the capacity of such phages to replicate and spread is limited. This restriction may ease public and regulatory concerns, but might also prove a disadvantage by curtailing some of the most potentially powerful aspects of phages that are being developed for use as antimicrobial agents.
Additional Challenges and Engineering Solutions
While phages offer many advantages as antimicrobial therapeutic agents, several challenges need to be addressed.

• **Specificity** - While the high specificity of phages can be advantageous, therapeutic agents need to be broad enough to target all strains of a particular bacterial pathogen causing an infection. Phages with broad genus-specific host ranges exist. Additionally, phage mixtures can provide broad-spectrum targeting and, through directed evolution, phages can be selected to provide broader coverage of targeted pathogens (See box “Directed Evolution”).

• **Immune Clearance** - The immune system can clear phages rapidly, potentially limiting their efficacy as antimicrobial agents. Phages with prolonged circulation lifetimes can be generated by directed evolution (See box “Directed Evolution”) or they can be genetically engineered to decrease their immunogenicity (58). Although dosing strategies might help in prolonging the half-lives of some antimicrobial phage agents, this shortcoming may limit phage therapy to use in treating acute systemic infections as well as mucosal and skin infections.

• **Delivery** - Because phages are not mobile and must somehow be delivered to the site of infection, easily accessible infections such as those involving the skin, gastrointestinal tract, and lungs—and those caused by pathogens such as *Klebsiella*, *Clostridium*, and *Pseudomonas*—are among the best early targets for establishing proof of principle. Intracellular infections appear to be more challenging for phage-based therapies.

Environmental and Prophylactic Applications
Phages may be used directly to treat patients with bacterial infections or prophylactically to prevent infections.

• **Food Production** - Foodborne pathogens are a major source of outbreaks, and strategies are already in place that use phages to kill *Salmonella*, *Listeria*, and *E. coli* during processing of meats, cheeses, and fresh produce. Some phages are administered to live animals, while others are used while meat is being processed. For example, a two-week phage treatment prior to cattle being slaughtered reduces the likelihood of beef from those treated animals becoming contaminated with *E. coli* O157:H7. Such phages are non-toxic and do not affect animal growth or select for antibiotic resistance.

• **Water Sources** - Phages can rid contaminated water sources of several specific bacterial pathogens that cause diarrheal disease. This application could be particularly valuable in public health terms for people in developing nations and during natural disasters, when *Vibrio* and *Shigella* can run rampant in drinking water supplies. Phages can be deployed quickly and inexpensively, with a small initial dose perhaps enough to amplify once established within the bacterial population being targeted.

• **Human Microbiome** - The human microbiome can be a source of pathogenic bacteria, including *Staphylococcus aureus*. While typically innocuous on the skin, *S. aureus* becomes problematic when it breaches the epithelial barrier—for example, through wounds such as surgical incisions. Phages could be used as a prophylactic treatment before surgery to decolonize patients and reduce the risk of these bacteria causing opportunistic infections.

During the 1930s, phages were used to treat parts of the Ganges River in India to control *V. cholera*, dramatically reducing cholera outbreaks among people bathing in the river and drinking its water. Phage treatment of water supplies might have prevented the Haitian cholera outbreak of 2010, but public opposition prevented such plans from being implemented.
VI. Challenges and Solutions Moving Forward

While many preclinical results from the testing of therapeutic microbes are encouraging, some major obstacles remain to be overcome before these strategies are ready to be evaluated clinically. These challenges include increasing safety, optimizing efficacy, and adapting treatments for use in human systems. Other challenges include educating clinicians, investors, government regulators, and the general public about these developments to build trust and interest in these potentially valuable agents for treating disease.

Tailoring Treatment Regimens for Efficacy against Cancer

Ideally a single, orally administered dose of a microbial therapeutic agent would provide full activity against all primary cancers and prevent the development of any metastases. Realistically, however, this goal is likely to be unattainable. How to develop and best use therapeutic microbes leaves several broad questions to address.

- **One Agent Fits All?** Some bacterial strains are effective against multiple tumor types in animal models, suggesting that a one-size-fits-all bacterial treatment for solid tumors may be a workable strategy. Following this approach would simplify clinical development and testing in the lead up to regulatory review. However, developing specialized microbial strains for use against specific tumor types might be a necessary alternative to generate more effective treatments.

- **Primary versus Metastatic Tumors?** Primary tumors and metastases pose different challenges and respond differently to traditional treatments. Whether therapeutic microbes can effectively treat both is under debate, and may depend on each agent being developed. Questionable potency of microbial agents against primary tumors suggests that established lesions may be more difficult to disrupt than microscopic metastases and that these microbial agents may need to be used in combination with traditional anticancer treatments. Even so, finding that these novel microbial agents are effective in treating metastases is an important step forward, as metastases are generally resistant to treatment and, thus, more aggressive and deadly than primary tumors themselves (40, 46, 47).

- **Preventive versus Therapeutic Treatment?** A therapeutic vaccine ideally would not only treat primary tumors but also prevent metastatic tumors. However, achieving both these goals together requires that the agent induce potent as well as lasting immune responses. Doing so will likely require strategic dosing to optimize the therapeutic window, with subsequent dosing to meet the preventive as well as therapeutic requirements.

- **Repeated Dosing?** Like many conventional vaccines, microbial anticancer vaccines used for treating cancers will likely need to be administered repeatedly to generate strong and lasting immune responses against tumors. For example, weekly administration of *Salmonella* dramatically enhances its efficacy when used as an antitumor immunogen.

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**Text box 4. Serving Developing Nations**

Access to medical treatments in developing nations is limited by cost, agent stability, and availability of medical care. Therapeutic microbes help to overcome these obstacles. Because microbes reproduce, they are less expensive to make than traditional drugs, costing as little as $0.002 per dose ($2/1,000 human doses). Also, in cases where the therapeutic microbes can amplify within the host, small and relatively inexpensive single doses may be sufficient to provide full efficacy. Meanwhile, traditional medications typically require expensive multiple doses (57, 64).

Therapeutic microbes are relatively stable and may better withstand prevailing conditions in the developing world. While many traditional drugs and vaccines require refrigeration, phages typically are stable for 14 months and endure 55°C temperatures when lyophilized and encapsulated in pill form (65). Meanwhile, the bacterium *C. novyi* can be stored and administered as spores, which are resistant to heat and desiccation (57, 64).

Therapeutic microbes that could be orally administered would make it easier to treat patients in areas where medical care is limited, while single-dose efficacy would help in dealing with patient adherence issues. Meanwhile, prophylactic phage treatment of environmental sources of disease can reduce the overall disease burden, shrinking the need for costly medical care (57, 64).
vaccine in mice, inhibiting primary tumor growth and also metastasis, doubling survival time and curing 40% of animals (59). Determining the doses and timing of boosters to optimize long-term protection is very much a trial-and-error process, and booster protocols will likely differ from one species to another; meaning that vaccines developed for humans will need to be optimized in humans.

- **Administration Routes?** Different means for administering such agents will have advantages and limitations, requiring evaluation before being selected for a particular clinical use. Intratumor injection delivers agents directly into tumors, minimizing immune clearance. This approach appears promising based on canine and human clinical trials (10).

However, this delivery route can be technically challenging when applied to some kinds of tumors. Injecting antitumor agents into the spleen or lymph nodes, for example, can induce adaptive antitumor responses as well as anti-metastatic effects. Intraperitoneal injections are not difficult technically, but can induce innate and adaptive immune responses. Meanwhile, intravenous injection can increase the danger of triggering sepsis and immune clearance responses. Oral administration remains the easiest and least invasive approach, and may be workable when *Salmonella* is serving as the therapeutic agent because it effectively invades through mucosal tissues to reach regional and systemic lymph nodes (29). However, oral administration of such agents might not achieve the uniformity of dosing that is possible through parenteral injections.

- **Pre-Clinical Animal Models versus Human Systems?** Striking the right balance between safety and efficacy is likely to differ when moving an agent from tests in animals to clinical trials in humans, due to differences in immune systems and how microbes adapt to specific hosts. Nonetheless, investigators should not immediately abandon treatment strategies that initially fail when they are moved from animal models into humans. For example, a modified dosing strategy might make all the difference between failure and success. Exercising such patience during this stage of development may, of course, prove challenging in the face of arguments that a particular project appears too costly or risky to be worth continuing.

**Combination Therapies**

Combining therapeutic microbes with traditional therapies may prove necessary when treating some cases in which cancer or antibiotic-resistant bacterial infections are particularly resilient. Moreover, clinical trial protocols likely will require testing therapeutic microbes in combination with standard of care treatments.

Combinations of therapeutic agents should be selected carefully to avoid risks from potentially cumulative toxicities, unusual side effects, or counteracting agents that undermine each other’s effects. When possible, agents should be selected for the potential of the combined agents to synergize, complement each other’s deficiencies, target independent mechanisms, and reduce side effects. For example, immune- and nonimmune-mediated treatments might be combined, perhaps within a single microbe.

Another promising combination would be to use therapeutic bacteria with conventional chemotherapeutic agents to treat solid tumors, relying on appropriate microorganisms to target the hypoxic core of those tumors, which chemotherapeutic agents have difficulty in penetrating. Pairing therapeutic microbial agents with chemotherapeutics that specifically inhibit vascularization would increase the hypoxic areas of the tumor in which those bacteria would grow, while pairing immune response-inducing microbes with chemotherapies that enhance host immune functions could lead to synergistic effects. Bacteria can also be used to enhance the delivery of chemotherapeutic agents. For example, *Clostridium* expresses a protein that disrupts cell membranes, and this property might be harnessed to release liposome-encapsulated drugs within tumors (60).

Therapeutic microbes could be paired with radiation therapy as a way of destroying radiation-resistant cells within the hypoxic core of tumors (38). Because radiation therapy draws macrophage-carrying bacteria to irradiated tumors, enhancing the immunostimulatory, antitumor effects of those bacteria. Using microbes in follow-up treatments to stimulate immune system responses against tumors is yet another promising approach.

Combining all three approaches—therapeutic microbes, chemotherapy, and radiation therapy—might be necessary in some cases to maximize impact. However, timing when to deliver particular components within such combinations could prove to be critical and might vary when moving from results in animal models to humans. In general, it appears that microbes should be administered to patients prior to treating them with chemotherapeutic agents or radiation, both of which are toxic to white blood cells and therefore curtail antitumor immune responses. However, microbes could be administered soon after starting chemotherapy,
taking advantage of infiltrating immune cells to recruit microbes to tumors.

The clinical use of such combinations undoubtedly will yield unexpected consequences. Some experts argue against using such combinations, saying that efforts to develop them will complicate a field that now needs to focus on identifying agents that will work reliably and on their own. Others argue that synergistic combination therapies may be what will work best in patients. Ultimately, trial and error through experiments will be the best way to address these uncertainties, an approach that has held up historically, particularly in the development of anticancer therapies.

Selecting Standards

It is essential to select standards to facilitate progress in this growing field. Microbial strains, animal models, and testing methods should be selected and characterized to serve as a baseline for making meaningful comparisons of specific agents and particular treatment strategies. Otherwise, conflicting results will impede progress. Choosing suitable standards, however, may prove a difficult challenge. Some experts suggest systematically screening microbial strains to evaluate their prospects as therapeutic agents, while others argue that freezing specific options too early would be a mistake and could impede progress. In any case, strains chosen early to serve as standards are not meant to be optimal for use in medical practice, but to be used in foundation research that will lead to improvements in the overall technology. *S. typhimurium* strain UK-1, which is considered a standard for recombinant vaccine research, may be a good candidate for use as a standard strain in this emerging field of research on therapeutic microbes.

Animal Models

Inconsistencies between results from testing experimental therapies in mouse models and in human clinical trials can be problematic. Inconsistencies stem from several sources, including from differences between the mouse and human immune systems and from the testing of experimental therapies for their effectiveness against human tumor xenografts in immunodeficient mouse hosts. Because so many experimental microbial treatments rely on stimulating immune host immune responses, the widespread use of immunodeficient mice could be seriously misleading.

Other, animal species, including rabbits and guinea pigs, could prove more helpful than immunodeficient mice in animal model studies. However, results from studying these or other animal models can also be misleading. In general, such models of cancers do not fully and accurately reflect their counterpart diseases in humans. For example, subcutaneous tumor xenografts have different vasculature structure and are more easily cured by a variety of agents. Some experts argue that it would be better to use animals that spontaneously develop tumors rather than to rely on animals whose tumors were induced in experiments (61, 62).

- **Companion animals** - Companion animals such as dogs and cats that develop spontaneous tumors offer an unusual opportunity for evaluating anticancer. Spontaneously arising tumors in these animals may provide better approximations to those that develop in humans than do comparable tumors that are being studied in rodent species. Moreover, studying how such tumors respond to therapeutic microbes might also better approximate how those agents will behave when used to treat comparable tumors in humans.

A recent report in which a therapeutic *Clostridium* strain was used to treat tumors in dogs lends support to this idea (10, 11). In contrast to immunocompromised mice, the dogs treated with that experimental strain of *Clostridium* had intact immune systems, an important factor in terms of tumor pathology and the effectiveness of that particular treatment. Further, the size of tumors in dogs—in contrast to those in mice or other rodents—makes them more comparable to those in humans, along with closely related, issues such as vascularization, hypoxia, and clonal variation. Moreover, dogs share a variety of environmental exposures with humans, and develop a similar spectrum of cancer types. Indeed, for many researchers, dogs are the animal of choice for studying cancer (59).

Testing of experimental therapies in dogs has a long history, and many drugs now used to treat humans were first tested and approved in veterinary medicine. Although veterinary trials of experimental products are less costly and cumbersome than are human clinical trials, they provide proof-of-concept studies and translational information that can help to drive human trials forward. Approvals of veterinary medicines and subsequent sales of such products can allow companies to generate revenue, attract investor support, and build enthusiasm among researchers, clinicians, and the public for the development of these and other novel clinical products (63). Tests in dogs may be useful not only for developing microbial antitumor agents but also phages as therapeutic agents to use against bacterial infections.

- **Livestock** - Phage treatments and vaccines can be directed against—and tested in—various pathogens that infect livestock, which provide yet another population in which to evaluate generate anticancer immune responses, without which some chemotherapies are ineffective (67). This phenomenon may explain why chemotherapy works only in some patients but not in others. Thus, probiotics or fecal transplants that alter the microbiome composition, especially in the context of antibiotic use, may be important adjuvants to chemotherapy. The influence of the microbiome on the efficacy of therapeutic microbes may be even greater, due to their immune-mediated antitumor effects. Diet and environmental exposure also affect the composition of the microbiome, and may influence disease pathogenesis and treatment. Cultural prevalence of comorbidities such as chronic infections with anti-inflammatory nematodes, pro-inflammatory gingivitis, or infection with *H. pylori* could play a role in cancer or in how individuals respond to infectious agents. These epidemiological differences may yield clues to pathogenic mechanisms, as well as guide prevention and treatment approaches within specific populations.
Harnessing the Power of Microbes as Therapeutics: Bugs as Drugs

There are four proteins known to have these dual activities: azurin from *Pseudomonas aeruginosa*; arginine deiminase from *Mycoplasma arginini*; Laz from *Neisseria meningitidis*; and MPT63 from *Mycobacterium bovis* BCG (1).

Azurin, which preferentially enters cancer cells where it interferes with multiple pathways, has been studied extensively. A 28-amino acid peptide fragment of azurin, called p28, completed a phase I clinical trial in which it was administered to five escalating doses, p28 treatment led to no observable toxicity even at the highest concentration. Clinically beneficial effects, including partial or complete regression of the drug-resistant tumors some types of microbial therapeutics. Preventing and treating such infections in livestock animals are of great importance to public health and the economy. Moreover, while treating humans with phages remains controversial, the use of such agents to treat livestock species is a widely accepted practice. Various phages that target pathogenic *E. coli*, *Salmonella*, and *Campylobacter* are being used to treat cattle, pigs, chickens, and lambs before slaughter. Hence, livestock animals offer another proving ground for evaluating many kinds of phage treatments (51).

Acceptance and Funding

Because support for studying therapeutic microbes is scarce, educational efforts are needed to persuade decision makers to provide additional resources to help in moving this field forward.

- **Scientists:** Although interest in therapeutic microbes is gaining momentum in the scientific community, misconceptions are still widespread. Educational efforts directed at colleagues can help to correct those misconceptions and to gain support for further research.

- **Industry:** Industry resources will be required to support the development of microbial therapeutics, including help in funding and conducting clinical trials. Attracting industry will depend, in part, on gaining support from other sources, including clinicians, insurance companies, and the public. It also will depend, in part, on sorting out patent and liability issues. However, other features of therapeutic microbes—they are relatively inexpensive and easy to manufacture, stable, and, in some cases, orally administered—should make them attractive to industry. Further, these agents could be used widely for treating a broad variety of important diseases, meaning the target population is nearly the entire global population (See box “Serving Developing Nations”). The high risk, high reward nature of therapeutic microbes should tempt investors as well as biotechnology and pharmaceutical companies to carefully evaluate this emerging area of technology.

- **Clinicians:** While some clinicians began using therapeutic microbes very early, others did not because of concerns for patient safety (4, 5). Now that bacteria are increasingly recognized for their health benefits in the microbiome and as probiotics, clinicians may be more amenable to considering therapeutic microbes as having a place in mainstream medical practice (46, 68). Efforts should be directed at the medical community to familiarize clinicians with research on therapeutic microbes and their potential advantages over traditional approaches to preventing and treating cancer and infections. The medical community is beginning to adopt several practices that reflect a new appreciation for the role of the microbiome in human health (See box “Microbiome Manipulation”).

- **Patients:** Ultimately, patients will need to overcome their fears and to be convinced of the benefits of therapeutic microbes. Patients who participate in experimental treatments often come forward as advocates when those treatments prove successful, sharing their views with news outlets, funding agencies, and policy makers and, thus, promoting new and successful developments in medicine (69, 70). Meanwhile, however, many members of the public have negative opinions regarding microbes, genetically modified organisms, and vaccines. Familiarity with probiotics and success in clinical trials involving fecal transplant procedures, along with stories circulated by the new media and through advertising, could ease the way toward eventual acceptance of now-experimental treatment procedures involving therapeutic microbes.
VII. Conclusion

Microbes offer a promising means for preventing and treating diseases that arise from uncontrolled cellular proliferation, including microbial infections and cancers, both of which can become resistant to conventional treatments. Despite great efforts, these diseases remain difficult and sometimes become impossible to treat, suggesting that unconventional strategies will be required to meet these challenges.

Therapeutic bacteria and viruses, including phages, offer potential advantages over traditional pharmaceutical products. Suitable microbes, with multiple targeting and cytotoxic mechanisms, are more difficult for tumors to evade than are conventional drugs. Unlike conventional drugs, microbes can counter-evolve to avoid resistance and maintain their long-term efficacy. In many cases, therapeutic microbes can be expected to replicate within the patient who is being treated at the site where that treatment is needed, effectively establishing the dose that is needed to treat the level of disease encountered, while sparing patients many unwanted side effects.

The immunostimulatory properties of therapeutic microbes are an equally powerful part of this overall strategy, recruiting a system within patients that is designed, when properly activated, to combat their diseases. Therapeutic microbes can help to coordinate innate and adaptive immune responses to help in eliminating primary tumors, while stimulating adaptive responses to prevent metastases and relapse. If therapeutic microbes can block or eliminate these aggressive manifestations of cancer while they are still at the microscopic stage and thus undetectable by other means, this approach would be a true breakthrough in cancer treatment.

Genetic manipulation provides powerful ways to improve the targeting, efficacy, and safety of therapeutic microbes now being developed. Preliminary studies involving treatment in animal models of cancer and infectious diseases offer examples of how therapeutic microbes can be far superior to traditional drug treatments (48, 51, 63, 69). However, these experimental therapeutic microbial agents still face significant hurdles before they can be considered fully ready to treat those diseases in humans.

References:

References


Further Reading


On the cover: T₄ bacteriophage. Computer artwork of a T₄ bacteriophage virus. The swollen structure at top is the head, which contains DNA inside a protein coat. Attached to this is the tail, consisting of a tube-like sheath and tail fibers (at bottom). T₄ bacteriophages are parasites of Escherichia coli, a bacteria common in the human gut. The virus attaches itself to the host bacteria cell wall by its tail fibers; the sheath then contracts, injecting the contents of the head (DNA) into the host. The viral DNA makes the bacteria manufacture more copies of the virus. Image retrieved from Pasieka/Science Source.