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Advancements in synthetic biology-based bacterial cancer therapy: A modular design approach

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ABSTRACT

Synthetic biology aims to program living bacteria cells with artificial genetic circuits for user-defined functions, transforming them into powerful tools with numerous applications in various fields, including oncology. Cancer treatments have serious side effects on patients due to the systemic action of the drugs involved. To address this, new systems that provide localized antitumoral action while minimizing damage to healthy tissues are required. Bacteria, often considered pathogenic agents, have been used as cancer treatments since the early 20th century. Advances in genetic engineering, synthetic biology, microbiology, and oncology have improved bacterial therapies, making them safer and more effective. Here we propose six modules for a successful synthetic biology-based bacterial cancer therapy, the modules include Payload, Release, Tumor-targeting, Biocontainment, Memory, and Genetic Circuit Stability Module. These will ensure antitumor activity, safety for the environment and patient, prevent bacterial colonization, maintain cell stability, and prevent loss or defunctionalization of the genetic circuit.

1. Introduction

Synthetic biology, or SynBio, seeks to program living bacterial cells with artificial genetic circuits rationally designed to perform user-defined functions (Wu et al., 2019, Prasad et al., 2023). One of the key features of SynBio is the application of engineering principles to build biological systems through a four-step cycle: (1) Design and modeling of the genetic circuit in silico; (2) Construction and implementation of the genetic circuit; (3) test of the system with in vivo experiments; and (4) learning process and enrichment of the system (Boada, 2018).

The foundations of SynBio are considered to be the design, construction, and implementation of (1) the first toggle switch (Buecherl and Myers, 2022), a bistable genetic circuit composed of two repressors and two promoters, each promoter is inhibited by a repressor transcribed by the opposing promoter (Gardner et al., 2000); and (2) a synthetic oscillatory network of transcriptional regulators also known as a repressilator since it was formed of a three transcriptional repressor system, the network oscillated in typical periods of hours slower than the cell-division cycle in order for the oscillator to be pass from one generation to another (Elowitz and Leibler, 2000). Since then, more complex systems have been developed (Cameron et al., 2014) with a

Abbreviations: Synbio, synthetic biology; DBTL, Design-Build-Test-Learn; NSCLC, STAT-3, non-small cell lung cancerSignal Transducers and Activators of Transcription-3; HVEM, Herpesvirus entry mediator; STING, Stimulator of IFN Genes; MTD, mitochondrial targeting domain; CPP, cell-penetrating peptide; BDEPT, bacterial directed enzyme/prodrug therapy; tkRNAi, Transkingdom RNA interference; QS, Quorum Sensing; AHL, Acyl homoserine lactone; TRIP, Transkingdom RNA interference plasmid; RBS, Ribosome Binding Sites; UBP, unnatural base pair; NSAA, non-standard amino acid.

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broad range of applications, such as industrial processes (Gao et al., 2019), agriculture (Wurtzel et al., 2019) or medicine (Leventhal et al., 2020).

Cancer is a current challenge in our society. With 18.1 million new cases and more than 9.5 million deaths in the year 2018 (Bray et al., 2018), cancer is a significant cause of morbidity and mortality worldwide, as well as the first cause of death in high-income countries (Dagenais et al., 2020). Moreover, lung cancer is the most common type of cancer, followed by breast, prostate, and colon cancer (Bray et al., 2018).

Bacteria have been frequently regarded as the causative agent of infectious disease, and previous studies have described the pathogenic role of some bacteria in cancer development (Dejea et al., 2018; Bertaglia et al., 2023). However, most bacteria are non-pathogenic and could potentially affect our health in a positive way. In fact, bacteria have been tested as a successful anticancer therapy (Piñero-Lambea et al., 2015b). The use of bacteria as a cancer treatment is not a new concept; German physician W. Busch seems to be the pioneer when in 1868 purposefully infected a woman with an inoperable sarcoma with *Streptococcus pyogenes*. The tumor shrunk, but the patient died from a bacterial infection nine days after, because *S. pyogenes* produce erysipelas. In 1883, Fehleisen reported the use of *Streptococcus* in a breast cancer patient who had a complete tumor regression, and six months later remained free of the malignancy (Coley, 1891). After that, hundreds of patients with sarcoma were treated with Coley's toxin (Coley (1898)), whose developer is now broadly recognized as the founder of cancer immunotherapy.

However, the use of bacteria originated infections, erysipelas and other malignancies, and many patients died as a result of these side effects. For this reason, the idea of using bacteria against cancer disappeared with the advance of chemotherapy, radiotherapy, and other techniques (Pawelek et al., 2003). Now, with the development of genetic engineering, synthetic biology and deeper knowledge about both microbiology and oncology, it is possible to improve bacterial therapies, making them safer and effective (Fig. 1).

Bacterial cancer therapies might overcome some drawbacks of the current cancer therapies such as (1) high cost, unlike bacterial therapies that are cost-effective (Jiang et al., 2010); (2) side effects derived from systemic drug action, in contrast bacterial therapies maximize drug accumulation within the tumor, minimizing off-target effects (Ozdemir et al., 2018) and (3) development of resistance to current therapies. Many bacterial cancer therapies under study have been shown to develop immunological memory and, consequently, resistance to secondary tumor challenge and longer cancer-free time intervals (Leventhal et al., 2020).

The rational design of microorganisms for biomedical applications requires the availability of gene synthetic modules with a predictable phenotype that could be integrated into complex cellular assemblies, that also have orthogonal behavior inherited through cell generations (Piñero-Lambea, 2014). The present work gains insight into the interface between SynBio and oncology, exploring the current situation of bacterial cancer therapies and combining current bacterial therapies under development with the study of novel systems. Some reviews about bacterial cancer therapies have been developed (Ozdemir et al., 2018; Marzhoseyni et al., 2021), but none of them have explored the wide variety of strategies that can be combined to develop new bacterial therapies, neither identify nor determine thoroughly the basic requirements to engineer bacteria for fighting cancer.

We propose a systematic modular design for bacterial therapy alongside all the necessary modules with the requirements that any microorganism should reach before to be considered as an effective bacterial cancer therapy. We analyzed each module and the systems proposed. Some limitations in current bacterial cancer therapies have been detected, like shortage of bacteria with a biocontainment module.

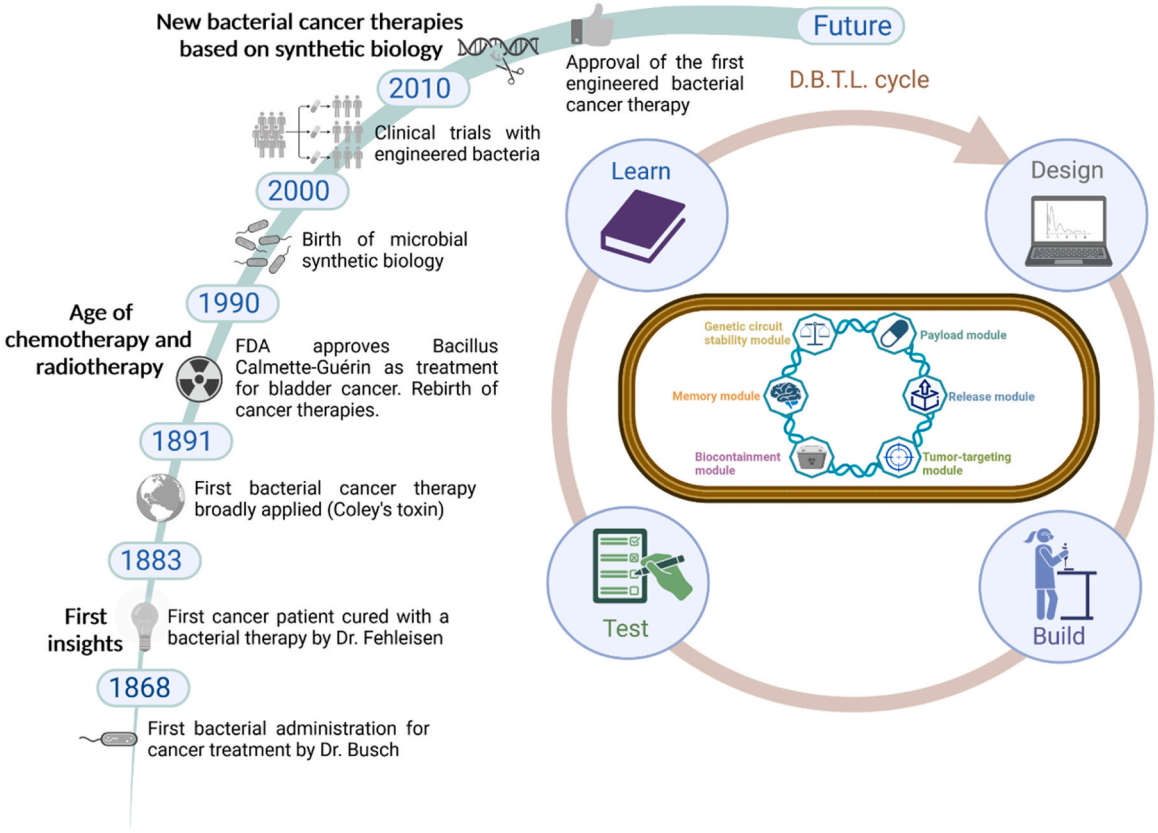

Fig. 1. Timeline of bacterial cancer therapies evolution. From the first bacterial administration to treat cancer to the use of synthetic biology and the Design-Build-Test-Learn cycle to create specifically engineered bacteria for precise type of cancer cell towards the future of bacterial cancer therapies.

This work aims to facilitate the development of new engineered bacteria against cancer by analyzing the various approaches and strategies available for each module, allowing the reader to choose the best option for the desired outcome while having no negative effects on the cancer patient or the environment.

Table 1
Necessary modules for a bacterial cancer therapy.

MODULES	APPROACH	STRATEGIES
PAYLOAD *antitumoral activity*	Stimulation of the immune system	Wild stimulation
		Immunomodulatory proteins enhancing stimulation
	Cytotoxic molecules	
	Transference of genetic material	
RELEASE *delivery of the payload onto the tumor tissue*	Secretion systems	
	Cytoplasmatic content release	Externally controlled lysis
		Cell-population density-controlled lysis
	Bacterial invasion of tumor cells	Cell invasion
		Synthetic Payload Delivery Device (PDD)
TUMOR-TARGETING *bacterial action on the tumor only*	Selective proliferation of bacteria within tumor tissue	Wild tumor tropism
		Adhesion molecules and synthetic adhesins
	Tumor tissue signals detection	Quorum sensing systems
		Inducible promoters
		Engineered chemoreceptors
		Toehold switch
		miRNA and p53 sensing
BIOCONTAINMENT *safety for the environment and the patient*	Preventing bacteria escape from the host	Auxotrophy
		Inducible suicide genes
	Suicide genetic circuits	Deadman kill switch
		Passcode kill switch
	GeneGuard plasmid system	
	Artificial strains unable to survive in natural environments	Minimal genomes Artificial genetic languages
		Altered chemical language
MEMORY *maintaining the target expression once the stimuli have disappeared*	Toggle switches	
	Epigenetic modifications	
	DNA-encoded memory	Synthetic Genetic Memory
		Synthetic logic circuits
		SCRIBE system
GENETIC CIRCUIT STABILITY *no loss of the gene circuit nor its functionality*	Limitation of target gene expression	Two layer circuit
	Plasmid control	Plasmid partitioning protein
		Low mutation rate bacterium
	Essential plasmids for bacteria survival	Antibiotic resistance genes
		Balanced-lethal host-vector system
		Post-segregational killing

2. Chassis selection

The microorganism that will host our synthetic genetic circuit is referred to as the chassis. It is critical to choose it carefully because this decision will have an impact on all of the modules developed, the engineering process, the clinical application, and the efficacy of the therapy in vitro assays, animal models, and clinical trials.

Many bacterial species, both Gram-negative and Gram-positive, have

been used. The first consideration is the need to avoid pathogenic strains that may lead to the development of other diseases. Some pathogenic bacteria have been used in attenuated strains, such as *Listeria monocytogenes*, which has progressed to phase II clinical trials as a promising treatment for non-small cell lung cancer (NSCLC) (NCT03847519, Clinicaltrials.Gov, 2019a). Furthermore, selecting a strain with wild tumor tropism may be advantageous for improving tumor-targeting effect (Piñero-Lambea et al., 2015b).

Many strains are being engineered with minimal genomes that contain only the genes required for self-replicable life (Choe et al., 2019), low-mutation rates (Csörgő et al., 2012) or artificial genetic and chemical languages (Fischer et al., 2020), which could improve the bacterial therapy and its safety. Nevertheless, developing this type of strain is expensive and not many strains are available.

Because there is a large library of cataloged genetic parts, it is common to use *E. coli* (Loessner et al., 2009; Ho et al., 2018; Ahmed and Lage, 2018) or other well-known and non-pathogenic bacteria as the chassis for our genetic circuit. Furthermore, other organisms, such as *Bacteroides*, can be used because they last much longer in the intestinal microbiota. However, they require extensive libraries and are more difficult to develop (Ozdemir et al., 2018).

Other bacteria that have also been widely employed are *Clostridium acetobutylicum* (Theys et al., 2001; Nuyts et al., 2001b), *Clostridium oncolyticum* (Nuyts et al., 2002) and *Clostridium novyi* (Roberts et al., 2014). *Clostridium* spp. is a strictly anaerobic, spore-forming Gram--positive bacteria whose spores have been used in many cancer therapies (Roberts et al., 2014).

Lastly, some bacteria have been found to be useful, despite not being widely used, such as *Salmonella enterica* (Royo et al., 2007), *Bifidobacterium infantis* (Zhu et al., 2011), and *Bifidobacterium longum* (Wei et al., 2016; Hu et al., 2009).

3. Modules for engineered bacterial cancer therapies

In this work, we have identified six modules or requisites that any microorganism must integrate to be suitable for clinical applications in cancer treatment. There is only one exception with the memory module, which is not compulsory but helpful for some approaches. These modules aim to develop engineered bacteria that can fight cancer effectively without risk to the patient or the environment. Each module plays an important and specific role (Table 1):

(1) Payload module, being responsible for the antitumor activity.
(2) Release module, that delivers the payload onto the tumor tissue.
(3) Tumor-targeting module, which directs the bacterial action only to the tumor cells, avoiding off-target effects.
(4) Biocontainment module, ensuring the safety of the therapy for the environment and the patient.
(5) Memory module, useful in some applications that require to maintain the target gene expression once a specific stimulus or inducer have disappeared.
(6) Genetic circuit stability module, to avoid the loss of the genetic circuit or its function.

3.1. Payload module

One of the most important modules in bacterial cancer therapies is the payload module, which is the mechanism or type of molecules that bacteria use to attack and kill tumor cells. There is a broad range of possibilities, but it depends on the specific tumor type being treated in many cases. However, bacteria could encode a cytotoxic payload for different types of tumors. We have identified three main approaches. (1) First, many bacteria have shown a wild stimulation of the immune system with an antitumor effect. They even can be engineered including immunomodulatory proteins to enhance this effect. (2) Second, the use of cytotoxic molecules can be produced directly by bacteria or through the enzyme/prodrug system, in which bacteria encode an enzyme that catalyzes the transformation of a non-toxic prodrug to a cytotoxic molecule. (3) Third, transference of genetic material from bacteria to tumor cells, such as micro-RNA (miRNA), small RNA that can bind to messenger RNAs (mRNAs) to block them from making proteins, to silence oncogenes or engineered DNA for its expression in tumor cells.

3.1.1. Stimulation of the inmmune system

3.1.1.1. Wild stimulation. The host organism detects bacteria as a threat, leading to the immune system stimulation (Fig. 2a) (Gurbatri et al., 2022). An example is *Bacillus Calmette-Guérin* (BCG) immunotherapy, broadly applied in the clinic to treat bladder cancer and as a vaccine for tuberculosis. These bacteria induce the up-regulation of the Programmed death ligand 1 (PD-L1), an "adaptive immune mechanism" of cancer cells to avoid anti-tumor responses that binds to its receptors and activate proliferative and survival signaling pathways consequently acting as a pro-tumorigenic factor in cancer cells (Han et al., 2020), on both macrophages and dendritic cells through the secretion of interleukins (IL-6 and IL-10) and the consequent activation of the Signal Transducers and Activators of Transcription-3 (STAT-3), leading to superior white blood cells (CD4 + T-cells) responses to recall antigen (Copland et al., 2019). A clinical trial has shown that Mixed Bacterial Vaccine (also called Coley's toxin) can lead to tumor regression by increasing interleukin 6 (IL-6), Tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ), and Interleukin-1 beta (IL1-β) (Karbach et al., 2012; NCT00623831, Clinicaltrials.Gov, 2008).

E. coli also has demonstrated moderate antitumor activity because of the immune system stimulation, causing an increment of IL-6 and TNFα levels inside the tumor environment (Leventhal et al., 2020). Additionally, *S. typhimurium* can infiltrate colon tumors causing an immune response with the activation of M1 macrophages that produce proinflammatory cytokines, phagocytize microbes, and initiate an immune response (Zheng et al., 2017). Although some bacteria have been designed with active compounds as the primary resource against cancer, most of them have shown to stimulate the immune response contributing to tumor regression (Loeffler et al., 2007; Loeffler et al., 2008; Zhu et al., 2011; Leventhal et al., 2020).

3.1.1.2. Immunomodulatory proteins enhancing stimulation. As previously discussed, bacteria stimulate an immune response originating tumor regression. This property can be strengthened using engineered bacterium that produces immunomodulatory proteins, whose function will be the attraction, activation, and stimulation of immune cells within the tumor microenvironment (Fig. 2b).

In cancer, immunomodulatory proteins have been produced using attenuated *Salmonella typhimurium*. LIGHT-expressing *Salmonella* inhibited the growth of primary tumors and the dissemination of lung metastases in a number of animal tumor models (Loeffler et al., 2007). TNFSF14, also known as Herpesvirus entry mediator (HVEM-L), is an immunomodulatory protein that promotes tumor rejection and long-lasting antitumor immunity by regulating tumor vasculature, promoting the neogenesis of tertiary lymphoid structures, and enhancing the infiltration of effector tumor-infiltrating lymphocytes (Skeate et al., 2020).

Salmonella typhimurium expressing the proapoptotic cytokine Fas ligand (FasL) has shown tumor regression in murine models of breast carcinoma, colon carcinoma, melanoma, and lung metastases (Loeffler et al., 2008). Likewise, IFN-γ has been produced by *S. typhimurium* and used against melanoma. By facilitating the attraction and activation of macrophages and Natural Killer cells, INF-γ reduced the development of tumor cells. Moreover, it improved antigen presentation and processing as well as T cell tumor recognition and eradication. By the induction of chemokines like protein-10 and monokines as well as the growth of INF-

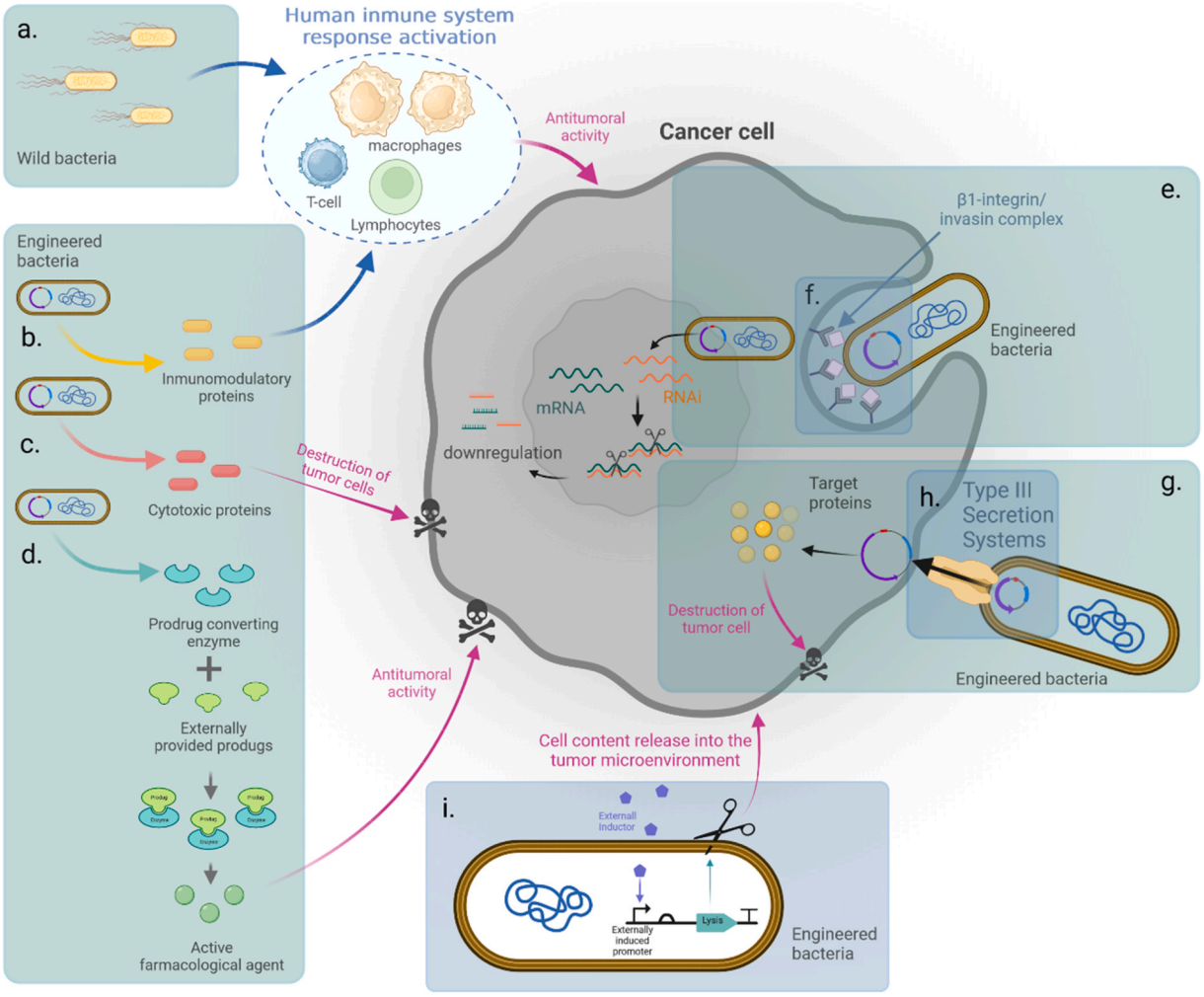

Fig. 2. *Payload / Release module.* WILD STIMULATION. **a)** The host organism recognizes bacteria as a threat, which stimulates the immune system. IMMUNOMODULATORY PROTEINS. **b)** The immune response induced by bacteria can be enhanced using an engineered bacterium that produces immunomodulatory proteins to attract, activate, and stimulate immune cells within the tumor microenvironment. CYTOTOXIC MOLECULES. **c)** Cytotoxic protein's coding genes can be engineered into bacteria's payload module to destroy tumor cells after being released. **d)** Bacterial directed enzyme/prodrug therapy (BDEPT). Engineered bacteria produces the enzyme that will catalyze the prodrug to become an active pharmacological agent. TRANSFERENCE OF GENETIC MATERIAL: **e)** Transkingdom RNA interference (tkRNAi). A short RNA molecule suppresses the expression of genes with a complementary sequence by breaking down the target mRNA and suppressing protein translation. To deliver the RNAi effector into the tumor cell, we could use **f)** The cell invasion strategy discussed in the Release module to encode the gene *Inv*, which produces Invasin, a bacterial surface protein that binds to the 1-integrin on the target cell surface, after which the bacterium is absorbed into the host cell via endocytic vesicles. **g)** DNA that encodes proapoptotic molecules or antigens to ensure that cancer cells are recognized by the immune system after being transferred into the cancer cell using **h)** Type III Secretion Systems (T3SSs). T3SS form a channel that connects the bacterial cytoplasm with the cytoplasm of the host cell and allows transkingdom interactions. The transferred DNA encodes proapoptotic molecules or antigens, ensuring cancer cells are recognized by the immune system. **i)** Externally controlled lysis. Bacterial lysis is a method to release bacterial content into tumor microenvironment, using bacteria encoding a lysis protein under an inducible promoter.

γ-dependent CD4 + T cells, it can also have an anti-angiogenic effect. The last impact of IFN-γ is the activation of macrophages, which results in the production of nitrite and Reactive Oxygen Species that have an antitumorigenic effect by inducing DNA damage (Yoon et al., 2017, Srinivas et al., 2019).

The Stimulator of IFN Genes (STING) agonists are another example of immunomodulatory proteins employed in bacterial cancer treatments. Activation of STING results in the stimulation of Antigen-Presenting Cells (APCs) and the production of IFNα and IFNβ (type I IFNs), which are required for the generation of antitumor CD8 + T cells. It has been demonstrated that the administration of STING agonists stimulates the reversion of immune suppression and, consequently, tumor regression (Jing et al., 2019). Leventhal et al. (2020), used this strategy in *E. coli* expressing *dacA* gene that encoded a CDA-producing enzyme (CDA, or cyclic di-AMP, is an agonist of STING). This strategy has also reached the clinical trials with an ongoing phase I study in patients with advanced/metastatic solid neoplasm and lymphoma (https://www.clinicaltrials.gov/ identifier: NCT04167137).

In TLR5-negative colon cancer murine models has been successfully treated with *Vibrio vulnificus* flagellin B (FlaB) (Zheng et al., 2017). According to Lauté-Caly et al. (2019), the flagellin of *Enterococcus gallinarum* MRx0518 can elicit a strong pro-inflammatory response. Patients with non-small cell lung cancer (NSCLC), renal cell carcinoma, bladder cancer, or melanoma are being evaluated in a phase II clinical trial using this immune-modulatory protein combined with Pembrolizumab (https://www.clinicaltrials.gov/identifier: NCT03637803). Moreover, MRx0518 is being tested in a phase I clinical study alongside radiotherapy for individuals with resectable pancreatic cancer (https://www.clinicaltrials.gov/ identifier: NCT04193904).

Finally, research has demonstrated the effectiveness of utilizing therapeutic nanobodies to block molecules found in cancer cells, such as CD47 (Chowdhury et al., 2019), PD-L1, or CTLA-4 (Gurbatri et al.,

2020), the latter exerts its inhibitory function primarily inhibiting CD28-positive costimulation by competing to bind to their shared B7 receptors (CD80/CD86) on the antigen-presenting cells (APC) (Zhao et al., 2018).

Nanobodies have a length of around 15 kDa and lack the Fc (Fragment, crystallizable) region that requires glycosylation by mammalian cells so that they can be produced by bacteria (Gurbatri et al., 2020) to act as an immunomodulatory protein.

3.1.2. Cytotoxic molecules

Genes encoding cytotoxic proteins can be engineered into bacteria and the payload module to destroy tumor cells when they are released (Fig. 2c). A payload protein must be produced, diffuse through tissue, and effectively destroy cancer cells for it to be successful (Jean et al., 2014). Since certain molecules have both therapeutic and cytotoxic effects, the distinction between immunomodulatory and cytotoxic proteins is not entirely obvious.

In this regard, proteins from the TNF (tumoral necrosis factor) family have been widely used. TNFα can induce both apoptotic and necrotic forms of cell death through the selective action on the neovasculature of tumors, stimulation of T cell-mediated immunity and direct cytotoxicity to tumor cells (Nuyts et al., 2001b). Another member of the TNF family is TRAIL (TNF-related apoptosis-inducing ligand), which binds to their membrane receptors (DR4 and DR5) and causes apoptosis through the activation of caspase-3. TRAIL has been expressed in non-pathogenic *S. typhimurium* reducing the risk of death by 76% in mice with mammary tumors (compared with irradiated controls) (Ganai et al., 2009).

E coli has been used to express the pore-forming toxin α-hemolysin from *Staphylococcus aureus* (SAH) in bearing mammary carcinomas mice (Jean et al., 2014). ClyA is another pore-forming toxin of *E. coli* that can disrupt the membrane of target cells. It has proven to have a positive effect against colon cancer and lung metastasis in combination with radiotherapy in mice (Benke et al., 2015). Noxa is a transcriptional target of the tumor suppressor gene p53 that activates mitochondrial damage and the intrinsic apoptosis pathway. Massive necrosis is caused by Noxa's mitochondrial targeting domain (MTD), which has been linked to a cell-penetrating peptide (CPP) and later inserted into *S. typhimurium* alongside a lysis system that has an anti-tumor impact (Jeong et al., 2014).

A prodrug converting enzyme is encoded by bacteria as part of a treatment method known as bacterial directed enzyme/prodrug therapy (BDEPT). Prodrugs are substances that must undergo chemical conversion through metabolic processes in order to become active pharmacological agents. The enzyme catalyzes the transformation of a prodrug into an antitumor compound (Fig. 2d). The advantage of this strategy is that the anticancer agents are usually toxic at therapeutic doses, so BDEPT, administers a prodrug with less toxicity. Afterward, it would be transformed into the anticancer agent only in tumor tissue, where bacteria are located because of the use of the Tumor-targeting module that will be explain later (section 2.2 Tumor-targeting module). In this regard, achieving higher doses of the anticancer agent in tumor tissue is possible without side effects in healthy tissues (Lehouritis et al., 2013; Friedlos et al., 2008). Various examples of this strategy with their enzyme, produg and active compound are listed in Table 2.

Table 2
Enzyme/prodrug systems.

Enzyme	Prodrug	Antitumor compound	References
Thymidine kinase	Ganciclovir	Unknown	(Tang et al., 2009)
CDase	5-Fluorocytosine	5-Fluorouracil	(Nuyts et al., 2002; Theys et al., 2001; Royo et al., 2007)
Myrosinase	Glucosinolate	Sulphoraphane	(Ho et al., 2018)
CPG2	Derivatives from Glutamic acid	DNA interstrand crosslinking	(Friedlos et al., 2008)
Nitroreductase	CB1954	DNA interstrand crosslinking	(Nuyts et al., 2002; Swe et al., 2012)
Nitrate / Nitrite reductases	NO_3 (and C_3N_4)	Nitric Oxide	(Zheng et al., 2018).
β-Glucuronidase	9ACG	9AC	(Cheng et al., 2008; Cheng et al., 2013).
PNP	6MePdR	6MeP	(Chen et al., 2012).

3.1.3. Transference of genetic material

The transference of the payload from the selected chassis microorganism to the target cells can be done using genetic material (either with RNA o DNA).

Transkingdom RNA interference (tkRNAi) are strategies that can modify gene expression within the host genome. They have been used to silence oncogenes that are responsible for a tumorigenic process (Xiang et al., 2006). This approach needs a Release Module that allows the transference of tkRNAi from bacteria to the cancer cells (Fig. 2e).

By the breakdown of the target mRNA and suppression of protein translation, a short RNA molecule downregulates the expression of genes with a complementary sequence. This phenomenon is known as RNA interference (RNAi) (Nguyen and Fruehauf (2009)). The biggest challenge with this phenomenon is getting the RNAi effector into the tumor cell. The solution is to engineer *E. coli* to encode the gene Inv that produces Invasin, a strategy further discussed in section 2.2 Release Module. The effectiveness of this method to deliver therapeutic short hairpin RNA (shRNA) encoding plasmidic DNA into target cells in order to hijack the cellular RNAi machinery has been successfully proven in oral squamous carcinoma cells in vitro (Ahmed and Lage, 2018) and against human colon cancer xenografts in mice (Xiang et al., 2006).

Transference of DNA with a therapeutic effect onto target cancer cells can be done using Type III Secretion Systems (T3SSs), a Gram-negative bacteria secretion method that forms a channel connecting the bacterial cytoplasm with the cytoplasm of the host cell, this strategy will be further discussed in 2.2 Release Module. The transferred DNA should be designed and expressed in mammalian cells encoding any of the previous active payload strategies, such as proapoptotic molecules (Fig. 2f). The critical point is to deliver killer DNA to cancer cells or an antigen so cancer cells can be recognized by the immune system (Castagliuolo et al., 2005).

IL-12 transgene-encoding plasmid DNA is delivered to cancer cells by genetically altered *Bifidobacterium longum*, causing the cancer cell to express IL-12 and triggering the host immune response. Phase I clinical trials for this bacterial cancer treatment were recently (2019) conducted in people (NCT04025307, Clinicaltrials.Gov, 2019b).

3.2. Release module

As previously stated, the Payload module will be the load carried by the chassis to exert the anticancer effect; however, it is also critical to engineer a module for the release of this payload, as most bacterial therapies require molecules to be released outside of the bacteria. But the Release module chosen is not arbitrary; it is determined by the type and mechanism of the payload that we are working. Some release systems may be ideal for specific bacterial cancer therapy while failing to function properly in others. Some of these systems have been developed over the years, and we have identified three major types of release strategies. The first is the secretion of molecules into the extracellular space or directly into the cytoplasm of the host cell via Type III Secretion Systems (T3SS). The second is the release of cytoplasmatic content via bacterial lysis, which is regulated by external induction or quorum sensing systems. Finally, the third is bacterial invasion of tumor cells to deliver their content.

3.2.1. Secretion systems

Because Gram-positive bacteria lack an outer membrane, they can secrete proteins directly into the extracellular medium. They do so via the General Secretory (Sec) route. Gram-negative bacteria, such as

E. coli, require more complex systems due to the outer membrane (De Keyzer et al., 2003).

The protein transport across cellular membranes in bacteria is a challenging biochemical feature. So far, seven types of secretion systems (TSS) have been described in bacteria. Five (T1SS, T2SS, T3SS, T5SS and T8SS) have shown their ability to secrete recombinant proteins (Burdette et al., 2018).

There are four types of engineering techniques for secretion systems. 1) Change in the secretion tag that directs the secretion of a protein; 2) Engineering the secretion system machinery; 3) Shifting a secretion system to a more suitable production strain; and 4) Managing the secretion system's genetic regulation (Burdette et al., 2018).

In addition to dividing bacterial secretion systems into four categories, secretion systems detailed in this section can be divided into two types based on whether their payload enters or bypasses: (1) One-step systems, which deliver the protein directly to the extracellular space, or (2) Two-step systems, which use an intermediate step in which the protein is exported to the periplasm first. For two-step systems, the first step is to export proteins to the periplasm via the general secretory (Sec) or twin arginine translocation (Tat) pathways (Sibbald et al., 2006). Almost all secretion systems require a signal sequence or a peptide secretion tag for protein release.

The secretion systems' efficacy has already been tested. In murine tumor models using the YebF secretion tag fused to the enzyme myrosinase to convert host-ingested glucosinolates, natural components of cruciferous vegetables, to sulphoraphane, an organic small molecule with known anticancer activity (Ho et al., 2018). As cancer immunotherapy, the pelB leader sequence (a sequence of amino acids that, when attached to a protein, directs the protein to the bacterial periplasm) was fused to *Vibrio vulnificus* flagellin B (FlaB) in tumor tissues (Yoon et al., 2017). Finally, IFN- conjugated to the N-terminus of SipB, a *Salmonella* surface protein required for mammalian cell invasion, used as a tumor-targeting anti-cancer agent (Yoon et al., 2017).

TYPE III SECRETION SYSTEM (T3SS). Among all the Secretion Systems mentioned above, type III secretion systems are one of the most used in engineered biotherapeutics and have been described in Gram-negative bacteria. T3SS form a channel that connects the bacterial cytoplasm with the cytoplasm of the host cell and allows trans-kingdom interactions (section 2.1.3 Transference of genetic material), such as the transference of proteins (Deng et al., 2017) (Fig. 2g).

T3SS has been applied in engineered *S. typhimurium* expressing immunomodulatory molecules to treat breast cancer, colon cancer, melanoma, and lung metastases (Loeffler et al., 2007; Loeffler et al., 2008). To improve this type of secretion system, genes responsible for T3SS formation could be under the control of an inducible promoter (Reeves et al., 2015).

3.2.2. Cytoplasmatic content release

3.2.2.1. Externally controlled lysis. Bacterial lysis is an immediate way to release all the bacterial content into the tumor microenvironment. This can be achieved with bacteria encoding a lysis protein under the control of an inducible promoter (Fig. 2h). For example, the *'Lys'* fragment of the phage iEPS5 is regulated by the P_{BAD} promoter of the *E. coli* arabinose operon, which is externally induced by L-arabinose (Jeong et al., 2014). A similar strategy for releasing bacterial content has been used with engineered *S. typhimurium* carrying the bacteriophage lambda lysis gene cluster SRRz, induced by anhydrotetracycline (Camacho et al., 2016). Bacterial lysis can also be externally induced with light using molecules like the aggregation-induced emission photosensitizer (AIE PS) with bioactive mitochondria (Mito-AIEgen-lipid) for photodynamic therapy (Liu et al., 2020).

3.2.2.2. Cell-population density controlled lysis. The principle of this gene synthetic circuit is based on the ability of bacteria to lyse themselves when the population grows and reaches a certain density threshold, hence being also called a population oscillator. This case of controlled lysis is activated by Quorum Sensing (QS) and does not need external induction (Din et al., 2016). This synthetic genetic circuit has four genes: (1) Lux I, an enzyme that synthetizes acyl homoserine lactone (AHL); (2) Lux R, an AHL-sensitive transcription activator; (3) ϕX174E; a bacteriophage lysis protein; and (4) the therapeutic protein.

The expression of the lysis protein is driven by a promoter activated by LuxR·AHL (Plux operon) so that when bacteria reach the tumor, the population level is low (AHL concentration is very low as well), and no self-lysis occurs. As bacteria proliferate, AHL accumulates until a specific density threshold is reached. AHL binds to LuxR, which activates the expression of the lysis protein releasing of all the bacteria's contents. The active transcription factor LuxR·AHL causes the expression of LuxI generating more AHL, producing positive feedback (Din et al., 2016). One of the aspects that makes this system so interesting is that the lysis circuit is activated only when the population density is high, and it becomes inactivated when the population decreases, so the population growth is in an oscillatory way. At the same time, the release cycles of antitumoral compounds occur through bacterial lysis, obtaining a treatment that can be maintained over time (Danino et al., 2010). Recently, a programable *E. coli* has been engineered with this lysis circuit to release an anti-CD47 nanobody, leading to tumor progression in murine cancer models of lymphoma, mammary carcinoma and melanoma (Chowdhury et al., 2019). Prindle et al. (2012a); Prindle et al. (2012b) have proposed another type of oscillator using the *LuxI* and *aiiA* genes, without bacterial lysis. LuxI generates AHL, while aiiA encodes an enzyme that hydrolyzes AHL.

3.2.3. Bacteria invasion of tumor cells

3.2.3.1. Cell invasion. Some payloads need to be delivered inside the tumor cells. To ensure that bacteria can penetrate the tumor cell, the *Inv* locus gene of *Yersinia pseudotuberculosis* must be expressed. This gene encodes Invasin, a bacterial surface protein that binds to the β1-integrin in the target cell surface (Huh et al., 2013; Ahmed and Lage, 2018) (Fig. 2i). Afterward, bacteria are introduced within the host cell through endocytic vesicles. Moreover, bacteria should also encode the *Hly* gene from *Listeria monocytogenes*, which produces listeriolysin O, a perforin cytolysin able to perforate phagosomal membranes after bacteria uptake. This allows the release of the bacteria content, such as a cytotoxic protein or a DNA designed for its expression inside the host cell (Castagliuolo et al., 2005; Xiang et al., 2006).

This release strategy has been used in *E. coli* to deliver *transkingdom* interference RNAi (tkRNAi) against oncogenic HPV16-E7 protein to oral squamous carcinoma cells (Ahmed and Lage, 2018). *Transkingdom* RNA interference plasmid (TRIP) has been developed to ease the transference of a short RNA (shRNA) to tumor cells. TRIP contains three key elements: the shRNA expression cassette, *Inv* and *Hly* genes (Nguyen and Fruehauf (2009)).

One of the advantages of this approach is that β1-integrins are overexpressed in several solid tumors, focusing their bacterial action on tumor cells and not in healthy tissues (Blandin et al., 2015). For a controlled regulation of cell invasion, the *Inv* gene can be expressed downstream using an inducible promoter activated by hypoxia or L arabinose and the Quorum Sensing system (Anderson et al., (2006)). In contrast, some bacteria with immunomodulatory payloads have been demonstrated to have an antitumoral effect in a phagocytosis-dependent manner, although the mechanism is not clear yet (Leventhal et al., 2020).

Another option for cell invasion is using cell-penetrating peptides (CPPs) fused to the target protein. CPPs are short and membrane-permeable peptides capable of targeting intracellular proteins and carrying cargo proteins into target cells (Hyang-Mi et al., 2021). The most studied CPPs are those derived from the TAT protein of HIV-1 (Patel

et al., 2019), and DS4.3 peptide derived from S5 subunit of a voltage-gated potassium channel (Kv2.1). The last one has been used successfully to deliver the prodeath mitochondrial targeting domain (MTD) of Noxa, a p53 transcriptional target that induces apoptosis by activating mitochondrial damage and the intrinsic apoptosis signaling pathway, to tumor cells in a murine mammary carcinoma model, using engineered *S. typhimurium* (Jeong et al., 2014).

3.2.3.2. Synthetic payload delivery device (PDD). The Payload Delivery Device (PDD), not to be confused with the Payload module, is an adaption of the previously stated cell invasion strategy. It increases payload delivery by invading the tumor cells, resulting in bacterial uptake into a vacuole. After recognizing the vacuole microenvironment, the bacterium lyses itself and the vacuole, delivering bacterial macromolecules into the cytosol enhancing bacterial content release (Huh et al., 2013).

The payload delivery device is formed of: (1) PLD or Payload Device; (2) ID or Invasion Device, formed by *Inv* gene constitutively expressed; (3) VSD or Vacuole Sensing Device, senses the vacuole microenvironment and detects when the bacteria have been internalized, activating the SLD and VLD; (4) SLD or Self-Lysis Device, produces bacterial lysis in response to VSD activation; and (5) VLD or Vacuole Lysis Device, that causes the lysis of the vacuole. Here, bacteria produce the payload first, and then invade the tumor cell. After that, the vacuole microenvironment is detected and the bacteria lyse themselves and the vacuole, releasing the payload into the mammalian host cell (Huh et al., 2013).

3.3. Tumor-targeting module

One of the most important advantages of bacterial therapies is their ability to avoid off-target effects and focus solely on tumor cells and metastases, which is accomplished through the tumor-targetin module. Several strategies have been developed to achieve that bacterium reaches the tumor, combining two or more systems to ensure and enhance the tumor-targeting effect. These strategies can be classified into two groups depending on their underlying mechanisms. First, we have systems focusing on the specific proliferation of bacteria within tumor tissue, such as wild tumor tropism, adhesion molecules, synthetic adhesins or quorum sensing systems. Second, some systems can detect molecules in tumor tissue and control the expression of the payload encoding gene only in tumor tissues; thus, if the bacterium enters healthy tissue, no expression occurs. Engineered chemoreceptors, inducible promoters, toehold switches, and miRNA and p53 sensing are among these systems.

3.3.1. Selective proliferation of bacteria within tumor tissue

3.3.1.1. Wild tumor tropism. Many bacterial strains have displayed a wild tropism for the tumor microenvironment, allowing them to avoid the problems associated with poor selectivity and limited tumor penetrability attributed with conventional cancer chemotherapies (Piñero-Lambea et al., 2015b). Over the years, many species of (facultative and strict) anaerobic bacteria have been reported to proliferate preferentially within solid tumors.

Several reasons may explain this wild tropism of bacteria to tumors. First, the tumor microenvironment is immune-deficient and lacks macrophage and neutrophil clearance mechanisms, helping bacteria to escape any immune system attack (Liang et al., 2022). Second, solid tumors are hypoxic environments (low levels of oxygen). Although hypoxia reduces the efficacy of some current anticancer strategies, like surgery, chemotherapy, and radiotherapy, many bacteria can benefit from it (Nuyts et al., 2002). Third, chaotic and leaky vasculature in tumors and the presence of chemotactic compounds increase bacterial entry and retention within the tumoral mass (Pawelek et al., 2003; Piñero-Lambea et al., 2015b).

Despite the immune-deficient tumor microenvironment, bacteria have been found to accumulate almost exclusively in large necrotic areas separated by a barrier of host neutrophils, a type of white blood cell that aids in the fight against infections but are unable to reach the necrotic core (Westphal et al., 2008). This could explain why the therapeutic effect of engineered bacteria can be enhanced by radiotherapy, which would destroy the tumor cells in the normoxic (normal level of oxygen) area, while bacteria cells destroy the tumor cells in the hypoxic area (Jiang et al., 2010).

Moreover, a lack of host neutrophils can increase the total number of bacteria within the tumor environment, improving bacteria-mediated tumor therapy. Engineering bacteria that secrete antibodies only inside tumor tissue could result in a local neutrophil shortage (Westphal et al., 2008); however, this process must be done carefully, or it could result in a systemic infection to the patient.

Some examples of bacteria that have exhibited wild tumor tropism are *Salmonella typhimurium* (Yoon et al., 2017; Ganai et al., 2009; Loeffler et al., 2007), *Salmonella enterica* (Royo et al., 2007), *Bifidobacterium infantis* (Zhu et al., 2011), *Bifidobacterium longum* (Hu et al., 2009), *Escherichia coli* (Jiang et al., 2010; Leventhal et al., 2020), *Clostridium acetobutylicum* (Theys et al., 2001) and *Clostridium oncolyticum* (Lambin et al., 1998).

3.3.1.2. Adhesion molecules and synthetic adhesins. Another tumor-targeting strategy is the expression of adhesion molecules in the bacterial surface to bind other target molecules from cancer cells. An ideal application of this system would be to target molecules that are overexpressed in tumor cells, but not in normal cells.

E. coli Nissle 1917 was recently engineered to express Histone-like protein A (HlpA), which binds to Heparan Sulphate proteoglycan, overexpressed by colorectal cancer cells. The HlpA fusion protein bound to both human and murine colorectal cell lines with sub-micromolar affinity, according to this study by Ho et al. (2018).

β1-integrin (responsible for integrating extracellular matrix signals inside the cell) is overexpressed in many types of solid cancers because it takes part in tumor progression, neoangiogenesis, migration, invasion into the surrounding stroma, extravasation through neoangiogenic vessels and homing new tissues (Blandin et al., 2015). Invasin is a protein encoded for the *Inv* gene of *Yersinia psudotuberculosis*. It binds to β1-integrins, leading to the phagocytosis of the bacterial cells allowing the delivery of the payload into the cancer cells. In fact, is commonly used in bacterial cancer therapies as a Release module approach (Ahmed and Lage, 2018), like we mention in section 2.2.3 Bacteria invasion of tumor cells.

Beyond natural molecules, synthetic adhesins can be designed to drive an effective and specific adhesion of bacterium to a tumor-target cell. The construction of synthetic adhesins is achieved by the fusion between the outer membrane anchoring β-domain of adhesin Intimin A with a VHH antibody (or nanobody) recognizing a specific antigen in the target cell's surface (Piñero-Lambea, 2014). The advantage of these engineered synthetic adhesins is the wide variety of nanobodies available, allowing the synthetic adhesin to target many molecules on the cancer cell surface. Particularly *E. coli* expressing synthetic adhesins have been tested in mice improving their colonization efficiency and specificity for tumors in vivo (Piñero-Lambea et al., 2015a).

3.3.2. Sensing of the molecules in the tumor tissue

3.3.2.1. Quorum sensing systems. Quorum sensing (QS) is a property discovered in *Vibrio fischeri* that acts as a density-dependent switch, where gene expression depends on bacterial population density. As bacterial cell accumulation is usually higher in tumor tissue than in healthy tissue, a system that only activates gene expression when a high population density is reached would help to restrict gene expression inside the tumor tissue, avoiding off-target effects (Swofford et al.,

2015).

Boada et al. (2017) proposed a gene synthetic circuit based on the LuxR·AHL Quorum sensing mechanism to activate the expression of a protein of interest only when LuxR·AHL activates the Plux promoter. The QS system is made up of a) LuxI, an enzyme that synthesizes the autoinducer or communication molecule N-Acyl Homoserine Lactone (AHL), b) LuxR, a protein that binds to AHL to form the transcription factor (LuxR·AHL), and c) the target gene with anticancer activity. LuxR is constitutively expressed, whereas the Plux promoter drives LuxI and target gene expression (Fig. 3a).

The QS system has been widely used in the field of cancer research, with engineered *Salmonella* expressing reporter proteins only in tumor microenvironments (Swofford et al., 2015). One more example is engineered *E. coli*, in which the QS system regulates the expression of a lysis protein, causing bacteria to lyse and release their content after accumulating in tumor tissue (Chowdhury et al., 2019). One of the drawbacks of the QS system, due to population heterogeneity, is that it activates one part of the bacterial population while a small portion remains nonactivated.

3.3.2.2. Inducible promoters. Genetic circuits with constitutive gene expression make it difficult to control the number of therapeutic compounds produced by bacteria because of aspects, like population density (One of the easier ways to regulate gene expression is using well-characterized promoters and Ribosome Binding Sites (RBS)) (Ozdemir et al., 2018). In fact, a wide range of genetic components are available, as well as characterization methods aim to describe a specific genetic part in a more detailed way (Boada et al., 2019). The development of libraries with standard and well-characterized genetic elements allows us to design, construct, and implement more complex systems to target tumor cells (Lou et al., 2012). However, with a constitutive gene expression, off-target effects may increase.

Further control can be achieved by inducible promoters, which only drive the expression of a target gene under specific conditions or in the presence of a particular trigger signal. The main benefit of inducible promoters is their ability to act as a "switch", allowing for temporal and spatial regulation of gene expression. Several inducible promoters have been developed and applied to bacterial therapies (Table 3).

Nuyts et al. (2001a) found that radiotherapy at the clinically relevant dose of 2 Gy (unit of radiation dose, expressed as absorbed energy per

Table 3
Inducible promoters and their inductors.

Promoter	Inductor	Reference
recA	Radiotherapy	(Ganai et al., 2009; Nuyts et al., 2001b)
P_{ompC}	Red light	(Tabor et al., 2009)
P_{BAD}	L-arabinose	(Jeong et al., 2014; Zheng et al., 2017)
P_{rhaBAD}	L-rhamnose	(Loessner et al., 2009)
P_{tet}	Anhydrotetracycline	(Loessner et al., 2009)
P_{fnrS}	Hypoxia	(Leventhal et al., 2020)
HIP-1	Hypoxia	(Mengesha et al., 2006)
$P_{IL\text{-}8}$	Inflammation	(Castagliuolo et al., 2015)
P_{norV}	Nitric Oxide (inflammation)	(Archer et al., 2012)
P_{phsA}	Thiosulphate	(Daeffler et al., 2017)
P_{ttrB}	Tetrathionate	(Daeffler et al., 2017)
P_{sal}	Salicylate	(Royo et al., 2007)

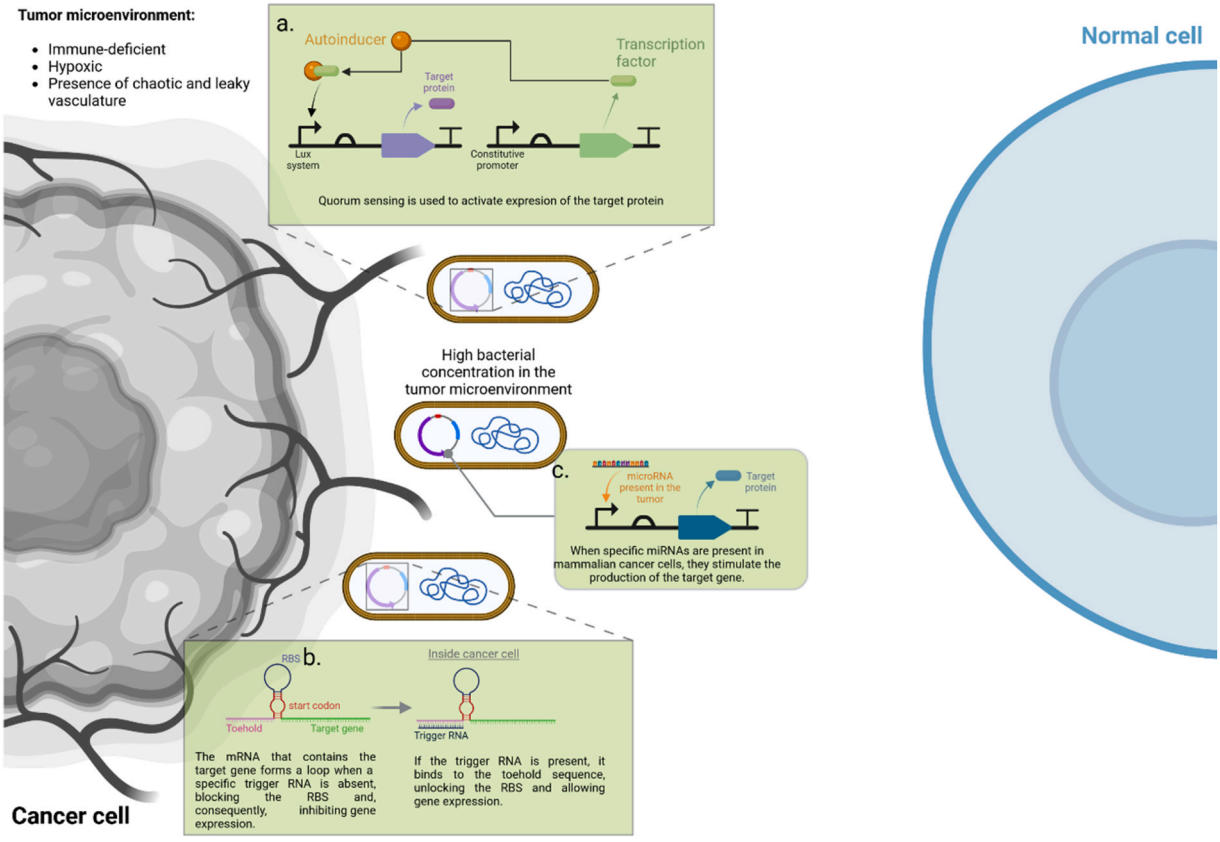

Fig. 3. *Tumor-targeting module.* **a)** Quorum sensing (QS) is useful in cancer research, as bacterial cell accumulation is typically higher in tumor tissue than in healthy tissue. Bacteria produce the transcription factor and the autoinducer, which freely diffuses though the bacterial membrane. When the population level is higher than a certain threshold, the internal concentration of the autoinducer is high enough to allow the formation of the transcription factor-autoinducer complex that drives the synthesis of the target protein. **b)** Toehold switch. Target gene expression is repressed due to an mRNA loop that blocks the Ribosome Binding Site; if a trigger RNA is present in the tumor cell, it unlocks the RBS, enabling gene expression. **c)** miRNA sensing. The target gene is expressed only when a certain combination of miRNAs is present in the tumor cell.

unit mass of tissue) increases anti-tumour necrosis factor α (mTNF-α) secretion by recombinant clostridia in 44%. To convert constitutive promoters into radio-inducible promoters, the Cheo Box sequence could be added. Radiotherapy activates the recA promoter and, at the same time, it has a cytotoxic effect because of the DNA damage it causes (Nuyts et al., 2001b) (Nuyts et al., 2002). However, bacteria may not suffer this DNA damage as a consequence of efficient DNA repair mechanisms, such as the SOS repair system (Baharoglu and Mazel, 2014).

Piraner et al. (2017) developed two orthogonal families of high-performance thermal bioswitches (TlpA- and TcI-based) with configurable thresholds between 32° and 46 °C. They characterized the effectiveness of six temperature-dependent transcriptional repressors (TlpA, TcI, TetR mutants (A89D and I193N) and LacI mutants (A241T and G265D)) and six heat shock promoters (GrpE, HtpG, Lon, RpoH, Clp and DnaK). Displaying the potential utility of these devices by using focused ultrasound, recognizing and responding to a fever, and self-destruction at ambient temperatures to prevent environmental contamination after leaving the intended host.

However, the usage of a single promoter might offer limited specificity and efficacy. More complex systems have been designed to address this issue, such as a dual-promoter integrator. The dual-promoter integrator includes three genes and their promoters, encoded on plasmids. The two fusion proteins are regulated by two duplicates of native promoters to control expression of the output gene, which is produced in proportion to the activity of the promoters chosen. To enable the expression of the output gene, the fusion proteins must combine to form a transcriptional complex (Nissim and Bar-Ziv (2010)). Finally, by combining inducible dual-promoters with an appropriate release module, such as those mentioned in section 2.2 Release Module, that allows the payload to be transferred into the mammalian cancer cell, we could create an engineered genetic circuit in which our target gene is expressed only when the activity of two cancer-specific synthetic promoters is high (Nissim et al., 2017).

3.3.2.3. Engineered chemoreceptors. Chemotaxis signaling pathways are used by bacteria to detect environmental changes. Chemoreceptors, which are highly specific signaling proteins, allow bacteria to respond to ambient chemoeffectors. Despite the large variety of chemoeffectors accessible, medical applications frequently demand innovative sensors with enhanced or new selectivity to the molecules of interest. Nowadays, tailored chemoreceptors are conceivable due to significant understanding about receptor sequences, functions, and mechanisms (Bi and Lai, 2015).

There are numerous approaches to creating novel chemoreceptors. Among these is 1) Site-directed mutagenesis rational design method, which involves both the availability of the protein structure and understanding of the link between sequence, structure, mechanism, and function. 2) Directed evolution method, that requires a library of diverse protein variations to test for favorable mutations. 3) Creating hybrid chemoreceptors by fusing the periplasmic sensing domain of a non-native protein, such as a foreign chemoreceptor or a histidine kinase, to the cytoplasmic signaling domain of the target chemoreceptor (Bi and Lai, 2015).

Some examples of chemoreceptors are modular transcription factors that repress the expression of a gene when a specific ligand is absent. These engineered transcription factors are composed of two domains: a ligand-binding domain and a DNA-binding domain. Changing the first domain can produce novel repressors, and changing the second one modifies the promoter that binds to the transcription factor (Shis et al., 2014).

Furthermore, to increase the repertoire of ligands that can be detected, is possible to develop a synthetic receptor through the fusion of a single domain antibody (ligand binding domain) and a DNA binding domain. This synthetic receptor forms a dimer after binding the ligand to the single-domain antibody (VHH). The dimerization of the receptor causes the activation of gene expression. The advantage of this method is that there are extensive libraries of nanobodies that can be used to relate a specific ligand with the trigger of a synthetic gene network. Lastly, these synthetic receptors can be applied to engineer cytosolic and transmembrane receptors, allowing the detection of intracellular and extracellular ligands to direct the bacterial action only to the tumor cells (Chang et al., 2018).

3.3.2.4. Toehold switch. Despite how many independent genetic switches have been engineered, the design of interconnected switches is more complicated because each switch is sensitive to its predetermined trigger compound (Ausländer and Fussenegger, 2014). Toehold switches are de-novo-designed prokaryotic riboregulators that control the expression of a target gene based on the presence/absence of a specific RNA (trigger RNA) in the host cell.

In this strategy, the mRNA that contains the target gene (switch RNA) forms a loop when a specific trigger RNA is absent. This loop blocks the bacterial RBS and, consequently, inhibits gene expression. In contrast, if the trigger RNA is in the cell, it binds to the Toehold sequence and prevents the loop formation, so the bacterial RBS is accessible and translation is allowed (Green et al., 2014) (Fig. 3b).

As this riboregulatory system works through predictable and designable base-pairs rules, it enables the rational design of genetic circuits with specific behavior in complex cellular environments (Green et al., 2017). Moreover, the response to the toehold switch can be modulated by introducing a supplementary inhibitory RNA hairpin that shortens the length of the unpaired toehold region in the switch RNA plus the control of the trigger RNAs expression with inducible promoters (Kim et al., 2019).

3.3.2.5. miRNA AND p53 sensing. MicroRNAs (miRNA) are post-transcriptional regulators with a distinct profile in cancer cells. As a result, this tumor-targeting strategy can be modified to only induce the production of a target gene when specific miRNAs are present in the mammalian cancer cell (Wu et al., 2019) (Fig. 3c). This strategy has been tried in HeLa cancer cells, producing apoptosis while having no effect on non-Hella cell types (Xie et al., 2011).

Synthetic circuits can also be designed to detect p53 levels. P53 is a tumor suppressor protein that regulates a wide variety of stress signals and prevents cellular transformation hence being known as "the cellular gatekeeper" or "guardian of the genome". p53 expression is kept at low intracellular levels under normal physiological conditions, but in response to cellular stresses such as DNA damage, oncogene activation, ribosomal stress, and hypoxia, p53 expression is rapidly induced and the protein is stabilized (Mircetic et al., 2017). Engineering a genetic circuit that drives the expression of a target gene in cells with high p53 levels can be used to direct bacterial action only to tumor cells.

3.4. Biocontainment module

Biocontainment is critical for ensuring the safety of our new engineered microorganisms and their use as anticancer therapies. There are two types of biocontainment approaches. The first is to prevent engineered bacteria from escaping the mammalian host, and the second one is to prevent gene transfer from the engineered strain to a wild one (Wang and You, 2020). Even if a bacterium is not viable, its DNA may represent a threat via horizontal gene transfer (Ozdemir et al., 2018). Therefore, biocontainment strategies are needed to prevent the unintended proliferation of Genetic Modified Organisms (GMOs) in natural ecosystems (Mandell et al., 2015).

3.4.1. Prevention of engineered bacteria from escaping the mammalian host

3.4.1.1. Auxotrophy. The use of auxotrophy is a widely used

biocontainment strategy. Here, a gene that encodes an essential metabolite has been removed from the bacteria's genome. This prevents the engineered bacteria to survive unless the metabolite is externally supplied, confining them to the regions in which they are intended to thrive (Brooks and Alper, 2021). However, the exchange of nutrients between organisms, also known as cross-feeding, in the environment must be considered when using this strategy (Germerodt et al., 2016).

A diaminopimelic acid (dap), a necessary component of the bacterial cell wall, auxotrophic strain would not be able to survive in a mammalian host environment because *dapA* gene is not present in eukaryotes. Furthermore, thymidine (*thyA* gene) auxotrophy is also an effective biocontainment mechanism because most extracellular spaces lack free thymidine. The use of these two auxotrophies strategies has been proven helpful in engineered bacteria to be used as cancer therapies (Leventhal et al., 2020).

3.4.1.2. Inducible suicide genes. Another less risky technique for killing and controlling bacteria populations to keep them from escaping the target host is to use inducible suicide genes, which can be activated at any time by administering an inductor or under certain conditions (Brooks and Alper, 2021). The benefits of this method over antibiotics include the potential absence of antibiotic-resistance genes in bacteria and faster mortality rates. Suicide genes can kill bacteria almost immediately after they are activated, whereas antibiotics require an extended period of time to have an effect (Loessner et al., 2009).

3.4.2. Suicide circuits

3.4.2.1. Deadman kill switch. The deadman kill switch is a biocontainment approach in which the repression of a toxin is linked to a specific environmental signal via a toggle design, as detailed more in section 2.5.1. If this signal is lost, the toxin is released, and the bacteria are quickly killed (Fig. 4a). Additional modification involves replacing the toxin with a protease whose target is an essential protein for the bacterium. When this protease is produced, the bacteria dies because the essential target protein is destroyed. Toxins and protease techniques could be coupled to provide a biocontainment strategy that is modular, adaptable, and extensible enough to be used in a wide range of industrial and biotherapeutic applications (Chan et al., 2016).

3.4.2.2. Passcode kill switch. The passcode kill switch strategy possesses single transcriptional architecture but responds to different environmental input combinations to control gene expression and cell survival. One example is to use hybrid LacI-GalR transcription factors to expand the range and complexity of environmental signals that define biocontainment conditions.

A series of passcode circuits containing these TFs were used to construct a series of Passcode circuits with a single transcriptional architecture, but that reacts to different combinations of environmental inputs. The toxin output module is controlled by a TF (hybrid C) whose expression is controlled by an AND gate (a logic circuit that needs all inputs to be TRUE at the same time in order for the output to be TRUE) formed by two TFs in the Passcode circuits (hybrid A and hybrid B) (Fig. 4b). This serial arrangement establishes the requirement that both of the inducers recognized by hybrid A and hybrid B (inputs a and b, respectively) be present in order for hybrid C to be expressed and repress toxin expression. The absence of inputs a and b, or the presence of input c, enables toxin expression, resulting in cell death (Chan et al., 2016).

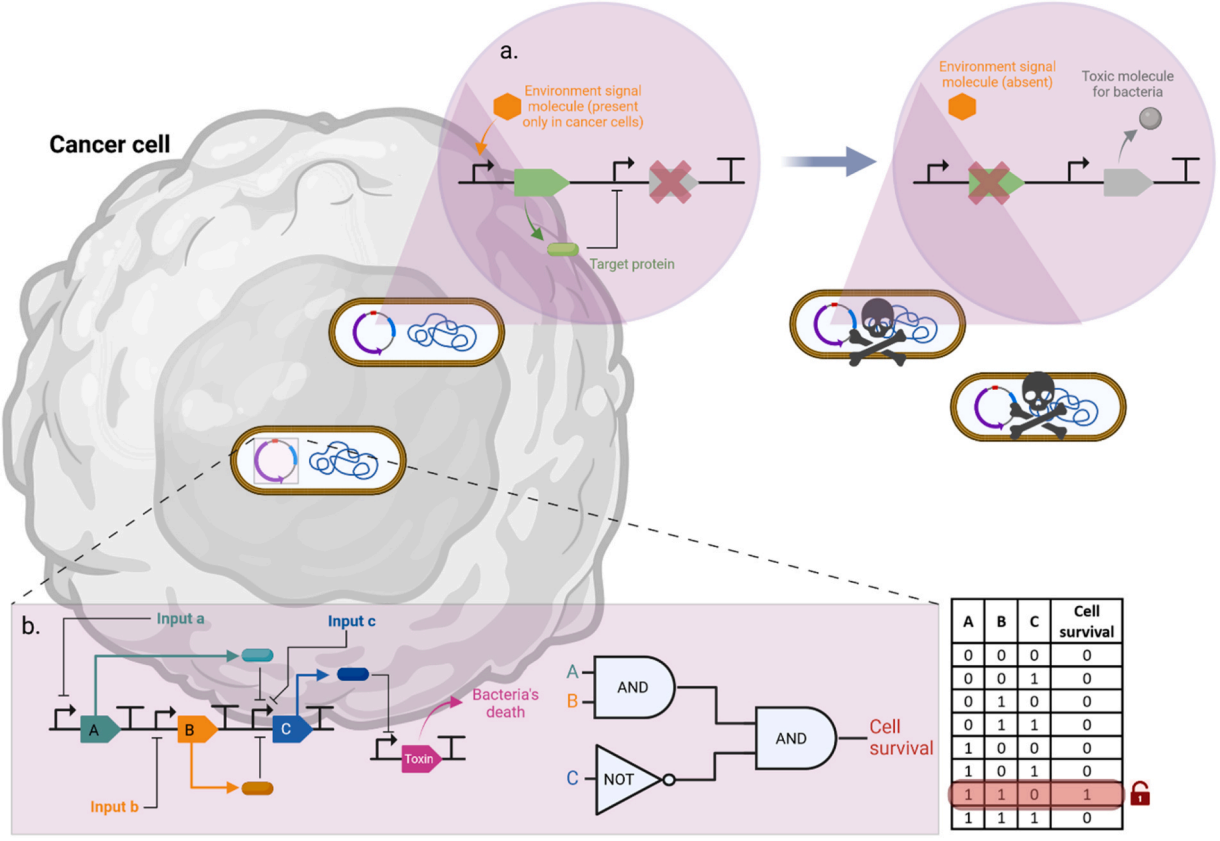

Fig. 4. *Biocontainment module.* **a)** Deadman kill switch, if the environmental signal molecule is present in the environment, the green molecule is expressed, inhibiting the expression of the protease or the toxin allowing bacterial survival. **b)** Passcode kill switch, based on three distinct 'input signals': input a, input b, and input c. Input a inhibits coding sequence A, while input b inhibits coding sequence B. Proteins A and B can both bind the C operator, thereby inhibiting protein C expression. Protein C suppresses toxin expression and can be suppressed by input c. The cell survives only in the presence of proteins A and B, and not in the absence of protein C.

3.4.3. Geneguard plasmid system

The GeneGuard plasmid system also allows for bacterial biocontainment and prevents plasmid transference via a mechanism in which the plasmid and host require each other to survive. More specifically, the host strain cannot survive without the plasmid, and the plasmid requires its intended host strain for replication. This is accomplished by combining conditional replication origins, rich-media compatible auxotrophies, and toxin-antitoxin pairs (Wright et al., 2014).

A plasmid with replication ignitors encoded only in the chromosome of a specific host, and either the R6K or ColE2 (-P9) conditional origin of replication (COR) are used. When the bacteria strain is transformed with the plasmid, the host's essential *thyA* or *dapA* gene is removed; therefore, needs the plasmid to encode one of these essential genes. A broad-host-range constitutive promoter to increase the likelihood of expression is used, and the genes for toxic protein ζ (from *Streptococcus pyogenes*) or Kid (from *E. coli*) are included. Because the antitoxin is expressed in the chosen strain, the vector is disadvantageous for other strains that do not express the antitoxins (Wright et al., 2014).

Bacteria were transformed using two plasmids, the Genomic cassette and the Vector cassette. The first one has the genes for the antitoxin and the COR replication initiator (which produces the protein necessary for the replication of the Vector cassette), flanked by the necessary 5′ and 3′ homology arms for *thyA* or *dapA* knockout creation. It also has λ Red-mediated genomic insertion method. Once this plasmid is inside the cell, the genes between the 5′ and 3′ regions are inserted into the bacterial chromosome, replacing the essential gene *thyA*. Moreover, the Vector cassette has the gene that complements the auxotrophy, the toxin gene and the COR. The genes for the antitoxin and the plasmid replication are introduced in the genome through the Genomic cassette (Wright et al., 2014). This system's main problem is that it fills too much genetic space and may limit the application of the other modules.

3.4.4. Artificial strains unable to survive in natural environments

3.4.4.1. Minimal genomes. Bacteria with minimal genomes are bacteria in which non-essential genes have been removed from their genome. This works by eliminating genes that are nonessential for cell growth under ideal conditions in the laboratory (Hutchison et al., 2016). Minimal genome encoding only the genes needed to sustain life might preclude the unexpected evolution of engineered bacteria and provide an excellent genetic chassis to implement the therapeutic gene devices (Piñero-Lambea et al., 2015b). Bacteria would thus be able to grow only in certain conditions, such as the tumor microenvironment, but if these conditions changed, they would be unable to adapt to the new environment and would die.

However, the line between essential and non-essential genes remains undetermined, because many quasi-essential genes are necessary for robust growth, but they are not critical for viability. Consequently, during genome minimization, there is a trade-off between genome size and growth rate (Hutchison et al., 2016). The most extended tools to identify essential genes are the single gene deletion libraries and the Trasposon-directed insertion site sequencing (TraDIS) (Goodall et al., 2018).

Many microorganisms with minimal genomes have been developed in the last decade. The 1079 kbp genome of *Mycoplasma mycoides* has been reduced to 531 kbp (473 genes) through improved transposon mutagenesis methods (Hutchison et al., 2016). Additionally, a recent pan-genomic analysis of 491 *E. coli* strains has identified 243 essential genes shared by all these strains. (Yang et al., 2019). Sometimes, genome-reduced bacteria have had growth problems, often originated from imbalanced metabolism. Adaptative Laboratory Evolution (ALE) is a method to the re-optimize growth performance of these bacteria and it usually rewires the metabolism with mutations in rpoD (RNA polymerase sigma factor) gene, altering promoter binding of RNA polymerase (Choe et al., 2019).

3.4.4.2. Artificial genetic languages. One of the most promising biocontainment strategies are artificial synthetic languages. At least since the Last Universal Common Ancestor, all the organisms have had their genomic information encoded with a two-base-pair genetic alphabet (A-T, G-C). Now, the thymine can be substituted in the *E. coli* genome if it is grown with the synthetic thymine analog 5-chlorouracil and the gene for thymidylate synthase has been removed from the bacterial genome (Patra et al., 2013).

In the last decade, some studies have expanded this alphabet to include several unnatural base pairs (UBPs). These unnatural base pairs are X-Y (Dien et al., 2018) and Z-P (Zhang et al., 2015), that is 1 Å wider than the base-pair G-C (Georgiadis et al., 2015). Six-base-pair *E. coli* has been engineered with a plasmid composed by the unnatural bases X and Y (and the four natural bases: A, T, G, C), whose nucleotides triphosphates are introduced inside the cell by a transporter of *Phaeodactylum tricornutum*, without generating a significant growth burden (Malyshev et al., 2014).

This expanding alphabet enables the production of a broader range of proteins and metabolites, but it could also be used as a biocontainment strategy because these synthetic bacteria require unnatural compounds for survival, which are not found in nature. Additionally, the UBPs can be included in the bacterial chromosome, plasmid, or both, depending on the objective. One of the most critical challenges with these UBPs is to achieve their availability inside the cells and their transcription and translation by the cellular machinery (Zhang et al., 2017). However, the ATGCXY combination has identified nine unnatural codons that can produce unnatural protein, through the incorporation of noncanonical amino acids (Fischer et al., 2020).

3.4.4.3. Altered chemical language. This biocontainment strategy proposed for bacteria, is based on modifying the host strain to redesign essential enzymes by substituting some amino acids with non-standard amino acids (NSAAs) (Tack et al., 2016; Praveschotinunt et al., 2018). The enzyme preserves its function, but bacteria need the non-standard amino acids to produce more enzymes and, consequently, to survive. These amino acids are not present in a natural environment, so it is necessary to supplement them to the bacteria. Therefore, bacteria have metabolic dependence on non-standard amino acids and cannot survive in a non-controlled environment if these amino acids are not provided. In addition, these strains exhibit resistance to evolutionary escape through mutagenesis and horizontal gene transfer (Mandell et al., 2015).

3.5. Memory module

So far, most of the genetic circuits exposed in this work showed a transitory activation, so they were activated when a specific stimulus was present and deactivated when it is not. However, in some cases, it may be necessary for the circuit to be activated by the stimulus and remains activated once it has disappeared. In such cases, including a Memory module would be advantageous but optional. This memory effect could be based on toggle switches, epigenetic modifications, complex logic gates using recombinases, or the SCRIBE system, that records a DNA mutation.

3.5.1. Toggle switch

One of the first built synthetic circuits was the toggle switch. Each promoter is inhibited by the repressor that is transcribed by the opposing promoter. The toggle is bistable across a wide range of parameter values, and the two states are resilient to the fluctuations inherent in gene expression (will not flip randomly between states) (Gardner et al., 2000) (Fig. 5a).

In order to detect and record antibiotic exposure while passing through the mouse gut, Kotula et al. (2014) genetically modified *E. coli* with a synthetic memory system (toggle switch) based on the bistable phage lambda cI/Cro genetic switch. This circuit was designed to start in

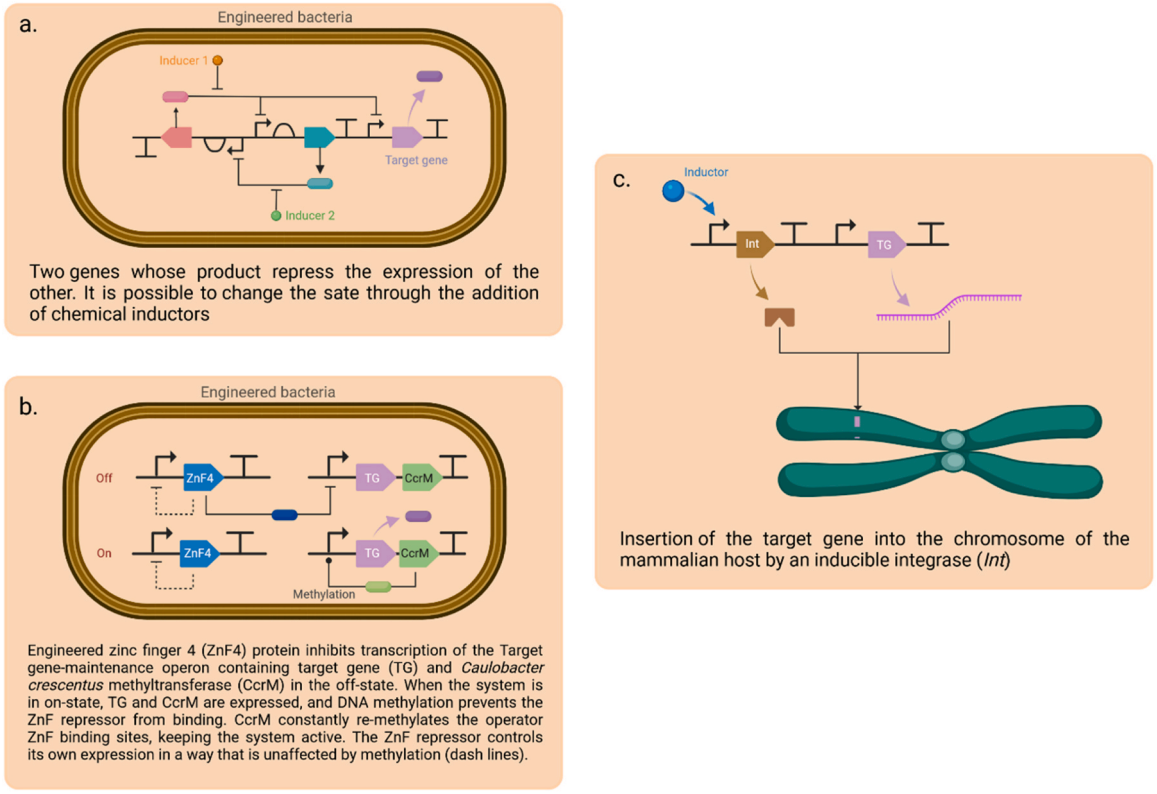

Fig. 5. *Memory module.* **a)** Toggle switch, consist in two genes whose product repress the expression of the other. Additionally, it is possible to change the state of the process with the addition of chemical inductors. **b)** Epigenetic memory, a genetic circuit where the Zinc finger (ZnF4) inhibits transcription of the reporter-maintenance operon containing the Target gene and methyltransferase (CcrM) in the off-state. When the system is turned on, the target gene and CcrM are expressed, and DNA methylation prevents the ZnF repressor from binding. CcrM constantly re-methylates the operator ZnF binding sites, keeping the system active. **c)** DNA-ENCODED MEMORY: Synthetic Genetic Memory, a permanent memory module can be created by controlling irreversible recombinases and integrases with an inducible system. This strategy could be used in engineered circuits for bacterial cancer therapy, reactivating therapeutic action when biomarkers for cancer increase.

the cI state and switch to the Cro state upon induction of a "trigger" element, which directs transcription of the acro gene via a tetracycline-responsive promoter. This demonstrates the use of the toggle switch as a Memory module approach in bacterial cancer therapies, by switching from an inactivated state when cancer cells disappear to an active state if the cancer cell reappears.

3.5.2. *Epigenetic memory*

Epigenetic signals are defined as heritable but also reversible, allowing for the design of circuits with stable switching and memory effects while remaining resettable. Maier et al. (2017) used synthetic circuit design and epigenetic mechanisms to build a synthetic epigenetic memory system in *E. coli* that can detect and remember temporary stimuli over many cell generations. This bistable memory system is made up of artificial regulatory networks that store information in the form of reversible DNA methylation patterns.

The system used an artificial adenine-N6 methylation dependent DNA binding protein with engineered zinc finger (ZnF) proteins and TALE repressors that are sensitive to cytosine-C5 methylation. Using this ZnF protein and the *Caulobacter crescentus* CcrM methyltransferase, which introduces DNA-(adenine N6)-methylation at GANTC sites, Maier et al. (2017) created a bistable basic module for epigenetic memory systems that can exist in two states, on and off.

In the initial off-state, the ZnF repressor binds to the promoter region of the *ccrM* gene and suppresses its expression. When the binding of the ZnF repressor is weakened, CcrM is expressed and can methylate its own promoter region. DNA methylation of the ZnF binding site weakens ZnF binding even more, resulting in a positive feedback loop (Fig. 5b). Additionally, mf-Lon protease degradable CcrM variant could be used to create reversible switching (Maier et al., 2017). This strategy can be applied in the field of biomedical therapy by developing sensor systems that respond to tumor biomarkers if they reappear after the tumor has disappeared.

3.5.3. *DNA-encoded memory*

The conversion of transitory information into long-term responses is a common feature of many biological processes and is critical in the development of a viable bacterial cancer therapy. Genomic DNA is an excellent medium for storing data in living cells. Because it is naturally propagated when cells divide and can be stable even after cell death Mimee et al. (2015).

3.5.3.1. Synthetic genetic memory. A permanent memory module can also be created by controlling irreversible recombinases and integrases with an inducible system. Serine integrases, which catalyze unidirectional inversion of the DNA sequence between two recognition sequences, can be used to provide our chassis with permanent genetic memory (Fig. 5c).

Mimee et al. (2015) cloned Integrase 12 (Int12) under the control of the rhamnose-inducible promoter to create an inducible memory switch in *B. thetaiotaomicron* with permanent genetic memory. The Int12 recombinase switch was activated by increasing concentrations of the inductor rhamnose, resulting in the inversion of a DNA memory array in the *B. thetaiotaomicron* chromosome between integrase binding sites (attB/attP) Li et al. (2018).

Using this strategy as part of an engineered circuit for bacterial cancer therapy could allow integrases to catalyze the inversion of a specific DNA memory array to reactivate the therapeutic action if the

presence of biomarkers increases after the cancer cells have disappeared. Keep in mind, however, that because DNA recombination is permanent, this memory strategy will only work once.

3.5.3.2. Synthetic logic circuits. Another memory strategy uses logic gates to drive the expression of two different recombinases that target genetic components for DNA inversion, resulting in conditional gene expression. AND, OR, FALSE, TRUE, NOT A, NOT B; A NIMPLY B and XOR are just a few examples of logic gates that can be created by combining different combinations of recombinase-invertible promoters, terminators, and output genes (Cubillos-Ruiz et al., 2021).

Siuti et al. (2013) discovered long-term memory maintenance for at least 90 cell generations in living *Escherichia coli* cells using synthetic genetic circuits that use recombinases to implement Boolean logic functions with stable DNA-encoded memory of events. This integrated logic and memory strategy can be used to implement complex state bacteria for therapeutic purposes.

3.5.3.3. Synthetic cellular recorders integrating biological events (SCRIBE). The SCRIBE strategy enables long-term cellular recording based on a gene regulatory signal that drives the expression of a single-stranded DNA (ssDNA) along with a recombinase. On the basis of sequence homology, ssDNAs address specific target loci and introduce precise mutations into genomic DNA, converting transient cellular signals into genomically encoded memory. As a result, precise mutations accumulate as a function of the magnitude and duration of signal exposure (Farzadfard and Lu, 2014).

3.6. Genetic circuit stability module

Synthetic circuits implemented in bacteria may suppose a strong metabolic burden for the host microorganisms. Consequently, by selective pressure, bacteria could eject the plasmids or accumulate mutations that lead to the genetic circuit's function lost (Sleight and Sauro (2013); Ozdemir et al., 2018). Several tools have been developed to make more accessible the design and optimization of genetic circuits (Nielsen et al., 2016). However, a framework to design synthetic circuits with low metabolic burden to express a heterologous protein remains open (Ceroni et al., 2015).

Several strategies have been developed to improve the stability of synthetic genetic circuits. The first is to restrict a target gene's expression until certain environmental conditions are met (two-layer circuit). The second method involves plasmid control through the use of low mutation rate bacteria to avoid mutation accumulation. Third, the most widely used strategy for genetic circuit stability is the use of plasmids encoding essential genes for bacteria survival, such as plasmids encoding antibiotic resistance genes or plasmids encoding essential genes only found on a specific strain that undergoes lysis in its absence (balanced-lethal host-vector system) or produces a toxin and antitoxin combination (Post-Segregational Killing).

3.6.1. Limitation of target gene expression

3.6.1.1. Two layer circuit. The possibilities of regulating gene expression through synthetic genetic circuits are enormous (Kent and Dixon, 2020). One of the most interesting is the two-layer circuit. The two-layer circuit aims to reduce synthetic genetic circuits' metabolic burden by decoupling the cell growth from metabolite production using integrated modules that monitor nutrients and substrate to autonomously regulate enzymatic pathways. This strategy is suitable for payload strategies based on enzymes (Jiang et al., 2020), such as the enzyme/prodrug strategy previously discussed in section 2.1.2. Cytotoxic molecules.

In this kind of gene circuit, the expression of the enzyme is dependent on two interconnecting sensing modules (the substrate and nutrient sensing modules), which allow the autonomous regulation of the substrate- and nutrient-dependent enzymatic pathways. The gene expression of the enzyme is repressed until the bacterial population is sufficiently high, the specific nutrients have been consumed, and the enzyme substrate is available to be catalyzed by the enzyme (Lo et al., 2016). The genetic stability of our engineered cancer therapy bacteria could be maintained by engineering host cells to delay enzyme expression until cells have exhausted essential nutrients and reached high cell densities.

3.6.2. Plasmid control

3.6.2.1. Plasmid partitioning protein. One of the reasons for the increased use of free-plasmid bacteria is unequal plasmid distribution during bacterial division, which results in one of the daughter cells not receiving a plasmid (Sheng et al., 2021). Actin-like protein 7 (Alp7), from the *Bacillus subtilis* plasmid pLS20, is one strategy for resolving this issue. This protein forms long filamentous structures when produced in *E. coli*. The dynamically unstable filaments interact with the plasmid and grow and shrink repeatedly. Elongation combined with dynamic instability allows the filament to search repeatedly for a second plasmid. If the free end of a filament comes into contact with another plasmid during cell division, continued elongation separates the two plasmids to the cell poles ensuring equal segregation in both daughter cells (Derman et al., 2009).

By producing signals that are easily detectable in urine, Danino et al. (2015) developed an oral diagnostic using *Escherichia coli* Nissle 1917 that can noninvasively indicate the presence of liver metasasis. They used a dual-maintenance vector to ensure a synthetic gene circuit that can be used for extended periods of time. The first maintenance device is a toxin-antitoxin system, which produces both a toxin (hok) and a short-lived antitoxin (sok), so that plasmid loss causes the cell to be killed by the long-lived toxin. The second device produced filaments using alp7 to ensure equal segregation during cell division.

3.6.2.2. Low mutation rate bacterium. In natural environments, molecular mechanisms that generate genetic variation are critical for organism evolution. Furthermore, in laboratory and clinical settings, these mechanisms are unnecessary and, in most cases, undesirable because they lead to defunctionalization of genetic circuits through mutation accumulation. Bacteria tend to lose the synthetic circuits to reduce their metabolic burden (Millan et al., 2018).

They were able to do this by getting rid of the DNA polymerase enzymes that cause induced mutagenesis and generate diversity. While maintaining normal growth, the resulting strain displayed a significant drop in overall mutation rates, especially under various stressful situations.

3.6.3. Plasmids essential for bacteria survival

3.6.3.1. Antibiotic resistance genes. Most bacteria are engineered with plasmids carrying a specific antibiotic resistance. Consequently, bacteria cannot survive without this plasmid because of the selective pressure exerted by the antibiotic in the environment. This technique is commonly used as a selection marker and a plasmid maintenance system (Fedorec et al., 2019; Chien et al., 2017). However, antibiotic resistances are unsuitable for clinical applications because of the potential horizontal gene transfer and the disruption of native microbiota (Kent et al., 2020).

3.6.3.2. Balanced-lethal host-vector system. Galán et al., (1990) addressed this issue developing a balanced-lethal strategy in which the *asd* gene of *St. mutans* was introduced in a plasmid that complements an *asd* mutation in the *Salmonella* strain's chromosome. The *asd* gene encodes aspartate-semialdehyde dehydrogenase, an enzyme required for the synthesis of diaminopimeic acid (DAP), an essential component of

Gram-negative bacteria's cell wall peptidoglycan. *Asd* mutants lyse rapidly in the absence of DAP (Jiang et al., 2010). Because DAP is not present in mammalian tissues, this balanced-lethal strategy ensures that all *asd* mutant bacteria that survive carry the recombinant plasmid (Fig. 6a).

This strategy has been used in *E. coli* engineered with *Staphylococcus aureus* α-hemolysin (SAH), a pore-forming protein, against mammary cancer in murine mammary carcinoma cells. SAH generated by bacteria increased necrotic tissue while rapidly decreasing tumor volume. As a result, SAH is an excellent agent for tumor-targeting bacterial delivery and a promising anticancer treatment (Jean et al., 2014).

Parallel results were achieved with the removal of the *glmS* gene in *E. coli* and *S. typhimurium*. This strategy ensured that bacteria without the plasmid undergoes lysis when there is a lack of D-glucosamine (GlcN) or N-acetyl-D-glucosamine (GlcNAc), nutrients necessary for peptidoglycan synthesis. (Kim et al., 2013; Jeong et al., 2014).

3.6.3.3. Post-segregational killing (Toxin-antitoxin (TA)). Plasmid-free bacteria can be killed by a process known as post-segregational killing. They are based on plasmids that encode both a long-lasting toxin and its short-lived antitoxin, hence being also known as toxin-antitoxin (TA) strategy (Harms et al., 2018). The toxin is produced and neutralized by the antitoxin while the plasmid is inside the cell. However, if a free-plasmid bacterium appears, the antitoxin is rapidly degraded. Any bacterium will die because the long-lasting toxin will remain inside the bacterial cell rather than the antitoxin (Fig. 6b). Axe/txe is an example of a proteic TA system derived from the axe-txe locus of the gram-positive *Enterococcus faecium* plasmid pRUM, whose toxin acts as a bacteriostatic agent (Gurbatri et al., 2020). Examples include hok/sok, a TA system whose toxin functions as a bactericidal (Fedorec et al., 2019), microcin-V, a low-molecular-weight toxic bacteriocin that kills through pore formation, and MazF/MazE, a TA system that responds to DNA damage stress and consists of the toxin MazF-dr and the antitoxin MazE (Dai et al., 2021).

4. Future perspectives

So far, several bacterial cancer therapies have demonstrated advances in developing more complex gene circuits, with increased efficacy and safety for the patient as well as the environment. This suggests that engineered bacterial therapy someday could become a standard cancer treatment, moving from research to clinic and the patient.

The development of new technology and new research being done around the world will provide more tools to keep sharpening bacterial cancer therapies specificity and sensitivity. In this aspect is important to mention that Synbio has also allowed the development of other replicating cancer therapeutics like engineered viruses (Tripodi et al., 2023). Oncolytic viruses (OVs) can serve as replicating vectors for cancer gene therapy (Cable et al., 2021). They are capable of reproducing inside tumor cells, leading to their destruction (oncolysis) (Wang et al., 2022). Furthermore, OVs can deliver therapeutic substances to modify the tumor microenvironment by affecting tumor blood vessels and altering the components of the extracellular matrix (Ajam-Hosseini et al., 2023, Shalhout et al., 2023). However, this remodeling process, particularly the activation of strong adaptive and innate immune responses, can sometimes impede the ideal spread of the virus within and between tumors (Azad et al., 2023).

As with many other cancer treatments, bacterial cancer therapies also have some challenges and limitations that have been identified. The simplicity and efficiency of the gene circuits engineered, the use of antibiotic resistances that are incompatible with clinical applications, the lack of strategies for genetic circuit stabilization and biocontainment, and the integration of all modules in the same bacterium are some of the challenges that scientists are currently facing. These limitations must be addressed from the initial design stage of any bacterial cancer treatment. Therefore, we outline a modular and systematic design of these therapies to facilitate recognition of these limitations in each of the proposed modules for an effective implementation of any future synthetic bacteria-based therapy.

Human cell-based therapies have already been approved by the US Food and Drug Administration (FDA) (Golchin and Farahany, 2019). However, no live microbial therapy has been approved yet, despite the fact that manufacturing and evaluation guidelines have been developed (Food And Drug Administration (2018); Brooks and Alper, 2021). Furthermore, several clinical trials with engineered bacteria against cancer are in the works (NCT00623831 (Clinicaltrials.Gov, 2008), NCT03637803 Clinicaltrials.Gov, 2018a, NCT03750071 (Clinicaltrials.Gov, 2018b), NCT03847519 (Clinicaltrials.Gov, 2019a), NCT04025307 (Clinicaltrials.Gov, 2019b), NCT04167137 (Clinicaltrials.Gov, 2019c), NCT04193904 (Clinicaltrials.Gov, 2019d)).

Even though synthetic biology applications in medicine are new, the rapid development of new systems and advances in their efficacy and security will lead to a rapid growth of this discipline in the coming years. It has the potential to be used to treat cancer as well as other malignancies such as infections and inflammatory, immune, or metabolic diseases. It is already possible to develop bacterial therapies and engineered bacteria for other fields (such as agriculture or diagnostics). It is expected that in the coming years, their use will expand and gain strength with the development and introduction of new systems to modify and control the behavior of bacteria at our leisure.

CRediT authorship contribution statement

Andrés Arboleda-García: Conceptualization, Methodology, Writing – review & editing, Visualization. **Ivan Alarcon-Ruiz:** Conceptualization, Methodology, Editing, Writing – original draft preparation. **Lissette Boada-Acosta:** Methodology, Writing – review & editing. **Yadira Boada:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision. **Alejandro Vignoni:** Conceptualization, Writing – review & editing, Supervision, Project administration,

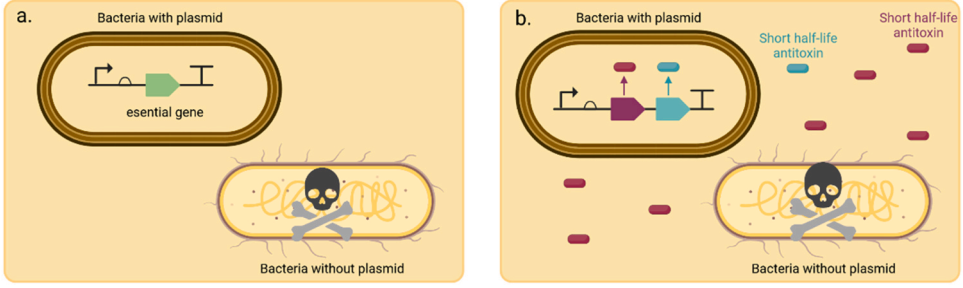

Fig. 6. *Genetic circuit stability module.* **a)** Balanced-lethal host-vector system. Bacteria without plasmid cannot proliferate because the essential gene is only encoded in the plasmid thus bacteria without the plasmid undergoes lysis in the absence of the plasmid. **b)** Post-segregational killing. The plasmid encodes a toxin with a long half-life and an antitoxin with a short half-life. When a bacterium ejects the plasmid or lacks it, the antitoxin degrades quickly, unlike the toxin, killing the bacteria.

Funding acquisition. **Eloisa Jantus-Lewintre:** Conceptualization, Supervision, Project administration, and Funding acquisition.

Declaration of Competing Interest

none.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2023.104088.

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