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Current Opinion in Biotechnology

Engineering bacteria for cancer immunotherapy

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Bacterial therapeutics have emerged as promising delivery systems to target tumors. These engineered live therapeutics can be harnessed to modulate the tumor microenvironment or to deliver and selectively release therapeutic payloads to tumors. A major challenge is to deliver bacteria systemically without causing widespread inflammation, which is critical for the many tumors that are not accessible to direct intratumoral injection. We describe potential strategies to address this challenge, along with approaches for specific payload delivery and biocontainment to ensure safety. These strategies will pave the way for the development of cost-effective, widely applicable next-generation cancer therapeutics.

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Current Opinion in Biotechnology 2024, **85**:103061

This review comes from a themed issue on **NanoBiotechnology**

Edited by **Annie Gai** and **Yvonne Yamanaka**

For complete overview of the section, please refer to the article collection, "NanoBiotechnology (2023)"

Available online 13 January 2024

https://doi.org/10.1016/j.copbio.2023.103061

Introduction

Bacterial infections have been known to result in spontaneous tumor regression for over 4000 years [1]. Bacterial lipopolysaccharide (LPS) is the major active component that triggers the release of potent cytokines, including TNF-α, which causes hemorrhagic necrosis of tumors [2]. Apart from their ability to stimulate the immune system, certain anaerobic bacteria possess the unique ability to selectively colonize tumors. This attribute makes them promising candidates for the delivery of therapeutic payloads to tumors [3–5]. When bacteria are administered intravenously, most are cleared from the blood within hours and only a few reach tumors and other tissues. Bacteria residing in healthy tissues are then cleared within a few days, whereas bacteria residing in tumors rapidly proliferate, reaching densities of 10^8–10^9 colony-forming units (CFUs) per gram of tumor tissue [6–8]. This CFU range is equivalent to the number of bacteria that are contained in 1 mL of an overnight *E. coli* culture [9].

When employing bacteria as a drug delivery system, their potent immunostimulatory effects that cause systemic inflammation are typically unwanted. Therefore, *Salmonella typhimurium* mutants that have a defect in LPS synthesis, causing minimal inflammation, have been selected for clinical developments [6,10]. Although intravenous administration of these live bacterial therapeutics was well-tolerated in cancer patients, efficient tumor colonization was not achieved. Out of 25 patients, only one exhibited substantial tumor colonization with a count of 10^9 CFUs per gram of tumor tissue [6]. However, despite successful colonization, the *S. typhimurium* strain used in this study did not demonstrate any therapeutic effects.

In this review, we will discuss strategies to improve the colonization of tumors with bacteria as well as approaches to engineer bacteria such that they stimulate antitumor immune responses for therapeutic applications. We will address safety aspects related to the selective release of therapeutic payloads in tumors as well as biocontainment strategies.

Strategies to improve tumor colonization

Live bacterial therapies can be delivered via intratumoral injection, but most tumors are not readily accessible. Therefore, intravenous administration is a more practical route. However, this method is associated with several challenges, including low bacterial survival in circulation and poor accumulation within tumors.

Targeting tumor vasculature

Tumor colonization by intravenously administered bacteria is typically efficient in preclinical rodent models, but clinical trials showed that human tumors are more difficult to colonize [6]. Factors contributing to the reduced efficiency in patients compared with mice may include differences in tumor architecture and vasculature [11]. Most preclinical studies use transplanted tumor models, in which cancer cells are injected into syngeneic mice and grow quickly into tumors with a large hypoxic and necrotic core. This core provides an immune-privileged environment where anaerobic

bacteria can thrive and become concentrated between live and necrotic tissue [8].

The vascular network of transplanted tumors in mice is typically more fragile than that of spontaneous tumors. Spontaneous tumors arise from genetic mutations or exposure to carcinogens and grow slowly over months, more closely resembling human tumors. A comparison of tumor colonization by intravenously administered bacteria between transplanted and spontaneous tumors showed that the latter contained 10,000-times fewer bacteria [12]. Interestingly, colonization of spontaneous tumors was significantly improved by administering a vasculature-disrupting agent (VDA), Combretastatin A4 Phosphate (CA4P), beforehand. The VDA facilitates bacterial escape from the vasculature into tumors and causes necrosis of tumor tissue, thereby expanding the niche in which bacteria can thrive (Figure 1). Similar observations were made in a rhabdomyosarcoma transplant model in rats [13]. While tumors larger than 3 cm^3 can be efficiently colonized, smaller tumors that inherently have less hypoxia and little or no necrosis are difficult to colonize. Administration of a VDA to the rats induced necrosis in small tumors and strongly improved their colonization with bacteria. There are different types of VDAs that specifically destroy existing tumor blood vessels. These include microtubule-destabilizing drugs, flavonoids with antivascular functions, and drugs targeting endothelial cell receptors [14]. Several VDAs, including the aforementioned CA4P, are being tested in clinical trials and may in the future potentially be combined with bacterial therapies.

To understand and further improve bacterial colonization of tumors, we can draw on concepts from the field of nanomedicine. For instance, the leaky vasculature and impaired lymphatic drainage of tumors, nanomedicines, or macromolecules in general, can accumulate in tumors,

Figure 1

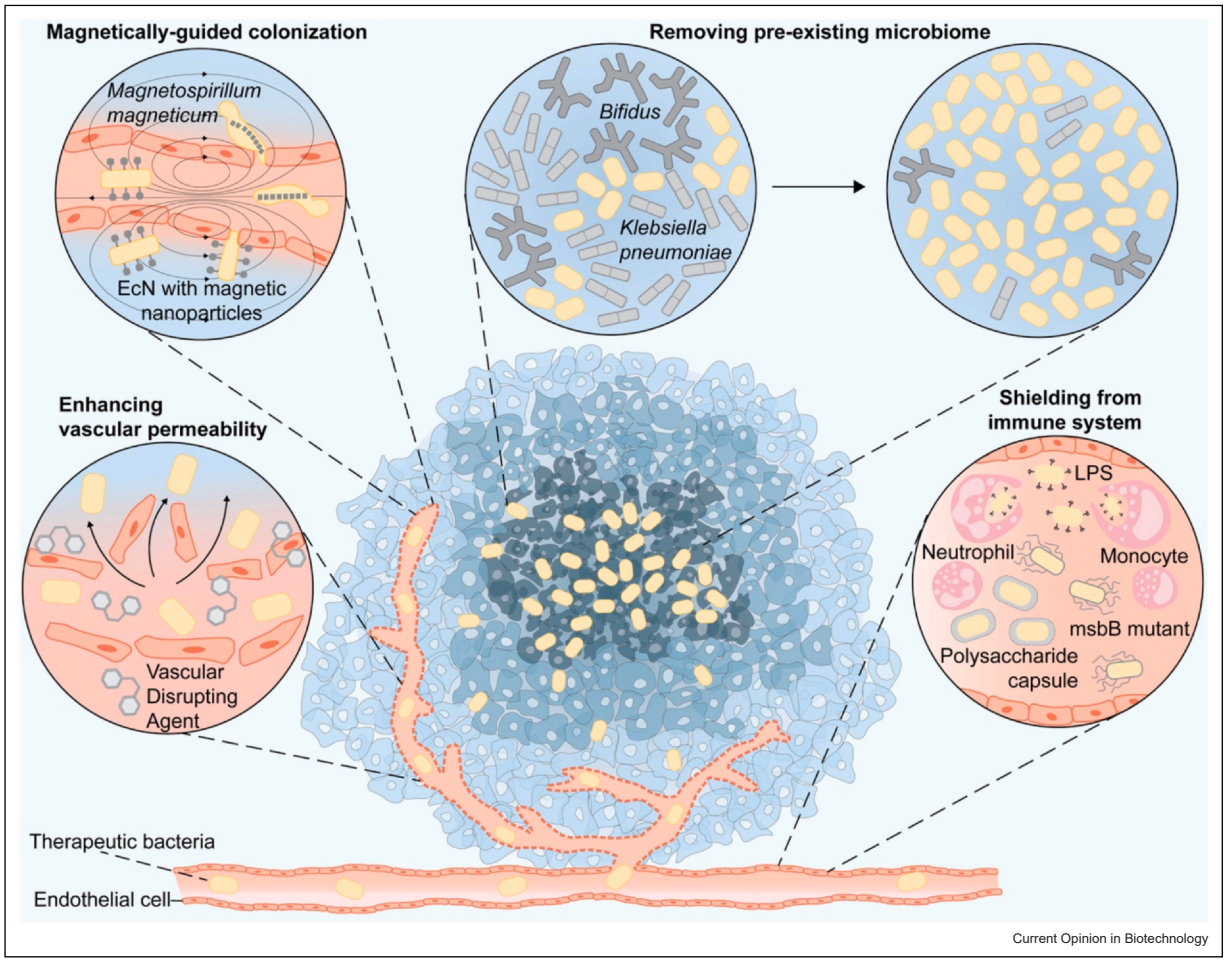

Illustration of different concepts to improve tumor colonization with therapeutic bacteria. Shown is a vascularized tumor with a necrotic core in which bacteria grow.

a phenomenon known as the *enhanced permeability and retention* effect (EPR) [15]. However, the EPR effect is highly variable between different tumor types with some spontaneous tumors and metastasis exhibiting less nanoparticle accumulation [16]. For example, stiff tumors in which blood vessels are squeezed and therefore poorly perfused do not readily accumulate nanoparticles. To overcome this challenge, focused ultrasound (FUS) in combination with contrast agents can be applied to achieve sonopermeation of solid tumors and metastases [17].

Removing the existing tumor microbiome

To harbor therapeutic bacteria, human tumors require hypoxic, immune-privileged regions. However, therapeutic bacteria probably need to compete for these niches, which may already be colonized by other microbiota. Indeed, recent studies demonstrated that human tumors are colonized by a diverse microbiome [18,19] consisting of intracellular and extracellular bacteria whose composition differs between tumor types and potentially influences disease progression [20]. Mapping the distribution of microbiota in human oral squamous cell carcinoma and colorectal cancer showed that bacteria colonize microniches that are poorly vascularized and immunosuppressive because T cells are kept out of these niches [21]. However, bacteria were also found in larger hypoxic areas of tumors. For example, needle aspiration biopsies of the necrotic centers of cavitating lung tumors contained pathogenic bacteria, including *Klebsiella pneumoniae* and nonpathogenic bacteria such as *Bifidobacterium* [22]. Given that tumors are most likely inhabited by a microbiome, removing it could create room for the colonization of therapeutic bacteria. Indeed, in one study, it was found that pretreatment of mice with a cocktail of antibiotics slightly enhanced the colonization of their tumors upon systemic administration of bacteria [23]. However, it remains to be determined whether improved colonization outweighs the potentially negative impact of antibiotics on cancer patients receiving immunotherapy [24].

Approaches to direct bacteria toward tumors

To improve tumor colonization, strategies are being developed to direct bacteria toward tumors by applying external magnetic fields. Magnetic responsiveness can be achieved by functionalizing commonly used strains such as *Escherichia coli Nissle* 1917 (EcN) with magnetic nanoparticles [25]. Another option is to use magnetotactic bacteria, such as *Magnetospirillum magneticum*, which naturally produce magnetic iron oxide nanocrystals. By applying rotating magnetic fields, bacterial torques are generated, causing the bacteria to tumble along blood vessels, which increases their chance of crossing the vascular endothelium and entering tumors [26]. This procedure increased the colonization of transplanted tumors threefold 24 h after intravenous injection of bacteria. Rotating magnetic fields can be generated at clinically relevant scales, which, in the future, may allow for the directing of magnetically responsive bacteria to deeply situated tumors.

Shielding bacteria from an immune attack

Using live bacteria as cancer therapeutics raises concerns about toxicity since bacterial LPS is a potent inducer of host-derived inflammatory mediators. With the discovery that a component of LPS, referred to as lipid A, is responsible for most of its inflammatory activity and the identification of the msbB gene that is involved in lipid-A synthesis [27], a mutant msbB *S. typhimurium* strain was developed that is far less immunogenic [10]. This strain was used in clinical trials and was well-tolerated [6]. Similarly, an EcN strain with a defect in the msbB gene was tolerated by Bagg Albino mice in tenfold higher doses than wild-type strains [28].

To temporarily shield EcN from an immune attack, elegant inducible synthetic gene circuits were developed that regulate bacterial encapsulation, a process by which bacteria produce a protective layer of polysaccharides that helps the bacteria to evade the immune system. Bacteria were designed to subsequently lose the capsule, which resulted in effective clearance in vivo [7]. This strategy enabled a tenfold increase in maximum tolerated dose of bacteria. Another approach to shield bacteria from the immune system is to pack them into apoptotic bodies, but this approach can only be applied to intracellular bacteria. When injected intravenously, apoptotic bodies containing bacteria are cleared much slower than bacteria that are not surrounded by a membrane. Apoptotic bodies also cause less inflammation and exhibit improved accumulation in tumors [29].

Bacterial chassis and controlled payload delivery

There are several facultative or obligate anaerobic bacteria that colonize tumors. *S. typhimurium* is perhaps the best-studied species and was used in most clinical trials involving bacteria for cancer treatment [30,31]. However, the probiotic EcN is emerging as a popular chassis [32]. Unlike *S. typhimurium*, which can to some extent also colonize healthy organs in mouse models, EcN exclusively accumulates in tumors [8] and has a well-established human safety record [33]. In addition, its ability to be readily engineered and its susceptibility to a broad range of antibiotics makes it a promising strain for therapeutic payload delivery.

EcN localizes and persists in tumors but has no or only moderate antitumor activity [34,35]. Therefore, EcN was engineered in numerous ways to enhance its antitumor activity. This includes the introduction of payloads such as cytotoxic factors that kill cancer cells [36], checkpoint

inhibitors that unleash immune responses to tumors [37–39], cytokines that activate immune cells [38,40], chemokines that attract immune cells to tumors [41], enzymes that produce agonists of innate immune receptors [35], synthetic antigens for Chimeric antigen receptor (CAR) T cells killing, [42], and neoantigens as a vaccine for antitumor immunity [43]. Payload protein release in these examples was achieved with a variety of methods, including secretion tags and programmed cell lysis. In addition, releasing a small molecule to metabolically modulate the tumor microenvironment can support effective antitumor immune responses [34]. In this study, EcN was engineered to continuously convert the metabolic waste product ammonia into L-arginine, which enhances the antitumor functionality of T cells synergistically with immune checkpoint blockade. In general, metabolic modulation of tumors is nontoxic and can be combined with the delivery of other therapeutic payloads. As research in this area continues, the list of payloads and strategies for engineering EcN is likely to expand and strains will be developed that combine several payloads and features.

Another chassis that recently gained interest is *Staphylococcus epidermidis*, a skin commensal that naturally colonizes the skin. When *S. epidermidis* is applied to the mouse skin, it drives a local increase of T cells preempting infections in colonized tissue [44]. In a recent study, *S. epidermidis* was engineered to induce tumor-specific immune responses within the context of natural skin colonization [45].

Genetic circuits for spatial control of payload delivery

Many of the payloads delivered by bacteria to tumors are toxic. Precise control over payload production and release could potentially maximize therapeutic effects while minimizing systemic toxicity (Figure 2). Inducible payload production can also provide an effective mechanism to separate efficient cell growth from high-level payload production [35,46]. The potentially burdensome effects of heterologous biosynthesis pathways are well-understood in the metabolic engineering field, and new inducible control strategies are being actively developed [47,48].

A variety of genetic circuits can be used to implement synthetic sense-and-respond programs for cancer targeting [46]. Tumor-specific growth or therapeutic payload production can be placed under control of promoters that respond to characteristic features of the tumor microenvironment, such as low O_2 levels, low pH, or lactate [49,50]. New strategies have also been reported for bacteria to detect specific, tumor-associated DNA sequences [51].

Alternatively, bacteria can be engineered to express or release their payload in response to external stimuli using chemically inducible or temperature-sensitive promoters. For example, bacteria colonizing a mouse tumor could activate reporter gene expression from an arabinose-inducible promoter when arabinose was injected intravenously [8]. Another approach involves engineering bacteria to sense local increases in temperature, which can be induced in tumor tissue by FUS. Temperature-actuated circuits have been developed to enable the expression and release of therapeutic nanobodies or Interferon-gamma in response to a brief thermal stimulus (42 °C) [39,40]. Additionally, bacteria can be functionalized with liposomes containing a chemotherapeutic and indocyanine green, which can absorb near-infrared light and convert it into heat. This process causes on-demand release of the chemotherapeutic through changes in the lipid membrane [25].

Figure 2

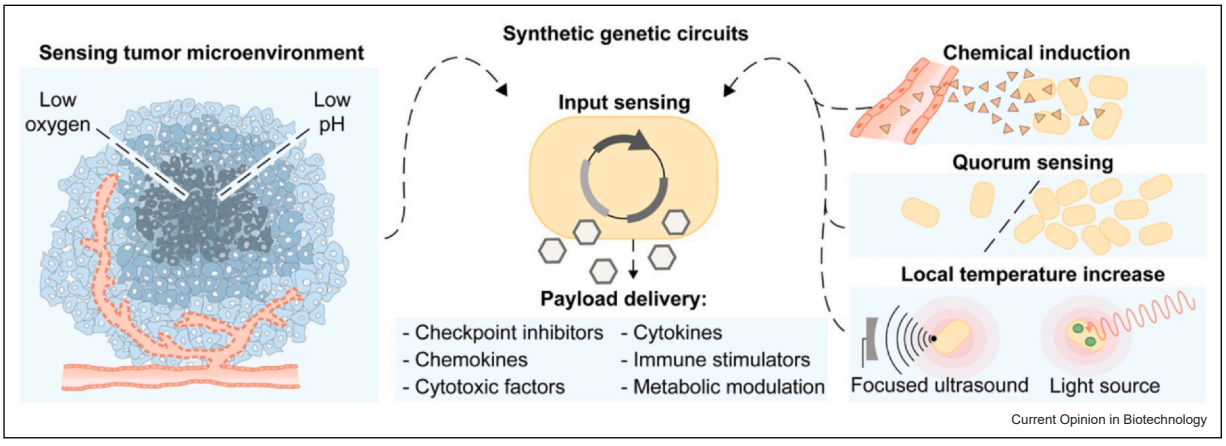

Illustration of concepts for spatial payload delivery.

Another promising approach is to leverage the mechanism by which bacteria sense population density, which increases upon successful colonization of tumors. To this end, a synthetic biology approach was used to engineer EcN with a synchronized lysis circuit that undergoes intratumoral quorum lysis to release its payload locally. This approach bypasses the difficulty of secreting proteins in *E. coli* [52], and ensures that cargos are only released where bacteria can grow to relatively high density, such as in a tumor. In preclinical transplant tumor models, this approach has demonstrated antitumor efficacy when used with anti-CD47 and anti-PD-1 nanobodies as therapeutic payloads [37,38], and with a synthetic antigen to tag tumor cells for Chimeric antigen receptor (CAR) T cells killing [42]. In these studies, mice tolerated the bacteria, and there was no indication of systemic toxicity from the therapeutic payload. Notably, in the synthetic antigen strategy, bacteria were successfully delivered both intratumorally and intravenously. However, humans have heightened immune responses to bacterial LPS compared with mice [53], and further optimization will likely be necessary for intravenous delivery in human systems.

Biocontainment for clinical applications

Biocontainment is critical for bacteria engineered to colonize human tissue (Figure 3). An effective biocontainment strategy should prevent escape into the environment or other human hosts, and should include kill switches to prevent uncontrolled growth in patients [54,55]. In principle, both goals can be accomplished with synthetic auxotrophies, where genetic modifications prevent the bacteria from synthesizing an essential metabolite. In practice, however, horizontal gene transfer between bacteria often allows escape. To overcome this challenge, a recent report used a dual-auxotrophy strategy with an engineered EcN strain (SYNB1891) that produces stimulator of interferon genes (STING) agonists [35]. Auxotrophy for thymidine prevents escape into the environment, while auxotrophy for diaminopimelic acid, a cell wall component, prevents proliferation in mammalian hosts. This strain can be injected intratumorally and functions as a short-term immunostimulant. In a Phase-I clinical trial, this strain was tolerated in patients and produced upregulation of immune activation genes [56].

Alternative biocontainment approaches could allow safe proliferation with long-term tumor colonization and continuous payload release. For example, genetically recoded *E. coli* can be engineered with multiple dependencies for the synthetic amino acid bisphenylalanine bisphenylalanine (bipA) [57]. The absence of bipA halts proliferation, and these bacteria show undetectable escape from bipA dependency. More recently, a recoded *E. coli* was engineered to eliminate horizontal gene transfer into and out of the engineered bacteria [58]. First, the Ser codons TCG and TCA were replaced with synonymous Ser codons across the entire genome and engineered Leu tRNAs were delivered that recognize TCG/TCA. Next, synthetic genetic constructs were coded using TCG/TCA for essential Leu residues. This approach ensures that any genetic material invading into the engineered strain will be mistranslated with S->L mutations. Further, synthetic genes transferred out to other organisms will be mistranslated with L->S mutations. In a therapeutic setting, this approach would ensure that genes coding for toxic payloads cannot escape into the native microbiome. While genome recoding provides robust biocontainment, these approaches were prototyped in an *E. coli* K12 derivative. Implementation in potential therapeutic strains such as EcN or *S. typhimurium* would require substantial strain engineering, although initial recoding efforts have been performed in *S. typhimurium* [59]. Alternative genetic kill switches could potentially be portable between multiple strains. For example, a chemically inducible Clustered Regularly Interspaced Short Palindromic Repeats-based kill switch was constructed in EcN using redundant circuits to achieve stability [60].

Future perspectives

Bacterial therapeutics can be engineered as multifunctional systems that target tumors, modulate the

Figure 3

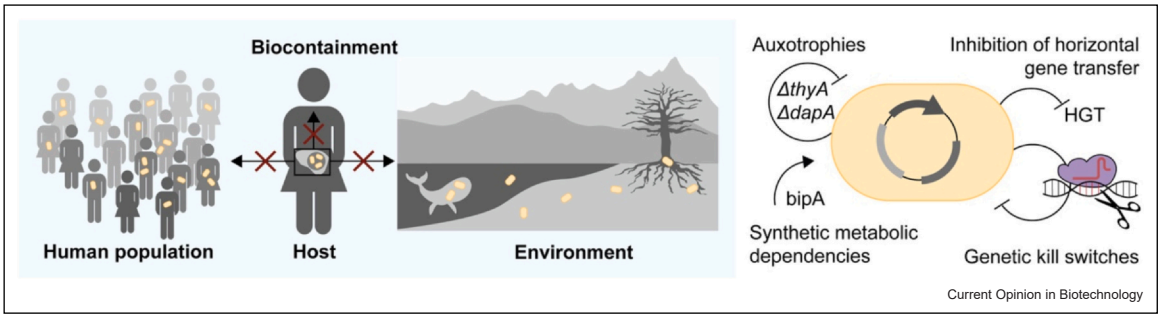

Illustration of strategies for biocontainment. HGT, Horizontal gene transfer. bipA, bisphenylalanine. delta-thyA, auxotrophy for thymidine. delta-dapA, auxotrophy for diaminopimelic acid.

metabolic environment, and selectively release anti-tumor drugs or immunostimulatory molecules within a single therapeutic agent. However, to fully harness the potential of bacterial therapeutics, several challenges need to be addressed, including efficient colonization upon intravenous administration, controlled payload delivery to minimize off-target toxicities, as well as biocontainment strategies.

To achieve successful tumor colonization upon intravenous administration, further research may focus on engineering bacteria for improved tumor homing. Different treatments may be explored to enhance bacterial escape into tumors and to expand hypoxic niches in tumors to provide an environment for bacterial growth. Emerging strategies to control bacteria with self-regulating genetic circuits, sense-and-respond functions, or external stimuli may prove instrumental for successful therapeutic applications.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The Geiger laboratory received research grants from Synlogic.

Acknowledgements

This work was supported by the Swiss National Science Foundation (310030_197737), the European Research Council (803150), by Swiss Cancer League (KFS-4593-08-2018), and by Synlogic. J.G.Z. was supported by the U.S. National Institutes of Health (R35 GM124773).

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