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Emerging Applications of Bacteria as Antitumor Agents

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Abstract

Bacteria are associated with the human body and colonize the gut, skin, and mucous membranes. These associations can be either symbiotic or pathogenic. In either case, bacteria derive more benefit from their host. The ability of bacteria to enter and survive within the human body can be exploited for human benefit. They can be used as a vehicle for delivering or producing bioactive molecules, such as toxins and lytic enzymes, and eventually for killing tumor cells. *Clostridium* and *Salmonella* have been shown to infect and survive within the human body, including in tumors. There is a need to develop genetic circuits, which enable bacterial cells to carry out the following activities: (i) escape the human immune system, (ii) invade tumors, (iii) multiply within the tumorous cells, (iv) produce toxins via quorum sensing at low cell densities, and (v) express suicide

genes to undergo cell death or cell lysis after the tumor has been lysed. Thus, bacteria have the potential to be exploited as anticancer agents.

Keywords: Cancer; *Clostridium*; quorum sensing; *Salmonella*; tumors

1. Introduction

Cancers manifest the disruption of cell growth by genetic predispositions (oncogenes and genes responsible for DNA repair, apoptosis, and tumor suppression) and environmental factors, including exposure to the dangerous chemicals present in tobacco smoke and ionizing radiation [1-3]. Human cancers represent genetic disorders of somatic cells, which are marked by several abnormalities. Autosomal genetic predispositions lead to a high risk of cancer; furthermore, approximately 7% of all human cancers are hereditary [4]. Individuals who have genetic alterations in *p53*, *BRCA1*, and *BRCA2* also exhibit a reduced ability to suppress the growth of cancer cells [5]. The rate of cancer occurrence is predicted to increase to 23.6 million cases within the next decade [6]. However, in the United States, the death rate due to cancer has dropped continuously over the past two decades due to increased public awareness [7, https://seer.cancer.gov/report_to_nation/statistics.html].

Despite continuous global research efforts, cancer therapies continue to rely on a combination of surgery, radiotherapy, chemotherapy, hormones, and immunological methods [6]. Cancer therapies often involve disease management with severe side effects, including drug resistance, the capacity to repair DNA defects, and their drug detoxifying potential that interferes with apoptosis [8]. Although gene therapy seems to be the most promising [9], the use of toxins, immunogens, cytokines, or enzymes as anticancer agents appears to be the more commonly used strategies [10]. Consequently, diverse biological gene delivery vehicles based on viruses and bacteria have been developed [6,11-13]. In this article, the potential opportunities and challenges of exploiting tumor-specific bacteria have been presented to treat cancer.

2. Bacteria used to target tumors

Human tumors are characterized by hypoxic, apoptotic, or quiescent physiological conditions, which prevent easy access to conventional drugs. Radiation therapy, which depends upon oxygen radicals to induce DNA damage, is rendered ineffective by the anaerobic conditions that prevail within tumors [14,15]. Early evidence of cancer patients exhibiting temporary recovery after bacterial infections dates back to 1867 [12]. Researchers developing strategies to circumvent these challenges have been fascinated by the bacterial ability to populate the human body and grow anaerobically, including tumors [16-19] (Fig. 1). Possible mechanisms for controlling bacterial accumulation in tumors include (i) entrapment in vasculature, (ii) entrance into tumors after an inflammatory reaction, (iii) chemotactic attraction towards compounds explicitly produced by tumors, (iv) growth in tumor-specific physiological conditions, and (v) protection from the immune system [17]. Since cancer patients are immunocompromised, the tumor environment is conducive for the growth of bacteria that would otherwise be eliminated by macrophages and neutrophils [20]. Concerted studies have revealed bacteria that could be used as anticancer agents [13,21-23] (Table 1 and Fig. 2).

2.1. Clostridium

Clostridium spp., *C. novyi*, and *C. sordellii*, being anaerobic and highly motile, can spread rapidly and extensively within the poorly vascularized tumor areas [14]. The spores of genetically engineered *C. novyi*-NT germinated well within the avascular regions of the tumors and effectively eradicated tumors in mice [24,25]. Genetically modified, *C. novyi*-NT is non-toxic. It can produce redox proteins during sporulation and secrete lipases even in a vegetative state. This feature allows the bacteria to survive exclusively in tumors [14,26]. Furthermore, *C. novyi* has been reported to

cause immunogenic tumor cell death by producing reactive oxygen species (ROS) [27]. *C. novyi*-NT is a critical potential bacterial therapeutic agent [24,28].

2.2. Salmonella

Salmonella spp., especially the *S. typhimurium* strain VNP20009, selectively invades tumor tissues in mice, resulting in dramatic tumor reduction or elimination [29-35]. *Salmonella* spp. are attracted to tumors by serine, aspartate, and ribose, and can thrive in the presence of nutrients derived from dying tumor cells, as seen in animal models [32,36,37]. *S. enterica* has endogenous promoters, *pflE*, and *ansB*, explicitly activated in a human PC3 prostate tumor and tumor-free nude mice [38]. Although colonization by *Salmonella* at low cell densities does not exhibit any intrinsic tumor cell toxicity in vitro, it has been shown to cause tumor apoptosis in vitro and in vivo by using a red fluorescent dye bound to caspase-3 [39-41]. Furthermore, *Salmonella* spp. colonization in tumors elicits an immunological response, whereby the influx of blood into tumors increases the concentration of tumor necrosis factor-α (TNFα), which could interfere with antitumor activity. The attenuated *Salmonella* strain, *msbB*, was found to have TNFα levels of approximately 10% [37]. This feature can be regarded as a significant advancement in the safe administration of these bacteria in humans.

Attenuated *S. typhimurium* has also been used as an agent for delivering the expression vector pSNhTS, which carries the activator of caspases (Smac) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) genes, which are regulated by the hTERT promoter. Smac increases TRAIL-induced apoptosis, while the hTERT promoter effectively permits the expression of specific genes in tumor cells, restricting tumor growth by up to 90% [42]. Genetically modified *Salmonella* expressing the murine cytokine interleukin (IL)-4 or IL-18 genes significantly

increased survival time in preclinical mouse cancer models, thus offering a safe and effective alternative to the systemic use of bioactive compounds [43-45]. The bacteria could inhibit pulmonary metastases, and the growth of primary subcutaneous tumors in immune-competent mice challenged with syngeneic multi-drug-resistant carcinoma cell line clones. These activities were attributed to (i) natural killer (NK) cells and T-lymphocyte accumulation; (ii) heavy granulocyte infiltration; and (iii) increased intra-tumoral cytokine production [46]. Mice were administered attenuated *Salmonella typhimurium* (SalpIL2), lacking the gene coding for human IL-2 (Sal-NG) to prevent the relapse of pulmonary metastases in osteosarcoma. A significant reduction in pulmonary metastases was recorded, with killer cell populations increasing by 3.96-fold due to SalpIL2 and 4.2-fold due to Sal-NG [47]. When co-cultured with colon carcinoma or melanoma cells, *Salmonella* transposon insertion mutants revealed that the bacterial genes *adiY, yohJ, STM1787, STM1791*, and *STM1793* could activate cancerous cells. A *Salmonella* strain regulated by the STM1787 promoter expressed luciferase and exhibited tumor-induced bioluminescence. The toxin gene expressed by the STM1787 promoter proved to be a selective antitumor agent [48]. Genetically engineered *S. typhimurium* strain A1-R-GFP was helpful against metastatic human cancers such as glioma, fibrosarcoma, and osteosarcoma in the nude-mouse model [49].

2.3. Bifidobacterium

Systematic screening of *Bifidobacterium* species was carried out to extend the limits of efficiency of chemotherapeutic agents. It revealed that intravenously injected *B. longum* precisely invaded and grew vigorously within the mice tumors [50]. Since the bacterium targets an anaerobic environment rather than tumor-specific receptors, it can reach solid tumors in different tissues and

sites [51,52]. *Bifidobacterium bifidum* surfaces conjugated with folic acids enabled them to bind to their respective tumor receptors and acted as a vehicle to carry semiconductor nanocrystals or quantum dots deep into tumor tissues [53]. Using a similar approach, *Bifidobacterium breve* and *Clostridium difficile* were designed as delivery systems to administer nanorods for imaging and for tumor ablation [54]. The therapeutic effect of recombinant *B. breve* expressing IL-24 in head and neck tumor xenografts was observed in mice. It was found to increase the inhibition of tumor growth and the induction of apoptosis [55]. Despite the complexities of genetically modifying *Bifidobacterium*, a unique Bifidobacteria Expression SysTem (BEST) has been developed, which allows the production and delivery of heterologous proteins to mucosal surfaces. Its function was validated by cloning murine IL-10, achieving seven-fold higher levels of IL-10 secretion [56]. A newer technique, using high-intensity focused ultrasound (HIFU) for the non-invasive destruction of cancerous cells, was limited by energy retention at low concentrations and short durations. The therapeutic efficiency of HIFU synergy was enhanced using lipid nanoparticles attached to *Bifidobacterium* by electrostatic adsorption [52].

Bacteria can be used as carriers for therapeutic agents targeting primary tumors and metastasis [57-60]. Since the formation of fresh blood vessels (angiogenesis) is necessary for the growth and metastasis of solid tumors, blocking this process has been envisaged as a good strategy to treat solid tumors. An attenuated auxotrophic strain of *Salmonella* and recombinant human endostatin (rhEndostatin) could significantly reduce the growth of murine malignant melanoma [61]. Endostatin produced by *B. adolescentis* and TRAIL can strongly inhibit angiogenesis and tumor growth [62,63]. Treatment of vesicular endothelial growth factor receptor 2 (VEGFR-2) in animal models of lung cancer, colorectal carcinoma, and malignant melanoma by using oral antiangiogenic bacterial vaccines was reported to be effective [64,65].

2.4. Escherichia

Monitoring the entry and location of *E. coli* and three attenuated pathogens (*Listeria monocytogenes, S. typhimurium*, and *Vibrio cholerae*) in live animal tumors by using GFP and luciferase luminescence revealed exciting results. It showed that conditions considered necessary were not critical for intra-tumoral replication and tumor specificity: (i) anaerobic growth conditions, (ii) the vaccinia virus lacking the gene coding for thymidine kinase, and (iii) auxotrophic mutations [57]. *E. coli*, expressing a model tumor antigen and listeriolysin-O (LLO), exhibited strong antitumor activity, which was attributed to the induction of cytotoxic T-lymphocytes and the restriction of Foxp3 T-regulatory cells. *Escherichia coli* induced a strong antitumor effect against WT1-expressing tumors by co-expressing LLO and Wilms tumor gene 1 (WT1), a clinically relevant tumor antigen associated with most adult leukemias, Injecting the NAPYLPSCL peptide with *E. coli*-LLO also exhibited antitumor effects, indicating the potential of *E. coli*-LLO as a vaccine [66]. The anticancer properties of *E. coli* K-12 and DH5α have been evaluated as they can be easily genetically modified [41].

2.5. Actinobacteria

Investigation of the bioactive molecules salinosporomide produced by the Actinomycetes *Sinomonas humi* strain MUSC 117^T, *Monashia flava* strain MUSC 78^T, and *Microbacterium mangrovi* strain MUSC 115^T revealed their anticancer properties against human cervical carcinoma cell lines (Ca Ski) and human colon cancer cell lines (HT-29) [67]. The chemical profiles of the bioactive compounds {19} and {21} were similar to those reported for myxobacteria *Stigmatella* [68]. Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- {16}, produced by the *S. humi* strain MUSC 117T, also reduced the expression of the serine/threonine kinase Akt.

It inhibits the proliferation of cells and promotes apoptosis in tumors [69-71]. *Streptomyces* spp. - *S. antibioticus* and *S. canaries* produce saphenamycin, which has an IC_{50} of 0.6 μg/mL in CCRF/CEM T cell leukemia cells [72]. Saphenamycin was also found to extend the lives of mice with leukemia cell implants [73].

2.6. Listeria

Attenuated *L. monocytogenes* (LM)-based vaccines have demonstrated efficacy against established B16F10 melanomas and metastatic breast cancer [74]. LM-expressing truncated LLO and amino acid fragments of tumor-associated antigens (TAA) have been shown to cause tumor cell death via high ROS levels [75]. The activation of CD8 T cells by *Listeria*-derived antigens significantly reduced metastases at a young age [76]. The highly attenuated *Listeria* vector, LmddA, which expresses a chimeric human Her2/neu (ChHer2) gene, effectively reduced immune tolerance, whereas the highly attenuated construct, DXS31-164, delayed tumor growth in Her2/neu transgenic animals. These findings support the use of this vaccine for treating malignant HER2/neu-overexpressing cells in pancreatic, colorectal, and breast cancers [77]. The blood of mice and humans affected by cancer contains many myeloid-derived suppressor cells (MDSCs). Using MDSCs as carriers, attenuated *Listeria* strains were delivered into tumor cells, resulting in significant reductions in the blood and primary tumor MDSC population accompanied by the conversion of the remaining subpopulation into immune stimulators. Consequently, high IL-12 concentrations were produced and a drastic reduction in tumor growth and the number of metastases was observed [78,79]. Mice with metastatic breast cancer (4T1 model) were treated with an LM-based vaccine, which expressed tumor-related antigen Mage-b and carried α-

galactosylceramide, which almost eliminated metastases without any evident toxicity. This live bacterial vaccine is another approach for treating metastatic breast cancer [80].

2.7 *Lactococcus*

Bioactive phenazine compounds, purified from *Lactococcus* spp., demonstrated strong antifungal properties against *Fusarium oxysporum*, *Penicillium chrysogenum*, and *Aspergillus niger* [81]. These substances exhibited selective cytotoxicity against HeLa and MCF-7 cancer cell lines with IC_{50} values of 20 and 24 μg/mL, respectively [81]. Phenazines have been reported to act by interfering with topoisomerase I and II in cancer cells [82].

2.8 Other Organisms

Intravenous injections of *Proteus mirabilis* strain RSM203 effectively treated Ehrlich carcinoma Line-1 [83]; however, the genus has not been explored extensively in recent years. The intra-tumoral injection of live *Streptococcus pyogenes* cells in a mouse model caused pancreatic cancer regression [84], whereas stimulating CD4+ lymphocytes with the *S. pyogenes* exotoxins SpeA, SpeB, and SpeC caused cytokine secretion [85]. Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene), a heterocyclic tripyrrolic toxin produced by *S. marcescens*, has been shown to exhibit antitumor activity [86] and is an effective apoptotic agent against different cancer cell lines [87,88]. Prodigiosin has been reported to trigger apoptosis in HCT-116 cells utilized as a model for colorectal cancer, reducing the growth rate in a dose (100, 200, and 400 nM)- and time (48 h)-dependent manner by 16, 42 and 54 %, respectively [89]. *Caulobacter crescentus* has demonstrated similar antitumor effects in murine tumor models [90], whereas *Rhodobacter sphaeroides* has recently been identified as a tumor-targeting bacterium [91].

3. Use of engineered strains to target tumors

Diffusion barriers and pressure gradients prevent bacterial entry into eukaryotic tissue. Once inside the tumor, bacteria still need to multiply within specific microenvironments. Furthermore, unwanted pathogenicity must be removed from strains derived from pathogenic bacteria.

3.1. Quorum sensing

Eukaryotic hosts can sense pathogen invasion and activate defense mechanisms. Free-living microbes produce toxins and antibacterials to attack other cells or resist antibacterials without estimating the extent of the attack on them; however, pathogenic microbes must evade host attacks and comprehend the ability of the host to resist their invasion [91-93]. At this point, microbes mount their counter-defense only after gauging the extent of attack by the host's immune system [94-96]. This microbial mechanism for communicating and activating specific pathogenic genes in a cell density-dependent manner is termed quorum sensing (QS) (Fig. 3). In QS, these signal molecules form a complex with the receptor to transcribe virulence genes. To exploit QSS and selectively kill tumor cells, bacteria that can target, invade, and effectively lyse tumor cells must be used (Table 2). These organisms can be modified as improved anticancer agents that overcome the limitations of current cancer therapies [97].

3.2. Cell invasion

The interaction between bacteria and cancer cells depends on the expression of invasion genes (Fig. 3). *E. coli*, engineered to express invasion genes from *Yersinia pseudotuberculosis*, was capable of invading cancer-derived cell lines such as HeLa, U2OS, and HepG2. Furthermore, InvC *E. coli* present the advantage of a single gene being able to initiate the adhesion and invasion of mammalian cells, including tumor cells [98]. Bacterial invasion efficiency was increased and better regulated when the invasion process was mediated by linking it to the hypoxia-responsive *fdhF* or arabinose-inducible *araBAD* promoters, especially under anaerobic conditions or in 0.02% arabinose, respectively. The whole circuit comprised the *lux* operon of *Vibrio fischeri* (for cell tracking), *inv* from *Y. pseudotuberculosis,* and the *fdhF* promoter or the arabinose operon. The

construct was activated in cervical carcinoma, hepato-carcinoma, and osteosarcoma cell lines [98,99]. After entering the tumor, the bacteria must perform specific functions, including producing antitumor agents, in a well-orchestrated manner [100,101]. Synthetic circuits can process the information being transmitted by environmental and nutritional signals, tuning the production of bioactive molecules via a range of systems, including oscillators, a toggle switch, or even a pulse generator [102-104].

3.3. Gene expression and circuits

Genetic circuits under QS regulation have been evaluated in colorectal and other human cancers. An engineered biological system integrating three different circuits was designed to kill colon cancer cells in the following manner: (i) detect colon cancer cells via their specific carcinoembryonic antigen (CEA), (ii) QS-mediated conversion of 5-fluorocytosine into toxic 5-fluorourocil along with the invasin gene, and (iii) a QS-mediated suicide cascade. These circuits allowed *Magnetospirillum magneticum* AMB-1 to invade targeted cancer cells and induce death in a density-dependent manner [105]. A genetic circuit that discriminates between cancerous and healthy cells via epidermal growth factor receptor (EGFR) expression on cancer cells has also been developed. It allows the chemotaxis of engineered bacterial (*E. coli*) cells towards them [106] and triggers an anticancer response above a threshold AI-2 density [107]. QS-mediated production of prodigiosin in engineered *Serratia marcescens* proved to be cytotoxic against various cancers [108].

Beyond designing an entire circuit [109], certain synthetic acylhomoserine lactones (AHL) analogs with 3-oxo substitutions, such as 3-oxo-12-phenyldodecanoyl-L-homoserine lactone (HSL) [12b], have demonstrated high activity against cancer cells, including prostate carcinoma

cell line, PC3, and the colorectal carcinoma cell lines, H630 and H630-1 [110]. Synthetic compounds 5 and 87, which are AHL analogs, inhibited the proliferation of human oral squamous carcinoma cells derived from tongue cancer SAS cells and gingival carcinoma Ca9-22 cells [111]. The antitumor activities of chemically synthesized QS signaling molecules were tested in chronic myeloid leukemia (CML) K562 cells. Importantly, two analogs of AHL caused apoptosis via the activation of JNK and the induction of p21. This analog induced caspase-independent apoptosis in CML K562 cells [112].

Synthetic AHL analogs examined against the cell lines of breast cancer (MCF-7), gastric cancer (MGC-803), hepatocellular carcinoma (SMMC-7721), and esophageal cancer (EC-9706), revealed that terminal phenyl groups with chalcone scaffolds had dramatically higher cytotoxicity than those with hydrophobic side chains. Compounds 10a–k and 14 with 4-amino chalcone scaffolds demonstrated high levels of inhibition against cancer cell lines and exhibited higher potency than 5-Fu and AHLs. The synthetic AHL analog compound 10i, which possessed a 3, 4, 5-trimethoxy group, was the most potent, whereas analog 11e arrested the cell cycle of MCF-7 cells in the G2/M phase and induced cellular apoptosis [113]. Cytotoxicity assays and the evaluation of NF-κB inhibitory activities against QS molecules and their analogs in Hodgkin's lymphoma cells (L428) revealed several compounds dose-dependently inhibited NF-κB signaling. Inhibitors such as ITC-12, ITC-Cl, and Br-furanone caused 50% NF-κB inhibition at concentrations between 4.1 and 12.8 μM, cytotoxicity against L428 cells with IC50 values between 3.1 and 18.3 μM, and A549 adenocarcinoma cell migration inhibition at concentrations between 2.6 and 7.9 μM. The inhibitory effects of these compounds on Hodgkin's lymphoma cells were attributed to the suppression of NF-κB subunits [114].

The use of AHLs in cancer therapy has also been demonstrated through TRAIL utilization [115]. It has been hypothesized that QS-mediated oligopeptides could be used in cancer therapy as agonists or antagonists, since they are recognized by eukaryotic cells. Furthermore, their small structure facilitates tissue invasion, and there are similarities between the behavior of metastatic cells and QS-mediated bacterial cells [116].

A QS-based circuitry was developed and expressed in *E. coli* to target and kill tumors [98]. The invasin gene (*inv*) from *Y. pseudotuberculosis* was used to induce the binding and invasion of β1-integrin-expressing tumors. Since cancer cells are hypoxic and have a high cellular density, the *inv* gene is regulated by the QS *lux* operon derived from *V. fischeri*; the system was induced under the anaerobic conditions necessary for formate dehydrogenase (*fdhF*) promoter induction. QS regulates the *lux* genetic circuit, with the promoter only triggering invasion under anaerobic conditions. The system was tested using metastatic breast cancer (HeLa), osteosarcoma (U2OS), and hepatocarcinoma (HepG2) cell lines to achieve toxin delivery to cancer cells [117]. A synchronized lysis circuit (SLC) was developed by integrating a QS autoinducer, AHL, and a lysis bacteriophage (φX174 E) in feedback loops in *S. enterica* and was tested using the HeLa cell line.

Farnesol, known for regulating QS in the fungal pathogen *Candida albicans*, has been shown to significantly limit the proliferation of oral squamous cell carcinoma (OSCC) lines and promote apoptosis via signaling pathways, rendering it a potential therapeutic agent [118-120]. Farnesol also activates innate immune cells but suppresses adaptive immunity [121].

3.4. Bio-toxins as antitumor agents

Natural toxins produced by QS can be utilized for cancer therapy. Bacteriocins such as colicin, nisin, pediocin, and pyocin produced by bacteria *Klebsiella*, *Pediococcus*, *Lactobacillus*, and

Pseudomonas, are biodegradable, non-immunogenic, and cause cancer cell-specific toxicity [122-126].

Bacterial toxins, such as cytolysinA (ClyA) from the *E. coli* strain K-12, form pores in mammalian cell membranes and induce apoptosis. ClyA production in *S. typhimurium* and *E. coli* reduced tumor growth in mice [60,127-129], whereas injection of murine tumors with *E. coli*, engineered with α-hemolysin from *Staphylococcus aureus*, induced regression and necrosis [130,131]. These proteins induce apoptosis in mammalian cells and have higher toxicity to cancer cells [129,132-135]. Various cytotoxic cytokines have been tested as therapeutics, including the FAS ligand TRAIL and TNFα, both of which have shown efficacy against a wide range of cancers, including lung, breast, pancreas, colon, prostate, bladder, kidney, brain, and ovarian cancers [133-138]. The major challenge to cytotoxic cytokine use is administration-associated toxicity; thus, an alternative strategy could be their localized production within tumors.

3.5. Selecting the cancer killer

A few criteria have been set for successful bacterial cancer therapy, some of which have been well defined, whereas others need to be improved upon [36]. The bioactive agent should be able to selectively invade the tumor, have high toxicity, be complementary to recognized therapies, have resistance to defense mechanisms (the immune system), be tunable, and should be degraded once its action is complete [17,139,140]. To date, a few bacteria have been identified with the ability to accumulate within cancer cells: (i) *Clostridium*, due to its obligate anaerobic nature, (ii) *Salmonella* and *Escherichia*, as facultative anaerobes, and (iii) *Listeria*, due to its ability to target immune cells. The ability of cancer cells to suppress the immune system prevents the clearance of bacterial cells [141].

3.6. Incorporating synthetic biology

Since the *inv* gene enables mammalian cell invasion, a *Bacillus* strain should be engineered with *inv*, its QSS, and lactonase to inactivate any non-cognate QS signals. Inducible phenotypes may be preferable to constitutive phenotypes; therefore, a bacterium with high plasticity for utilizing endogenous mechanisms could be a safe and effective anticancer therapy [142]. Synthetic biology has enabled the creation and exploitation of cells in a predictable manner; genomic reduction creating knockout mutants has been reported in a variety of bacteria, including Gram-negative *E. coli* K-12, *Haemophilus influenzae*, *P. aeruginosa* PA14, and *Acinetobacter baylyi* ADP1, as well as *Corynebacterium glutamicum* (Actinobacteria) and *Bacillus subtilis* (Firmicutes) [143]. The most intricate aspect of genomic reduction is predicting genes whose deletion will not prove detrimental; *E. coli* mutants lacking up to 29.7 % of the parental chromosome have been thoroughly evaluated, whereas only 271 genes of the total *B. subtilis* genome (4.2 Mbp) have proven essential. In *B. subtilis*, a 24.7 % reduction in the DNA content has been achieved and may produce bacterial cells with industrial applications [144-147]. *Bacillus* species, which are generally recognized as safe, have the potential to act as an alternative to *E. coli* [147]; however, their half-life and the burden of their uncontrolled growth must be reduced [99].

3.7. Fate of the killer organism

An attractive anticancer therapeutic strategy is to restrict the expression of the killer gene after eradicating the cancer via a second 'population control' QS circuit, which can self-regulate the population density of the anticancer bacterium. To do so, de novo gene circuits must be designed to inhibit killer gene expression. Since bacteria possess suicide machinery activated by stress and

starvation, for example, the QSS-regulated coordinated killing of *Streptococcus pneumoniae* sub-populations (Fig. 3) [148,149]. Although high concentrations of the killer protein cause cell death, low QS signal expression can help target private goods. A circuit has been constructed composed of two plasmids, pLuxRI2 and pluxCcdB3, in which the *ccdB* killer gene encodes a fusion protein that leads to cell death by inhibiting DNA gyrase. Since AHL serves as an external inducer to regulate this circuit, it can be influenced either by hydrolytic enzyme activity or by the increased pH of the medium [150].

3.8. Intracellular QS mediated anticancer activity

Bacterial QSS operates at the levels related to: (i) public goods - production of extracellular enzymes, and (ii) private goods – production of molecules within the cell [151]. QS mediated utilization of different carbon sources: (i) adenosine, and (ii) bovine serum albumin (BSA) [152]. BSA gets metabolized by QS dependent elastase produced extracellularly and can prove beneficial to the entire population (public goods) [153]. On the other hand, degradation of adenosine takes place with the help of LasR dependent nucleoside hydrolase (Nuh) in the periplasmic space [154,155]. It is thus useful only to producer cell and act as private goods. Bacteria after invading cancerous cell can produce a basal quantity of QS signal (AHLs), enough to activate the QSS related to metabolism of nucleoside (adenosine) and meet its energy needs. The signal, instead of being transported outside the cell can interact with the receptor to transcribe a toxin gene for producing toxin in quantities enough to lyse only one host cell. At this point, the bacteria can be made to activate the promoter of a “killer” gene, whose merchandise will be suicidal to the bacterial cell. The host organism will thus get rid of cancerous cell also the infectious bacteria.

4. Opportunities

Bacterial therapies act via direct oncolysis or by modulating the immune system. Their major mechanism of action is exotoxin secretion or nutrient competition [37]; however, bacteria can also kill tumor cells by multiplying vigorously, which bursts the host cell, or by inducing apoptosis [49]. There are a few areas where opportunities exist for further exploitation: (i) genetically modified bacteria with the ability to colonize the tumor microenvironment [41,156], (ii) oncolytic bacteria, which induce cell death [130], and (iii) the induction of antigen responses [157-160]. Therefore, microbes are well-suited as therapeutic agents for metastatic disease.

Since certain bacteria proliferate specifically in the necrotic or hypoxic tumor regions, they can be combined with cytotoxic therapies to achieve synergistic benefits [14,20,28]. Bacterial enzymes that activate prodrugs in the tumor microenvironment can also be utilized [161]. For example, genetically modified *Salmonella* expressing effector genes, such as herpes simplex virus thymidine kinase, are able to convert the prodrug ganciclovir into its toxic form by suppressing tumor growth. *Listeria* has also been used for the delivery of prodrug-converting enzymes, such as yeast cytosine deaminase, uracil phosphoribosyl transferase, and purine-nucleoside phosphorylase [162]. Recently, *Bifidobacterium* has been used as a system for delivering ILs and quantum dots, thus increasing the number of bacteria that can be used for targeting tumors [52-56].

5. Clinical trials

Considerable effort has been invested in testing anticancer agents; however, most research has been limited to model cancer lines [10]. Progress has been made in developing improved strategies, with many potential therapeutics moving toward clinical trials [17,163]. To date, genetically

modified bacteria have proven effective at inhibiting tumors in mice [134], whereas attenuated bacterial strains have also shown positive results in tumorous and non-tumorous animals [24]. Of these attenuated microbial therapies, the intravesical Bacillus Calmette-Guérin (BCG) vaccine has proven to be the most effective in reducing the recurrence and progression of non-muscle-invasive bladder cancer [164].

The ability of *Clostridium* spores to specifically target and colonize cancer cells was first demonstrated over six decades ago. Clinical trials of bacterial vaccines and bacteria for targeting tumors have provided vital information on systemic responses in cervical, liver, lung, metastatic colorectal, oropharyngeal, ovarian, pancreatic, and prostate cancers, as well as in solid tumors [165-167]. An intratumoral injection of *C. novyi*-NT spores had surprising survival benefits in a murine orthotopic brain model [168]. In contrast, other studies have demonstrated the induction of tumor regression by bacteria [27,169]. These preclinical experiments have enabled phase 1 investigational clinical studies in cancer patients, with promising data generating confidence among patients and doctors that this treatment will become a reality [170]. The use of *Clostridium*-directed enzyme prodrug therapy (CDEPT) is expected to circumvent issues related to wild-type strain utilization. The clinical evaluation of transferable genes with the ability to target the immunosuppressive hypoxic tumor microenvironment is likely to provide synergy with other immune therapies [171].

Salmonella strain VNP20009 administered to cancer patients with metastatic melanoma could not colonize in most cases in clinical trials. Thus, microbial-associated molecular patterns (MAMPs) like LPS, flagella, and CpG, supplied from sites in the tumor region might be the cause of the antitumor response, which was quite poor in the patients. The immunogenic response due to purified LPS or dead bacteria varies from high, in cases of colon carcinoma CT26, to low, in

the case of RenCa (a renal adenocarcinoma) [172]. Thus, the efficacy of a therapy depends on the potency of the bacterial infection and the immunogenicity of the tumor. A comparison of bacterial clearance from host systems showed that bacteria are more resistant to murines than humans, which encourages their trials in the latter [172]. In fact, per-exposure to *S. typhimurium* enables humans and dogs to generate immunity to some extent, whereas mice have been treated in pathogen-free conditions [173]. Furthermore, human beings are generally treated with chemotherapy, which is also likely to affect the immune system, leading to reduced responsiveness. Patients in the late stage of cancer are also highly immune-compromised.

Nevertheless, only a few immune therapeutics are available in the clinic to date. In the first clinical trial on 39 human subjects affected by five different cancers, tumor shrinking was seen in five of them over two years. In 2010, chimeric antigen receptor therapy, or CAR therapy, targeted tumor cells by genetically modifying the patient's T cells. This resulted in the disintegration of leukemic cells [174]. Immunotherapeutic agents have been well recognized because of their therapeutic responses; however, their success at the clinical level has been limited [175]. The potential of clinical responses to immunotherapy using engineered T lymphocytes based on adoptive cell transfer has been successful enough to evaluate their efficacy in the treatment of other malignancies [176].

L. monocytogenes selectively infect antigen-presenting cells, delivering tumor antigens to activate long-lasting, tumor-targeting cytolytic T lymphocyte (CTL)-mediated immunity. In preclinical cancer models, LM-based systems have demonstrated highly significant therapeutic efficacy [158-160,177-179]. Coupling 188-rhenium with *Listeria* via antibodies to deliver radioactivity reduced the cancerous cell mass of highly metastatic mouse pancreatic tumors [180,181]. In addition, the scope of bacterial anticancer therapies has been extended to naturally

occurring canine tumors [182,183]. However, there have been clinical trials with ambiguous outcomes [168,182].

6. Microbiota

There is a diverse range of bacteria present within the human gut. Many intestinal bacterial communities are dysbiotic in various diseases, including colorectal cancer [184]. *Helicobacter pylori* is widely known to cause deadly infectious disease; however, it can also act as an anticancer agent, although the mechanism is still unknown [185]. In eubiosis, microbiota leads to homeostasis through two mechanisms: (i) the generation of metabolites such as short-chain fatty acids (SCFA), and (ii) by participating in immune responses [186,187]. Recently, two SCFA-producing strains present in the intestinal microbiota, *Faecalibaculum rodentium* and *Holdemanella biformis*, have been reported to show an anti-tumorigenic effect by inhibiting the activation of NFATAc3 and calcineurin [188]. Gut microbiota can also be used to overcome hypoxic conditions, invading and stimulating the innate immune system, increasing their efficacy as anticancer agents [12,189]. The microbiome of the human gut and breast tissue encounter many infectious bacteria, including *Pseudomonas, Vibrio, Clostridium, Yersinia*, and *Streptococcus* spp. [190,191]. The *Pseudomonas aeruginosa* QSS signal 3-oxo-C_{12}-HSL blocks proliferation and induces apoptosis in human BC cell lines [192]. Furthermore, 3-oxo-C_{12}-HSL has been documented to influence mammalian cell viability by downregulating thymidylate synthase and reducing the growth of H630 (human colorectal cancer) [193,194]. It inhibits cancer growth by increasing the activity of 5-fluorodeoxyuridine, taxol, and tomudex, which regulate tubulin expression [195]. 3-oxo-C_{12}-HSL also triggers cell death in mammalian lymphocytes. It is integrated into the plasma membrane and causes the dissolution of lipid domains, which leads to caspase-mediated apoptosis [196]. A few

gut microbiota oligopeptides also promote angiogenesis and influence metastases, a property that can be exploited to effectively treat cancer [197,198]. Therefore, probiotics could be used to modify the microbial composition and thereby help develop this strategy for anticancer therapy [199-201].

7. Bacteria-based microrobots

Bacteria-based microrobots have been proposed to treat cancers. Biomedical microrobots have been developed by integrating micro-electromechanical systems (MEMS) with nano- and biotechnologies [202,203]. In this innovative theranostic methodology, bacteria are used as microactuators and microsensors to deliver drugs for treating solid tumors [204]. The therapeutic *S. typhimurium* strain was encapsulated in a biodegradable alginate microbead, and its flagellated strain was immobilized on its surface [204]. It protects bacteria from the host immune system [205].

8. Alternatives to live bacterial therapies

In addition, to live bacterial cells, non-living cell-based therapies using bacterial minicells, outer-membrane vesicles, and cell-wall complexes also appear to be beneficial [206-208]. For example, bacterial minicells with restricted metabolism can provide an effective platform for introducing limited genetic material, such as QS machinery and genes for producing predefined quantities of toxins. The concept of using an acute bacterial infection for the induction of a strong antitumor immune response has been gaining interest for improving treatment strategies; however, it has been challenging to provoke a safe and consistent therapeutic response. Subcutaneous administration of the immunotherapeutic peptide QBKPN, derived from inactivated *Klebsiella*,

induced antitumor innate immunity in a lung cancer model. The peptide worked alongside NK cells and the NKG2D pathway to increase the production of cytotoxic molecules, with a patient trial demonstrating the efficacy, safety, and tolerability of the system [209].

9. Challenges in using bacteria as antitumor agents

The critical aspects of cancer management include (i) surgery, (ii) radiation, (iii) chemotherapy, and (iv) immunotherapy [13]. The FDA has approved the use of an immune-checkpoint blockade (ICB) monotherapy to control several types of cancers; however, some patients do not respond to ICB agents. The basic reasons for this hindrance are: (i) the genetic make-up of the cancer cells, (ii) the host (immunosuppressive) environment, and (iii) poor anticancer cellular responses [13,210-214]. A few issues, which have been identified as critical for the success of microbial therapy, are the development of: 1) a predictive animal model, 2) cancer prophylactic therapies, and 3) applying Good Manufacturing Practices [12], which are a balance between cost, accuracy, and efficiency.

Another major challenge is regulation; while developing microbial therapies, it is important to restrict the spread of the therapeutic organism and post-treatment infections [12, https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm404087].

The use of microbes for cancer treatment holds great promise; however, implementation is hindered by: (i) lack of specificity, (ii) short half-life of the vector, (iii) limited tropism for cells presenting antigens, (iv) host immunity, and (v) development of antibodies towards viral coat proteins. For example, *Mycoplasma hyorhinis* and species of *Shigella, Escherichia, Klebsiella, Salmonella, Citrobacter,* and *Serratia* expressing cytidine deaminase (CDDL) have shown to

transform the chemotherapeutic drug gemcitabine into its inactive form, which contributes to resistance to cancer therapies [215]. Gut bacteria can modulate anticancer immune responses, tumor pathogenesis, chemotherapy resistance, and the adverse effects of chemotherapy [216,217].

10. Concluding remarks and future directions

The feasibility of using bacteria as anticancer agents and replacing traditional cancer drugs remains unclear. The goal, although apparently straightforward, has remained elusive due to the high genetic and phenotypic variability of tumors. The areas requiring most attention include immune stimulation, controlled delivery, and the efficacy and safety of the approaches. Additionally, a greater synergy with other associated fields is necessary for successful implementation. An essential strength of these bacterial therapies is their potential to overcome drug resistance, a factor that limits the applicability of small-molecule anticancer therapies despite their long-term benefits.

Conflict of interest

Authors declare no conflict of interest.

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Figure legends

Fig. 1. Quorum sensing-mediated killing of cancer cells by bacteria.

Fig. 2. Diversity of bacteria as potential anticancer agents; their targets and actions.

Fig. 3. Proposed mechanism of cancerous cell death by quorum sensing-mediated bacterial toxin. **A**, Tumor. **B**, Bacterial cells at low cell density inside the tumor. **C**, Induction of quorum sensing in bacterial populations at the high cell density and expression of QS-mediated toxin production. **D**, Initiation of cancerous cell death by bacterial toxins. **E**, Lysis of cancerous cells and the initiation of bacterial suicide by the activation of killer genes. **F**, Complete lysis of tumor and bacteria.

Fig. 1

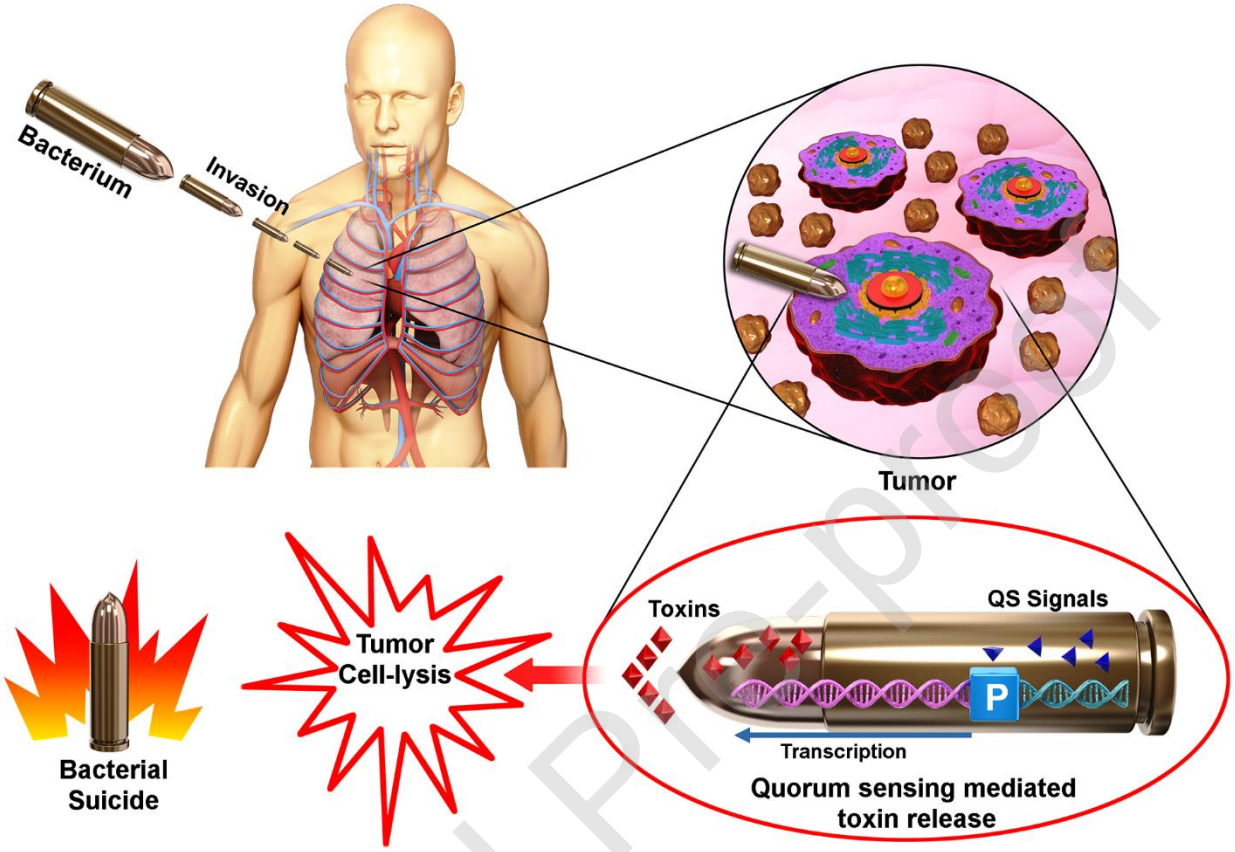

Fig. 2

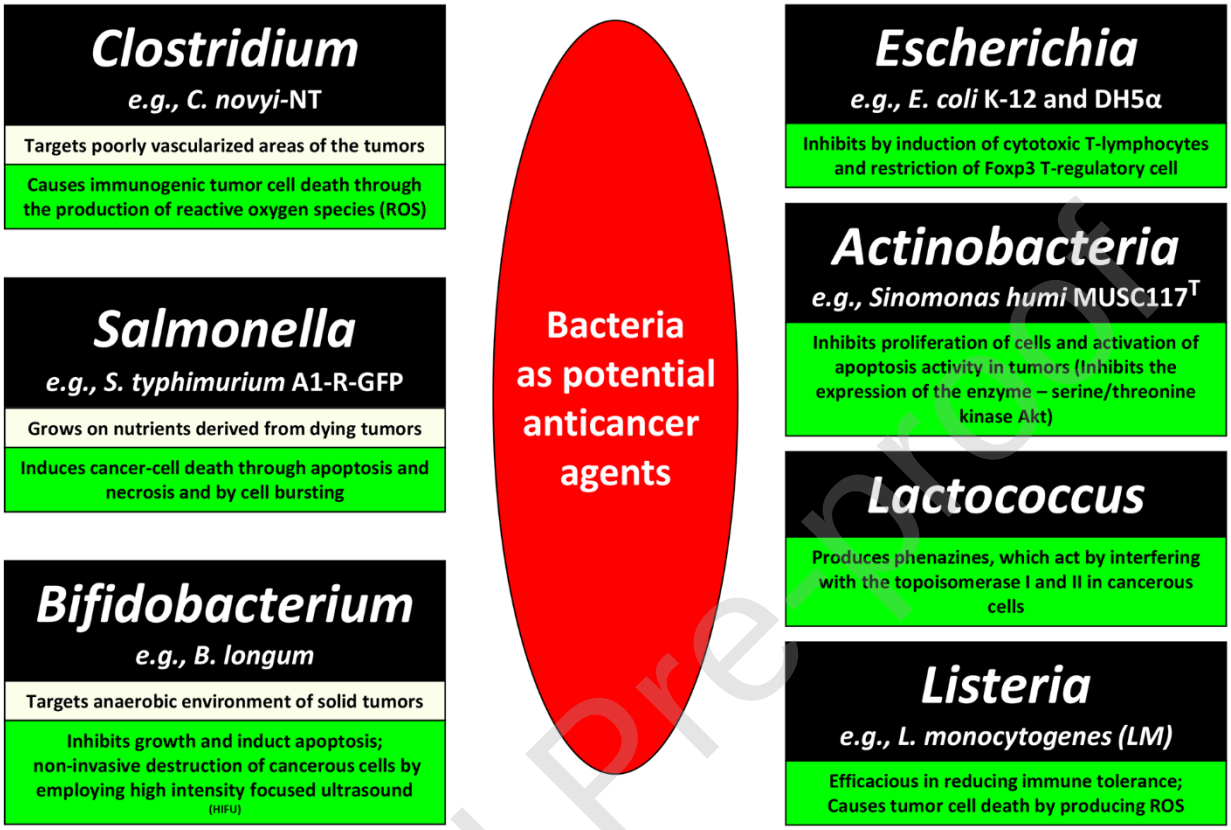

Fig. 3

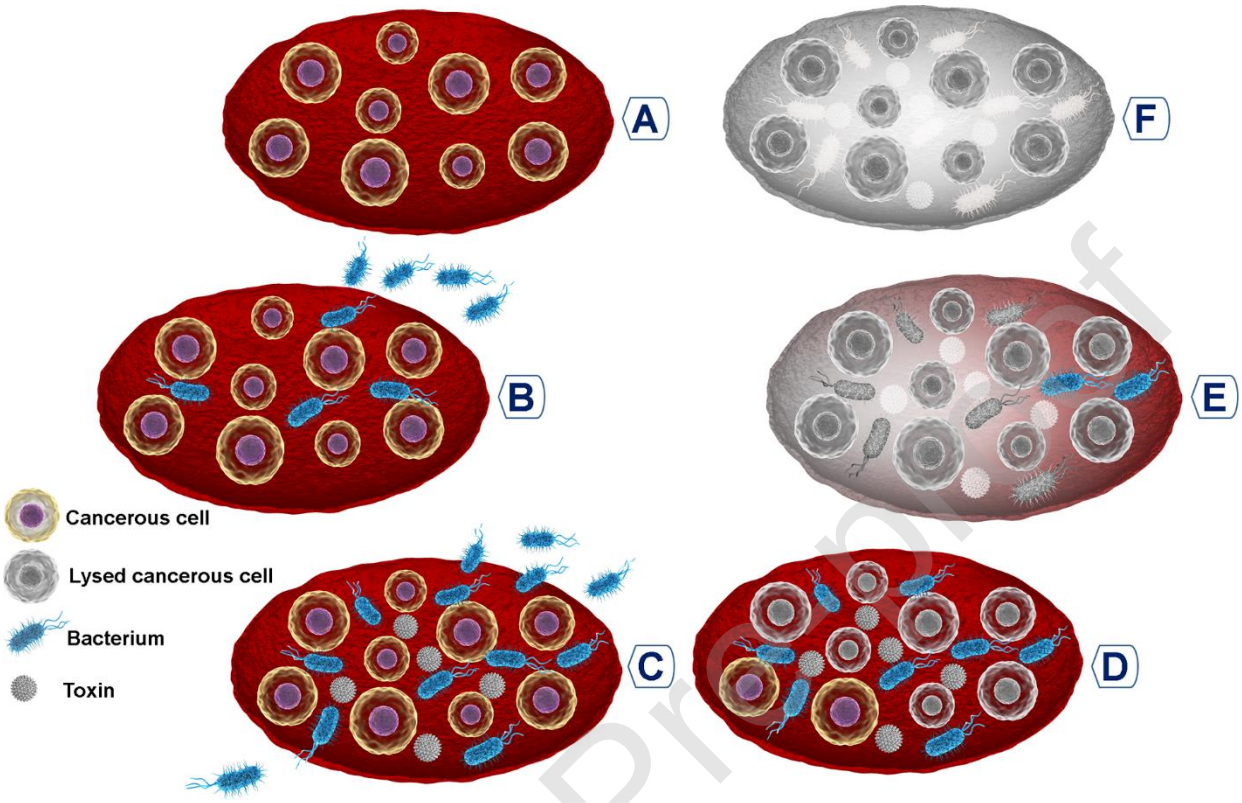

Table 1 Bacteria used for inhibiting tumors

Organisms	Host	Tumor	References
Clostridium novyi (NT)	BALB/c Nude	HCT116	[28]
	C57BL/6	B16	[28]
	BALB/c	CT-26, RENCA	[27]
	NZW	VX2	
Salmonella typhimurium strain VNP20009	Mice (Nude)	Melanoma, breast, lung, colorectal cell lines	[29,141]
S. typhimurium strain LH430	BALB/c Nude	SiHa (cc Xenograft)	[34]
	BALB/c	CMS5 (Sarcoma- Syngeneic) expressing human NY-ESO-I	[30]
	C57BL/6	H22 (HCC- Syngeneric), RM1	[33,35]
S. typhimurium (Auxotrophs)	BALB/c	CT-26	[20]
	DBA/2	P815	[21]
	Nude mice	MARY-X	[32]
	C57BL/6	B16G3.26	[64]
	BALB/c	CT-26	
Rec. *S. typhimurium* producing toxin-HlyE	BALAB/c	CT-26,4T1	[127,129]
Rec. *S. typhimurium* producing toxin - Stx2	Nude mice	B16, HCT1 16, HeLa	[48]
Rec. *S. typhimurium* producing antigen – *Listeria monocytogenes* $lap_{217-225}$ (LM-p60)	BALB/c	WEHI-164 cells expressing Lm-p60	[31]
Rec. *S. typhimurium* secreting murine death inducer – FasI	BALB/c	CT-26, D2F2	[133]
Rec. *S. typhimurium* secreting murine death inducer – Trail	BALB/c	4T1	[134]
Escherichia coli strain K-12	BALB/c	CT-26, 4T1	[60,128]
E. coli strain Nissle 1917	BALB/c	4T1	[135]
	C57BL/6	B16	
Rec. *E. coli* expressing toxin - LLO[a]	C57BL/6	MBL2, TRAMP-C	[66]

[a] Listeriolysin-O

Table 2 Biotechnological applications of microbial quorum sensing systems as anticancer agents

Organisms	QSS/molecule involved	Approaches	Applications	References
Salmonella (Non-pathogenic)	LuxI/LuxR (*Vibrio fischeri*)	QS genes fused with invasion gene from *Yersinia pestis* and anticancerous protein	Targeted killing of tumor cells	[142]
Escherichia coli	AI-2[b] producing anti- Epidermal Growth Factor receptors nanofactories	Bacteria by chemotaxis action of AI-2 colonizes cancerous cells and produces toxin	Targeted killing of cancerous cells	[107]
	3-oxo-C_6-HSL[c] synthesizing and detecting QSS[d]	Cancer stickybots: QS circuit fused with a toxin and a colon cancer cells specific carcino embryonic antigen detecting system	Specific killing of colon cancer cells	[106]
Serratia marcescens	SmaI/SmaR	AHL mediated prodigiosin synthesis	Treatment of cancer	[108]
Salmonella typhimurium	LuxI/LuxR (*V. fischeri*)	Arabinose induced P_{BAD} promoter under QSS to produce anti-cancer drug molecules	Inducible and specific anti-cancerous system	[109]
Pseudomonas aeruginosa	3-oxo-C_{12}-HSL	Downregulation of signal transducer and activator of transcription (STAT3) - Breast cancer (BR293, MCF-7, and MDA-MB-468)	Cancer therapy	[192]
		Cytoskeletal modifications resulting insusceptibility to 5-fluoroUracil in Human colorectal cancer (H630)	Mammalian cell viability	[193]
Synthetic AHL[a]	3-oxo-C_{12}-phenyldodecanoyl-L-HSL (Compound 12b)	Human colorectal carcinoma cell lines: (i) H630 (parental), (ii) H630-1 (5-fluorouracil resistant), and (iii) human prostate carcinoma cell line (PC3)	Cancer therapy	[110]
	AHLs (Compounds 5 and 87)	Tongue cancer cell line (SAS) and human gingival carcinoma cell line (Ca9-22)	Cancer therapy	[111]
Candida albicans	Farnesol	Oral squamous cell carcinoma (OSCC) lines	Anticancer therapeutic agent	[119,121]

[a] Acylhomserine lactone
[b] Auto-inducer
[c] Homoserine lactone
[d] Quorum sensing system