Bacteria for Targeting Tumors and Treating Cancer

FIELD OF THE INVENTION

The present invention relates to bacteria, a drug delivery composition, and methods of using the same for uses such as treating cancer.

BACKGROUND

Most antitumor drugs act against all actively dividing cells, resulting in serious or even lethal side effects. Targeted therapy has to be able to discriminate tumor from non-tumor tissues when systemically administered, so that both primary and disseminated tumors are treated.

Previous targeted therapy has relied on abiotic drugs. When systemically delivered, the abiotic drugs are dramatically diluted in the bloodstream, with only a small fraction being available for tumors. Moreover, the abiotic drugs depend on tumor vasculature for delivery and thus cannot effectively diffuse to poorly vascularized and hypoxic tumor tissues. Therefore, a variety of obligate or facultative anaerobes that are capable of post-delivery reproduction and prefer poorly vascularized tumor tissues, have been evaluated for their safety and efficacy in targeting tumors over the last decades. However, despite the increasing attention towards the bacterial therapy of cancers, its anticancer efficacy has so far been unsatisfactory.

In view of the demand for increasing the anticancer efficacy, more treatment methods and therapeutic agents that target tumors more specifically and kill tumors effectively are desired.

SUMMARY

One example embodiment of the present invention provides a series of bacteria for targeting tumors and treating cancer. Each of the bacteria includes a nucleic acid system and a gene encoding a cytotoxin which kills tumor cells but does not affect viability of the bacterium. The nucleic acid system includes a first DNA fragment encoding a toxin which kills the bacterium, a second DNA fragment encoding an antidote which negates the toxin, a promoter of an antidote gene, and a constitutive promoter of a toxin gene.

In some embodiments, the gene encoding the cytotoxin is a homologous gene. In some embodiments, the gene encoding the cytotoxin is a heterologous gene. In some embodiments, the bacterium for targeting tumors and treating cancer is a genetically modified bacterium.

In some embodiments, the bacterium includes a constitutive promoter of a cytotoxin. In some embodiments, the bacterium includes an inducible promoter of a cytotoxin such that the cytotoxin are expressed in tumor tissues but silenced in non-tumor tissues. In some embodiments, the bacterium includes a repressible promoter of a cytotoxin such that the cytotoxin are repressed in non-tumor tissues but expressed in tumor tissues.

In some embodiments, the second DNA fragment is transcribed at tumor tissues but not transcribed at non-tumor tissues. The promoter of the antidote gene operably linked to the second DNA fragment, represses transcription of the second DNA fragment under control of a glucose level such that the antidote is expressed at the tumor tissues but not expressed at the non-tumor tissues. The constitutive promoter of the toxin gene operably linked to the first DNA fragment, causes constitutive transcription of the first DNA fragment such that the toxin is expressed at the tumor tissues and the non-tumor tissues.

In some embodiments, the promoter of the antidote gene controls transcription of the antidote gene such that glucose represses the transcription of the antidote gene. In some embodiments, the constitutive promoter of the toxin gene causes constitutive expression of the toxin gene.

In some embodiments, the first DNA fragment is transcribed at non-tumor tissues but not transcribed at tumor tissues. The constitutive promoter of the antidote gene operably linked to the second DNA fragment, causes constitutive transcription of the second DNA fragment. The promoter of the toxin gene operably linked to the first DNA fragment, causes transcription of the first DNA fragment under control of a glucose level. Expression of the toxin is higher than constitutive expression of the antidote under the control of the glucose level such that the toxin kills the bacterium at the non-tumor tissues.

In some embodiments, the promoter of the toxin gene controls transcription of the toxin gene such that glucose induces the transcription of the toxin gene. In some embodiments, the constitutive promoter of the antidote gene causes constitutive expression of the antidote gene.

In some embodiments, the bacterium for targeting tumors and treating cancer grows at tumor tissues but does not grow at non-tumor tissues.

Another example embodiment provides a drug delivery composition which includes the bacterium for targeting tumors and treating cancer. Another example embodiment provides a method of treating cancer by administering the bacterium or the drug delivery composition.

In some embodiments, the cytotoxin is selected from a group consisting of Pseudomonas aeruginosa exolysin, Bacillus cereus non-hemolytic enterotoxin, Vibrio cholera hemolysin A and Escherichia coli alpha-hemolysin.

Another example embodiment relates to introducing a heterologous gene into a tumor-targeting bacterium or causing a tumor-targeting bacterium to

overexpress a homologous gene. The heterologous or homologous gene encodes a cytotoxin that kills tumor cells. In some embodiments, the cytotoxin encoded by a heterologous gene is selected from a group consisting of Pseudomonas aeruginosa exolysin, Bacillus cereus non-hemolytic enterotoxin, Vibrio cholera hemolysin A; and the cytotoxin encoded by a homologous gene is Escherichia coli alpha-hemolysin. Examples of tumor-targeting bacteria include, but not limited to bacteria that selectively colonize solid tumors and leave non-tumor tissues intact by sensing signals of tumor microenvironment, such as hypoxia, glucose deprivation and acidification, and accordingly control their own viability. These tumor-targeting bacteria may be natural or genetically modified bacteria.

Other example embodiments are discussed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows a toxin-antidote genetic system that targets a bacterium to low-glucose environments in accordance with an example embodiment.

Figure 1B shows a schematic diagram for constructing a nucleic acid system that targets *Escherichia coli* to low-glucose environments using CcdB as a toxin and CcdA as an antidote in accordance with an example embodiment.

Figure 1C shows a drug delivery composition that includes a genetically engineered bacterium that delivers anti-cancer drugs to solid tumors in accordance with an example embodiment. The anti-cancer drugs include but are not limited to anti-cancer molecules or compounds produced by the engineered bacterium.

Figure 1D shows clones that are streaked on M63 agar with different concentrations of glucose from 0 mM to 4 mM to search for those that fail to grow in the presence of glucose but glow in the absence of glucose in accordance with an example embodiment.

Figure 2A shows colonization of the genetically engineered bacterial strains JY1 and JY6, and an unmodified wild-type E. coli strain named MG1655 in CT26 (a murine colorectal cancer cell line) tumors of Bagg albino/c (BALB/c) mice 15 days after intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 2B shows colonization of the bacterial strains JY1, JY6, and MG1655 in the liver of BALB/c mice with CT26 tumors 15 days after intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 2C shows percentage of livers colonized by the bacterial strains JY1, JY6, and MG1655 in BALB/c mice with CT26 tumors 15 days after

intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 2D shows colonization of the bacterial strains JY1, JY6, and MG1655 in tumors in nude mice with HCT116 (a human colorectal cancer cell line) tumors 7 days after intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 2E shows colonization of the bacterial strains JY1, JY6, and MG1655 in the liver of nude mice with HCT116 tumors 7 days after intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 2F shows percentage of livers colonized by the bacterial strains JY1, JY6, and MG1655 in nude mice with HCT116 tumors 7 days after intravenous injection of the bacteria (10^7/mouse) in accordance with an example embodiment.

Figure 3A shows colonization of the bacterial strains JYH1 and SH1*hly* in liver and spleen of nude mice carrying subcutaneous SW480 (a human colorectal cancer cell line) tumors in accordance with an example embodiment.

Figure 3B shows percentages of liver and spleen infected by the bacterial strains JYH1 and SH1*hly* in nude mice carrying subcutaneous SW480 tumors in accordance with an example embodiment.

Figure 3C shows liver abscess developed in a mouse treated with the bacterial strain SH1*hly* in accordance with an example embodiment.

Figure 3D shows microscopic images of Hematoxylin and Eosin (H&E)-stained liver sections from nude mice carrying subcutaneous SW480 tumors that are treated with Phosphate-buffered saline (PBS), strains SH1*hly* and JYH1 in an accordance with an example embodiment.

Figure 4 shows the targeting efficacy of intravenously injected strains JYH1 on CT26 tumors of immunocompetent BALB/c mice in accordance with an example embodiment.

Figure 5 show a method to construct a genetically engineered bacterial strain that targets solid tumors by selectively living and growing in glucose-deprived regions in accordance with an example embodiment.

Figure 6A shows the inhibitory effect of intravenously injected *E.coli* JYH1 on the growth of HCT 116 tumors in nude mice in accordance with an example embodiment.

Figure 6B shows the inhibitory effect of intravenously injected *E.coli* JYH1 on the growth of SW480 tumors in nude mice in accordance with an example embodiment.

Figure 6C shows a representative photo of SW480 tumors treated by intravenous injection of PBS in mice from 0-55 days in accordance with an example embodiment.

Figure 6D shows a representative photo of SW480 tumors treated by intravenous injection of JYH 1 in mice from 0-90 days in accordance with an example embodiment.

Figure 7 shows an *in vitro* cytotoxicity assay in accordance with an example embodiment illustrating that the plasmid-derived production of cytotoxins ExlA, Nhe, E. coli alpha-hemolysin and V. cholera hemolysin A are cytotoxic to the cancer cell line B16F10. pBAD, an empty plasmid serving as a negative control; hlyCABD, the operon encoding for E. coli alpha-hemolysin and its secretion system; Vhly, the gene encoding V. cholera hemolysin A. Error bar, SD.

Figure 8 shows hemolytic activity of the plasmid-derived E. coli alpha-hemolysin and V. cholera hemolysin A in accordance with an example

embodiment. HlyCABD, the operon coding for E. coli alpha-hemolysin and its secretion system; Vhly, the gene encoding V. cholera hemolysin A. All the plasmids were introduced into a non-pathogenic and non-hemolytic E. coli reference strain TOP10.

Figure 9 shows that cytotoxins enhance the inhibitory ability of E. coli on murine syngeneic tumors in immunocompetent mice. A single dose of E. coli with or without expressing various cytotoxins was injected into murine melanoma B16F10 tumors in C57BL/6N mice. (A) Body weight of the mice over time. Error bar, SEM. (B) Effects of intratumorally injected bacteria (5 10^7/mouse) on tumor growth. The mice treated with bacteria carrying the empty plasmid pBAD and those treated with PBS (negative controls) were euthanized on day 9 after tumor measurement due to excessive tumor growth. Error bar, SEM. *, $P < 0.05$. (C) Representative photos of the B16F10 tumors injected with E. coli expressing the cytotoxins tested or E. coli carrying an empty vector pBAD on day 9 (i.e. 9 days after tumor challenge).

Figure 10 shows that production of E. coli alpha-hemolysin from the single copy of the hlyCABD operon in the chromosome is not required for the anticancer efficacy of hemolytic E. coli. A single dose of the bacteria ($1x10^7$ cfu per tumor) was injected into murine melanoma B16F10 tumors of C57BL/6N mice. hly+, an hemolytic E. coli strain carrying the hlyCABD operon in the chromosome; hly-, its isogenic mutant deleted for the entire hlyCABD operon. Error bar, SEM.

DETAILED DESCRIPTION

Example embodiments relate to a nucleic acid system. The nucleic acid system is introduced into a bacterial strain so that a genetically engineered bacterial strain targets solid tumors but leaves normal tissues intact. The genetically engineered bacterial strain grows at tumor tissues but die at non-tumor tissues.

Other example embodiments relate to a bacterium for targeting tumors and treating cancer. The bacterium includes a heterologous or homologous gene encoding a cytotoxin which kills tumor cells, and the nucleic acid system. It shall be understood that the genetic modifications made to create the bacterium for targeting tumors and treating cancer can be done in any order. For example, insertion of the nucleic acid system into the bacterium can be performed first, followed by insertion of the cytotoxin-encoding gene to kill tumor cells. Alternatively, insertion of the heterologous or homologous gene that encodes the cytotoxin that kills tumor cells can be performed first, followed by insertion of the nucleic acid system.

The cytotoxin cannot be expressed at non-tumor tissues because the bacterium does not survive or live at the non-tumor tissues. In some embodiments, the bacterium grows at tumor tissues but does not grows at non-tumor tissues.

Hypoxia is the most commonly utilized feature of tumor microenvironments for targeting bacteria to solid tumors. Obligate anaerobes that strictly target hypoxia, however, are confined to necrotic regions of solid tumors, whereas facultative anaerobes colonize throughout solid tumors but infect normal tissues due to its loose control of hypoxia targeting. Example embodiments in accordance with the invention solve these technical problems by introducing into bacteria a nucleic acid system that improves the tumor specificity of bacteria by regulating glucose-dependent viability of the bacteria.

Bacteria can produce various cytotoxins which destroy mammalian cells in diverse mechanisms. Some cytotoxins efficiently kill most epithelial and endothelial cells. However, if a bacterium is administered to a patient, the cytotoxin expressed by the bacterium may not only damage tumor cells but also normal cells. Example embodiments solve these technical problems by introducing a cytotoxin-encoding gene into a genetically engineered bacterium or a natural bacterium with tumor specificity such that the cytotoxin only damages tumor cells, but keeps normal cells intact. Alternatively, damage of normal tissues by cytotoxins may also be avoided by inducibly expressing the cytotoxins under some inducible or repressible promoters, such as glucose-repressible promoters, so that the cytotoxins are expressed only in tumor tissues but silenced in non-tumor tissues. Further, the cytotoxin-encoding gene confers the bacterium additional ability to combat cancer.

Cytotoxins of an example embodiment include but are not limited to Pseudomonas aeruginosa exolysin, Bacillus cereus non-hemolytic enterotoxin, Vibrio cholera hemolysin A and Escherichia coli alpha-hemolysin.

Exolysin (ExlA) is a pore-forming toxin excreted by Pseudomonas aeruginosa. ExlA has lytic capacity on most cell types such as epithelial, endothelial and fibroblastic cells and macrophages but is poorly hemolytic. Non-hemolytic enterotoxin (Nhe) is the major toxin found in Bacillus cereus. Nhe induces cell lysis by triggering pore formation on the membrane of mammalian cells, induces cell cycle arrest at G0 /G1 phase and provokes cell apoptosis, but does not cause hemolysis. Vibrio cholera hemolysin A causes cell lysis by forming pores on eukaryotic cell membrane. In contrast to ExlA and Nhe, Vibrio cholera hemolysin A is not only cytolytic but also hemolytic. Escherichia coli alpha-hemolysin is produced by uropathgenic E. coli. Similar to Vibrio cholera hemolysin A, E. coli alpha-hemolysin is both cytolytic and hemolytic. It is cytotoxic to not only epithelial cells but also macrophages and natural killer cells, counteracting the host defense against E. coli.

Another embodiment provides a method of treating cancer in a patient in need thereof comprising the steps of administering to the patient a bacterium for

targeting tumors and treating cancer. In some embodiments, the expressed cytotoxin causes tumor cell lysis so as to treat the cancer. In some embodiments, the bacterium is administered in combination with one or more additional cancer therapies, such as surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, hormone therapy, or a stem cell transplant. When a therapy is administered in combination with another, the administration can be sequential or can be co-administered. In one embodiment, the cancer is melanoma.

Another embodiment provides a method of lysing tumor cells comprising the steps of contacting a bacterium which targets tumors and treats cancer with a tumor cell. In some embodiments, the method is an *in vitro* method. In other embodiments, the method is an *in vivo* method.

Figure 1A shows a nucleic acid system 100 including a toxin-encoding gene that is constitutively expressed, and an antidote-encoding gene under the control of a glucose-repressed promoter. The nucleic acid system confers bacteria the ability to target low-glucose environments in accordance with an example embodiment.

The toxin-antidote genetic system enables bacteria to selectively grow in glucose-deprived environments but die in the presence of glucose. As glucose deprivation is a feature of solid tumor microenvironments, the bacteria equipped with the nucleic acid system can specifically target solid tumors when applied systemically. Tumor cells are commonly deprived of glucose due to fast cell growth and excessive glucose consumption as well as inadequate blood supply. A glucose concentration is 0.123-0.424 mM in tumor tissues, and a glucose concentration is 1.22-1.29 mM in normal tissues, assuming 1g of tissues is 1ml. The toxin-antidote nucleic acid system enables the bacteria to selectively grow under low-glucose environments.

The nucleic acid system confers the bacteria the ability to selectively grow under low-glucose condition, which is a feature of tumor microenvironment. The bacteria such as *E. coli* have the intrinsic ability of preferentially growing

in solid tumors and colonizing normal tissues to a lesser extent, due to the heavily immune-suppressed microenvironment of tumors. The nucleic acid system that targets low-glucose environments confers higher tumor selectivity to the bacteria such as *E. coli* that is not sufficiently tumor specific to be used on its own, improving safety of bacterium-mediated tumor therapy.

In an example embodiment, the nucleic acid system that is a tumor-targeting system, is integrated into the chromosome of the bacteria, such that the bacteria do not solely depend on their natural ability to target tumors and in turn the safety of the bacteria is improved. In an example embodiment, the nucleic acid system is inserted into a plasmid. In an example embodiment, the nucleic acid system is a glucose-sensing system or module.

In an example embodiment, the bacteria that carry the nucleic acid system strictly colonize solid tumors by targeting low-glucose environments.

In an example embodiment, the nucleic acid system includes a toxin-encoding gene, an antidote-encoding gene, a glucose-repressed promoter that controls the transcription of the antidote-encoding gene, and a constitutive promoter that causes constitutive expression of the toxin-encoding gene.

In an example embodiment, in environments with physiological levels of glucose, the toxin is constitutively expressed whereas the antidote expression is repressed by glucose under the control of the glucose-repressed promoter. The bacteria that carry the nucleic acid system do not grow in environments with physiological levels of glucose because the antidote is not expressed to neutralize the toxin. In low-glucose environments, both the toxin and the antidote are expressed. The bacteria that carry the nucleic acid system grow in low-glucose environments because the antidote neutralizes the toxin.

In an example embodiment, the nucleic acid system includes a toxin-encoding gene, an antidote-encoding gene, a glucose-induced promoter that controls the transcription of the toxin-encoding gene, and a constitutive promoter that causes constitutive expression of the antidote-encoding gene.

In an example embodiment, in environments with physiological levels of glucose, the antidote is constitutively expressed whereas the toxin expression is induced by glucose under the control of the glucose-induced promoter. The bacteria that carry the nucleic acid system do not grow in environments with physiological levels of glucose because the toxin is expressed to a level higher than the expression of the antidote and thereby kills the bacteria. In low-glucose environments, the toxin is not expressed so that the bacteria live and grow.

In an example embodiment, the low-glucose environments include glucose at a concentration lower than 0.424 mM. In an example embodiment, the high-glucose environments include glucose at a concentration higher than 1.22 mM. In an example embodiment, the low-glucose environments have glucose at a concentration of 0.123-0.424 mM. In an example embodiment, the high-glucose environments have glucose at a concentration of 1.22-1.29 mM.

In an example embodiment, in solid tumors, a level of the expressed antidote is higher or equivalent to that of the expressed toxin so that the toxicity of the toxin is antagonized by the antidote.

In an example embodiment, the tumor-targeting nucleic acid system is a nucleic acid system that includes a first DNA fragment that encodes a toxin gene that expresses a toxin, a second DNA fragment that encodes an antidote gene that expresses an antidote that negates the toxin, a first promoter (i.e. a promoter of antidote gene) and a first constitutive promoter (i.e. a constitutive promoter of a toxin gene). The first constitutive promoter causes constitutive expression of the toxin gene. The first promoter regulates transcription of the second DNA fragment under the control of glucose concentration, such that the second DNA fragment is transcribed under low-glucose environments or in the absence of glucose, but not transcribed in the presence of glucose or under high-glucose environments. In an example embodiment, the second DNA fragment is transcribed in the absence of

glucose but not transcribed under high-glucose environments whose concentration is equal to or higher than 1 mM in M63 medium.

In an example embodiment, the first promoter (i.e. the promoter of the antidote gene) controls transcription of the antidote gene, such that glucose represses the transcription of the antidote gene. The second DNA fragment is transcribed in solid tumors but not transcribed in non-tumor tissues.

In an example embodiment, the tumor-targeting nucleic acid system is a nucleic acid system that includes a first DNA fragment that encodes a toxin gene that expresses a toxin, a second DNA fragment that encodes an antidote gene that expresses an antidote that negates the toxin, a second promoter (i.e. a promoter of a toxin gene) and a second constitutive promoter (i.e. a constitutive promoter of an antidote gene). The second constitutive promoter causes constitutive expression of the antidote gene. The second promoter regulates transcription of the first DNA fragment under the control of glucose concentration, such that the first DNA fragment is transcribed under high-glucose environments or in the presence of physiological levels of glucose, but not transcribed in the absence of glucose or under low-glucose environments. In an example embodiment, the first DNA fragment is not transcribed in the absence of glucose but transcribed under high-glucose environments whose concentration is equal to or higher than 1 mM in M63 medium.

In an example embodiment, the second promoter controls transcription of the toxin gene, such that glucose induces the transcription of the toxin gene. The first DNA fragment is transcribed in the non-tumor tissues but not transcribed in the solid tumors. In an example embodiment, the expression of the toxin is higher than the constitutive expression of the antidote at the non-tumor tissues.

In an example embodiment, the first DNA fragment is shown as SEQ ID No.1. In an example embodiment, the second DNA fragment is shown as SEQ ID No.2.

In an example embodiment, the first DNA fragment is located upstream of the second DNA fragment. In an example embodiment, the first DNA fragment is located upstream of the first promoter. In an example embodiment, the first DNA fragment is located downstream of the second DNA fragment. In an example embodiment, the second promoter is located upstream of the first DNA fragment. In an example embodiment, the first constitutive promoter is located upstream of the first DNA fragment. In an example embodiment, the second constitutive promoter is located upstream of the second DNA fragment. In an example embodiment, the first promoter is shown as SEQ ID No.3.

In an example embodiment, the nucleic acid system includes a random sequence that consists of 5 – 6 nucleotides, and is located immediately upstream of the second DNA fragment and downstream of the first promoter. In an example embodiment, the random sequence is GCCTT or TGTCT.

In an example embodiment, the nucleic acid system includes a random sequence that consists of 5 – 6 nucleotides, and is located immediately upstream of the first DNA fragment and downstream of the second promoter.

In an example embodiment, the nucleic acid system includes a random sequence that consists of 5 – 6 nucleotides, and replaces original or native 5 – 6 nucleotides of the bacteria that are located immediately upstream of the second DNA fragment.

In an example embodiment, the nucleic acid system includes a random sequence that consists of 5 – 6 nucleotides, and replaces original or native 5 – 6 nucleotides of the bacteria that are located immediately upstream of the first DNA fragment.

In an example embodiment, the nucleic acid system includes a random sequence that consists of 5 – 6 nucleotides, and replaces original or native 5 – 6 nucleotides of the first promoter. In an example embodiment, the nucleic

acid system includes a random sequence that consists of 5 – 6 nucleotides, and replaces original or native 5 – 6 nucleotides of the second promoter.

In an example embodiment, the random sequence is located downstream of the first promoter. In an example embodiment, the random sequence is located immediately upstream of the second DNA fragment.

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In an example embodiment, the nucleic acid system includes a third DNA fragment that encodes a selectable marker. In an example embodiment, the marker is chloramphenicol selectable marker (Cm^R). In an example embodiment, the selectable marker is a chloramphenicol resistance cassette. In an example embodiment, the third DNA fragment is shown as SEQ ID No. 4.

In an example embodiment, the third DNA fragment is located downstream of the first DNA fragment and upstream of the first promoter. In an example embodiment, the third fragment is located downstream of the second DNA fragment and upstream of the second promoter.

In an example embodiment, the nucleic acid system includes SEQ ID No.1, SEQ ID No.2, SEQ ID No.3, and SEQ ID No.4. In an example embodiment, the nucleic acid system includes a constitutive promoter to drive expression of ccdB as shown in SEQ ID No. 5. In an example embodiment, the nucleic acid system includes a rrnB transcription termination region as shown in SEQ ID No. 6. In an example embodiment, the nucleic acid system is shown as SEQ ID No. 7. In an example embodiment, the nucleic acid system is shown as SEQ ID No. 8.

In an example embodiment, the nucleic acid system includes a toxin gene, an antidote gene, a first promoter that controls transcription of the antidote gene,

and a constitutive promoter for the toxin gene. In an example embodiment, the nucleic acid system includes a toxin gene, an antidote gene, a second promoter that controls transcription of the toxin gene, and a constitutive promoter for the antidote gene.

In an example embodiment, the bacterial strain is a Gram-positive bacterial strain. In an example embodiment, the bacterial strain is a Gram-negative bacterial strain.

In an example embodiment, the bacterial strain is *Escherichia coli*. In an example embodiment, the bacterial strain is selected from a group consisting of *Escherichia coli* MG1655 and *Escherichia coli* SH1.

Figure 1B shows a schematic diagram 110 for constructing a nucleic acid system that targets *E. coli* to low-glucose environments in accordance with an example embodiment. Figure 1B shows that a randomized, glucose-repressed lactose promoter (Plac) and a CcdA/CcdB toxin-antidote pair are employed to construct a nucleic acid system that targets *E. coli* to low-glucose environments.

CcdB is a toxin that kills host bacteria, and CcdA is an antidote to counteract CcdB. In the tumor-targeting nucleic acid system, CcdB is constitutively expressed whereas CcdA expression is repressed by glucose under the control of the glucose-repressed lactose (lac) promoter. In low-glucose environments, bacteria carrying this nucleic acid system shall grow well because the antidote CcdA is de-repressed to neutralize CcdB. In the presence of physiological levels of glucose, CcdA expression is turned off and CcdB is freed up to kill the bacteria.

In an example embodiment, in the tumor-targeting nucleic acid system, CcdA is constitutively expressed whereas CcdB expression is induced by glucose under the control of the glucose-induced promoter. In low-glucose environments, bacteria carrying this nucleic acid system shall grow well

because the toxin CcdB is repressed. In the presence of physiological levels of glucose, CcdB expression is turned on to kill the bacteria.

As shown in Figure 1B, a randomized fragment composed of 5 – 6 nucleotides (nnnnn) replaces the original or native 5-6 nucleotides immediately upstream of the start codon of the ccdA gene (one example of the second DNA fragment). Different sequences in this randomized fragment result in different levels of the ccdA expression. With some sequences, the glucose levels in tumors are low enough to activate the expression of CcdA to antagonize the toxicity of CcdB, and the glucose levels in normal tissues are high enough to shut down the CcdA expression under the control of the lac promoter. A selectable marker, such as chloramphenicol selectable marker (Cm^R) is also used in the nucleic acid system of Figure 1B.

The nucleic acids systems with different sequences in the randomized fragment (a random library of the nucleic acid systems or DNA pool) are inserted into the chromosome of *E. coli* and the bacteria are then streaked on lysogeny broth (LB) agar plates with glucose (Glc (+)) or without glucose (Glu (-)) to screen those that fail to grow in the presence of glucose but grow in the absence of glucose. The arrow 112 indicates a clone that grows in glucose-negative medium but does not grow in medium with glucose. In an example embodiment, the concentrations of glucose on LB agar plates are 0 mM or 5 mM.

Figure 1C shows a drug delivery composition 120 that includes a genetically engineered bacterium 122 that not only specifically targets solid tumors but also delivers to solid tumors anti-cancer drugs 124 by producing anti-cancer molecules or compounds.

The genetically engineered bacterium 122 delivers the drug 124 to solid tumors and kills the tumor cells. The genetically engineered bacterium 122 includes the nucleic acid system discussed herein, such that the bacterium grows in the solid tumors but not grow in non-tumor tissues.

The following examples are provided illustrating various embodiments.

Example 1

Materials and Methods

Construction of a random library of engineered *E. coli* that targets low-glucose environments:

The tumor-targeting nucleic acid system designed in this example was composed of a constitutively expressed ccdB gene and a glucose-repressed ccdA gene under the control of a lac promoter. The antidote CcdA is repressed in the presence of physiological levels of glucose so that the toxin CcdB kills the bacteria. In contrast, the bacteria are alive under the low-glucose growth conditions because the expression of CcdA is de-repressed and counteracts the action of CcdB. To improve the capacity of CcdA in antagonizing CcdB under the control of the lac promoter under the low-glucose conditions or enhancing the ability of CcdB to kill bacteria in the presence of glucose, a random library of the tumor-targeting nucleic acid system was constructed by randomizing the 5 nucleotides immediately upstream of the start codon of the ccdA gene. To facilitate the genetic engineering on the chromosome by the λ-Red recombination technique, a selectable marker, chloramphenicol selectable marker (Cm^R), was included in the tumor-targeting nucleic acid system as illustrated in Figure 1B.

Specifically, a set of DNA fragments (i.e. tumor-targeting nucleic acid systems) that contains the ccdB gene under the control of a constitutive promoter, a selectable marker (such as the loxP-cat-loxP cassette), a 5 nucleotides (5nt)-randomized region, and the ccdA gene under the control of a glucose-repressed promoter (such as a lac promoter) was generated by overlapping polymerase chain reaction (PCR) as shown in Figure 1B. The 5nt-randomized region allows for generating a random library of the nucleic acid systems. The selectable marker makes it possible to insert the nucleic acid systems into the chromosome of bacteria by recombineering. Then, the library of the nucleic acid systems was inserted into the chromosome of *E. coli*, using the λ-Red

recombineering technique. After 1h-recovery in glucose-deprived LB, the bacterial culture was spread on glucose-deprived LB agar supplemented with antibiotics (12.5 μg/ml chloramphenicol if the loxP-cat-loxP cassette was used as the selectable marker). After overnight culture at 32ºC, individual colonies formed on the agar. Each colony is derived from replication of a single *E. coli* that has the potential to selectively grow under low-glucose conditions. These colonies formed a random library of putative tumor-targeting bacteria. Here, the CcdB-CcdA pair could be replaced by other toxin-antidote pair.

Library screen for bacteria targeting glucose-deprived environment:
Glucose-deprived LB medium was used for library screen for bacteria that selectively grew under low-glucose conditions. To screen the random library, each of the clones was streaked both on the glucose-deprived LB agar and LB agar plus 5 mM glucose. After overnight culture at 37ºC, clones that were found to grow readily on the glucose-deprived LB agar but not to grow on glucose-positive LB agar were further assessed using the minimal M63 medium agar. The M63 agar was supplemented with increasing concentrations of glucose in addition to 30 mM glycerol. Here, bacteria strains other than E. coli MG1655 could be used for screening for tumor-targeting bacteria using the same strategy.

In vivo assessment of the tumor-targeting efficacy of engineered bacteria:
Six- to eight-week-old nude mice were used for tumor implantation of human cancer cell lines, and six- to eight-week-old immunocompetent BALB/c mice were used for tumor implantation of murine derived cell lines. 1×10^7 of bacteria were injected into the tail vein of each mouse. Tumor size was measured using digital calipers every three days following the bacterial injection. At the end of the experiments, mice were euthanized and their tumors and organs were removed for determination of colony forming unit. Specifically, 1 gram of tissues was homogenized in 1 ml of Phosphate-buffered saline (PBS) buffer. The resulting tissue suspensions were serially diluted and plated, and colony forming units of the diluted suspensions were counted. The number of bacteria in each tissue was calculated according to

dilution ratio. Bacteria were regarded as being able to specifically target tumors if they were present in tumors but absent from organs.

In an example embodiment, the tumor-targeting nucleic acid system is composed of the constitutively expressed ccdB gene, a Cm^R cassette and lac promoter-controlled ccdA with a 5nt-random sequence being located immediately upstream its start codon. These elements are not necessarily placed in the order as shown in Figure 1B. In an example embodiment, the tumor-targeting nucleic acid system is composed of the constitutively expressed ccdA gene, a Cm^R cassette and a glucose-induced promoter-controlled ccdB with a 5nt-random sequence being located immediately upstream its start codon. The ccdB-ccdA pair can be replaced by other toxin-antitoxin pair. Cm^R can be replaced by other selectable markers. The number of nucleotides in the random sequence is not confined to five.

Gene cloning:

Genes encoding cytotoxins P. aeruginosa ExlA, B. cereus Nhe, V. hemolysin A and E. coli alpha-hemolysin were synthesized and cloned in a pBAD plasmid using CloneEZ seamless cloning technology by GenScript. All the recombinant plasmids were verified by sequencing analysis.

In vitro cytotoxicity assay:

Each of the cell line tested was seeded in 96-well plates at 1 X 10^4 cells per well in appropriate growth medium. When the cells grew to 80% confluency, they were co-cultured with E. coli strains tested at a moi of 100 (i.e. 100 bacteria per cell). As controls, the cells were also co-cultured with PBS alone. After 4-12 hours of incubation in antibiotic-free medium, the cells were washed thrice with PBS and stained with 1% crystal violet for 5 min. Only viable cells were stained because dead cells were removed by washing. The stained cells were gently washed with PBS and then destained with 95% ethanol. The amount of the crystal violet stain, which reflects the quantity of viable cancer cells, in the destaining solution was measured with a microtiter plate reader at 595 nm. Percentage of cells killed by the co-cultured bacteria

was calculated using the formula: (control - treat)/control x 100. All the experiments were performed in quadruplicate on two independent occasions.

Hemolysis assay:

Overnight cultures of bacteria were dropped on LB agar supplemented with sheep blood and then incubated at 37°C for 8 - 10 hours. Hemolysis as a result of breakdown of red blood cells was revealed by clearing of the agar.

In vivo assessment of efficacy of engineered E. coli on tumors:

Six- to eight-week-old female C57BL/6N mice were used for subcutaneous tumor implantation of human or murine cancer cell lines. 10^5-10^6 cells of the cell line tested were injected subcutaneously into the flank of each mouse. 10-15 days after the cell line injection when the average volume of tumors reached about 150-300 mm^3, 10^7 of bacteria were injected into the tail vein of each mouse or 5 x 10^7 of bacteria were directly injected into each tumor. Tumor volume and body weight were measured every three days following the bacterial injection. Tumor volume was calculated with the formula (longest diameter) x (shortest diameter)^2 x 0.52. Body weight without tumor weight was calculated by subtracting estimated tumor weight from body weight (1000 mm^3 of tumor tissue was assumed as 1 g).

Example 2

E. coli MG1655 was used in this example. The constitutively expressed CcdB, the lac promoter-controlled CcdA, the lac promoter, and Cm^R were used to construct the tumor-targeting nucleic acid system. The ccdB gene was located upstream of the Cm^R cassette that was located upstream of the lac promoter. A random library of the putative tumor-targeting bacteria was generated by inserting a randomized fragment (5 nucleotides in this example) in the lac promoter immediately upstream of the ccdA gene. The random library was chromosomally established in the *E. coli* strain MG1655. In this random library, each *E. coli* MG1655 variant carried in the chromosome a lac promoter variant with a distinctive 5 nucleotide-sequence in the randomized domain. The random library was screened to search for bacterial clones that selectively grew under low-glucose conditions. Specifically, the library was

established on LB agar depleted for glucose. The resulting *E. coli* clones were then individually streaked on both LB agar with 5 mM glucose and LB agar without glucose to screen for those that failed to grow in the presence of glucose but grew in the absence of glucose. Approximately 1500 clones were screened and 6 clones were found to preferentially grow in glucose-negative medium.

Figure 1D shows drawings 130 for the sensitivity of six *E. coli* clones to glucose on minimal medium M63 agar. The 6 clones were purified, incubated overnight, and serially diluted. 10 μl of each diluted suspension was dropped on minimal medium M63 supplemented with increasing concentrations of glucose (Glu) from 0mM Glu to 4mM Glu to verify their phenotype. Among the 6 clones, the 1st and the 6th clones grew well on glucose-negative medium agar, but grew poorly in the presence of glucose. In the contrast, the 2nd, 3rd, 4th and 5th clones displayed considerable growth in both glucose-negative and glucose-positive medium agar. Given this, the 1st and the 6th clones (boxed in figure 1D) were candidates of tumor-targeting bacteria and named JY1 and JY6 (also named JY8), respectively. If more library screening was carried out, more glucose-sensing clones could be identified.

The random sequence upstream of the ccdA gene in the chromosome of the clone JY1 is GCCTT. The nucleotide sequence of JY1 includes a sequence as shown in SEQ No. 5. The random sequence upstream of the ccdA gene in the chromosome of the clone JY6 is TGTCT.

The strain JY1 was deposited at the China General Microbiological Culture Collection Center (CGMCC) under deposit no. 14577 with deposit date of 30 August 2017. The strain JY6 was deposited at the CGMCC under deposit no. 14578 with deposit date of 30 August 2017.

Example 3

The engineered *E. coli* variants JY1 and JY6 were sensitive to glucose and failed to grow in the presence of glucose *in vitro*. This example provides *in*

vivo experiments and data showing that glucose levels in tumors were low enough for JY1 and JY6 to survive and grow. JY1 and JY6 were separately injected into the tail vein of immunocompetent BALB/c mice with CT26 (a murine colorectal cancer cell line) tumors (10^7 cfu/mouse). The parental strain MG1655 was employed as a control. 15 days after the tail vein injection, the bacteria were analyzed for their distribution in tumor and liver. The liver was chosen for the analyses because it is more vulnerable to bacterial infection than other organs.

Figures 2A-2F show *E. coli* JY1 and JY6 specifically targeted tumors in mice. Error bar, SEM. *P < 0.05, **P < 0.01.

Figure 2A shows colony forming unit (CFU) 200 per gram of CT26 tumors in BALB/c mice. As shown in Figure 2A, JY1, JY6, and MG1655 comparably colonized the CT26 tumors in the BALB/c mice and their levels in the tumors were over 10^8 cfu/g. These data showed that both JY1 and JY6 were good colonizers in the CT26 tumors carried by the immunocompetent BALB/c mice.

Figure 2B shows CFU 210 per gram of liver in BALB/c mice with CT26 tumors. As shown in Figure 2B, JY1 did not colonize the liver of BALB/c mice with CT26 tumors. JY6 colonized the liver of BALB/c mice with CT26 to a less extent than MG1655 did. Figure 2C shows percentages 220 of livers colonized by JY1, JY6 and MG1655 in BALB/c mice with CT26 tumors. As shown in Figure 2C, JY6 was detected in the livers of 20% of the BALB/c mice with CT26 tumors (1 out of 5) and JY1 was not detected in the liver of any mouse, while MG1655 colonization occurred in the livers of 60% of the mice (3 out of 5). These showed that JY1 and JY6 are more specific to tumors than MG1655 and that JY1 is more specific to tumors than JY6.

Further plating analyses showed that JY1 was also absent from blood and organs including spleen, heart, lung and kidney of the immunocompetent mice. Although JY1 and JY6 displayed comparable ability to colonize the CT26 tumors, JY1 was superior in specifically targeting the tumors than JY6 in the immunocompetent mice.

Similar experiments were performed in immunocompromised nude mice carrying subcutaneous HCT116 (a human colorectal cancer cell line) tumors. 7 days after the tail vein injection of the bacteria (10^7 cfu/mouse), the bacteria were analyzed in their distribution in tumor and liver. Figure 2D shows CFU 230 per gram of the HCT116 tumors in nude mice. As shown in Figure 2D, JY1, JY6, and MG1655 colonized the HCT116 tumors in the nude mice. Figure 2E shows CFU 240 per gram of liver in nude mice with HCT116 tumors. As shown in Figure 2E, JY1 did not colonize the liver of nude mice with HCT116 tumors. JY6 colonized the liver of nude mice with HCT116 tumors to a less extent than MG1655 did. Figure 2F shows percentage 250 of livers colonized by JY1, JY6 and MG1655 in nude mice with HCT116. As shown in Figure 2F, JY6 and MG1655 were detected in the livers of 28.57% (2 out of 7) and 85.71% (6 out of 7) of the nude mice, respectively. Again, JY1 was not present in the liver of any mouse (n=6). These showed that JY1 and JY6 are more specific to tumors in immunocompromised mice than MG1655 and that JY1 is more specific to tumors than JY6 in immunocompromised mice.

To ensure that JY1 did not infect normal tissues, blood and homogenized suspensions of the spleen, heart, lung and kidney of each mouse in the bacteria-treated group were further examined. All these were cleared of JY1. Although JY1 avoided infecting organs, it readily colonized the HCT116 tumors and its levels in the tumors reached 3.79 x 10^7 cfu/g (Figure 2D). Taken together, the *in vivo* data demonstrate that the tumor-targeting nucleic acid system carried by the bacteria JY1 and JY6 enables them to specifically target solid tumors in both immunocompetent and immunocompromised mice, and JY1 was superior to JY6 in the ability of targeting solid tumors.

Example 4

The glucose-targeting nucleic acid system carried by JY1 was next grafted into the chromosome of *E. coli* SH1, to show that this nucleic acid system is not confined to a particular bacterial strain.

E. coli SH1 was isolated from a stool sample provided by a healthy female volunteer. The stool sample was resuspended in PBS buffer and spread on LB agar supplemented with 1mM isopropyl β-D-thiogalactoside (IPTG) and X-gal (0.06 mg/ml). *E. coli* formed blue colonies and were discriminated from other bacteria species. SH1 is one of the fecal *E. coli* isolates. The strain SH1 was deposited at the CGMCC under deposit no. 14580 with deposit date of 30 August 2017.

The resulting recombinant *E. coli* strain was referred to as JYH1. The strain JYH1 was deposited at the CGMCC with deposit no. 14579 with deposit date of 30 August 2017.

JYH1 was then intravenously injected into nude mice carrying subcutaneous SW480 (a human colorectal cancer cell line) tumors. The mice were analyzed for bacterial colonization in tumors and organs 90 days after the intravenous injection of the bacteria. Because the tumors of four JYH1-treated mice were completely cured, only two tumors in this group were available for analysis. JYH1 was detected from one of the two tumors, reaching 1.8 x 10^8 cfu per gram.

It shows that when the module or the nucleotide system is introduced into *E. coli* SH1, the resulting strains can not only target tumors but also treat tumors.

Figures 3A and 3B show *E. coli* JYH1 specifically targeted SW480 tumors and did not colonize normal tissues in nude mice, while its isogenic strain SH1*hly* that was not engineered by the tumor-targeting nucleotide system colonized both tumors and normal tissues. *P < 0.05. Error bar, SEM.

Figure 3A shows CFU 300 per gram of liver and spleen tissues in nude mice with SW480. Figure 3B shows percentage 310 of liver and spleen organs infected by JYH1 and SH1*hly*. As shown in Figures 3A and 3B, JYH1 did not colonized liver or spleen. The livers, spleens, hearts, lungs, kidneys of all the JYH1-treated nude mice were all cleared of JYH1, indicating that the glucose-sensing module is able to confine JYH1 to tumors and prevent it from

spreading to distant organs for a long time. In contrast to JYH1, its isogenic strain SH1*hly* that is not equipped with the glucose-sensing module colonized not only tumors ($9.35 \times 10^8 \pm 5.97 \times 10^8$ cfu/g, mean ± SEM) but also organs. The livers ($6.0 \times 10^{10} \pm 6.0 \times 10^{10}$ cfu/g, mean ± SEM) and spleens ($1.85 \times 10^5 \pm 9.74 \times 10^4$ cfu/g, mean ± SEM) of four SH1*hly*-treated mice (80%, 4 out of 5) were infected by SH1*hly* when analyzed on day 82.

Among the infected mice, one mouse developed liver abscess as shown in Figure 3C. The picture 320 was taken on day 82 when the mouse was euthanized for analysis.

Tumor specificity of JYH1 and its requirement of the glucose-sensing, tumor-targeting nucleic acid system were also confirmed by Hematoxylin and Eosin (H&E) staining of liver sections 330, which showed that the liver of JYH1-treated mice was normal whereas massive inflammatory infiltration and abscess occurred in the liver of SH1*hly*-treated mice as shown in Figure 3D (Scale bar, 200 μm). Taken together, these data demonstrate that the glucose-sensing tumor-targeting nucleic acid system optimizes tumor specificity of JYH1 in nude mice.

Example 5

The ability of JYH1 in specifically colonizing tumors in immunocompetent mice was tested. JYH1 was intravenously administered to immunocompetent BALB/c mice carrying CT26 tumors. 14 days after the bacterial injection, all the mice were euthanized due to excessive tumor growth. Figure 4 shows CFU 400 per gram of normal tissues and CT26 tumors in BALB/c mice. Plating analysis of homogenized tissues showed that the intravenously injected JYH1 did not colonize any organs tested including the liver, spleen, heart, lung and kidney of the immunocompetent mice on day 14. In contrast, levels of JYH1 in the tumors reached 4.67×10^7 cfu per gram ($\pm 1.62 \times 10^7$ cfu/g) as shown in Figure 4. These together with the data from the nude mice demonstrate that JYH1 specifically targets solid tumors regardless of the integrity of the immune system.

Figure 5 shows a method 500 of constructing a genetically engineered bacterial strain that targets solid tumors by selectively growing in glucose-deprived environments.

Block 510 states inserting into a bacterial strain a random library of a nucleic acid system.

In an example embodiment, the nucleic acid system includes a first DNA fragment that encodes a toxin, a second DNA fragment that encodes an antidote that negates the toxin. The nuclei acid system also includes a first promoter that controls transcription of the second DNA fragment. The nucleic acid system also includes a first constitutive promoter that causes constitutive expression of the first DNA fragment.

In an example embodiment, the nucleic acid system includes a first DNA fragment that encodes a toxin, a second DNA fragment that encodes an antidote that negates the toxin. The nuclei acid system also includes a second promoter that controls transcription of the first DNA fragment. The nucleic acid system also includes a second constitutive promoter that causes constitutive expression of the second DNA fragment.

In an example embodiment, the toxin-antidote pair includes but is not limited to the CcdB-CcdA pair. Other toxin-antidote pairs such as AvrRxo1-Arc1, Hha-TomB, and PaaA2-ParE2 can be used to replace the CcdB-CcdA pair. In an example embodiment, the first promoter includes but is not limited to the lac promoter. The lac promoter can be replaced by other glucose-repressed promoters such as the promoters of gltA, sdhADC or tnaB. In an example embodiment, the second promoter includes but is not limited to the promoter of ptsG, the promoter of fruB and the promoter of ackA.

In an example embodiment, a random sequence that consists of 5 – 6 nucleotides is inserted to replace the native 5-6 nucleotides immediately upstream of the start codon of the ccdA gene and downstream of the first promoter.

In an example embodiment, a random sequence that consists of 5 – 6 nucleotides is inserted to replace the native 5-6 nucleotides immediately upstream of the start codon of the ccdB gene and downstream of the second promoter.

Block 512 states culturing the random library of the clones of the genetically engineered bacterial strain.

In an example embodiment, the nucleic acid system is grafted into the chromosome of the bacterial strain. In an example embodiment, the nucleic acid system is grafted into a plasmid and the plasmid is inserted into the bacterial strain. In an example embodiment, the bacterial strain includes but is not limited to *Escherichia coli* MG1655. Other *Escherichia coli* strains such as DH5α and CFT073 and other Gram-negative bacterial species such as salmonella and Shigella may be used to replace MG1655. In an example embodiment, clones of the bacterial strain that includes the nucleic acid system are cultured on LB agar with or without glucose.

Block 514 states selecting the clones that grow in the absence of glucose but do not grow in the presence of glucose, thereby obtaining the genetically engineered bacteria strain that targets the tumors.

In an example embodiment, the clones that grow in LB agar without glucose but do not grow in LB agar with 5 mM glucose are selected and identified as potential candidates of tumor-targeting bacteria.

In an example embodiment, the clones that grow in M63 agar without glucose but do not grow at glucose concentrations of 1-4 mM are confirmed as potential candidates of tumor-targeting bacteria.

In an example embodiment, the method further includes generating a random library of the nucleic acid system by inserting a random sequence that

consists of 5 – 6 nucleotides to replace native nucleotides that are located immediately upstream of the second DNA fragment, when the nucleic acid system includes the first promoter.

In an example embodiment, the method further includes generating a random library of the nucleic acid system by inserting a random sequence that consists of 5 – 6 nucleotides to replace native nucleotides that are located immediately upstream of the first DNA fragment, when the nucleic acid system includes the second promoter.

Example 6

In this example, the inhibitory effects of intravenously injected *E. coli* JYH1 on tumor growth *in vivo* were evaluated.

Figure 6A shows the inhibitory effect 700 of intravenously injected *E.coli* JYH1 on the growth of HCT116 tumors in nude mice. Intravenous injection of JYH1 repressed growth of HCT116 tumors in nude mice. In contrast, JY1 had little effects on the tumor growth compared to the PBS control. The HCT116 tumors of JY1-treated mice grew equally well with those of PBS-treated controls, whereas the HCT116 tumors of JYH1-treated mice grew relatively slowly and 50% of them (5 out of 10) started to regress 18-26 days after the intravenous injection of the bacteria (10^7/mouse). JYH1 displayed inhibitory effects on HCT116 tumor growth of 100% of the tested mice (n=10), demonstrating a significantly better antitumor efficacy than JY1 (Fisher's Exact test, $p < 0.0001$).

Figure 6B shows an analysis 702 for the inhibitory effect of intravenously injected *E.coli* JYH1 on the growth of SW480 tumors in nude mice. Figure 6C shows representative photos 704 of a SW480 tumor in PBS-treated mice from 0 and 55 days. Figure 6D shows representative photos 706 of a SW480 tumor in JYH 1-treated mice from 0 and 90 days. Growth of the SW480 tumors was monitored for as long as 90 days. Tumors of the JYH1-treated mice (n=6) regressed 26-52 days after the intravenous injection of JYH1, with the effective percentage being 100%. Among these, tumors of 66.7% of the mice

(4 out of 6) disappeared and did not relapse by the end of the experiments. The tumor of one of the remaining two mice was not cured but kept quiescent. The tumor of only one mouse relapsed (16.67%, 1 out of 6). In contrast, none of the PBS-treated tumors regressed or disappeared. These data further showed that JYH1 has significant repressive effects on tumor growth *in vivo*.

Therefore, in an example embodiment, bacteria that are toxic to both tumor cells and normal tissue cells can become specific to tumors and repress tumor growth without affecting normal tissues, when the bacteria are equipped with the tumor-targeting nucleic acid system.

Example 7

In this example, the cytotoxin-encoding genes (i.e. the genes encoding cytotoxin) are cloned in a pBAD plasmid. Genes encoding each of the cytotoxin were individually cloned into a pBAD plasmid. In the case of exlA of P. aeruginosa, nhe of B. cereus and hlyA of V. cholera (hereafter referred to as VhlyA), the pelB leader sequence was fused in frame to the upstream of the target genes to allow for the excretion of the encoded cytotoxins. A constitutive promoter was used to drive the transcription of the fused DNAs. In the case of the hlyCABD operon (hereafter referred to as hlyCABD) coding for E. coli alpha-hemolysin, the entire operon was cloned into the pBAD vector. The pelB leader sequence was not employed for the hlyCABD operon in that the products of the operon include not only the hlyA hemolysin but also the secretion system required for the hemolysin secretion. Sequencing analyses of resulting recombinant plasmids verified that all the four genes were correctly cloned.

In one example embodiment, the pelB leader sequence is shown in SEQ ID No. 9. The sequence of exlA with the pelB leader is shown in SEQ ID No. 10. The sequence of Nhe with the pelB leader is shown in SEQ ID No. 11. The sequence of hlyA of Vibrio cholera with pelB leader is shown in SEQ ID No. 12. The sequence of hlyBACD operon of E. coli is shown in SEQ ID No. 13.

Example 8

In this example, the cloned genes of Example 7 were shown to produce functional cytotoxins and kill cancer cells *in vitro* assays.

Each of the recombinant plasmid was introduced into an E. coli reference strain TOP10. This strain per se does not cause cell lysis and, therefore, any killing action has to be attributed to the toxin production from the plasmid that the strain carries. The murine melanoma cell line B16F10 was used for the *in vitro* cytotoxicity assay. The B16F10 cells were co-cultured with E. coli TOP10 carrying each of the four recombinant plasmids at a moi of 100 (i.e. 100 bacteria per cell). As controls, the cells were also co-cultured with TOP10 carrying an empty pBAD plasmid or PBS. After 12 hours of co-incubation, TOP10 carrying recombinant plasmids with the toxin-encoding genes displayed significant cytotoxic effects on B16F10 cells whereas TOP10 with the empty plasmid had little effects on the cell viability as shown in Figure 7.

These *in vitro* data verified that the genes cloned into the pBAD plasmid successfully produced cytotoxins to kill cancer cells. Among the four cytotoxins, the two hemolysins are hemolytic whereas the other two toxins are not. In agreement with this, TOP10 with pBAD-hlyCABD and TOP10 with pBAD-VhlyA caused hemolysis on blood agar while TOP10 carrying pBAD-exlA or pBAD-nhe did not (as shown in Figure 8). The hemolysis assays confirmed the functionality of the genes cloned in the plasmids.

Example 9

In this example, the cytotoxins are shown to enhance anticancer efficacy of bacteria *in vivo*.

The anticancer ability of bacteria that were transformed to overexpress each of the above-mentioned cytotoxins was assessed. JYH1 is an E. coli strain with intrinsic ability to moderately repress tumor growth. Each of the four recombinant plasmids was separately introduced into JYH1 to analyze if any of them enhanced the anticancer efficacy of JYH1.

In C57BL/6N mice, subcutaneous B16F10 tumors that were intratumorally injected with JYH1 overexpressing any of the four cytotoxins grew much more slowly than JYH1 carrying the empty plasmid (all $P < 0.05$). The cytotoxin-treated tumors regressed during the first 6 or 9 days after the treatment, while those treated with phosphate buffer saline (PBS) or JYH1 carrying the empty plasmid grew readily without regression (as shown in Figure 9).

Among the four cytotoxins, alpha-hemolysin is naturally produced by some E. coli strains. Deletion of the chromosomal gene encoding alpha hemolysin did not impair the ability of E. coli to repress tumor growth (as shown in Figure 10). This indicates that the intrinsic anticancer action of the hemolytic E. coli strains such as JYH1 is not dependent on their natural production of alpha-hemolysin from the chromosome. However, plasmid-derived overproduction of alpha-hemolysin enhanced the anticancer efficacy of these bacteria, as shown in Figure 9. alpha-hemolysin produced from a single copy of the operon in the chromosome is insufficient for tumor inhibition but its overproduction from a multi-copy plasmid is sufficient for the cytotoxin to significantly repress tumor growth.

Cytotoxins Pseudomonas aeruginosa exolysin, Bacillus cereus non-hemolytic enterotoxin and Vibrio cholera hemolysin A are not naturally produced by E. coli, but engineering E. coli to produce these cytotoxins enhances anticancer efficacy of E. coli.

In an example embodiment, E. coli was made to produce the non-E. coli derived cytotoxins by introducing plasmids carrying the genes encoding the cytotoxins into E. coli. In an example embodiment, the cytotoxin-encoding genes are inserted into the chromosome of E. coli to increase the anticancer efficacy. In an example embodiment, the cytotoxins improve the anticancer efficacy of bacteria other than E. coli.

As used herein, the term "treat," "treating" or "treatment" refers to methods of alleviating, abating or ameliorating a disease or condition symptoms, preventing additional symptoms, ameliorating or preventing the underlying

metabolic causes of symptoms, inhibiting the disease or condition, arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition either prophylactically and/or therapeutically.

As used herein, "immediately", "immediately upstream" or "immediately downstream", means that there are no other nucleotides between one DNA fragment and another DNA fragment.

As used herein, "system" refers to a combination or a genetic circuit that includes the toxin gene, the antidote gene and their respective promoters. The toxin gene and the antidote gene of the system may be placed in any order. The toxin gene and the antidote gene of the system may be located in the same molecule or in different molecules. In an example embodiment, one of the toxin gene and the antidote gene may be in the chromosome of a bacterium while the other gene may be in a plasmid in the same bacterium.

As used herein, "at the tumor tissue" can be interchanged with terms such as "in the tumor", "at the tumor site", "inside the tumor", "at areas of the tumor tissue". These terms refer to being located in an area where tumor cells are present and where the local environment is sufficiently low in glucose to support tumor cell survival.

CLAIMS

[Claim 1]

A bacterium for targeting tumors and treating cancer, each comprising:

a nucleic acid system; and

a gene that encodes a cytotoxin that kills tumor cells,

wherein the nucleic acid system comprises:

a first DNA fragment that encodes a toxin that kills the bacterium;

a second DNA fragment that encodes an antidote that negates the toxin, the second DNA fragment being transcribed at tumor tissues but not transcribed at non-tumor tissues;

a promoter of an antidote gene that is operably linked to the second DNA fragment and represses transcription of the second DNA fragment under control of a glucose level such that the antidote is expressed at the tumor tissues but not expressed at the non-tumor tissues; and

a constitutive promoter of a toxin gene that is operably linked to the first DNA fragment and causes constitutive transcription of the first DNA fragment such that the toxin is expressed at the tumor tissues and the non-tumor tissues.

[Claim 2]

The bacterium according to claim 1, wherein the cytotoxin that kills tumor cells is selected from a group consisting of Pseudomonas aeruginosa exolysin, Bacillus cereus non-hemolytic enterotoxin, Vibrio cholera hemolysin A and Escherichia coli alpha-hemolysin.

[Claim 3]

The bacterium according to any one of claims 1 - 2, wherein the tumor tissues have a glucose concentration lower than 0.424 mM, and the promoter of the antidote gene initiates the transcription of the second DNA fragment when the glucose level is lower than 0.424 mM.

[Claim 4]

The bacterium according to any one of claims 1 - 3, wherein the non-tumor tissues have a glucose concentration higher than 1.22 mM, and the promoter of the antidote gene represses the transcription of the second DNA fragment when the glucose level is higher than 1.22 mM.

[Claim 5]
The bacterium according to any one of claims 1 - 4, wherein the promoter of the antidote gene is located immediately upstream of the second DNA fragment.
[Claim 6]
The bacterium according to any one of claims 1 - 5, wherein the constitutive promoter of the toxin gene is located immediately upstream of the first DNA fragment.
[Claim 7]
The bacterium according to any one of claims 1 - 6, wherein the promoter of the antidote gene is selected from a group consisting of a lac promoter, a gltA promoter, an sdhADC promoter and a tnaB promoter.
[Claim 8]
The bacterium according to any one of claims 1 - 7, wherein a pair of the toxin and the antidote is selected from a group consisting of a CcdB-CcdA pair, an AvrRxo1-Arc1 pair, a Hha-TomB pair, and a PaaA2-ParE2 pair.
[Claim 9]
The bacterium according to any one of claims 1 - 8, wherein the first DNA fragment is shown as SEQ ID No. 1, and the second DNA fragment is shown as SEQ ID No.2.
[Claim 10]
The bacterium according to any one of claims 1 - 9, wherein the promoter of the antidote gene is shown as SEQ ID No.3.
[Claim 11]
The bacterium according to any one of claims 1 - 10, wherein the bacterium further includes a random sequence that consists of 5 – 6 nucleotides, and replaces original 5 – 6 nucleotides of the bacterium that are located immediately upstream of the second DNA fragment.
[Claim 12]
The bacterium according to claim 11, wherein the random sequence is GCCTT or TGTCT.
[Claim 13]
The bacterium according to any one of claims 1 - 12, wherein the bacterium further includes a third DNA fragment that encodes a chloramphenicol

resistance cassette, wherein the third DNA fragment is shown as SEQ ID No. 4.

[Claim 14]

The bacterium according to any one of claims 1 - 13, wherein the bacterium is derived from a bacterial strain selected from a group consisting of *Escherichia coli*, salmonella and Shigella.

[Claim 15]

The bacterium according to any one of claims 1 - 14, wherein the bacterium is derived from *Escherichia coli* MG1655.

[Claim 16]

The bacterium according to any one of claims 1 - 15, wherein the nucleic acid system is shown as SEQ ID No. 7 or SEQ ID No.8.

[Claim 17]

A bacterium for targeting tumors and treating cancer, comprising:

a nucleic acid system; and

a gene that encodes a cytotoxin that kills tumor cells,

wherein the nucleic acid system comprises:

a first DNA fragment that encodes a toxin that kills the bacterium, the first DNA fragment being transcribed at non-tumor tissues but not transcribed at tumor tissues;

a second DNA fragment that encodes an antidote that negates the toxin;

a constitutive promoter of an antidote gene that is operably linked to the second DNA fragment and causes constitutive transcription of the second DNA fragment; and

a promoter of a toxin gene that is operably linked to the first DNA fragment and causes transcription of the first DNA fragment under control of a glucose level, wherein expression of the toxin is higher than constitutive expression of the antidote under the control of the glucose level such that the toxin kills the bacterium at the non-tumor tissues.

[Claim 18]

The bacterium according to claim 17, wherein the cytotoxin that kills the tumor cells is selected from a group consisting of Pseudomonas aeruginosa exolysin,

Bacillus cereus non-hemolytic enterotoxin, Vibrio cholera hemolysin A and Escherichia coli alpha-hemolysin.

[Claim 19]

The bacterium according to any one of claims 17 – 18, wherein the tumor tissues have a glucose concentration lower than 0.424 mM, and the promoter of the toxin gene represses the transcription of the first DNA fragment when the glucose level is lower than 0.424 mM.

[Claim 20]

The bacterium according to any one of claims 17 – 19, wherein the non-tumor tissues have a glucose concentration higher than 1.22 mM, the promoter of the toxin gene initiates the transcription of the first DNA fragment and the expression of the toxin is higher than the constitutive expression of the antidote when the glucose level is higher than 1.22 mM.

[Claim 21]

The bacterium according to any one of claims 17 – 20, wherein the promoter of the toxin gene is located immediately upstream of the first DNA fragment.

[Claim 22]

The bacterium according to any one of claims 17 – 21, wherein the constitutive promoter of the antidote gene is located immediately upstream of the second DNA fragment.

[Claim 23]

The bacterium according to any one of claims 17 – 22, wherein the promoter of the toxin gene is selected from a group consisting of a ptsG promoter, a fruB promoter and an ackA promoter.

[Claim 24]

The bacterium according to any one of claims 17 – 23, wherein a pair of the toxin and the antidote is selected from a group consisting of a CcdB-CcdA pair, an AvrRxo1-Arc1 pair, a Hha-TomB pair, and a PaaA2-ParE2 pair.

[Claim 25]

The bacterium according to any one of claims 17 – 24, wherein the first DNA fragment is shown as SEQ ID No. 1, and the second DNA fragment is shown as SEQ ID No.2.

[Claim 26]

The bacterium according to any one of claims 17 – 25, wherein the bacterium further includes a random sequence that consists of 5 – 6 nucleotides, and replaces original 5 – 6 nucleotides of the bacterium that are located immediately upstream of the first DNA fragment.

[Claim 27]

The bacterium according to any one of claims 17 – 26, wherein the bacterium further includes a third DNA fragment that encodes a chloramphenicol resistance cassette, wherein the third DNA fragment is shown as SEQ ID No. 4.

[Claim 28]

The bacterium according to any one of claims 17 – 27, wherein the bacterium is derived from a bacterial strain selected from a group consisting of *Escherichia coli*, salmonella and Shigella.

[Claim 29]

The bacterium according to any one of claims 17 – 28, wherein the bacterium is derived from *Escherichia coli* MG1655.

[Claim 30]

The bacterium according to any one of claims 1 - 29, wherein the bacterium is derived from a strain JY1 deposited at the China General Microbiological Culture Collection Center (CGMCC) under deposit no. 14577, a strain JY6 deposited at CGMCC under deposit no. 14578, a strain SH1 deposited at CGMCC under deposit no. 14580, or a strain JYH1 deposited at CGMCC under deposit no. 14579.

[Claim 31]

A drug delivery composition, comprising a bacterium of any one of claims 1 - 30.