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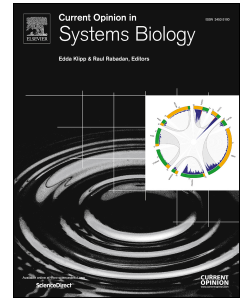
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Advances in Bacteria Cancer Therapies using Synthetic Biology

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ABSTRACT

Synthetic biology aims to apply engineering principles to biology by modulating the behavior of living organisms. An emerging application of this field is the engineering of bacteria as a cancer therapy by programming of therapeutic, safety, and specificity features through genetic modification. Here, we review progress in this engineering including the targeting of bacteria to tumors, specific sensing and response to tumor microenvironments, remote induction methods, and controllable release of therapeutics. We discuss the most prominent bacteria strains used and their specific properties, and the types of therapeutics tested thus far. Finally, we note current challenges such as genetic stability that researchers must address for successful clinical implementation of this novel therapy in humans.

INTRODUCTION

Synthetic biology is a rapidly growing discipline that aims to rationally design the behavior of living organisms. Much of the field's focus has been on implementing genetic circuits, in which inputs are transformed in a cell into digital or analog outputs, in a manner analogous to a computer program executing an algorithm [1,2]. The field began with construction of the repressilator and toggle switch circuits in bacteria [3,4]. Shortly after the field's inception, researchers envisioned applications to cancer therapies by programming bacteria to sense and respond to a particular cancer disease state [5]. Since then, significant progress has been made in the design of genetic circuits for behavior, ranging from counting and pattern formation to oscillations and complex logic operations [2,6-8]. As advances in engineering bacteria behaviors emerge, more complex forms of bacteria cancer therapies can be developed.

Although the application of synthetic biology to cancer therapy is quite new, bacteria have been explored as cancer treatments for over a century. In 1890, William Coley induced tumor regression via administration of a cocktail of *Streptococcus* and other strains collectively known as "Coley's Toxins" [9]. This approach was thought to stimulate or activate the immune system and is considered one of the early forms of immunotherapy. Later, bacterial strains such as the obligate anaerobe *Clostridium novyi* were shown to grow selectively in hypoxic regions of solid tumors [10-12]. Several facultative anaerobic bacterial strains were also demonstrated to localize and grow in tumors rather than healthy tissue, presumably due to decreased immune surveillance in the ne-

crotic core of tumors [13,14]. Interest in bacterial cancer therapy waned in the 20th century because of toxic side-effects resulting from inability to modify and control bacteria, as well as the advent of radiotherapy and chemotherapy [10]. Although these latter treatments became the mainstays of cancer treatment, more recently their toxicity and lack of specificity has become limiting, and more targeted approaches have gained traction [15]. With recent advances in cancer research and the newly available tools of synthetic biology, researchers are beginning to envision engineering bacteria to create potent cancer therapy. Improving upon the natural ability of bacteria to preferentially colonize tumors and implementing genetic circuitry can create a precisely controlled and highly specific delivery vehicle. A comprehensive review of bacterial therapies for cancer is given elsewhere [16]. Here, we focus specifically on the application of synthetic biology in bacteria to engineered bacterial cancer therapies, highlighting major instances of engineering in the last decade (Figure 1).

Engineered bacteria cancer therapies

Traditional approaches to genetic engineering involve limited modification to natural bacteria functions. The synthetic biology approach utilizes bacteria as a modular platform for engineering, in which components like genes and promoters can be interchanged and combined to create nuanced and complex circuits. Here, we highlight several examples of genetic circuits designed to program bacteria for therapeutic benefits in cancer treatment.

Targeting and Guiding

One way bacteria can naturally localize to tumors is by entering through the extensive tumor vasculature. Once inside, they can colonize the necrotic core, an immune-privileged environment protecting them from immune surveillance by macrophages and neutrophils [14,16]. This natural colonization process has the potential to be augmented by adding targeting or guiding mechanisms, which can also reduce the possibility of off-target colonization. One targeting method is engineering bacteria to express tumor homing proteins or peptides on the outer membrane (Figure 2A). Targeting motifs used so far include affibodies (proteins designed to bind targets such as upregulated receptors in cancer cells (e.g. HER2)), synthetic adhesion molecules which mimic immunoglobulin fragments and recognize antigen receptors, and known tumor-targeting peptides such as RGD [17-19]. In a recent example, Piñero-Lambea et al. administered bacteria expressing synthetic adhesion molecules termed adhesins to tumor-bearing mice and observed more efficient tumor colonization than by wild type bacteria. In addition, they saw reduced off target colonization of the spleen or liver due to the lack of adhesion binding on those tissues compared to tumor cells. Their work created a modular platform, in which different synthetic adhesins can be used for targeted localization to a variety of tumors. Another approach to increase tumor localization is to use external cues to guide bacteria to the tumor site. Felfoul et al. engineered a strain of magnetotactic bacteria to carry drug-loaded nanoliposomes. After injection of grafted tumors in mice, bacteria were guided by the application of a magnetic field in the tumor [20]. Whether through engineering targeted or remotely guidable bacteria, enhancing tumor localization has the potential to improve colonization efficiency and off-target effects and may have a significant impact on achieving successful clinical use of these therapies.

Tumor sensing and logic circuits

The tumor microenvironment presents several unique chemical and physical signatures that bacteria can be programmed to sense, in order to limit their action to specific settings (Figure 2B). For example, groups have engineered bacteria to sense glucose gradients and hypoxia, known tumor environment cues. To sense glucose gradients, Panteli et al. utilized a previously established synthetic hybrid receptor, containing the periplasmic domain of the *Trg* chemotactic receptor, to induce GFP expression based on glucose concentration [21]. The programmed bacteria sensed glucose concentration gradients over tumor cell masses within a microfluidic chamber. Their approach can be used to characterize the glucose profiles and metabolic activity over different tumor types [22]. To sense hypoxia, researchers have utilized known oxygen sensitive promoters, such as the synthetic FF20 or the endogenous promoter *pepT*, which are activated by oxygen binding Fumarate and Nitrate Reduction (FNR) regulatory proteins [23]. These promoters were fused to either the production of a therapeutic molecule (for targeted delivery) or the expression of an essential gene such as *asd* (for containment of bacteria in the hypoxic tumor area) [24,25]. To find a collection of promoters that respond to tumor conditions, several groups have sequenced tumor-resident bacteria in mouse models [26,27]. Leshner et al. injected *S. typhimurium* containing a promoter library driving GFP into mouse tumor models. By sorting and sequencing GFP expressing bacteria, they were able to identify tumor specific promoters [27]. In a follow-up paper, Deyneko et al. then adapted these promoters and incorporated hypoxia sensing elements to build a synthetic tumor specific hypoxic promoter [28].

Since bacteria can grow to a higher density in tumor environments than in healthy tissue, quorum (density) sensing can be used as a tumor sensitive switch (Figure 2C) [29-32]. In an application for cancer, Swofford et al. demonstrated activation of protein expression based on quorum sensing when bacteria population reached a critical density in mouse tumor models [31,33]. Quorum sensing has been multiplexed with additional input signals as well [29,30]. For example, Anderson et al. pioneered the use of AND gates for bacterial therapies in this context, sensing acyl-homoserine lactone (AHL) and Mg⁺ [30]. In the broader context of synthetic biology, groups have built XOR, NAND, and more complex circuitry to tightly regulate microbial sensing and computing [1,2,34,35]. As knowledge of tumor conditions and biomarkers improve, and the ability to construct bacterial logic systems increases, bacterial cancer therapies will be able to utilize these frameworks for elevated safety and specificity.

Remote inducibility

Inducible systems use external cues, allowing for an additional level of control over production of a therapeutic or genetic circuit of interest. Chemical inducers are commonly used *in vitro* to control bacterial promoters. In the context of bacterial cancer therapies, researchers have used chemical inducers such as L-arabinose, salicylic acid, Isopropyl β -D-1-thiogalactopyranoside (IPTG), AHL and tetracycline to remotely activate bacteria resident in tumors [36-41]. Chemical induction allows for actuation of dose and timing and requires little genetic modification. However, due to the unknown structure of tumor

vasculature a priori and different tissue diffusivities of chemical inducers, local inducer concentration is often difficult to estimate and may result in inaccurate and non-uniform induction of bacteria inside of tumors. Additional challenges include being able to sustainably induce gene expression over time, although this challenge could be mitigated by toggle-switch circuits that require a single pulse of inducer [42].

In addition to chemical cues, inducible expression from electromagnetic or light waves has also been tested in the context of bacterial therapies (Figure 1). For instance, one approach is to use γ -irradiation to indirectly activate the inducible *recA* bacterial promoter [43-45]. The γ -irradiation causes DNA damage, which then promotes the degradation of the RecA repressor LexA. Removal of the repressor LexA allows transcription of downstream genes (Figure 2D) [43]. The advantage of using gamma waves is deep penetration of tumor tissue. However, the method also induces DNA damage, which can be toxic to nearby healthy cells and possibly introduce unwanted mutations in bacterial genes encoding therapeutics. Other types of wave modulation may include utilization of ultrasound waves or optogenetic approaches that control gene expression with visible light [46-48]. These modes of induction can provide more exact the spatial and temporal control of microbes, although they may require specialized instruments or infrastructure for use.

Release of Bacterial Therapeutics

While bacteria can produce a wide variety of therapeutics, an ongoing challenge is the effective release of therapeutics from bacteria into the microenvironment. One method,

lysis of bacteria has been utilized by expressing specific phage lysis genes or changing culturing conditions [43,49]. Pijkeren et al. administered ampicillin to lyse bacteria and thus release plasmids for tumor cell uptake [50]. Instead of utilizing antibiotics directly for lysis, Chamacho et al. placed an adapted bacteriophage lambda lysis operon under a tetracycline inducible promoter to better control cell lysis. In the same bacteria, they also programmed a salicylic acid inducible protein production cascade to cause accumulation of therapeutics before lysis [51]. In addition to releasing therapeutics, lysis provides two other advantageous properties: (1) release of bacterial adjuvants that may stimulate immune responses, and (2) pruning of the population growth over time. Repeated lysis of bacteria can lead to oscillations in population growth. Din et al. engineered a circuit termed the synchronized lysis circuit (SLC) in which a lysis gene, as well as production of a therapeutic compound, was regulated by quorum sensing. The growth of SLC bacteria would trigger rhythmic bacterial death (Figure 2C) [29]. The SLC circuit led to reduction in tumor activity *in vitro* and *in vivo*, where it slowed tumor growth while controlling bacterial growth. Additionally, mice were healthier when treated with the lysis circuit bacteria than from bacteria with a constitutively produced therapeutic. Dynamic circuits such as these capable of driving periodic drug delivery may have unique implications, as the timing of drug administration has recently been shown to be important to therapeutic efficacy and chemoresistance development [52,53].

Bacterial secretion is another promising means of therapeutic delivery to tumor micro-environment [40,43,54]. Secretion can be achieved by use of a leader signal sequence, a a short peptide fused to the N-terminus of the protein of interest [40,55,56]. These

leader signal sequences are analogous to zip codes that traffic the translated protein to the bacterial periplasm followed by secretion outside of the cell [57]. The limitation of secretion methods is that the amount of protein delivered depends on the secretory pathway, which can be limited to certain organisms (e.g. *E. coli* does not naturally secrete proteins) [58]. Interestingly, some groups have demonstrated successful delivery of cargo to tumor sites despite lysis or secretion, presumably due to basal lysis of bacteria in the tumor microenvironment [59-61]. In addition, mere release of therapeutics in extracellular space may not be therapeutically effective if the released protein has an intracellular anti-tumor effect. Utilizing cell-penetrating peptides (CPP), other cell invasion mediated strategies or type III, secretion systems to traffic therapeutic cargo into cells are possible ways to overcome this challenge [5,62,63].

Strains and Clinically Relevant Properties of Bacteria in Use

One important consideration for engineered bacterial therapies is the species and strain of bacteria used. Some of the different bacteria utilized so far include *S. typhimurium*, *E. Coli*, *Bifidobacteria*, lactic acid bacteria such as *Streptococcus* and *Lactobacillus*, *Listeria*, and *B. subtilis*. Each of these strains has its own unique properties affecting its potential use for cancer therapy including its tumor colonization ability (Figure 1), ability to invade tissue, interaction with the immune systems, and ease of genetic manipulation.

Gram Negative Bacteria

Currently, the two most used bacteria for engineered bacterial cancer therapy are *S. typhimurium* and *E. coli*. Both are Gram-negative bacteria that have been shown to colonize tumors in mice at high ratios compared to normal tissues [13]. As Gram-negative bacteria, they naturally contain lipopolysaccharide (LPS, a 3-part phosphoglycolipid found in the cell wall), which can be responsible for immune system stimulation (Figure 1) [64]. However, these strains have several differences affecting their potential use for cancer therapy.

S. typhimurium (*Salmonella enterica serovar Typhimurium*) is the most widely studied engineered bacterial cancer therapy and has been used for a variety of applications, reaching as far as clinical trials. As a facultative anaerobe, it can grow in both the hypoxic core of tumors as well as the non-hypoxic regions. The most prominent strains of *S. typhimurium*, all genetically attenuated for safety, are VNP20009 and A1-R, and recently other strains have been investigated such as SL7207 and CRC2631 [4]. When administered, VNP20009 and other strains preferentially colonize tumors over healthy tissues at ratios greater than 1000:1 [15]. Interactions of the strain with the host immune system are also important; it has been shown that *Salmonella* is capable of recruiting immune cells such as T-cells, resulting in immune cell/T-cell-mediated tumor killing [65]. While pathogenic strains like *S. typhimurium* must be attenuated (modified) for safe clinical use, such as the mutation of an LPS gene in VNP20009, these modifications may reduce their clinical efficacy [64,66,67].

E. coli, a model organism extensively studied in synthetic biology, is the next most widely used bacteria in the field of cancer therapeutics. While it is also a Gram-negative fac-

ultative anaerobe, unlike *S. typhimurium*, it has non-pathogenic variants naturally found in the human gut (commensal strains, some of which are probiotics that have a positive effect on health when given) [68]. The ability to make use of these strains without further attenuation and their status as clinically approved probiotics makes them attractive candidates for use in therapies [69]. The most common probiotic strain in use is *E. coli* Nis-1917, but alternative strains have been explored [41,69]. While *E. coli* has been used more than *S. typhimurium* in the overall field of synthetic biology, Prindle et al. demonstrated that significant genetic circuits built in *E. coli* could be transferred to the more clinically used *S. typhimurium*, noting that these two species have similar ease of engineering as they have fully sequenced genomes, knockout collections, and easily used tools for genetic manipulation [70].

Gram Positive Bacteria

Gram-positive bacteria have been explored as cancer therapies, but have been engineered to a lesser extent than *S. typhimurium* and *E. coli*. *Clostridium novyi* was one of the early strains shown to have an anticancer effect, and recently attenuated *Clostridium novyi*-NT has garnered renewed interest [50,54,71,72]. Other Gram-positive bacteria that have been used include lactic acid bacteria, *Bifidobacteria*, *Listeria*, and *B. subtilis* [50,73-75]. *Bifidobacteria* and *Lactobacillus* have probiotic strains naturally found in the human gut and are already in use for other diseases, making them a popular choice for therapeutic delivery [76,77]. While *Listeria* has been investigated mostly as a vaccine, some groups have used it as a gene delivery vector for cancer therapy due to its intracellular life cycle [50,78,79]. Currently, engineering of Gram-positive bacteria is lim-

ited by the lack of synthetic biology tools available. *Streptococcus*, for example, is difficult to transform—a fundamental procedure in synthetic biology [80]. *B. subtilis* is naturally competent and its full genome has been sequenced, although genetic circuitry has not been as developed. New tools are being developed for genetic engineering in this species and others, though much work remains to be done [75,81-83].

Box 1: Anti-Cancer Therapeutics Delivered by Bacteria

A major consideration in bacteria cancer therapy is the choice of therapeutic. While in some cases the bacterium itself is the therapeutic, bacteria are most often used to locally and specifically deliver a therapeutic molecule of interest, as controlled local delivery can mitigate unwanted off-target effects compared to delivering a therapeutic systemically [15,16,69]. Many reviews have detailed therapeutics used; they broadly fall under two types: proteins and nucleic acids [84-86]. Proteins include cytotoxic agents, immune-stimulatory molecules, growth pathway regulators, and prodrug enzymes [55,87,88]. One example of a cytotoxic agent is HlyE, a pore-forming toxin, which has been used by several groups [23,29]. Immunostimulatory molecules can include cytokines, antibodies and tumor-specific antigens. Recently, delivery *in vivo* of *S. typhimurium* expressing flagellin B, a structural component of the flagellum from *Vibrio Vulnificus* was shown to lead to recruitment of macrophages and tumor regression, possibly via activation of the toll like receptor pathways [88]. DNA encoding for cytokines and bacteria antigens can be delivered for gene transfer via a co-opted invasion system from *Listeria*, or shRNA can be delivered for RNAi-mediated gene knockdown [61,85]. Recently, some studies have also used bacteria as carriers for traditional chemotherapies, such

as “bacteribots” and “nanoswimmers” loaded with liposomes and nanoparticles, respectively, of doxorubicin [89,90].

CONCLUSIONS

Engineered bacterial cancer therapy promises controllable, targeted delivery of therapeutics to tumors, mitigating some of the major issues of current therapies. There are several current challenges researchers must address to develop bacteria as a successful therapy.

An inherent issue in using engineered bacteria is the potential for mutation or plasmid loss, which could cause loss of therapeutic production or reversion of the safety modification. While antibiotics can be used to maintain selection for plasmids *in vitro*, use of such methods presents a challenge *in vivo* because they may lead to resistance development and dysbiosis. One approach to mitigate plasmid loss is genomic integration, which is known to be fairly stable. Clairmont et al. showed VNP20009 strain, with several chromosomal modifications for safe attenuation, was genetically stable over many generations *in vitro* and *in vivo* [93]. Integration is less convenient for rapid construction of variants, although many systems such as lambda red, phage integration, and CRISPR have been developed [91,92]. However, these systems typically have lower copy number and hence lower redundancy, leaving strains susceptible to loss of function mutations. Alternatively, stabilizing elements can be incorporated into engineered plasmids, such as plasmid segregation, toxin-antitoxin systems, and auxotrophy, includ-

ing balanced-lethal systems [29,94,95]. In some applications, plasmid loss may in fact be desirable so that the toxin production function is not maintained.

Future applications in programming bacteria may include more complex feedback systems which could respond to cell death or self-regulate. As the field of cancer biology advances, more potential therapeutic targets will be discovered, allowing an ever-widening range of therapeutics to be engineered into bacteria. Finally, the efficacy of bacteria in clinical trials must be demonstrated for eventual use in patient treatment. While *Bacillus Calmette-Guerin* therapy is approved for use in bladder cancer, no engineered bacterial cancer therapy is clinically approved as of yet [96]. Currently, there are several clinical trials that utilize bacteria for cancer therapy. One example is Marina Biotech's CEQ508 bacteria, which delivers RNAi to treat a condition underlying colon cancer; additionally, Aduro is testing attenuated *Listeria* treatment and BioMed Valley Discoveries has a clinical trial for attenuated *Clostridium* [97-99]. Although clinical trials are in early stages, as more and more therapeutics reach the clinical trial phase, the successful use of engineered bacteria for cancer therapy may be just over the horizon.

FIGURE LEGENDS

Figure 1: Overview of Engineered Bacterial Cancer Therapeutics. Synthetic biology is capitalizing on bacteria's natural ability to colonize immunoprivileged, hypoxic core regions of tumors through escaping from leaky vasculature. A variety of strategies such as targeting, inducing gene expression, quorum-sensing, expressing and releasing cytotoxics, and intracellular gene delivery have been engineered to control the behavior of these bacteria and produce anti-tumor effects.

Figure 2: Engineered Circuits for Bacterial Cancer Therapy. **(A)** Targeting to tumor cells can be achieved by expression of tumor-specific peptides on the bacterial outer membrane. **(B)** Tumor microenvironments can be sensed specifically by AND logic gates, which expresses an output such as a therapeutic only if all required inputs or markers of the tumor microenvironment are present. **(C)** In many cases lysis of bacteria must be induced to release therapeutics into the tumor; one relevant circuit is the synchronized lysis circuit which uses quorum sensing. **(D)** Therapeutics or other actions can be driven by inducing promoters through chemical inducers or radiation.

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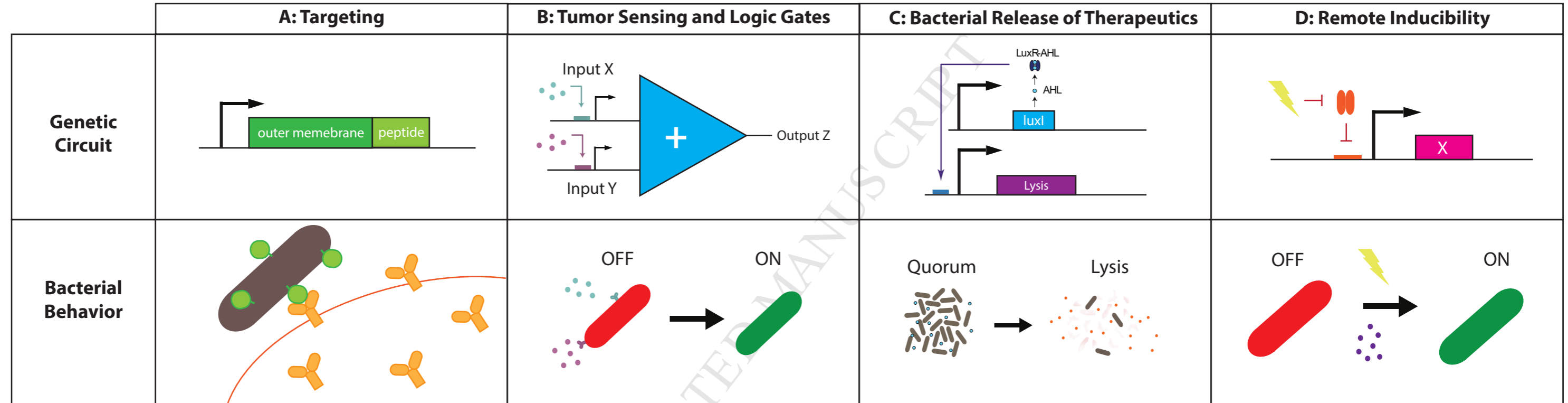
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Advances in Bacteria Cancer Therapy Using Synthetic Biology

HIGHLIGHTS

- Use of synthetic biology approaches can engineer bacteria for cancer therapy
- Engineered bacteria express and release therapeutics, target and sense tumors
- With more genetic engineering tools, properties of different bacteria can be used
- Current challenges include mutation, plasmid stability, demonstrating clinical efficacy