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Review

Engineered bacteria as drug delivery vehicles: Principles and prospects

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ABSTRACT

The development of drug delivery vehicles is in significant demand in the context of precision medicine. With the development of synthetic biology, the use of genetically engineered bacteria as drug delivery vectors has attracted more and more attention. Herein, we reviewed the research advances in bioengineered bacteria as drug carriers, with emphasis on the synthetic biology strategies for modifying these bacteria, including the targeted realization method of engineered bacteria, the designing scheme of genetic circuits, and the release pathways of therapeutic compounds. Based on this, the essential components, design principles, and health concerns of engineering bacteria as drug carriers and the development prospects in this field have been discussed.

1. Introduction

More than a century ago, Paul Ehrlich proposed an idea of a drug that would work as a "magic bullet," which could go straight to its intended cell-structural targets and selectively eliminate the diseased cells yet remain harmless to the surrounding normal cells [1]. Since then, the field of targeted drug delivery has progressed considerably. The drug delivery system is a critical component in developing new drugs and modifying old drugs. The limits and challenges associated with old drugs include limited bioavailability, non-specific targeting, limited absorption, and safety. Nevertheless, developing a new drug is time-consuming and laborious, and the process is often unsuccessful. Many strategies have been developed for this purpose, including individualized drug therapy, nanoparticle-based delivery systems, drug-conjugate delivery systems, therapeutic drug monitoring, and stimulus-sensitive targeted therapy [2]. Drug targeting is based on delivering a high drug concentration to the targeted site, such as a specific organ or organs, a single cell or cells, or blood vessels while minimizing drug concentration in the non-target areas. The specific targeting of a drug helps optimize the therapeutic effect while reducing the side effects due to multi-target interactions, high doses, and off-target concentrations. A carrier is a specially designed system essential for efficiently delivering an encapsulated drug to the targeted site. Drug targeting complexes should be non-toxic, non-immunogenic, biochemically inert, biodegradable, biocompatible, and physicochemically stable *in vivo* and *in vitro* [3]. The carrier should have a predictable and controllable drug release pattern, reproducible and cost-effective preparation, easy bodily elimination, and minimal drug leakage during transport.

The most common strategy over the past few decades is based on using nanotechnology to modify drug delivery systems [4]. The advantages of nanoparticles include the enhancement of the therapeutic efficacy by augmenting the cell uptake efficiency and a more comprehensive targetability of cellular and intracellular targets based on their size and mobility, improved target specificity, and flexibility [5]. Although nanotechnology exhibits several advantages, the limitations include aggregation and blockage of capillaries. The instability of the drug delivery systems could also lead to the premature release or leakage of the drug before reaching the diseased site [6].

Synthetic biology employs artificially designed biological systems as an alternative method for targeted drug delivery. The field of synthetic biology emerged around the millennium after the complete genome sequencing of bacteria back in the 1990s and the field has proliferated [7]. One of the early examples of the utilization of therapeutic strategy was using a synthetic circuit for bacterial invasion of tumor cells [8]. In

Abbreviations: AHL, acyl-homoserine lactone; AI, autoinducer; AIP, autoinducing peptides; ANN, artificial neural network; aTc, anhydrotetracycline; CAP, capsular polysaccharide; DSF, diffusible signal factor; FDA, U.S. Food and Drug Administration; FP, fusion peptides; GFP, green fluorescent protein; GIT, gastrointestinal tract; GM, genetically modified; HA1, hemagglutinin 1; HIV, human immunodeficiency virus; IPTG, isopropyl β-d-1-thiogalactopyranoside; LAB, lactic acid bacteria; MV, membrane vesicle; NICE, nisin-controlled expression; NRPS, non-ribosomal peptide synthase; OMV, outer membrane vesicle; PD-L1, programmed death-ligand 1; PEDV, porcine epidemic diarrhea virus; PKS, polyketide synthase; PQS, Pseudomonas quinolone signal; QS, quorum sensing; RiPPs, ribosomal synthesized and post-translationally modified peptides; SARS, severe acute respiratory syndrome; SLIC, synchronized lysing integrated circuit; SP, signal peptide; STING, stimulator of interferon gene; SUSAR, suspected unexpected serious adverse reaction.

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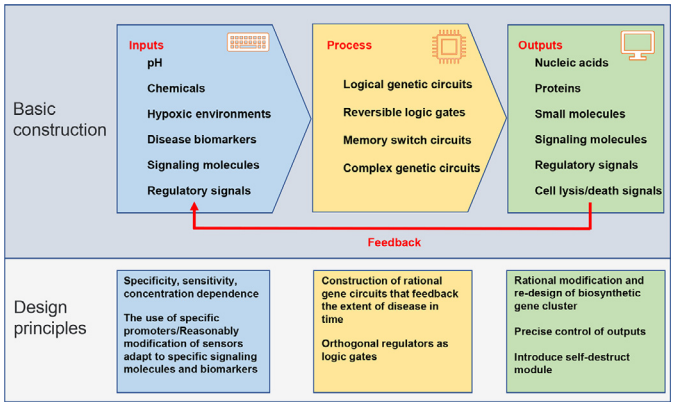

Fig. 1. Basic construction and design principle in the development of therapeutically engineered bacteria.

the recent reports, synthetic bacteria were used to enhance radiotherapy and immunotherapy for cancer treatment [9, 10]. Therefore, microorganisms can be combined with synthetic biology to achieve an effective drug targeting strategy.

With recent advancements in science, more sophisticated strategies have been developed for designing drug delivery vehicle with specific targeting. Synthetic biology approaches include using bacteria as an individual micro-machine for performing programmed tasks in the medical field [11]. These modifications allow it to be used as a vehicle for delivering pharmaceutical molecules or proteins and transfecting nucleic acids into the host cells. Synthetic bacteria's characteristics and easy accessibility offer potential applications in preventing, diagnosing, and treating various diseases [12–15]. In this review, we have discussed the principles and advancements associated with engineered bacteria as drug delivery vehicles, its remaining shortcomings and safety concerns, and its future development prospects.

2. Progress in the recent years

In Table 1, we listed some recent representative research in this field. A well-designed engineered bacteria for drug delivery applications consists of three essential components: the targeting of the diseased tissue and regulatory input signal, the intracellular genetic circuit part, and the active compound delivery component [16] (Fig. 1). In general, engineered bacteria can target diseased tissues or organs, and sense specific biomarkers in the diseased environment or induce specific conditions. Furthermore, an elaborately designed intracellular metabolic pathway can activate or inhibit the expression of related genes, synthesize biologically active therapeutic molecules, and release drug payloads precisely in diseased tissues or organs. These components are interdependent and mutually restricted, as well as have multiple functions.

3. Regulatory input signal and targeting of engineered microorganisms

Numerous studies have illustrated the effectiveness of several synthetic biological components for modifying bacterial signaling pathways. The bacteria transmit information via the classical two-component system [17], quorum sensing (QS) system [18], transcriptional regulators [19], and extracytoplasmic function sigma factors [20]. A typical two-component regulatory system consists of histidine kinases on the cell membrane and effector proteins downstream [21]. It is theoretically possible to artificially engineer histidine kinases and acquire the ability to sense specific external environments through the directed evolution of proteins. Additionally, the downstream regulators can be modified to reprogram the signaling pathways that lead to different genetic events. In a QS system, bacteria produce an organic molecule called "autoinducer" (AI) to perceive their population density, and the concentration of these compounds was found to regulate the expression of related genes [22]. Moreover, the feedback of the target gene expression levels of the autoinducer biosynthetic gene cluster regulates the concentration of the autoinducer. Many species of autoinducer molecules have been identified, and the best-known example is the acyl-homoserine lactones (AHLs) that are frequently used by gram-negative bacteria. The autoinducing peptides (AIPs) are the major autoinducers produced by gram-positive bacteria. The other AIPs include Pseudomonas quinolone signals (PQSs), indole, and diffusible signal factors (DSFs) [21, 23, 24]. These small molecule compounds can be linked to other biomarkers through chemical methods to guide the engineered bacteria into the specific tissues (Fig. 2A) [25].

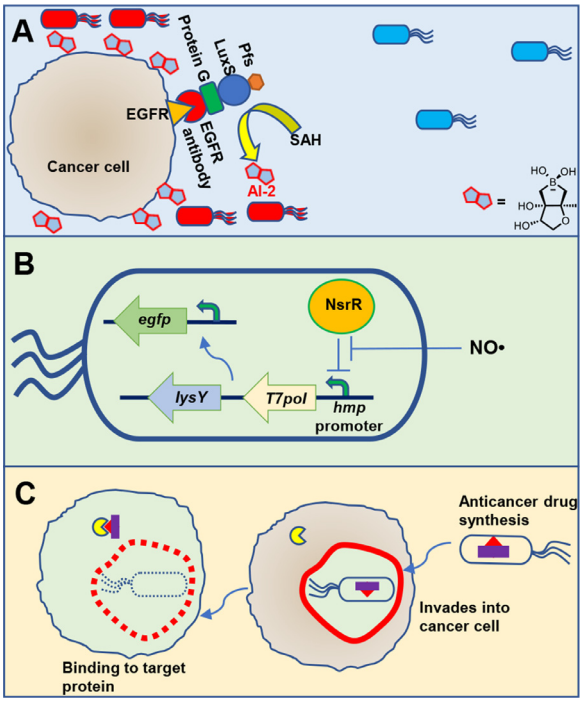

Fig. 2. Example of targeting and signal input implementation. A: Fusion protein-synthesizing bacterial AI-2 is targeted to the epidermal growth factor receptor on the surface of tumor cells. AI-2 molecules are produced from the cell surface and recognized by engineered bacteria based on the AI-2 level to control the antitumor drug biosynthesis (blue: uninduced; red: induced). Adapted from Ref [25]. B: The engineered bacteria respond to nitric oxide (NO) and produce a homogeneous fluorescent response. The system uses a NO-response promoter to amplify the original signal into a second vector responsible for GFP expression and uses co-expressed T7 lysozyme (*lysY*) to repress T7Po to regulate *T7lac* promoter activity. Adapted with permission from Ref [27]. Copyright © 2018, American Chemical Society. C: The regulatory protein complex FlhDC promotes Salmonella to invade tumor cells and release therapeutic payloads such as nanobody. Adapted from Ref [42].

The host cells or organs can produce a variety of biologically active molecules, such as signaling molecules, cytokines, and enzymes. At the same time, pathological changes are associated with environmental changes, such as temperature, pH, and oxygen concentration. Therefore, the response of the designed engineered bacteria should be highly specific, sensitive, and concentration-dependent to the targeted organs or tissues and capable of detecting or sensing specific biomarkers associated with the lesion. For example, the high NO environment is a characteristic marker of the inflammatory tissues and is often targeted by engineered bacteria. The NO sensing system, NorR, is designed to target engineered bacteria in the inflamed tissues (Fig. 2B) [26, 27]. Tetrathionate, a thiosulfate sensor, is a biomarker of inflammation, and a modified tetrathionate sensor was used for diagnostics and therapy. For example, *E. coli* was used to detect gut inflammation [28], and engineered *E. coli* containing a biosynthetic gene cluster was controlled by

Table 1
Representative research in recent years using bioengineered bacteria as drug delivery vehicles.

Bacterial strains	Target or Usage	Function	Refs
E. coli	Cancer	Controlled adhesion to target tumors	[107]
E. coli Nissle 1917	Phenylketonuria	Insertion of phenylalanine ammonia lyase and *L*-amnio acid deaminase in genes for releases of SYNB1618	[108]
E. coli	Inflammation	Report Inflammation by sensing NO	[27]
E. coli	Cancer	Specifically lyse within the tumor microenvironment and release an encoded nanobody antagonist of CD47	[44]
E. coli Nissle 1917	Cancer	Controlled production and intratumoral release of nanobodies targeting PD-L1 and CTLA-4	[49]
E. coli Nissle 1917	Cancer	Temporarily evading immune attack, loss of encapsulation results in effective clearance	[48]
E. coli	Vaccine	Expressing SARS-CoV-2 and porcine epidemic diarrhea virus (PEDV) fusion peptide on the cell surface	[78]
E. coli Nissle 1917	Cancer	Delivery of p53 and Tum-5 to solid tumors	[109]
E. coli Nissle 1917	Cancer	Continuously converts ammonia to *L*-arginine, increase the number of tumour-infiltrating T cells and had marked synergistic effects with PD-L1 blocking antibodies in the clearance of tumours.	[62]
E. coli Nissle 1917	Cancer prevention	Transform host-ingested glucosinolates to sulphoraphane	[110]
E. coli Nissle 1917	Cancer	Targets STING-activation to phagocytic antigen-presenting cells (APCs) in the tumor and activates complementary innate immune pathways	[111]
E. coli Nissle 1917	Hyperammonemia	Converts NH_3 to *L*-arginine	[112]
E. coli Nissle 1917	Colitis	Produce 3-hydroxybutyrate	[113]
E. coli Nissle 1917	Gut	Delivery of matrix-tethered therapeutic domains to the gut	[114]
E. coli Nissle 1917	VRE infection	Produce and secrete Enterocin A, Enterocin B, and Hiracin JM79	[115]
E. coli Nissle 1917	Salmonella infection	Produce Microcin H47	[29]
E. coli Nissle 1917	Diagnose	Thiosulfate and tetrathionate sensors for detecting gut inflammation	[28]
E. coli Nissle 1917	Cancer	Induce the regression of colorectal cancer through production of 5-aminolevulinic acid	[91]
S. typhimurium	Cancer	Expressing and secreting IFN-γ for killing melanoma cancer cells	[116]
S. typhimurium	Cancer Vaccine	Secreting Vibrio vulnificus flagellin B (FlaB) for tumor suppression	[117]
S. typhimurium	Cancer	Expressing Neoantigen for immunotherapy	[77]
S. typhimurium	Cancer	Release nanobody against target protein	[42]
S. typhimurium	Cancer	Expressing cytolysin A (ClyA) to target cancer stromal cells and cancer cells	[118]
S. typhimurium	Cancer	Delivery of the anti-angiogenic agent endostatin and to inhibit tumor growth	[119]
S. typhimurium	Cancer Vaccine	Tumor vaccine to express VEGFR2 and increase VEGFR2-specific T cell responses	[120]
S. typhimurium	Cancer	Boost photodynamic therapy and systemic anti-tumor immunity for synergistic cancer treatment	[121]
S. typhimurium	Cancer	Angiogenic inhibitors delivered by the type III secretion system	[99]
S. typhimurium	Cancer	To deliver the anti-angiogenic agent "endostatin"	[119]
S. typhimurium	Cancer	Encoding DNase I	[122]
S. typhimurium	Cancer	Under light irradiation, the encoded VEGFR2 gene was released and expressed in tumor tissues	[123]
S. typhimurium	Cancer	Deliver Human IL-2	[124]
S. typhimurium	Cancer	Deliver siRNAs against PD-1	[81, 82]
S. typhimurium	Cancer	Deliver PD-1 siRNA	[125]
S. typhimurium	Cancer	Targeting indoleamine 2,3-dioxygenase restructures the immune contexture	[126]
L. lactis	Infection	Expressing and secreting cLFchimera peptide with antimicrobial properties	[127]
L. lactis	Inflammation	Producing Hsp65 to reduce severity of inflammation	[128]
L. lactis	Vaccine	Displaying EG95, an immunogenic antigen from the *E. granulosus* as a vaccine	[129]
L. lactis	Inflammation	Expressing TGFβR2 extracellular domain to reduce hepatic fibrosis	[130]
L. lactis	Infection	Secreting endolysin and VAPGH to inhibit the growth of *S. aureus*	[131]
L. lactis	Diabetes	Delivery of proinsulin and IL-10	[132]
L. rhamnosus	Vaccine	Antigen display for mucosal immunization	[76]
L. lactis	-	DNA Delivery in eukaryotic cells	[133]
L. salivarius	Inflammation	Surface display IL-17, IL-23 and TNFα for the treatment of Inflammatory Bowel Disease	[134]
L. lactis	Inflammation	Producer/secretor of IL-23 protein blockers into the gut	[135]
L. lactis	Inflammation	Secreting bovine lactoferricin-lactoferrampin	[136]
L. lactis	Inflammation	Anti-TNF scFv expression	[137]
L. lactis	Diabetes	Expressing bioactive exendin-4 to promote insulin secretion and beta-cell proliferation	[138]
B. animalis	Diabetes	Improving hepatic insulin sensitivity	[102]
B. longum	Inflammation	Expressing rhMnSOD to suppress colitis.	[139]
L. monocytogenes	Cancer	Stimulating innate and E7 antigen-specific immune responses	[103]
L. monocytogenes	Cancer Vaccine	Inducing pore-forming protein gasdermin C (GSDMC)-dependent pyroptosis	[140]
C. sporogenes	Cancer	The expressed NmeNTR can induce the metabolism of prodrug PR-104A into the active form	[105]
Outer membrane vesicles	Cancer Vaccine	Specific anti-tumor immune response via specifically presenting antigens onto OMV surface.	[86]
Yeast	Inflammation	Express a human P2Y2 purinergic receptor	[141]
S. boulardii	Infection	Against *Clostridioides difficile* infection	[106]

a tetrathionate-induced promoter that produces the Microcin H47 for treating Salmonella infections [29].

Signal input or targeting is relatively common in engineered living bacteria as drug delivery vehicles, and three bacterial chassis are most widely used. One of the most popular engineered microbes used in synthetic biology originated from natural human commensals, such as lactic acid bacteria (LAB), a general term for bacteria that can ferment carbohydrates into lactic acid [30]. LAB strains can survive the harsh conditions in the gastrointestinal tract (GIT) and colonize specific intestinal tissues [31]. Due to the strong resistance to the harsh conditions, recombinant LAB is an excellent candidate for mucosal delivery of antigen proteins, drug molecules, and foreign DNA. Therefore, they can be used in vaccine development, drug delivery, gene therapy, and other popular fields.

For example, engineered *Lactococcus lactis* attacks *Enterococcus faecalis*, a gut bacterium that causes infections, by secreting antienterococcal peptides only when *L. lactis* detects a sex pheromone, cCF10, produced by *E. faecalis*. When cCF10 is present, the promoter triggers production of the three bacteriocin genes to synthesize enterocin A, hiracin JM79 and enterocin P, that have antimicrobial activity against *E. faecalis* [32].

Mucosal delivery is the most common method for introducing LAB into the human body. LAB has evolved specific mechanisms to reach the epithelial cells through the mucus layer [33]. One of the examples

of utilizing LAB through a mucosal delivery system is to prevent the entry of HIV. Many commensal bacteria in the vaginal mucosa of healthy women consist of H_2O_2-producing Lactobacillus, which plays a protective role in preventing urogenital infections. Cyanovirin-N (CV-N), isolated from the cyanobacterium *Nostoc ellipsosporum*, is a potent CCR5 and CXCR4-tropic HIV inhibitor that blocks multiple steps leading to membrane fusion and viral entry. *Lactococcus jensenii* isolated from the vagina was engineered to secrete CV-N that effectively inhibited both CCR5 and CXCR4-tropic HIV [34]. This study showed that Lactobacillus-derived CV-N could inhibit CCR5-tropic HIV_{BaL} infectivity *in vitro* at a concentration of 0.3 nM and produce full-length CV-N when administered intravaginally to mice during the estrus phase [35]. This work proved that bioengineered bacteria secreting natural products inexpensively and durably ceases heterosexual transmission of HIV in females.

Unlike LAB, Salmonella is a foodborne pathogenic bacteria that cause food poisoning [36, 37]. Salmonella must overcome the conditions adapted by humans to inhibit and kill foodborne pathogens, such as low pH, hypertonic treatment, low temperature, and stress. Therefore, Salmonella has evolved a network to cope with the challenges of a changing environment to protect itself from stressful conditions. Thus, the genetically modified (GM) Salmonella can be used as an anticancer therapeutic by secreting vesicles with anticancer drugs [38].

The critical cancer pathways cannot be effectively targeted as the solid tumor membranes are difficult to penetrate. In this regard, bioengineered Salmonella is a perfect candidate for delivering therapeutic drugs by targeting cancer cells' necrotic/hypoxic regions [39, 40]. Salmonella is capable of multiplying selectively in tumors and inhibiting their growth. Meanwhile, they have been demonstrated to colonize and destroy tumors and have emerged as biological gene vectors to the tumor microenvironment [41]. Recent research has led to the development of a highly modified strain of Salmonella that uses three genetic circuits to precisely control drug production, cell invasion, and protein release. The control of invasion with the master regulator, *flhDC,* increases delivery efficiency by over 500 times. A study found that about 70% of Salmonella can enter the tumor cells (Fig. 2C), allowing therapeutic proteins to accumulate directly and autonomously within the cancerous cells. After removing the tumor cells, the bacterium will be eliminated automatically without affecting normal cells [42].

Due to the ease of maintenance, high yield, and well-established system, *E. coli* is one of the most dominant prokaryotic systems used in gene expression and is widely used in drug targeting and delivery. *E. coli* has been favored by academia and industry laboratories because it proliferates on cheap media to a high cell density, and its genetics are better understood than any other microorganism. *E. coli* can be genetically engineered to fit with sensors that pick up specific signals such as AIs and can be transformed into a drug carrier that has the property of controlled lysis near target cells. For example, CD47 is one of the effective antitumor targets discovered in recent years. Blocking the CD47 "Don't eat me" signaling pathway can enhance the targeting of macrophages to tumor cells and eliminate the tumors through the STING signaling pathway [43]. In January 2022, the FDA partially suspended Gilead's Magrolimab after an unexpected severe adverse reaction (SUSAR) occurred in a clinical trial. The safety of CD47 target drugs was questioned again. However, the latest research shows that engineered *E. coli* as a drug delivery carrier can significantly alleviate the toxic and side effects [44]. In this research, an *E. coli* strain that contained a synchronized lysis circuit produced a quorum sensing molecule, AHL, as a signal to control the growth and lysis of *E. coli,* followed by the release of encoded nanobody antagonists of CD47 in the tumor cells.

4. Construction of intracellular metabolic pathways in engineered microorganisms

The process of the metabolic pathway is similar to the CPU. After sensing the specific signals, bacteria start to process the information by activating downstream gene clusters, finally leading to the release of drugs for the treatment or bacteria lysis. Due to the complexity of the human internal environment, the introduction of new genetically engineered microorganisms will cause disturbance in the internal environment. Therefore, designing these microbes with specific turning on or off the expression of target genes at the right time is essential. The development of this field is mainly focused on gene level regulatory design. Modern computing processes are based on temporal logic, in which the state of a circuit depends on the current input signal and past input signals. In synthetic biology, implementing temporal logic requires feedback regulation embedded in the genetic circuitry. However, feedback regulation is not only difficult to design but also difficult to scale up. In recent years, scientists have at least tried to implement logic AND, OR, and NOT gates through regulation at the genetic level [45]. A recent study applied this idea in bacteria using an artificial neural network (ANN) type architecture to demonstrate logical reversibility in single and ensemble living cells [46]. In this work, a synthetic genetic reversible Feynman gate was constructed, the commonly used organic molecules isopropyl β-d-1-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc) were used as inputs, and the fluorescence proteins EGFP and E2Crimson were designed as outputs (Fig. 3A). The results showed that the expression pattern of the EGFP and E2Crimson as a function of aTc and IPTG matched with the Feynman gate truth table. The input signal is applied to the engineered invading *E. coli*, and the information is delivered to the HeLa cells via shRNA. The output is expressed by inhibiting two native genes in HeLa cells, AKT1 and CTNNB1. Another example of this system is the measurement of bacterial responses in the gut. The memory switch can be turned on when transcriptional trigger is activated. This memory-on state can remain in the gut for more than a week. In this case, the memory-on and memory-off states of the switch

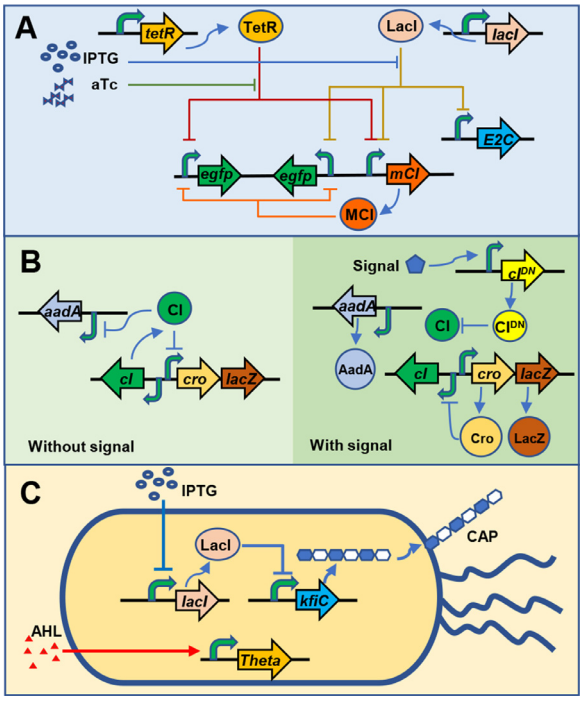

Fig. 3. Example of some complex genetic circuits A: Synthetic genetic design of Feynman gate in engineered *E. coli* processing two input chemicals IPTG and aTc. Adapted with permission from Ref [46]. Copyright © 2022 American Chemical Society. B: HTMS circuit design in memory-off and memory-on states. Adapted from Ref [47] under the Creative Commons Attribution 4.0 International license. Copyright © 2019, Naydich et al. C: Programmable system for control immune evasion and clearance through the bacterial CAP levels and controlled release of anti-tumor drugs. Adapted from Ref [48] under the Creative Commons CC BY license. Copyright © 2022, Harimoto et al.

correspond to the mutually repressive proteins, Cro and CI can be regulated by the promoters (Fig. 3B) [47].

Well-designed genetic circuits can help engineered bacteria become more adaptable as drug delivery vehicles. As for the bacteria themselves, their toxicity to the host limits the tolerable dose and efficacy. In a recent study, based on a small RNA screen of capsular biosynthesis pathways, the researchers constructed inducible synthetic gene circuits that regulate bacterial encapsulation in *Escherichia coli* Nissle 1917 [48]. Specifically, they constructed a programmable bacterial surface capsular polysaccharide (CAP) expression system that regulates the bacterial surface. This controls the bacteria's immunogenicity and *in vivo* viability and increases their drug loading and in situ trafficking, enhancing their therapeutic efficacy and improving safety. This study constructed a programmable CAP expression system that switches CAP biosynthetic genes "on" or "off" depending on the concentration of the inducer IPTG. Animal studies have shown that this system can protect the bacteria from immune system attack at the beginning to achieve therapeutic concentration rapidly and allow the bacteria to translocate to the distal tumor site. The bacteria can be quickly removed after the treatment is complete without causing severe toxic side effects such as sepsis. Combined with a cluster of antitumor drug synthesis genes regulated by AHL to control payload release, this genetically engineered bacterium achieves clearance of distant metastatic tumors (Fig. 3C).

Although these successes are still relatively rare in synthesizing therapeutic microorganisms, promoter engineering is fundamental in designing intracellular signaling pathways. Therefore, the specific signals these promoters are regulated by, the intensity of promoters and the signal feedback mode of transcriptional termination are essential. An engineered bacterium with a self-feedback pathway can determine its fate depending on the situation. For example, an ideal engineered bacterium for tumor treatment should start the expression of relevant genes and release compounds with antitumor activity after infecting tumor cells. When the tumor is cleared, it receives a signal for completing the mission and clears itself so as not to infect the healthy cells. One of the gene circuits for tumor therapy involves the tumor-selective bacterial generation, population restriction, and periodic therapeutic release. Once a critical density or colony is reached, the colony splits, effectively releasing its therapeutic payload. In a recent study, the transcription of quorum sensing gene, *luxI,* and phage lysis gene, *ϕX174E,* were controlled by quorum sensing gene promoters. The release of PD-L1 and CTLA-4 nanobodies were optimized using the synchronized lysing integrated circuit (SLIC) platform to confine the bacterial population to the tumor site, thereby minimizing the risk of systemic toxicity [49, 50].

Matching a specific promoter and adjusting the strength for the expression of multiple or a particular gene is critical in constructing intracellular signaling. In this regard, we explored the example of LAB. The nisin-controlled expression (NICE) system is often used in synthetic biology. Once the LAB population is increased, nisin produced by LAB accumulates and triggers the downstream transcription. A low concentration of nisin in the range of ng/mL can fully trigger the promoter [51]. This high sensitivity allows the production of target proteins through a tightly controlled gene expression. One example utilizing the NICE system was to develop an oral LAB-based vaccine against the H1N1 virus. *L. lactis* contained the hemagglutinin 1 (HA1) antigen fused to the nisP anchor protein for surface display of the HA1 antigen and can be induced by nisin. The results showed that specific anti-HA1 sIgA antibodies were produced in BALB/c mice by oral administration of recombinant *L. lactis* strains than in the control group [52]. Moreover, some specific promoters strongly promote the expression of downstream genes in specific environments. For example, the hypoxia-responsive *fdhF* promoter and the anaerobically inducible *nirB* promoter can be used to recognize hypoxic environments such as tumors [8, 53].

On the other hand, constitutive gene expression can be activated to regulate an intracellular metabolic pathway. The advantage of this system is that it does not have endotoxins, inclusion bodies, spores, or extracellular proteases and can be universally used in many other bacteria. For example, the constitutive expression of the fusion protein GST-SUMO-MT was activated in recombinant *L. lactis* strain pGSMT/MG1363. The expression of GST-SUMO-MT was found to inhibit the increase in the levels of lead in the blood of rats, a potential therapy for preventing lead poisoning via GIT [54].

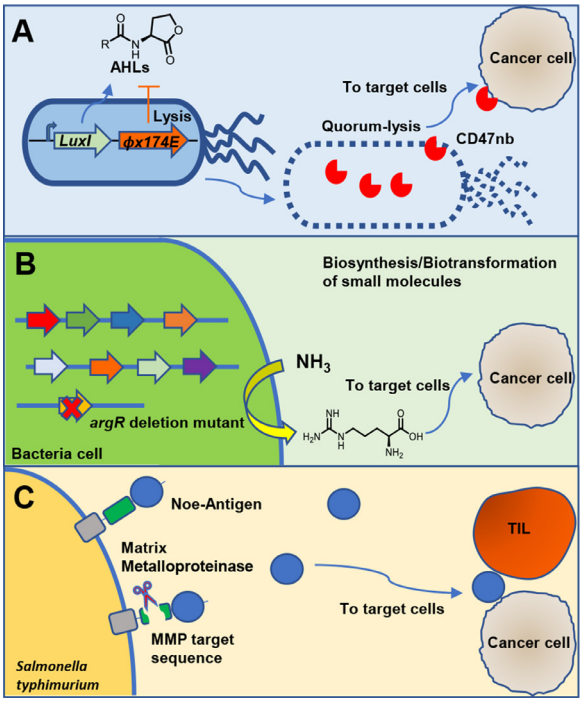

Fig. 4. Example of several payload output methods. A: Engineered *E. coli* reaches a quorum and induces the phage lysis protein *ϕ*X174E leading to bacterial lysis and release of anti-CD47 nanobody, which binds to CD47 on the tumor cell surface. Adapted from Ref [44]. B: Engineered *E. coli* Nissle 1917 not only colonizes the tumor but also converts excess ammonia produced by tumor metabolism into *L*-arginine, thus facilitating the effect of immunotherapy. Adapted from Ref [62]. C: After Neoantigen displayed on the surface of Salmonella was cleaved by matrix metalloproteinase (MMP), the cancer cell was detected by tumor-infiltrating lymphocytes using cleaved Neoantigen. Adapted with permission from Ref [77]. Copyright © 2021, American Chemical Society.

5. Biosynthesis and delivery of therapeutic compounds

The most important property of engineered bacteria as drug delivery vehicles is the production of active therapeutic substances. Many strategies have been developed where the bioengineered bacteria can deliver the small molecules, desired proteins, and genes to their targeted sites. From the current research trends, these molecules are mainly focused on the heterologous expression of therapeutic proteins or enzymes. Limited research focused on the expression of small molecule and biosynthetic gene clusters, including using gene-editing tools like Cas13 in engineered bacteria by heterologous expression techniques [55, 56]. In addition, although the membrane vesicles produced by bacteria do not belong to the bacteria itself, they can also be designed as an effective drug carrier [57, 58]. Even though the expression of protein molecules is probably mature, it faces several problems, such as cryptic regulatory elements caused by under-expression or unpredictability [59, 60]. This requires us to reconfigure the entire gene clusters from scratch, including the proper collocation of various regulatory genes and promoters.

After an engineered bacterium has synthesized a therapeutic molecule, it must be transported out of the bacterial extracellular space. In addition to the direct lysis release discussed above (Fig. 4A), genomes encoding small molecules often have dedicated transporter proteins that secrete synthesized compounds such as ABC transporter. Although ex-

amples of the use of engineered bacteria to deliver small therapeutic molecules are lacking, researchers have attempted to control the QS of pathogens and thus reduce their virulence by delivering an autoinducer. A prime example is a study by Duan et al. that used *E. coli* producing CAI-1 ((*S*)-3-hydroxy-4-one) to reduce the virulence of *Vibrio cholerae*. CAI-1 is an AI molecule originally produced by *Vibrio cholerae* that inhibits bacterial virulence by being sensed by the CqsS kinase on the cell membrane and causing phosphorylation of the downstream regulatory protein LuxO. Animal experiments have shown that ingestion of *E. coli* can produce CAI-1 and reduce the mortality caused by *Vibrio cholerae* [61]. Using engineered gene clusters to convert one chemical into another active compound in a given environment has also shown some advantages. In one study, the method was used to continuously convert ammonia from a tumor into L-Arginine (Fig. 4B), which increased the number of tumor-infiltrating T cells and had marked synergistic effects with PD-L1 blocking antibodies in the clearance of tumors [62]. For protein drugs, a bacterial transport signal peptide (SP) is needed so that the bacteria can secrete the active substances out of the bacteria [63, 64]. SPs are short peptides with 16–30 amino acids and are N-terminal extensions of secreted proteins. They act as a target and recognition signal for signal peptidases which remove SPs from the translocated protein across the cytoplasmic membrane and cell wall. This process will result in the extracellular release of the mature protein or peptide [65]. *Lactobacillus casei* strain Shirota (*Lc*S) was selected as a bacterial carrier for developing live mucosal vaccines against coronavirus for delivering proteins. The S glycoprotein was cloned into *Lc*S to express and secret the glycoprotein S by SPs. The oral immunization of BALB/c mice showed the immune responses against coronavirus with constitutive expression of recombinant *Lc*S [66].

The recombinant proteins with surface display systems in bacteria have been used in various biotechnological applications [67]. The surface display system was developed about 40 years ago, mostly in gram-negative bacteria [68–71]. Later, the strategy was applied in gram-positive bacteria with only one cytoplasmic cell membrane and relatively thick cell wall making them better candidates for anchoring opportunities and surface display of proteins [69, 72]. There are different types of surface anchoring domains, but the most commonly applied anchoring domains can be distinguished by either covalent binding to surface components, such as the LPXTG sequence of the M6 protein, or by non-covalent binding to the cell membrane or cell wall, such as the C-terminal domain of endogenous AcmA (cAcmA) [63, 73, 74]. These anchor proteins can display heterologous proteins on the cell surface of bacteria, providing a strategy for developing vaccines, biocatalysts, and whole-cell absorbents [75, 76]. For instance, Neoantigen was designed using a surface display to treat cancer through S. Typhimurium as a carrier (Fig. 4C) [77]. Neoantigen displayed on the surface of Salmonella was cleaved by matrix metalloproteinase (MMP, highly concentrated in the tumor). The cancer cell detected by tumor-infiltrating lymphocytes could use cleaved Neoantigen. Not only that, but the surface display can also be used to produce antigens to develop vaccines. In a recent study, researchers used genome-reduced *E. coli* to express SARS-CoV-2 and porcine epidemic diarrhea virus (PEDV) fusion peptides (FP) on their surface and evaluated their efficacy as killed whole-cell vaccines. The efficacy of the PEDV FP and SARS-CoV-2 FP vaccines was tested in a PEDV-challenged pig model. Both vaccines were found to induce an effective asymptomatic response upon viral challenge, enhance interferon-γ responses, reduce viral RNA load in jejunal tissue, and provide significant protection against the disease [78].

The technique of using bacteria for delivering genes directly into the tissue, organ, or organism is known as bactofection [79]. One example is using *E. coli* to deliver shRNA into HeLa cells for anticancer activity [46]. Another example of gene delivery is when Salmonella choleraesuis was used to carry the thrombospondin-1 (TSP-1) gene for melanoma and pulmonary metastasis treatment. The vector was constructed by cloning TSP-1 into the pTCY vector under the control of a rat β-actin promoter. The results showed that modified S. choleraesuis could target tumors for gene delivery, and the release of TSP-1 expression in tumors decreased intratumoral microvessel density and contributed to the antitumor activity [80]. In addition, siRNA delivery by Salmonella has also been reported [81, 82].

Lastly, an interesting phenomenon is that some bacteria can produce double lipid-layered nanoparticles called bacteria-derived membrane vesicles (MVs) [83]. As hollow sacs, MVs can carry active molecules such as secondary metabolites, endotoxins, proteins, peptides, DNA, and RNA [84]. However, the production capacity of bacterial MVs is limited, and it needs to be stimulated by chemical induction or genetic engineering. MVs produced by different bacteria are different and can target various tissues or organs and release the active substances loaded on them, which have good application potential in treating tumors, infections, and other diseases. The types of drugs delivered by MVs can range from small molecule chemotherapeutic drugs, radiotherapy drugs, and drugs affecting diagnostics, including peptides, proteins, and nucleic acids. Given the abundance of data on using MVs to deliver chemotherapeutic agents [85], they will not be explored in detail here.

Advances in synthetic biology have brought new prospects into the application of MVs. For example, one study elicited a specific antitumor immune response by genetically engineering the display of antigens specifically on the surface of OMVs [86]. In this study, tumor antigens fused with the ClyA proteins displayed on the surface of OMVs. Animal experiments showed that engineered MVs loaded with tumor antigens inhibited the metastasis of melanoma and the growth of colorectal cancer.

6. Discussion and prevision

Although bioengineered bacteria have raised the interest among researchers, employing this method in clinics raises controversial arguments. An important consideration for the use of genetically engineered microorganisms in safety of humans [87]. The research of genetically modified (GM) *Bacteroides ovatus* demonstrates that GM *B. ovatus* could survive under thymine starvation and transfer horizontal genes in the mammalian GIT resulting in transgene-carrying wild-type bacteria [88]. These results raised concerns about the uncertainty of GM bacteria after entering the human system and how they interact with other bacteria existing in the body. Those uncertainties need to be better studied before popularizing and promoting this method. To avoid horizontal gene transfer, using free plasmids directly in engineered bacteria is not desirable. This requires integrating the target gene into the genome and removing the integrase when modifying the bacterial genome. Some strains present in the human body are very easy to colonize after being GM and ingested by the human body, with few reports of the side effects [87, 89, 90]. However, this must be considered in the future before we can use an engineered microorganism in humans. Will the human body release genetically engineered microbes into the natural environment through interspecies microbial exchange with other animals such as pets? To minimize concerns like these, we need to design bacteria with appropriate genetic modules so that they can only survive in the human body or even in specific tissues or organs of the human body, and they cannot survive when they leave these specific environments.

The engineered bacteria have been reported to deliver large proteins such as antibodies and small molecules through biotransformation [91]. In addition, the use of bacteria-produced AI molecules to construct feedback genetic circuits has been used to construct therapeutic engineered bacteria [25, 44, 48]. However, in many cases, this system is preferred to deliver small therapeutic drugs. It is tantalizing that a large proportion of the drugs used in the clinic are derived from natural products synthesized by the biosynthetic gene clusters [92, 93]. Using engineered bacteria as a “vehicle” to precisely deliver biosynthetic gene clusters that encode small compounds to target proteins and turn the gene clusters on sounds like a fantastic story. Although we have made significant progress in the biosynthesis of natural products [94], direct delivery of natural products or natural product-like biosynthetic gene clusters

to target sites has not been reported. Unlike macromolecular proteins, these antibiotic biosynthetic gene clusters are often too large for common heterologous expression techniques to predict eventual issues [95]. Moreover, these sizable biosynthetic gene clusters, such as polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS), are often accompanied by many post-modification and regulatory genes [23]. Hypothetically, it is possible to completely reconfigure and simplify these biosynthetic gene clusters and precisely design gene regulatory pathways that work in the target tissue/target organ environment. However, this work appears to be very challenging in every way. A class of natural products has attracted our interest. Unlike other sizable biosynthetic gene clusters, the ribosomal synthesized and post-translationally modified peptides (RiPPs) are encoded by a relatively small cluster of biosynthetic genes [96, 97]. These compounds have a wide range of biological activities and are readily amenable to structural modifications at the gene level [98]. We believe that this class of compounds can be easily expressed and targeted for delivery in engineered bacteria through rational design. Furthermore, how to deliver these macromolecules/small molecule compounds out of bacteria is also worth investigating. In addition to signal peptides, some secretion systems, such as the type III secretion system of Salmonella, can be modified to secrete target proteins [99] or even to directly import these therapeutic proteins into target cells [100]. For small molecule compounds, modifying the original antibiotic delivery pump or transporter protein [101] of bacteria is necessary to deliver therapeutic compounds.

The main engineered microorganisms used as drug carriers are Lactococcus, Salmonella, and *E. coli*. As mentioned earlier, Lactococcus is a very widely used and well-known probiotic, and much work has been done on the genetic modification of Lactococcus. Salmonella has been used to release anticancer compounds within tumors, given its ease of colonization within the tumor environment. *E. coli* has also been studied more because it is the basic engineered bacterial chassis. Other strains such as *Bifidobacterium animalis* [102], *Listeria monocytogenes* [103], *Staphylococcus epidermidis, Staphylococcus lugdunensis* [104], *Clostridium sporogenes* [105] and some yeast [106] are also promising. Since the research utilizing synthetic biology to modify bacteria as drug delivery vehicles is not well developed, there is no well-established and mature system yet.

In summary, research using synthetic bacteria as drug delivery vehicles is still in its infancy. Most of these studies are currently focused on the expression of therapeutic proteins in the vicinity of target cells, while the delivery of small molecule therapeutic compounds is rare. These components' elemental composition and design principles are described in Fig. 1. Regardless of the microbial chassis on which it is based and the type of compound to be delivered for diagnostic, therapeutic, or prophylactic functions, a rationally designed engineered bacterial delivery vehicle must contain three components: signal input, signal processing, and integration, and compounds output. With further development, this system will undoubtedly become more complex and precise in the foreseeable future and can be applied in many aspects such as prevention, diagnosis, and treatment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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