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Engineering the perfect (bacterial) cancer therapy

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Abstract

Bacterial therapies possess many unique mechanisms for treating cancer that are unachievable with standard methods. Bacteria can specifically target tumors, actively penetrate tissue, are easily detected and can controllably induce cytotoxicity. Over that last decade, *Salmonella*, *Clostridium* and other genera have been shown to control tumor growth and promote survival in animal models. In this Innovation article I propose that synthetic biology techniques can be used to solve many of the key challenges associated with bacterial therapies such as toxicity, stability and efficiency; and can be used to tune their beneficial features, allowing the engineering of 'perfect' cancer therapies.

Introduction

Bacteria have unique capabilities that make them well-suited as 'perfect' anticancer agents. Because their genetics can be easily manipulated, bacteria can be engineered to overcome the limitations that hamper current cancer therapies. Many current treatments, including chemotherapy and radiation, are toxic to normal tissue and cannot completely destroy all cancer cells[1]. Three major causes of these problems are incomplete tumor targeting, inadequate tissue penetration and limited toxicity to all cancer cells[1–3]. These drawbacks prevent effectual treatment and are associated with increased morbidity and mortality.

Using a top-down engineering approach, the ideal cancer therapy can be envisioned: it would be tiny programmable *robot factories* (Figure 1A) that specifically target tumors, are selectively cytotoxic to cancer cells, are self-propelled, are responsive to external signals, can sense the local environmental and are externally detectable. Specific targeting would permit the use of more toxic molecules without systemic effects. Self-propulsion would enable penetration into tumor regions that are inaccessible to passive therapies. Responsiveness to external signals would enable precise control of the location and timing of cytotoxicity. Sensing the local environment would permit "smart," responsive therapies that can make decisions about where and when drugs are administered. Finally, the ability to be externally detected would provide critical information about the state of the tumor, the success of localization and the efficacy of treatment.

Bacteria can be viewed as these perfect robot therapies because they have biological mechanisms to perform all of the ideal functions mentioned above (Figure 1B. Over the last century, many genera of bacteria have been shown to preferentially accumulate in tumors, including *Salmonella*[4], *Escherichia*[5] , *Clostridium*[6–7] and *Bifidobacterium*[8]. *Caulobacter*[9], *Listeria*[10–11], *Proteus*[12] and *Streptococcus*[13] have also been investigated as anticancer agents. For propulsion and sensing, bacteria have flagella that enable tissue penetration[14]

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and chemotactic receptors that direct chemotaxis towards molecular signals in the tumor microenvironment[15–16]. For example, the TAR receptor detects aspartate secreted by viable cancer cells and the TRG receptor promotes migration towards ribose in necrotic tissue[16]. Selective cytotoxicity can be engineered by transfection with genes for therapeutic molecules, including toxins[17–19], cytokines[20–21], tumor antigens[22] and apoptosis inducing factors[23–27]. External control can be achieved using gene promoter strategies that respond to small molecules[17, 28–29] or radiation[23, 26–27, 30]. Bacteria can also be detected using light[5, 31–32], magnetic resonance imaging (MRI)[33] or positron emission tomography (PET)[34–36]. Finally and most importantly, the ease of genetically manipulating bacteria is the feature that will have the greatest effect on therapy development because it enables precise tuning and limitless functional combinations.

Once fully implemented and tested, the unique capabilities of bacterial therapies will change the way cancer is treated. Manufacture of drugs within tumors would beneficially shift temporal drug concentration profiles compared to intravenous administration (Figure 2). Because bacteria can migrate and accumulate far from vasculature, more of the therapeutic would be present in distal regions for longer periods of time compared to small molecules that only diffuse passively. Intratumoral production would be more toxic to cancer tissue and less toxic to normal tissue. This inversion of drug localization would eliminate tumors from the inside out, and would have the simultaneous effects of increasing efficacy and decreasing damage to normal tissue.

To date many different bacterial strategies have been implemented in animal models (Tables 1 and 2) and some human trials have been carried out (Table 3). Using these strategies, many researchers have observed experimental success, with reduced tumor volume, increased survival and treatment of metastatic disease (Table 1). Success has also been shown treating multiple tumor sites (Table 1); the most notable is pancreatic cancer[13, 37], for which new targeted treatments could dramatically improve the poor current prognosis of less than 25% five-year survival. Since the mid 1990's, the number of published bacterial therapy papers has increased with a doubling time of 2.5 years (Figure 1C). This rapid rise has been driven almost entirely by increasing use of *Salmonella* as a delivery vector (Figure 1C). This Innovation article will describe many of the advances that have fuelled this enthusiasm including, specific bacterial targeting of tumors; intratumoral penetration; native bacterial cytotoxicity; expression of anticancer agents; gene triggering strategies; and detection of bacterial therapies.

Bacterial targeting of tumors

One of the major advantages of bacterial therapies for cancer is the ability to specifically target tumors. The mechanisms of bacterial accumulation in tumors differ depending on oxygen tolerance. Obligate anaerobes (e.g. *Clostridium, and Bifidobacterium*) cannot survive in oxygen and injected bacterial spores can only germinate in anoxic regions of tumors[38–39]. Completely deoxygenated tissue is unique to tumors and is not present in most other organs of the body. Obligate anaerobes are therefore highly effective at accumulating in the large hypoxic regions of tumors[14]. This absolute specificity was demonstrated early by Malmgren et al. who injected *Clostridium* into tumor-bearing mice and showed that only the mice with tumors died from the infection[7].

Facultative anaerobes (e.g. *Salmonella* and *Escherichia*) use a more complex set of mechanisms to target tumors. Five interacting mechanisms are thought to control the accumulation of facultative anaerobes in tumors: entrapment of bacteria in the chaotic vasculature of tumors[40], flooding into tumors following inflammation[41], chemotaxis toward compounds produced by tumors[15–16], preferential growth in tumor-specific

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microenvironments[15, 31], and protection from clearance by the immune system[42]. These mechanisms enable *Salmonella* to accumulate in tumors at ratios greater than 1000:1 compared to organs rich in reticuloendothelial cells (such as the liver and spleen) and even greater in other organs[40, 43–45].

When injected systemically, *Salmonella* attach to the walls of tumor vasculature with a low but measurable frequency (~0.035% of bacteria in the blood)[40]. In addition, the number of bacteria that adhere is dependent on blood velocity, suggesting that hemodynamics play an important role in the initial interaction of bacteria with tumors[40]. Similarly, the accumulation of *Salmonella* is associated with an influx of blood into tumors, caused by an immunologically induced rise in the blood concentration of tumor necrosis factor-α (TNFα)[41]. This mechanism would be reduced for attenuated *msbB*⁻ strains that elicit much lower (~10%) TNFα levels[46]. The production of TNFα immediately after injection therefore has contradictory effects; it promotes accumulation in tumors but is also the primary cause of bacterial toxicity due to septic shock[46]. This dependence on an immune response to promote targeting could also reduce the utility of repeated dosing with bacteria, which is a limitation that does not affect bacteria delivered as spores[47].

In *in vitro* tumor models, *Salmonella* identify and penetrate tumors by detecting and chemotaxing towards small molecule gradients of serine, aspartate and ribose[15–16]. In addition, the growth rate of *Salmonella* is greater in *in vitro* tumors when dying cells are present[15], a phenomenon which is also observed in animal tumor models[40–41, 46]. The importance of this mechanism for promoting accumulation is supported by the increased tumor specificity of auxotrophic *Salmonella* that require leucine and arganine, which are nutrients derived from dying tumor tissue[31, 48].

Because tumors are immune-privileged environments[49], bacteria can replicate unimpeded by the macrophage and neutrophil clearance mechanisms that normally serve to eliminate them[50]. In this way, the immune system plays a complicated role in bacteriolytic therapy; it provides a mechanism to guide bacterial accumulation, but also impedes dispersion and efficacy. The interaction between bacteria and the immune system also works in reverse; many bacterial therapies sensitize the immune system to induce tumor clearance[51–52].

Intratumoral penetration

Intratumoral targeting is an essential characteristic of an optimized cancer therapy (Figure 1). Compared to normal tissue, tumors have chaotic vasculature and large intercapillary distances, impeding delivery of therapeutic molecules[3, 53]. This reduces therapeutic efficacy by creating cellular regions that have low drug concentrations and reduced nutrient supply[1, 3]. Low levels of oxygen and glucose create quiescent cells that are unresponsive to chemotherapeutics designed to target rapidly growing cells. Proper intratumoral targeting enables drug delivery directly to these distal, unresponsive cells that are far from tumor vasculature (Figure 2). In this way, the metabolic heterogeneity of tumors is both a blessing and a curse; molecular gradients reduce therapeutic efficacy but also create unique environments that can be targeted.

Motility is the key feature of bacterial therapies that enables intratumoral targeting. Bacteria can actively swim away from vasculature and penetrate deep into tumor tissue (Figure 2). Because bacteria are complex living organisms that can acquire energy from their environment, their transport is not entropically limited. This contrasts to the concentration of passive molecules, which drops with distance from vasculature. Because bacteria are self-propelled, their density can be higher far from the vascular source. It has been shown that bacteria that can disperse throughout tumor tissue have a greater ability to regress tumors[14]. *Salmonella* have also been shown to chemotax towards molecules produced by dying tumor

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tissue[15–16]. *Salmonella* contain chemoreceptors that sense small molecules in the local environment. For example, using knockouts, it has been shown that the aspartate receptor intiates chemotaxis towards viable tumor tissue; the serine receptor induces tissue penetration; and the ribose receptor directs migration toward necrotic tissue[16].

In addition to intrinsic motility, the host immune system plays a critical role in preventing bacterial dissemination throughout tumors. Neutrophils have been shown to prevent bacteria from spreading from necrotic into viable tumor tissue[50]. This containment is one possible reason that attenuated *Salmonella* had limited success reducing tumor growth in human trials[54–56]. Depleting host neutrophils increases tumor bacterial densities and enables spread throughout viable tumor tissue[50].

Native bacterial cytotoxicity

Many successful experiments have shown that the natural toxicity of bacteria is sufficient to regress tumors (Table 1). Native bacterial cytotoxicity is caused by sensitization of the immune system and competition for nutrients[42]. Although some organisms naturally produce toxins, these are typically removed to prevent pathogenicity[14]. Much early work on bacterial therapies relied on natural toxicity because direct genetic modification was not possible. The ability of bacteria to regress tumors has been recognized since the early 1800's[57]. In the time before strict antiseptic technique, tumor regression was occasionally observed following severe bacterial infection[57]. This observation led to the development of Coley's toxin, a bacterial extract that stimulates a general immune response[57–59]. Because of this early success, this approach persists in many contemporary strategies[20, 60] that are similarly designed to stimulate immune responses (Table 2). The idea that living bacteria could be anticancer therapeutic agents was first advanced in the middle of the 20th century[6–7]. The increased availability of antibiotics and the discovery that tumors contain anoxic regions[61] spurred multiple investigations[6, 62] which showed that *Clostridium*, an obligate anaerobe, could regress tumors in mice (Table 1). There was sufficient enthusiasm to initiate a small clinical trial, and oncolysis was observed in three out of five patients following injection with *C. butyricum*[63] (Table 3).

More recently, *Salmonella* has been tested for its anti-cancer properties[4, 46], and similar to *Clostridium*, *Salmonella* is naturally cytotoxic and has been shown to regress tumors when administered alone (Table 1A). Immunosensitization is one of the key mechanisms of *Salmonella* cytotoxicity; accumulation of *S. choleraesuis* in tumors induces neutrophil infiltration and antitumor immune responses[64]. When investigated in human trials, *Salmonella* with a modified lipid-A (strain VNP200009) was found to be non-toxic and tumor colonization was observed[55]. In dogs administered VNP200009, colonization was also observed and complete cure was seen in 4 of the 35 animals[65]. There is also potential that *Salmonella* could be delivered orally to reduce toxicity. Following oral administration in mice, *Salmonella* preferentially accumulated in tumors and maintained its anticancer effects[66] with very low toxicity[67]. Oral delivery may be different in humans, where bacterial escape from the gut into the circulation occurs less often than in mice[68].

Expression of anticancer agents

Another advantage of bacterial anticancer agents is that they can be genetically modified to increase their effectiveness. Many strategies have been employed (Tables 1, 2) and two major mechanisms have been studied: the direct expression of proteins that have physiological activities against tumors and transfer of eukaryotic expression vectors into infected cancer cells. For both of these mechanisms, three categories of anticancer agents have been investigated: cytotoxic agents that directly kill cancer cells, cytokines that stimulate immune cells to kill cancer cells, and tumor antigens that sensitize the immune

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system against cancer cells. Prodrug strategies have been reviewed previously[69–70] and will not be discussed here.

Cytotoxic agents

Bacterial toxins are the most obvious cytotoxic agents because these genes are native to bacterial physiology. Cytolysin A (ClyA or HlyE) is a bacterial toxin that acts by forming pores in mammalian cell membranes and inducing apoptosis[18–19]. ClyA is a native bacterial protein that is ready transported to the bacterial surface and secreted without modification[17–18]. Multiple groups have shown that treating mice with *E. coli* or *S. typhimurium* expressing ClyA reduces tumor growth[17–19].

Three of the cytotoxic agents are members of TNFα family: FAS ligand (FASL), TNF-related apoptosis-inducing ligand (TRAIL) and TNFα[23–27]. These proteins selectively induce apoptosis via death receptor pathways, which activate caspase-8 and caspase-3, an important apoptotic mediator[23]. All three are selectively cytotoxic to cancer cells compared to normal cells[23–24]. FASL specifically induces apoptosis in cells that possess the FAS receptor[24]. TNFα and TRAIL have been shown to be cytotoxic towards colon, breast, lung, prostate, renal, ovarian, bladder, glioma and pancreatic tumors[23, 71]. When systemically administered as protein drugs, all three members of this family have two deficiencies that are overcome by bacterial delivery: hepatotoxicity and a short circulatory half-life[23, 25–27]. Producing these proteins *in situ* would maintain a higher continual concentration in tumors compared to delivery to the circulatory system (Figure 2), and would reduce the systemic toxicity associated with their administration as small molecules. FASL is also immunologically active: it attracts tumor rejecting granulocytes, induces interleukin (IL23) production by dendritic cells and stimulates proliferation of T cells — three mechanisms that may culminate in specific killing of cancer cells[24].

Cytokines

Bacteria can also be engineered to deliver specific cytokines that have anti-tumor effects (Table 2). Cytokines induce immune cells to clear tumors by stimulating multiple mechanisms such as immune cell activation, proliferation and migration. When administered as a small molecule, IL2 activates the cytolytic function of natural killer (NK) and lymphokine-activated killer cells[72] and promotes lymphocyte proliferation[73]. Similar to IL2, IL18 (also known as IFNγ-inducing factor) induces T and NK cell proliferation and enhances their production of cytokines[74]. IL18 also suppresses angiogenesis by inhibiting fibroblast growth[74]. CCL21 controls migration of immune cells and may prevent tumor-induced immunosuppression[21]. LIGHT (also known as TNFSF14 and HVEM-L) is a TNF-family cytokine homologous to lymphotoxin that induces dendritic cell (DC) growth[20].

IL2 is the most extensively studied bacterially delivered cytokine[72–73, 75–80]. Reports describing IL2 delivery by *Salmonella* were the first to suggest that this genus could be effectively used as an anticancer agent[73, 80]. Oral administration of *Salmonella* expressing IL2 has been shown to function prophylactically and prevent tumor formation[79]. Despite multiple anticancer effects, IL2 and IL18 have had limited success as chemotherapeutics because of severe systemic toxicity[72–74]. Similar to the TNFα-family agents, local production of these cytokines within tumors would limit toxicity while stimulating tumor-infiltration by lymphocytes[72]. Treatment with *Salmonella* expressing LIGHT or CCL21 has been shown to induce leukocyte and neutrophil infiltration and inhibit tumor growth[20–21].

Tumor-specific antigens and antibodies

The expression of tumor-specific antigens is another bacterial strategy that utilizes the host immune system (Table 2). It functions by sensitizing immune cells and preventing the

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formation of tumors that present those antigens[22, 60, 81–82]. For example, RAF1 (also known as c-RAF) is a transcription factor upregulated in many tumors[22]; prostate-specific antigen (PSA) is upregulated in many prostate tumors[60]; and NY-ESO-1 (also known as CTG1B) is a germ cell protein often expressed by tumor cells[82]. To induce a more efficient immune response, PSA has been fused to cholera toxin subunit B (CtxB), a mucosal adjuvant[60]. Alternately, a non-specific immune response can be induced by the expression of a potent antigen, e.g. canine parvovirus (CPV)[81]. To facilitate interaction with immune cells, different protein secretion systems have been employed: for example, RAF1 and CtxB-PSA were fused to the α-hemolysin secretion signal[22, 60] and CPV was bound to OmpA, a membrane protein that forms outer membrane vesicles[81]. Because these strategies rely on a systemic immune response, it is not necessary for these antigens to be expressed in tumors[82]. Also, because the response is retained by the immune system, these bacterial therapies could be used for prevention or as treatment vaccines.

Alternatively, bacteria can be engineered to express single chain antibodies to inhibit proteins necessary for tumor cell function. For example, *C. novyi* has been modified to express single chain antibodies that bind the hypoxia inducible factor 1α (HIF1α) antigen[83]. HIF1α is an important target because it is associated with resistance to radiotherapy and chemotherapy and poor clinical outcome[83]. Preliminary studies have shown that bacterially produced antibodies bind the HIF1α epitope[83].

Gene transfer

The ability of therapeutic bacteria to transfer genetic material to mammalian cells was first reported in 1995, when it was shown that *Shigellae* could transfer plasmid DNA into baby hamster kidney cells[84]. Soon after, it was shown that *Salmonella* could also be used for trans-kingdom DNA transfer[85–86]. These reports generated significant enthusiasm for using bacteria (specifically *Salmonella*) to transfer the genes for cytotoxic and immunological agents into cancer cells (Table 2). Compared to direct expression, this approach has benefits as well as drawbacks. Gene transfer, which utilizes more permanent mammalian systems, may produce stronger, more stable expression. However, expression of the transferred genes may be harder to control[87]; expression could be limited by poor transfer efficiency; transferred genes may be heterogeneously distributed in tissues; and the genes could transfer to tissues other than those they are targeted towards.

Many of the same strategies have been attempted with gene transfer as with direct expression: cytotoxic agents, cytokines and tumor antigens (Table 2). Two early reports describe the transfer of the anti-angiogenic genes, endostatin[44] and thrombospondin 1[51], which kill tumors by preventing new blood vessel formation and cutting off the nutrient supply[44]. Although direct administration of endostatin to cancer patients showed only minimal antitumor activity, transfer of endostatin from *Salmonella* reduced microvessel density, decreased VEGF expression, and slowed tumor growth in mice[44]. Using a similar strategy as direct expression, reduction of tumor growth was shown by transferring the genes encoding TRAIL and SMAC (also known as DIABLO) into tumor cells from *Salmonella*[88].

The anti-tumor effects of three cytokines and growth factors have been explored by bacterial gene transfer: IL12[89–91], granulocyte/macrophage colony-stimulating factor (GM-CSF)[90], and Fms-like tyrosine kinase ligand (FLT3L)[92]. Similar to bacterially expressed cytokines, these molecules stimulate NK, T and DC cells[89–91]. In addition, IL12 induces IFN-γ production and GM-CSF activates neutrophils and macrophages to lyse tumor cells[90]. When expressed together, IL12 and GM-CSF significantly reduce tumor growth in mice, while limiting the systemic toxicity associated with systemic cytokine injection[90].

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The transfer of genes for two tumor antigens has been shown to be effective at reducing tumor growth in mouse models: α-fetoprotein (AFP) and vascular endothelial growth factor receptor 2 (VEGFR2, also known as FLK1)[93–95]. Antibodies against AFP, an embryonic protein overexpressed in hepatocellular carcinoma and not present in normal adult tissue, prevents formation of liver and colon tumors[95]. VEGFR-2 is an endothelial cell receptor that controls angiogenesis and antibodies against VEGFR2 have been shown to prevent angiogenesis and tumor growth in glioblastoma[94] and lung cancer[93] models.

Gene silencing

A complementary strategy to bacterial induction of gene expression is gene silencing. Silencing is achieved by transferring plasmids encoding small hairpin RNAs (shRNA) from *Salmonella* into cancer cells[96–97]. Gene-specific shRNAs are processed by the enzyme Dicer into small interfering double-stranded RNAs (siRNAs) that induce the degradation of target mRNAs[96]. To date, two genes have been silenced using this technique, signal transducer and activator of transcription 3 (*Stat3*)[96] and *Bcl2*[97]. Both factors inhibit apoptosis and STAT3 promotes cancer cell growth; overexpression of these factors has been associated with many tumor types, including prostate cancer and malignant melanoma[96–97]. Silencing of *Stat3* has been shown to prevent prostate tumor and metastasis formation in mice[97].

Gene triggering strategies

Control of gene expression is critical for managing the timing and location of drug production. Incorporation of specific promoter sequences upstream of genes that encode anticancer proteins enables control of transcription by external signals. Precise triggering of expression can be used to induce greater intratumoral effects while minimizing systemic toxicity[23]. Some gene products require tighter control than others; for example, cytotoxic molecules and cytokines that are known to be toxic cannot be constitutively expressed but tumor-specific antigens do not need to be expressed in tumors and so tight control of the genes expressing these antigens is not necessary[82].

There are two categories of gene triggering strategies: extracellular triggers and environmental sensors (Table 2). Three external triggers have been investigated: L-arabinose, salicylate and γ-irradiation (Figure 3). The pBAD system utilizes the regulatory protein AraC to respond to extracellular L-arabinose[17, 28–29] and is very tightly regulated[98]. The salicylate system is also tightly regulated and its cascade amplifies gene expression, producing induction ratios of 20–150 fold *in vitro*[99]. Both L-arabinose and salicylate are suitable and non-toxic biological triggers. In mouse models, it has been shown that intravenous administration of l-arabinose can activate gene expression in colonized tumors[29].

The *RecA* mechanism utilizes γ-irradiation as a trigger of gene expression (Figure 3) and is based on the SOS DNA repair system[23, 26–27, 30]. Irradiation has a major advantage over molecular triggers because it can directly penetrate tumor tissue and is not restricted by diffusion limitations[2]. γ-irradiation causes DNA damage and activates the protein RecA[23], which promotes autoproteolysis of the repressor LexA. The lysis of LexA, a repressor of the *recA* promoter, induces gene expression. This system is amplified by self induction of RecA when LexA is cleaved. To reduce basal expression and increase radiation responsiveness an extra Cheo box has been incorporated into the *recA* promoter, which has been shown to increase expression ten-fold[100].

To date, all environmental triggering strategies have been designed to sense hypoxia using the fumarate and nitrate reduction (FNR) regulator (Figure 3)[19, 101]. FNR is an oxygen-responsive transcription factor naturally present in *Salmonella*[19, 101–102]. In the absence of

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oxygen, iron-sulfide clusters induce the formation of FNR homodimers that bind to specific DNA sequences and promote transcription[19, 101]. In the presence of oxygen, the clusters and FNR homodimers disassemble, reducing transcription. Two artificial promoters have been developed that contain FNR-binding sites: FF+20* [19] and hypoxia inducible promoter-1 (HIP1[101]; Table 2). These two promoters were created by random[19] and directed[101] mutagenesis to amplify expression in hypoxia and reduce expression in normoxia[19]. To identify bacterial promoters that could be used in environmental triggering strategies, Arrach et al. develped a reporter system that they tested in tumor-bearing mice[103]. The two most active promoters, pflE and ansB, both contained FNR-binding sites and are known to be oxygen dependent[103]. These experiments did, however, identify other promoters that were not oxygen dependent and may rely on alternative environmental triggers.

Detection

Being able to locate colonized bacteria is clinically important because it enables the detection of obscured tumors and metastases. Four different strategies have been implemented to identify bacteria in tumors: bioluminescence, fluorescence, magnetic resonance and positron emission (Table 2). Bioluminescent bacteria are generated by transformation with plasmids containing the luxCDABE operon from *Photobacterium leiognathi*[5, 17, 104–106], and fluorescent bacteria are generated by transformation with plasmids containing the gene for green fluorescent protein (GFP)[5, 31–32]. Both of these mechanisms have proven to be very efficient at identifying tumors in mice using whole mouse imaging[5, 17, 31–32, 104–106]. These light-based mechanisms may have limited clinical application, however, because of the poor penetration of visible light through tissue.

Alternately, magnetotactic bacteria could be injected and detected by MRI. For example, *Magnetospirillum magneticum* produces magnetite (Fe_3O_4) particles and has been shown to accumulate in tumors[33]. For improved tumor targeting, the genes for magnetite production could be transferred into other bacterial strains[33]. Two different methods that have been used to detect bacteria with PET are expression of an exogenous viral tyrosine kinase[34–35] and reliance on endogenous protein kinases[36]. When herpes simplex thymidine kinase (HSV1-TK) is expressed in *Salmonella*, it selectively phosphorylates and traps the detectable marker 2’-fluoro-1-β-D-arabino-furanosyl-5-iodouracil (FIAU)[35]. Alternately, the endogenous protein kinases of *E. coli* Nissle 1917 have been shown to phosphorylate and trap [^{18}F]-2’-Fluoro-2’deoxy-1-β-D-arabino-furanosyl-5-ethyl-uracil ([^{18}F]-FEAU)[36]. Both these methods have successfully been shown to identify bacteria accumulated in mouse tumors[34–36].

Conclusions and future perspectives

Recently, many experiments have shown that bacterial therapies can successful regress tumors and promote survival in mice. However, numerous challenges remain before bacteria can be used in the clinic, including limited drug production, intrinsic bacterial toxicity, targeting efficiency, genetic instability and combination with other therapies. Tuning drug production is necessary to synthesize drugs at high enough concentrations to induce therapeutic effects but not so high that they cause systemic toxicity (see Figure 2). Controlling bacterial toxicity will be critical to ensure safety and permit regulatory approval. Both *Clostridium* and *Salmonella* have been shown to be non-pathogenic in multiple animal species[46, 65] and in human trials[54–56, 63], but any retained virulence could be problematic for immunocompromised late-stage cancer patients. Variable targeting efficiency could lead to poor efficacy for large groups of patients and will affect which sites could be effectively treated with bacteria. Targeting efficacy will also play a large role in the treatment of metastatic disease because, to be effective, bacteria will have to colonize a high percentage

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of distal sites. Genetic instability is a potential problem because mutations could create ineffective or harmful phenotypes. The rate of mutation will specify the upper time limit that bacterial colonies could be allowed to remain in tumors. Finally, determining the correct combination of bacteria and other cancer therapies (Tables 1 and 2)[14, 18, 107–110] will be critical for creating strategies that can completely clear tumors and metastases. Solving these challenges could overcome the limitations that have previously been seen in the clinic[54–56] (Table 3): reduced toxicity will increase the maximum-tolerated dose; improved targeting will increase tumor colonization; and efficient drug production will promote tumor regression.

All these challenges can be addressed using synthetic biology techniques. Rates of protein drug production can be optimized by manipulating multiple factors[111], including gene copy number, promoter strength, optimized codons, bacterial metabolism, mRNA secondary structure[112] and synthetic ribosome binding sites[113]. Both toxicity and targeting are affected by the immune response following injection and innate bacterial virulence. Determining which virulence factors are essential for targeting and which introduce unnecessary toxicity can be achieved by screening knockouts of the pathogenicity genes that, for example, enable evasion of the immune system, induce uptake into cells, promote intracellular replication and stimulate cytokine synthesis[114]. Other targeting mechanisms can be enhanced by genetic manipulation of endogenous chemoreceptors[16], selective control of bacterial proliferation in tumors, and strategies to avoid sequestration by neutrophils. Similarly, genetic stability could be enhanced by incorporating engineered genes on the bacterial chromosome and limiting homologous recombination and horizontal gene transfer.

This moment in history is a turning point for bacterial therapies. The preliminary proof-of-concept experiments have demonstrated the vast capacity of bacteria for treating cancer and illustrated the large number of effective tools that these robot factories possess. The ultimate bacterial therapy will consist of a collection of strains designed for specialized purposes rather than a single perfect strain. Successful treatment could utilize these strains cooperatively and in combination with molecular chemotherapy: a detectable facultative anaerobe could be used for diagnosis; an engineered immunogenic stain could be used to sensitize the immune system; an obligate anaerobe could be used to treat inoperable primary tumors; and a motile *Salmonella* strain that controllably produces a cytotoxic agent could be used to treat diffuse tumors and metastatic disease. All bacterial therapies will be in used in combination with other therapeutics (Tables 1 and 2)[14, 18, 107–110], which will have a synergistic effect: small molecules would kill cancer cells close to blood vessels and bacteria would kill cells far from vessels (Figure 2). The greatest strength of bacterial therapies is their genetic flexibility, which enables tuning for individualized therapy, targeting to multiple tumor sites and precise control of cytotoxicity. Once perfected, anticancer bacteria are expected to be an essential clinical tool, which can perform functions unachievable by other therapies, and can detect, prevent, and treat tumors and metastases.

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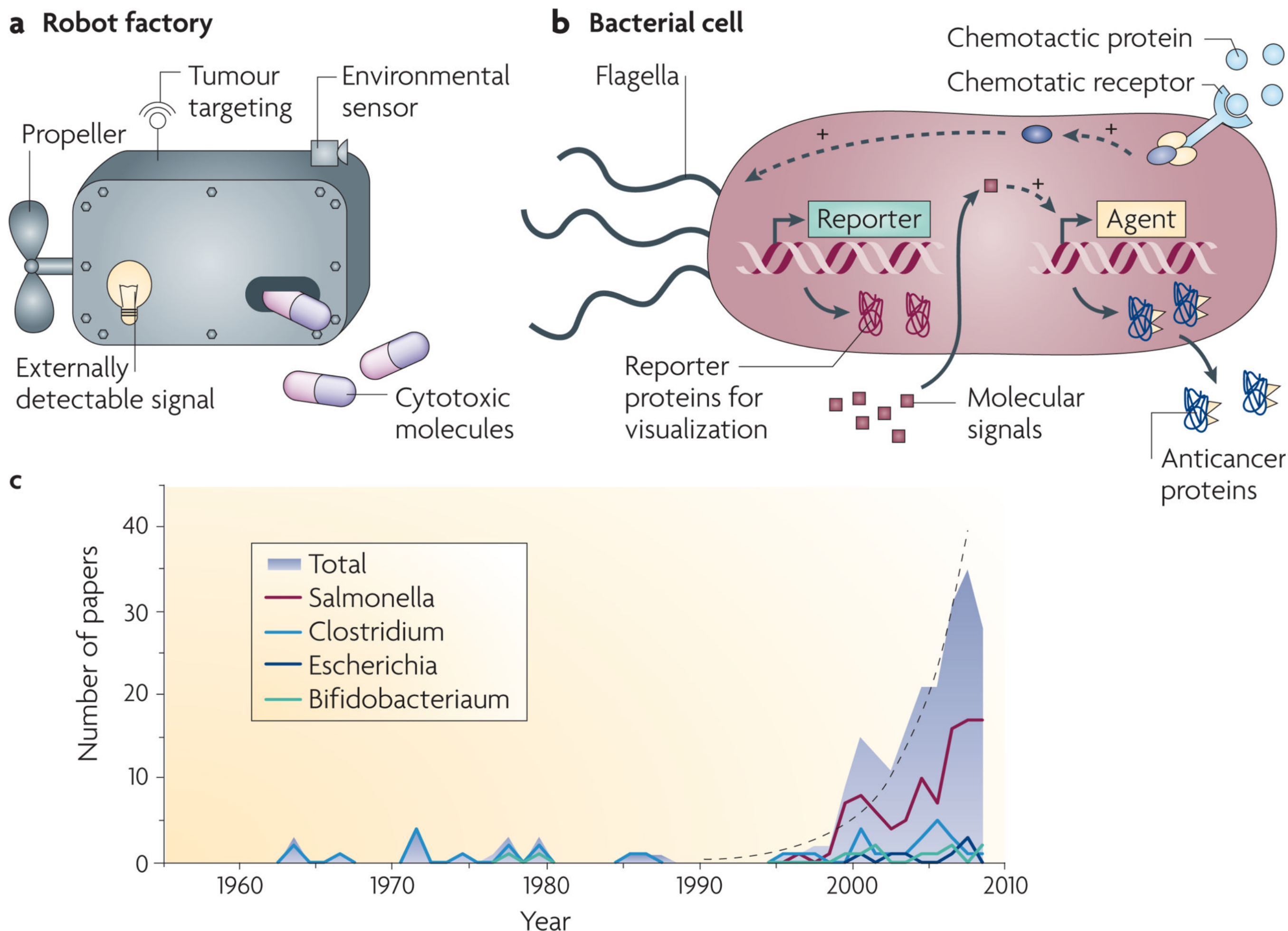

Figure 1. Bacteria are the optimal *robot factory* cancer therapies

A) The perfect cancer therapy would be able to perform six important functions: target tumors, produce cytotoxic molecules, self-propel, respond to triggering signals, sense the local environment and produce externally detectable signals. *B*) Bacteria have biological mechanisms to perform these functions: gene translation machinery to produce anticancer proteins (green); flagella to chemotax,;specific gene promoter regions to respond to molecular signals (purple cubes);chemotaxis receptors (orange);and 5) machinery to produce detectable molecules (red). *C*) The number of papers describing bacterial anti-cancer therapies has grown exponentially (black line) since the mid-1990s.

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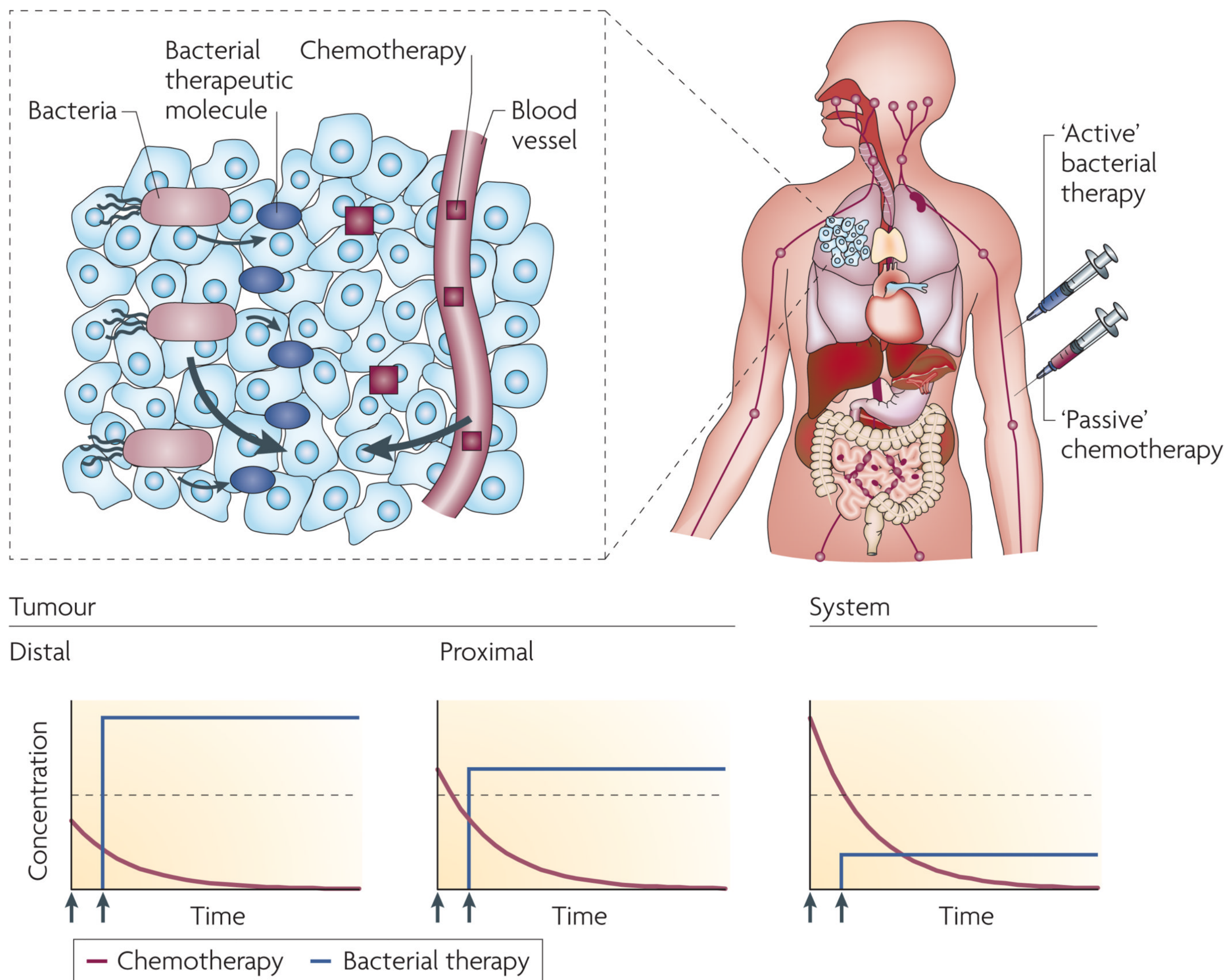

Figure 2. The transport properties of bacterial therapies produce preferable drug concentration profiles

When injected systemically, bacteria (red syringe, green organisms), specifically accumulate in tumors and migrate to distal regions far from vasculature (brown cells). These distal regions are typically hypoxic and hypoglycemic and contain quiescent and necrotic cells. Once triggered (small red arrows), bacteria begin to produce therapeutic molecules (red ovoids) that diffuse (large red arrows) into viable tissue (clear cells). Systemically injected (small blue arrows), passive chemotherapeutic molecules (blue cubes) diffuse into tumor tissue from blood vessels (large blue arrows). The concentration of bacterially produced molecules (red lines) is greatest in distal tumor regions and would remain constant as long as expression of these proteins continues. The concentration of chemotherapeutic molecules is greatest in systemic blood and drops as it is cleared by the liver or kidneys. Based on these profiles, bacterially produced molecules will be more cytotoxic (dotted line) in the distal regions of tumors and less systemically toxic. The profile of passive molecules is less favorable, with more systemic toxicity and less efficacy deep in tissue.

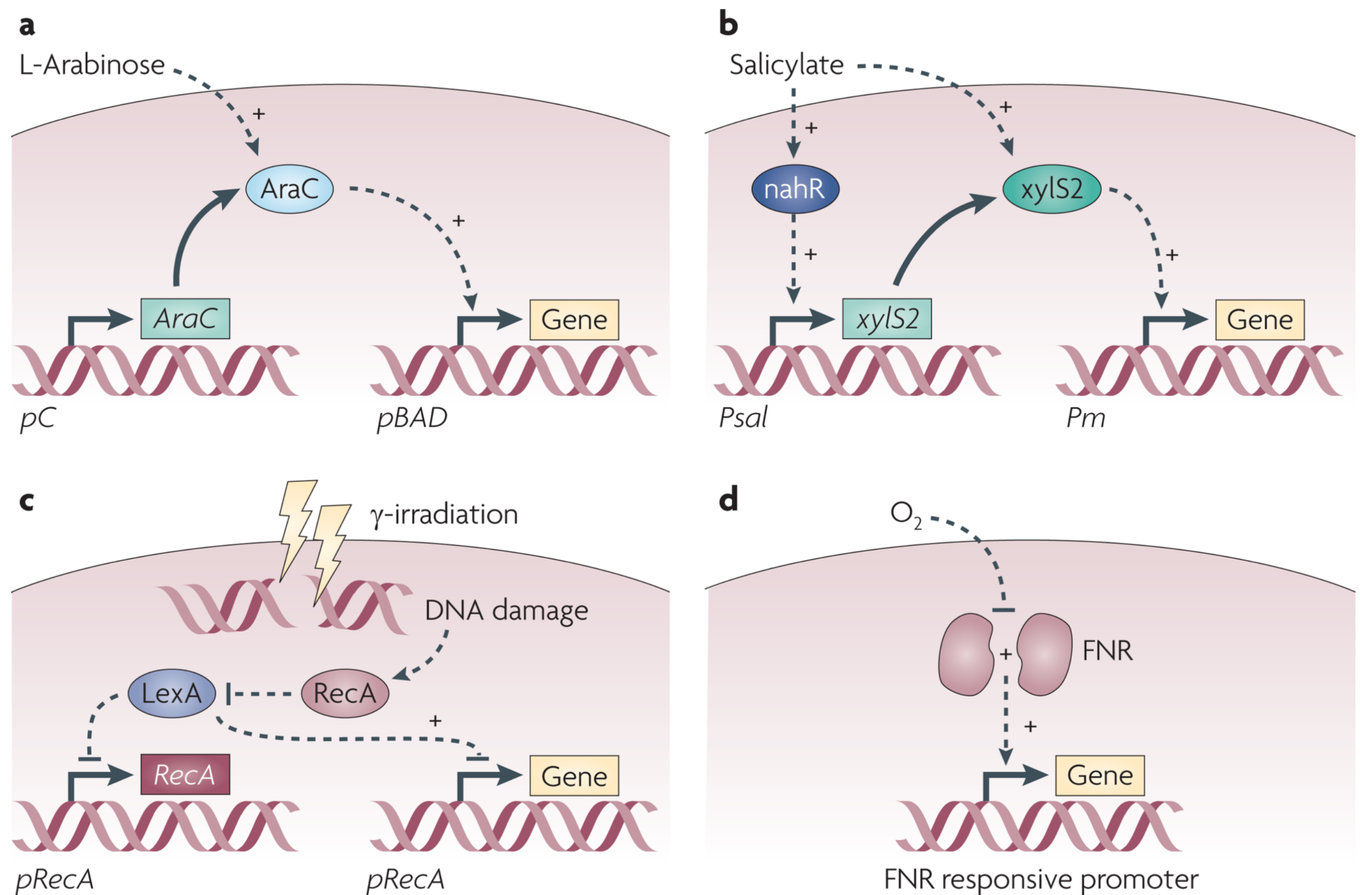

Figure 3. Gene triggering systems

A) The pBAD system, which responds to extracellular l-arabinose, contains two components: the arabinose sensitive protein AraC and the *pBAD* promoter. Constitutively expressed regulator AraC induces transcription by binding to the *pBAD* promoter. AraC is a positive and negative regulator of *pBAD*: it activates transcription in the presence of arabinose and represses transcription in its absence. *B*) The salicylate cascade system utilized a two salicylate-sensitive regulator proteins, nahR and xylS2 to maintain tight regulation. In the presence of salicylate, nahR activates transcription from the promoter *Psal*, leading to the expression of XylS2. XylS2, which is also sensitive to salicylate, activates transcription from the promoter *PmC*) The *RecA* system senses γ-irradiation, which causes DNA damage. This damage activates RecA, which induces autoproteolysis of LexA. Transcription is induced when LexA, a repressor of the *recA* promoter, releases from DNA. Feed-forward regulation increases the RecA concentration when the system is active. *D*) The FNR system turns on in hypoxic environments. The absence of oxygen promotes dimerization of FNR, which induces transcription. Multiple promoters bind FNR, including FF+20*, HIP-1, pflE and ansB.

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Table 1

Efficacy of bacterial therapies and strategies in animal models

A. Strategies showing tumor regression and/or increased survival	
Native bacterial toxicity	
Bifidobacterium	8
Caulobacter	9
Clostridium	6, 62, 115–117
Escherichia	118
Listeria	10–11
Proteus	12
Salmonella	4, 37, 46, 48, 64–67, 119–122
Streptococcus	13
Combination with other therapies	
Clostridium	14, 107–110
Escherichia	18
Salmonella	52, 123–125
Agents with control of expression	
Salmonella	19, 23
Expression of Anticancer Agents	
Escherichia	18
Salmonella	20–22, 24, 74–79, 81–82, 126
Gene Transfer	
Salmonella	44, 51, 88–95
RNAi	
Salmonella	96
Prodrug cleavage	
Clostridium	47, 127–129
Salmonella	130–134
B. Strategies that reduced metastatic burden or prevented metastasis formation	
Native bacterial toxicity	
Salmonella	121, 135–136
Expression of Anticancer Agents	
Escherichia	18
Salmonella	20–21, 24, 74–76, 93
C. Sites targeted showing either tumor regression or increased survival	
Breast cancer	48, 131
Colon cancer	131
Hepatocellular carcinoma	64
Melanoma	130–131
Neuroblastoma	78
Pancreatic cancer	13, 37
Prostate cancer	96

Spinal cord glioma	122

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Table 2

Bacterial strategies

A. Expressed anticancer agents		
Cytotoxic agents		
Cytolysin A (ClyA, HlyE)		17–19
Fas Ligand		24
TNFα		25–27
TRAIL		23
Cytokines		
CCL21		21
Interleukin 2 (IL-2)		72–73, 75–80
Interleukin 18 (IL-18)		74
LIGHT		20
Antigens and antibodies		
C-Raf		22
CtxB-PSA fusion protein		60
CPV-OmpA fusion protein		81
NY-ESO-1 tumor antigen		82
Single chain HIF-1α antibodies		83
B. Genetic transfer		
Cytotoxic and antiangiogenic agents		
Endostatin		44
Thrombospondin-1		51
TRAIL and Smac		88
Cytokines and growth factors		
Interleukin 12 (IL-12)		89–91
GM-CSF		90
Flt3 Ligand		92
Tumor antigens		
α-fetoprotein (AFP)		95
Flk-1		93–94
Gene silencing (shRNA)		
Stat3		96
Bcl2		97
C. Gene triggering strategies		
Signal	*Promoter*	
γ-irradiation	pRecA	23, 26–27, 30
L-arabinose	pBAD	17, 28–29
Oxygen (FNR)	FF+20*	19
	HIP-1	101
	pflE and ansB	103
Salicylate	XylS2-dependent *Pm* promoter	99

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D. Combinations with other treatments	
Anti-vascular agents	14, 123
Chemotherapeutic drugs	14, 51, 108, 110
Heat shock proteins	125
Heavy metals	107
Radiation	18, 109, 124
E. Imaging strategies	
Bioluminescence	5, 17, 104–106
Fluorescence	5, 31–32
Magnetic resonance (MRI)	33, 137
Positron Emission (PET)	34–36

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Table 3

Published human trials using bacterial cancer therapies

Strain	Cancer type	*n*	Responses	Ref.
C. butyricum M-55	Squamous cell carcinoma, metastatic, malignant neuroma, leiomyosarcoma, melanoma, sinus carcinoma	5	Oncolysis (3)	63
C. butyricum M-55	Vascular glioblastoma	49	Oncolysis	138
S. typhimurium VNP20009	Metastatic melanoma and renal cell carcinoma	25	Focal tumor colonization (3)	55
S. typhimurium VNP20009	Metastatic melanoma	4	Tumor biopsy culture positive for VNP20009 (1)	54
S. typhimurium VNP20009 TAPET-CD	Squamous cell carcinoma, adenocarcinoma	3	Intratumoral bacterial colonization (2)	56