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Biosystems engineering of prokaryotes with tumor-killing capacities

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Abstract

Certain bacteria selectively attack tumor tissues and trigger tumor shrinkage by producing toxins and modulating the local immune system, but their clinical utility is limited because of the dangers posed by systemic infection. Genetic engineering can be used to minimize the risks associated with tumor-targeting pathogens, as well as to increase their efficiency in killing tumor cells. Advances in genetic circuit design has led to the development of bacterial strains with enhanced tumor-targeting capacities and ability to secrete therapeutics, cytotoxic proteins and prodrug-cleaving enzymes, which allows their safe and effective use in the treatment of tumors. The present review details the recent advances in the design and application of these modified bacterial strains.

Keywords: Synthetic biology, cancer therapy, live vaccines, prodrug cleavage, *Salmonella*, *Clostridium*, *Bifidobacterium*

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INTRODUCTION

The parasites and commensals of the human body are uniquely suited to survive in an environment that is hostile to any other form of life: having evolved in parallel with the mammalian immune system, these organisms are capable of avoiding the native defense mechanisms of their host and modulating its physiological responses to ensure their dispersal and reproduction. In addition, human parasites are often specific to a limited number of organs or tissues, with some targeting different tissue types at different stages of their life cycle. As such, they must identify and selectively infiltrate one or more target tissues to maximize their chances of survival – a goal that they share with various therapeutic delivery vesicles, which are inferior in their targeting performance and may benefit much from imitating their natural, parasitic counterparts. Although their activities are ordinarily detrimental, the sensitivity of human pathogens to tissue metabolic activity, in conjunction with their natural ability to evade the immune system, makes them ideal models for the development of novel therapeutic strategies against cancer and other diseases.

While delivery agents often use antibodies and similar proteins for selective targeting, an alternative approach proposes the use of pathogens as anticancer agents, utilizing the natural propensity of bacteria such as *Salmonella* and *Clostridium* to preferentially localize in tumor tissues [1]. Genetic engineering methods can be used to modulate the toxin production capacities and further augment the tumor-killing efficiency of these bacteria, creating biological anticancer agents that can be administered to eliminate tumors with minimal damage to the rest of the body [2]. While conventional delivery agents suffer from issues such as limited shelf-lives, high production costs, batch-to-batch variance, low circulation times, accumulation in non-specific tissues, immunogenicity and potential acquisition of drug resistance by cancer cells, tumor-killing bacteria can be used as a more reliable alternative for cancer targeting, or serve as a complementary therapy to increase the efficiency of well-established treatments [3-8].

The present review will detail recent efforts in the engineering of bacteria for the treatment of cancer, with emphasis on the mechanisms by which pathogens localize in tumor environments,

bacterial species that are suitable for cancer treatment and the genetic engineering systems used to increase their effectiveness for this purpose.

SELECTIVE TARGETING OF CANCER BY BACTERIAL AGENTS

Tumors are characterized by an altered set of molecular and physiological conditions resulting from the expression of oncogenes by cancer cells and the direct consequences of unregulated cellular division on the host tissue. Solid tumors are therefore associated with their own *tumor microenvironment* (TME), a heterogenous cellular niche that favors the proliferation of tumor cells and exhibits a distinct metabolic profile that can be used to identify tumor presence. In particular, tumors often grow faster than the blood vessel network that surrounds them, leading to low oxygen concentrations, nutrient deprivation and increased acidity in the TME [9-11]. Each of these effects can be used to selectively target tumors while minimally damaging the surrounding tissue; however, these properties may also interfere with the function of antitumor agents, as the low-oxygen conditions interfere with the generation of reactive oxygen species during radiotherapy and the lack of adequate vascular supply limits the entry of therapeutic chemicals into the tumor. The resistance of cancer cells to conventional treatment is further enhanced by the ability of the TME to stimulate angiogenesis and locally suppress the immune system, facilitating the generation of aggressive cancer phenotypes.

Consequently, TME targeting and tumor cell infiltration is of considerable importance for cancer therapy, and a great number of delivery vesicles based on liposomes, dendrimers, hydrogels, supramolecular assemblies, metal particles and other nanomaterials have been developed for this purpose. These carrier systems typically localize in tumor sites with the help of antibodies or other molecular targeting agents; however, alternative approaches that utilize the distinct physiological features of the TME have also been reported in the literature: hypoxia- or pH-activated drug carriers, for example, selectively deposit their cargo in tumor sites due to the low-oxygen, high-carbonic acid profiles of these regions. Bacteria with antitumor activities fundamentally belong to this second group, as their propensity to target tumor environments stems from the anoxic, immuno-

privileged nature of the TME, which makes it ideal for the growth of pathogenic or opportunistic anaerobes. As such, bacteria that have been reported to infiltrate tumors are typically anaerobic or facultatively anaerobic [9].

Several reasons exist for using these bacteria for cancer treatment over conventional drug delivery vesicles, principal among which are the self-motility and tumor-targeting capabilities that are inherent to bacterial agents and can be further augmented by the expression of therapeutic proteins [8, 12]. Furthermore, bacteria can assist the immune cells in recognizing and killing tumor cells prior to metastasis [13], and can easily be eliminated from the body by antibiotic treatment at the end of the therapy [8]. While the TME normally suppresses the immune system, prevents apoptosis and enhances angiogenesis at the tumor site [14], bacterial cells can assist in the recognition of tumor cells by stimulating the host immune response, recruiting immune cells (*e.g.* macrophages, neutrophils and lymphocytes) to the tumor site and reversing the immunosuppressive properties of the TME [1, 8, 9]. For instance, endotoxins from gram-negative bacteria can trigger the secretion of TNFα (tumor necrosis factor α) from immune cells [15] and bacterial presence can likewise induce IFN-γ (interferon gamma) production in tumors, thereby activating cytokine-mediated pathways to destroy tumor cells [16].

The antitumor activities of bacteria and immune cells have been reported to be synergistic: neutrophils have been shown to be activated against tumor cells by bacteria [17], while bacteria themselves exhibit enhanced antitumor activity in the presence of cytotoxic T cells (CD8+ T cells) [18]. In addition to modulating the local inflammatory response, bacteria can also function as vectors to deliver cytokines [19] or tumor-specific antigens into antigen-presenting cells, priming the systemic immune response against tumors [20]. Furthermore, bacterial cells can be used to inhibit tumor-supporting processes such as angiogenesis, and their cytotoxicity against non-tumor cells can be avoided by attenuation, ensuring the site-specific growth and toxic activity of the engineered strain [21]. However, pathogenic bacteria do not natively exhibit all of these properties, and must be modified to maximize their antitumor efficiency while minimizing the potential

complications associated with the administration of pathogenic species into the human body. Biosystems engineering is therefore necessary to produce modified bacteria with the capacity to express therapeutic proteins, toxins or prodrug-converting enzymes at a specific target site [8]. Methods used for this purpose are detailed below.

BIOSYSTEMS ENGINEERING OF BACTERIA WITH ANTITUMOR ACTIVITY

Modulation of toxicity and immunogenicity

Salmonella, *Clostridium* and *Bifidobacterium* are the most frequently used bacteria for the development of *in vivo* tumor therapies, while the model bacteria *Escherichia* and *Bacillus* are utilized to a lesser extent (Table 1). As *Salmonella* and *Clostridium* species typically produce toxins, reducing the virulence of these bacteria to tumor environments or otherwise preventing them from exerting their systemic toxicity is critical for successful antitumor applications. This is typically performed by the attenuation of these bacteria to either eliminate virulence-related genes entirely or to limit their production to the anoxic tumor microenvironment. Low *et al*., for example, reported that a knockout mutant of the *Salmonella msbB* gene, which is involved in lipid A synthesis and TNF-α induction, exhibits a 10000-fold decrease in virulence while retaining its tumor-inhibitory effects in mice [22]. Likewise, a strain of *Clostridium novyi* lacking α-toxin expression was able to destroy tumor cells and generate a strong antitumorigenic immune response without exhibiting systemic effects when injected to tumor cells [23].

Conversely, the toxin-producing and immune system-activating properties of bacteria may also be enhanced to improve the antitumor response. CD4- and CD8-dependent tumor inhibition was observed in a *Salmonella enterica* serovar *typimurium* strain modified to express the chemokine CCL21, while the expression of IL-18 in the same species also created an effective tumor response based on the recruitment of T-cells, NK-cells and granulocytes [24, 25]. *S. enterica* serovar *typimurium* expressing the TNF-family protein FasL and *Escherichia coli* strain K-12 expressing cytolysin A were also used to effectively inhibit the growth of tumors, the former by

itself and the latter in combination with radiation therapy [26, 27]. In addition, immunogenic agents can be provided in limited doses alongside an attenuated strain to provide greater control over the ensuing immune response: while *msbB*-deficient *Salmonella* is capable of inhibiting tumor growth in mice, its ability to colonize human tumors is limited in the absence of a lipid A-mediated TNF-α response, and the tumor-colonization efficiency of the strain can be improved by externally administering lipid A to generate a controlled immune response without systemic effects [28].

Facultative intracellular parasites such as *Listeria*, *Mycobacterium*, *Yersinia*, *Shigella* and *Salmonella* are able to infiltrate bulk tumors or other cell types [29-31], and this ability can also be conferred to non-pathogenic bacteria: Laboratory strains of *E. coli* have been modified with the *Yersinia pseudotuberculosis* invasin gene to selectively enter immune cells following oral administration, and further modifications may allow these modified bacteria to serve as antigen-presenting agents [32, 33]. The tumor-specificity of invasive bacteria can also be improved by hypoxia-responsive elements; as low oxygen concentrations are one of the primary hallmarks of solid tumors. Non-pathogenic *Clostridium* strains have been engineered to produce a single chain antibody that inhibits HIF-1α, a regulatory transcription factor of tumor cells, in response to the hypoxia [34]. Likewise, a pro-apoptotic protein (TRAIL), expressed under control of a hypoxia-inducible (nirB) promoter in *Salmonella*, has been reported to show promise in mouse melanoma models [35, 36]. Clinical trial studies for VNP20009 (genetically attenuated *Salmonella*; *msbB-, purl-*) proved to be effective at localizing at the tumor site, shows immune activation and is safe enough for use as a vaccine; however, phase II clinical studies are necessary to further demonstrate its effectiveness in humans [37].

In situ drug activation and antigen secretion

Bacteria can also be introduced alongside a prodrug to facilitate its activation directly at the tumor site, which of considerable advantage for drugs with short plasma half-lives or severe side effects. Herpes simplex thymidine kinase (HSV1-TK) and *E. coli* cytosine deaminase (CD) systems are the most popular enzymes for this approach, the former phosphorylating Ganciclovir into

Ganciclovir triphosphate and the latter cleaving 5-fluorocytosine into 5-fluorouracil [38]. *S.enterica* serovar *typimurium* strains engineered with herpes simplex thymidine kinase were shown to be effective in the treatment of melanoma-bearing mice, while the spores of *C. sporogenes* transformed with the *E. coli* CD enzyme were able to reduce tumor formation to a greater extent than 5-fluorouracil itself when administered with 5-fluorocytosine, the non-toxic prodrug of this molecule [39, 40]. The purine nucleoside phosphorylase/6-methylpurine 2'-deoxyriboside (PNP/6MePdR) system was also used for the treatment of melanomas in the mouse model [41]. In addition to prodrug converting enzymes, proteins or protein fragments that exhibit drug activity in themselves can also be expressed in tumor-colonizing bacteria: *Salmonella* and *Bifidobacterium* strains have been modified to produce endostatin, a well-established anti-angiogenic agent that facilitates tumor shrinkage by inhibiting the formation of blood vessels at the tumor periphery, thereby blocking the supply of oxygen and nutrients into cancer cells [42, 43].

CB1954 is another prodrug for the treatment of cancer and can be converted into its toxic form by the activity of nitroreductase (NTR) enzymes. The activation of this prodrug was tested on human colorectal carcinoma-injected nu/nu mice with the co-administiration of NTR-expressing *Clostridium sporogenes*, which was able to significantly reduce tumor volume following repeated treatment [44]. DNA and RNA-based agents can also be delivered through tumor-invading bacteria, which eliminates the problems associated with the short plasma half-lives inherent to these drugs. *Salmonella* engineered with a short hairpin RNA (shRNA) that silences the immusuppressive molecule STAT3 was able to improve the effectiveness of conventional vaccines against highly immunosuppressive cancer cells [45]. In addition to vaccine therapy, the efficiency of the antiangiogenic peptide endostatin can also be increased through co-delivery with shRNAs: attenuated *Salmonella enterica* serovar *typhimurium* have been engineered to produce endostatin and a shRNA specific to STAT3 and shown to synergistically inhibit metastasis in mouse tumors [44].

Tumor-colonizing bacteria typically trigger a local immune response that may assist in the recognition of cancer cells, and this effect can be further enhanced if the resident bacteria also secrete tumor-associated antigens. These so-called "live vaccines" are advantageous in that they are able to directly deliver their cargo into the target site, bypassing the immune privilege of the tumor microenvironment. Two pathways exist for the internalization of bacterial antigens: Bacteria may be phagocytosed by immune system cells, escape the phagocytotic machinery and secrete their antigen within the cytosol, or deliver their cargo on or inside target cells through the type 3 secretion system, which is ordinarily used for the delivery of toxins. Either way, the antigen of interest is delivered to an antigen presenting cell, which will then affect the production of an immune response. In addition to the delivery of bacteria-produced antigens, this method can also be used to introduce the cDNA of an antigen into the body, allowing its production (and subsequent immune recognition) within the cells of the host [46].

Although tumor cells are associated with the overexpression of certain antigens, these are ordinarily not recognized by the immune system. Live vaccines are able to reverse this effect by directly presenting immune cells with tumor antigens, bypassing the immunosuppressive properties of the tumor environment. c-Raf, a serine-threonine kinase of the Raf family, is an example of such an antigen, and a *Salmonella enterica* serovar *typhimurium* strain (*aroA* SL7207) expressing c-Raf antigen was able to reduce Raf-induced lung adenoma growth in mice [47]. In addition, since bacteria are able to express multiple proteins in tandem, the immunostimulatory effects of the secreted antigens can be enhanced through the co-expression of adjuvant proteins. Such an approach has been tested by Fensterle *et al.* by using a recombinant *S. enterica* serovar *typhimurium aroA* SL7207 strain, which co-expresses a tumor-associated antigen (PSA) and an adjuvant protein (CtxB) and allowed the efficient prevention of prostate tumors in mice [48]. More recent studies have also used recombinant strains of bacillus Calmette-Guérin (rBCG) [49], *S. enterica* serovar *typhimurium* LB5000 and attenuated *S. typhimurium* SL3261 [50], and *Listeria monocytogenes* [51] as live cancer vaccines.

Bacterial imaging and multi-modal approaches

The ability of anaerobic bacteria to target tumor tissues can also be used for tumor diagnostics through their transformation with reporter genes. Both primary and metastatic tumors have been visualized under *in vivo* conditions using light-emitting proteins such as GFP and luciferase, expressed in bacteria such as *E. coli*, *S. enterica*, *Vibrio cholerae* and *L. monocytogenes*. The safety of these bacteria have also been enhanced by attenuation and, in *S. enterica* serovar *typhimurium* A1, auxotrophic dependence on the amino acids found in the tumor environment, which ensures that the bacteria can proliferate only within the TME [52-54]. Magnetotactic bacteria can also colonize tumors and enhance MRI imaging through the magnetite crystals they contain: *Magnetospirillum magneticum*, for example, was shown to enhance the T1-weighted imaging of solid tumors derived from human embryonic kidney cells in the mouse model [55]. Similarly, *S. enterica* serovar *typhimurium* VNP20009-TK, an attenuated strain expressing HSV1-TK, was able to localize to tumors and accumulate the radioactive nucleoside analogue 2'-fluoro-1-beta-D-arabino-furanosyl-5-iodo-uracil (FIAU) by converting it into $FIAU-PO_4$, which is unable to pass through the cell membrane and allows the highly sensitive PET imaging of the tumor site [56].

The synergistic use of multiple methods can further increase the efficiency of treatment and eliminate potential issues with metastasis and relapse, while the inclusion of imaging modalities into treatment options can also allow physicians to make better-informed decisions. This form of approach, called theranostics or multi-modal therapy, is especially common with nanomaterials, which can be functionalized with several distinct targeting, imaging and therapeutic molecules. However, similar methods are also applicable to bacteria, which can be modified to express multiple genes for enhanced tumor invasion, immune response activation, anticancer activity and imaging capacity (Figure 1). A combination of the *Yersinia* invasin gene and two genetic sensory systems (*fdhF* and *araBAD*, inducible by low-oxygen conditions and arabinose), for example, has been used to produce a strain of *E. coli* that can invade cells under hypoxia or in the presence of a co-delivered chemical. The same bacterium was also modified with a quorum sensing system

(*Vibrio fischeri lux* quorum sensing circuit) that ensures that invasion will occur only after a sufficient number of cells have localized to the tumor tissue [57]. Another environmental factor-dependent expression circuit was developed on a *Bifidobacterium* strain, in which the expression of *E. coli* CD enzyme was modulated by salicylate-activated regulator elements (the gene *xylS2* and the *xylS2*-dependent promoter *Pm*). CD expression and 5-fluorocytosine-to-5-fluorouracil conversion occurs only in the presence of acetyl salicylic acid, allowing cells to exhibit antitumor activity following the administration of this compound [58].

CONCLUSION

The administration of pathogenic bacteria into the human body is obviously a dangerous prospect; however, the risks posed by aggressive cancers and the lack of options for their treatment already necessitate the use of therapies with severe side effects. In addition, bioengineering can be utilized to minimize the dangers associated with human pathogens while retaining or enhancing their capacity to eliminate tumor tissue, creating treatments with greater specificity and higher safety than current chemo- and radiotherapy options. While such methods are currently limited to proof-of-concept demonstrations and pre-clinical studies, they would be greatly advantageous for the treatment of solid tumors and especially skin and gastrointestinal tract cancers, as these regions are already densely colonized by native microfauna. Indeed, probiotic bacteria are prime candidates for the engineering of novel bacterial treatments, as probiotic bacteria are already known to protect the gastrointestinal system against tumors and prevent the growth of existing tumors. The use of anaerobes also reduces the risk of systemic infection in the bacterial treatment of solid tumors, and genetic switches can be used to ensure that injected bacteria survives and produces toxins only in the presence of cancer cells. Combined treatments that either deliver bacteria in tandem with conventional anticancer therapies or modulate the secretion of multiple therapeutics may also reduce the risk of cancer recurrence and metastasis by delivering two or more highly toxic agents directly into the tumor site.

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Conflicts of interest

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. The authors declare no conflict of interest.

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Table 1. Selective targeting of tumors by bacteria

Tumor Targeting Bacteria	Modification	Tumor Model	References
Bifidobacterium breve	Exression of *lux* gene	Murine B16 melanoma	[59]
Bifidobacterium longum	Expression of endostatin gene	HepG2 human liver cancer, Yac-1 mouse lymphoma, L929 human fibre cell	[42]
Clostridium beijerinckii	Expression of pro-drug converting enzyme CD	Murine EMT6 carcinoma	[60]
Salmonella enterica serovar *typhimurium*	Attenuation by chromosomal deletion of the *purI* and *msbB* genes	Clinical trial (metastatic melanoma and metastatic renal cell carcinoma)	[61]
S. enterica serovar *typhimurium* 14028	Expression of HSV-TK gene	Murine B16 melanoma	[39]
S. enterica serovar *typhimurium*	Expression of IL-2 gene	Murine K7M2 osteosarcoma	[19]
S. enterica serovar *typhimurium*	Expression of STAT3-specific shRNA	Murine B16 melanoma	[45]
Salmonella choleraesuis	Expression of endostatin gene	Murine MBT-2 bladder cancer, MBT-2 bladder tumor, ML-1 hepatoma	[43]
S. choleraesuis	Expression of TSP-1 gene	Murine B16 melanoma	[62]
Bifidobacterium adolescentis	Expression of endostatin gene	Murine Heps liver cancer	[63]
Clostridium acetobutylicum	Expression of mTNF-α and *E. coli* CD	Rat rhabdomyosarcoma R1	[64]
Clostridium sporogenes	Expression of *E. coli* CD	SCCVII tumours in mice	[40]
C. acetobutylicum	Expression of IL-2 gene	Rat rhabdomyosarcoma	[65]
C. sporogenes and *Clostridium novyi*-NT	Expression of pro-drug converting enzyme NTR	Human colorectal carcinoma (HCT116)	[66]
S. enterica serovar *typhimurium* A1-R	Making quiescent tumor cells susceptible to cytotoxic agents	Quiescent MKN45 stomach adenocarcinoma in mice	[67]
S. enterica serovar *typhimurium* A1-R	Making quiscent tumor cells susceptible to cytotoxic agents	Human soft-tissue sarcoma growing in nude mice	[68]
S. enterica serovar *typhimurium* A1-R	Making quiescent tumor cells susceptible to cytotoxic agents	Human HER-2 positive cervical cancer in nude mice model	[69]
Salmonella enterica serovar *typhimurium*	Co-expression of endostatin and STAT3-specific shRNA	RM-1 mouse prostate cancer cell line	[44]
Escherichia coli K-12	Expression of *ClyA*	Murine CT26 colon cancer	[70]
Mycobacterium bovis bacillus Calmette-Guérin (BCG)	Expression of Th1 cytokines	Human bladder cancer cell lines (T24, J82, 5637, TCCSUP and UMUC-3)	[71]

S. *enterica* serovar *typhimurium*	Expression of immunostimulatory cytokine, CCL21	Multi-drug-resistant CT26 colon carcinoma, D2F2 breast carcinoma and B16 melanoma in mice	[24]
S. *enterica* serovar *typhimurium*	Expression of proapoptotic cytokine, FasL	Murine D2F2 breast carcinoma and CT-26 colon carcinoma	[26]
S. *enterica* serovar *typhimurium*	Expression of IL-18	Multi-drug resistant murine CT26 colon carcinoma and D2F2 melanoma	[25]
S. enterica serovar *typhimurium aroA* SL7207	Expression of a fusion protein of PSA and CtxB	Murine prostate cancer	[48]
S. enterica serovar *typhimurium aroA* SL7207	Expression of c-Raf antigen	Murine lung adenoma	[47]
E. coli	Expression of *inv* under the control of the quorum sensing *lux* operon	HeLa, HepG2, and U2OS cell lines	[57]
S. enterica serovar *typhimurium* VNP20009	Attenuation by chromosomal deletion of the *purI, xyl* and *msbB* genes with an increased resistance to EGTA	Bl6-F10 murine melanoma, LOX human melanoma and DLD-1 human colon carcinoma	[72]

lux, luciferase; CD, cytosine deaminase; HSV-TK, herpes simplex virus thymidine kinase; IL, interleukin; STAT3, signal transducer and activator of transcription 3; shRNA, short hairpin RNA; TSP-1, thrombospondin-1; TNF-α, tumor necrosis factor-α; NTR; nitroreductase; *ClyA*, Cytolysin A; FasL, FAS ligand; CCL21, collagen crosslinking 21; PSA, prostate-specific antigen; CtxB, cholera toxin subunit B; *inv*, invasin
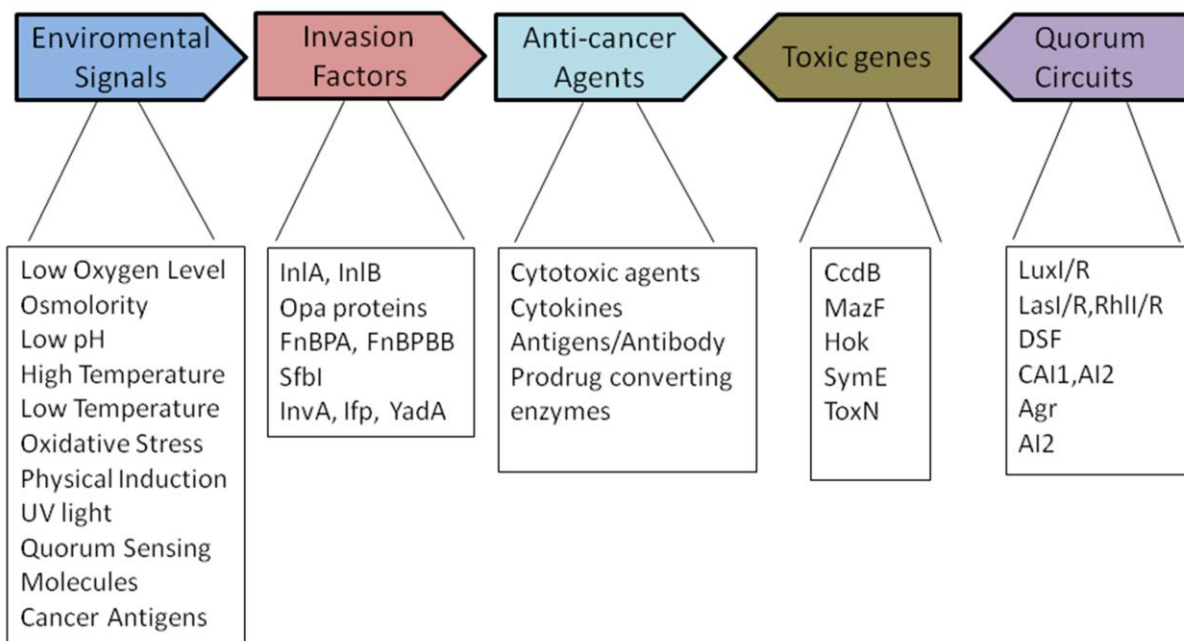
Fig. 1 Genetic elements used in the engineering of tumor-killing bacteria [73-78]. (Inl: Internalin; Opa: Opacity-associated proteins; Fn: Fibronectin; FnBP: Fibronectin-binding protein; SfbI: *S. pyogenes* fibronectin-binding protein I; InvA: Invasin A; YadA: *Yersinia* adhesin A)