

E. COLI MEDIATED GENE SILENCING OF BETA-CATENIN

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to, and the benefit of, U.S. Patent Application No. 61/114,610, filed November 14, 2008, the contents of which are herein incorporated by reference in their entirety.

BACKGROUND

10 Gene silencing through RNAi (RNA-interference) by use of short interfering RNA (siRNA) has emerged as a powerful tool for molecular biology and holds the potential to be used for therapeutic gene silencing. Short hairpin RNA (shRNA) transcribed from small DNA plasmids within the target cell has also been shown to mediate stable gene silencing and achieve gene knockdown at levels comparable to those obtained by transfection with chemically synthesized
15 siRNA (T. R. Brummelkamp, R. Bernards, R. Agami, *Science* 296, 550 (2002), P. J. Paddison, A. A. Caudiy, G. J. Hannon, *PNAS* 99, 1443 (2002)).

Possible applications of RNAi for therapeutic purposes are extensive and include silencing and knockdown of disease genes such as oncogenes or viral genes. One major obstacle for the therapeutic use of RNAi is the delivery of siRNA to the target cell (Zamore PD, Aronin N. *Nature*
20 *Medicine* 9,(3):266-8 (2003)). In fact, delivery has been described as the major hurdle now for RNAi (Phillip Sharp, cited by Nature news feature, Vol 425, 2003, 10-12).

Therefore, new methods are needed for the safe and predictable administration of interfering RNAs to mammals.

SUMMARY OF THE INVENTION

25 The present invention provides at least one invasive bacterium, or at least one invasive bacterial therapeutic particle (BTP), comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one Inv locus and at least one HlyA gene, wherein said siRNAs interfere with an mRNA of a gene target of
30 interest and wherein said invasive bacterium has reduced RNase III activity when compared to wild-type bacterium. Preferably, the invasive bacterium is an invasive *E. coli* bacterium. Preferably, the siRNAs interfere with the mRNA of β -catenin. The present invention also provides at least one prokaryotic vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one Inv locus and at least one HlyA gene, wherein said siRNAs
35 interfere with an mRNA of a gene target of interest. Preferably, the siRNAs interfere with the mRNA of β -catenin.

The present invention also provides methods of using the various invasive bacterium, BTP and vectors provided in the invention. For example, the present invention provides methods of delivering one or more siRNAs to mammalian cells. The methods include introducing at least one invasive bacterium, or at least one invasive bacterial therapeutic particle (BTP), comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one *Inv* locus and at least one *HlyA* gene, wherein said siRNAs interfere with an mRNA of a gene target of interest and wherein said invasive bacterium has reduced RNase III activity when compared to wild-type bacterium. Preferably, the invasive bacterium is an invasive *E. coli* bacterium.

The present invention also provides methods of regulating gene expression in mammalian cells. The method includes introducing at least one invasive bacterium, or at least one invasive bacterial therapeutic particle (BTP), comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one *Inv* locus and at least one *HlyA* gene, wherein said siRNAs interfere with an mRNA of a gene target of interest and wherein said invasive bacterium has reduced RNase III activity when compared to wild-type bacterium, where the expressed siRNAs interfere with at least one mRNA of a gene of interest thereby regulating gene expression. Preferably, the invasive bacterium is an invasive *E. coli* bacterium. Preferably, the siRNAs interfere with the mRNA of β -catenin.

The present invention also provides methods of treating or preventing a disease or disorder in a mammal. The methods include regulating the expression of at least one gene in a cell known to cause a disease or disorder (e.g., known to increase proliferation, growth or dysplasia) by introducing to the cells of the mammal at least one invasive bacterium, or at least one invasive bacterial therapeutic particle (BTP), comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one *Inv* locus and at least one *HlyA* gene, wherein said siRNAs interfere with an mRNA of a gene target of interest and wherein said invasive bacterium has reduced RNase III activity when compared to wild-type bacterium, where the expressed siRNAs interfere with the mRNA of the gene known to cause a disease or disorder. Preferably, the invasive bacterium is an invasive *E. coli* bacterium. Preferably, the siRNAs interfere with the mRNA of β -catenin.

The expressed siRNAs can direct the multienzyme complex RNA-induced silencing complex of the cell to interact with the mRNA of one or more genes of interest (e.g., β -catenin). Preferably, the expression of β -catenin is reduced as compared to wild-type β -catenin expression or as compared to the expression of β -catenin prior to the administration or treatment with an invasive bacterium or BTP containing one or more DNA molecules encoding for one or more siRNAs. The reduced expression of β -catenin can be reduced expression of β -catenin mRNA or reduced expression of β -catenin protein. Preferably, the expression of β -catenin is reduced at least 50% as compared to wild-type β -catenin expression (when compared to a normal, healthy cell) or as compared to the expression

of β -catenin prior to the administration or treatment with an invasive bacterium or BTP containing one or more DNA molecules encoding for one or more siRNAs; more preferably the expression of β -catenin is reduced at least 75% as compared to wild-type β -catenin expression or as compared to the expression of β -catenin prior to the administration or treatment with an invasive bacterium or BTP containing one or more DNA molecules encoding for one or more siRNAs; most preferably the expression of β -catenin is reduced at least 90% as compared to wild-type β -catenin expression or as compared to the expression of β -catenin prior to the administration or treatment with an invasive bacterium or BTP containing one or more DNA molecules encoding for one or more siRNAs.

Preferably, the disease or disorder can be, but is not limited to, a disease or disorder associated with the over expression of β -catenin. That is, a disease or disorder characterized by an increased expression (DNA, RNA or protein) of β -catenin in a cell or in a mammal in need of such treatment when compared to a normal (non-diseased) or wild-type cell or mammal. Preferably the disease or disorder to be treated is selected from the group consisting of colon cancer, rectal cancer, colorectal cancer, Crohn's disease, ulcerative colitis, familial adenomatous polyposis (FAP), Gardner's syndrome, hepatocellular carcinoma (HCC), basal cell carcinoma, pilomatricoma, medulloblastoma, and ovarian cancer.

The present invention also provides a composition containing at least one invasive bacterium or BTP and a pharmaceutically acceptable carrier.

The invasive bacterium or BTPs of the present invention can be attenuated, non-pathogenic or non-virulent bacterium

The mammalian cells can be *ex vivo*, *in vivo* or *in vitro*. The mammalian cells can be, but are not limited to, human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken, and primate cells. Preferably, the mammalian cells are human cells. In some preferred embodiments, the mammalian cells can be, but are not limited to, colon epithelial cells, rectal epithelial cells, intestinal epithelial cells, hepatocytes, skin epithelial cells, hair cells, neural cells, and ovarian cells.

The mammalian cells can be infected with about 10^3 to 10^{11} viable invasive bacterium or BTPs (or any integer within said ranges). Preferably, the mammalian cells can be infected with about 10^5 to 10^9 viable invasive bacterium or BTPs (or any integer within said ranges). The mammalian cells can be infected at a multiplicity of infection ranging from about 0.1 to 10^6 (or any integer within said ranges). Preferably, the mammalian cells can be infected at a multiplicity of infection ranging from about 10^2 to 10^4 (or any integer within said ranges).

The mammal can be, but is not limited to, human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken, and primate. Preferably, the mammal is a human.

The invasive bacterium comprises a deletion of a gene encoding RNase III. Preferably, the invasive bacterium comprises a deletion of an *rnc* gene encoding RNase III. Preferably, the RNase

III activity of the invasive bacterium is reduced at least 90% when compared to wild-type bacterium; more preferably the RNase III activity of the invasive bacterium is reduced at least 95% when compared to wild-type bacterium; most preferably the RNase III activity of the invasive bacterium is reduced at least 99% when compared to wild-type bacterium. Preferably, the invasive bacterium is an
 5 invasive *E. coli* bacterium.

The one or more DNA molecules can be transcribed into one or more shRNAs within the invasive bacterium. Preferably, the one or more shRNAs comprise a 3' overhang or a blunt end. Preferably, the one or more shRNAs do not comprise or include a 5' overhang (have a blunt end). Preferably, the one or more shRNAs comprise a 3' overhang of 2-5 base pairs; more preferably, the
 10 one or more shRNAs comprise a 3' overhang of no more than 2 base pairs (one or two base pair overhang); most preferably, the one or more shRNAs do not comprise or include a 3' overhang (have a blunt end). The one or more shRNAs are processed into one or more siRNAs. Preferably, the one or more shRNAs are processed into one or more siRNAs within the mammalian cell.

The prokaryotic vector comprising the one or more DNA molecules encoding the one or more
 15 siRNAs can include one or more promoter sequences, enhancer sequences, terminator sequences, invasion factor sequences or lysis regulation sequences. The promoter can be a prokaryotic promoter. Preferably, the prokaryotic promoter is a T7 promoter, a P_{gapA} promoter, a P_{araBAD} promoter, a P_{lac} promoter, a P_{lacUV5} promoter, or a *recA* promoter. Preferably, the promoter is a prokaryotic promoter. Preferably the prokaryotic promoter is a modified P_{lacUV5} promoter. The modified
 20 modified P_{lacUV5} promoter can comprise the sequence of SEQ ID NO:573. Preferably, the modified P_{lacUV5} promoter can comprise an UP element. The UP element can comprise nucleotides 7-26 of SEQ ID NO:573. Preferably, the prokaryotic vector further comprises at least one terminator sequence. Preferably the terminator sequence comprises a consecutive series of thymidine base pairs. More preferably, the terminator sequence can comprise at least 5 consecutive thymidine
 25 base pairs. The terminator sequence preferably comprises less than 20 consecutive thymidine base pairs. The prokaryotic vector can further comprise a second terminator sequence. Preferably, the second terminator sequence can be an *rrnC* terminator sequence. Preferably, the *rrnC* terminator sequence can comprise the sequence of SEQ ID NO:30-31 or SEQ ID NO:574. Preferably, these two terminator sequences are adjacent in the prokaryotic vector (they are consecutive sequences). More
 30 preferably, the two terminator sequences are separated. Preferably, the prokaryotic vector comprises the sequence for verified pMBV43. Preferably, the sequence for verified pMBV43 is SEQ ID NO:564.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.
 35 In the specification, the singular forms also include the plural unless the context clearly dictates otherwise. Although methods and materials similar or equivalent to those described herein can be

used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing a comparison between CEQ200 and CEQ221 at three doses in COS7 cells.

Figure 2 is a schematic showing the RNase III substrate hairpin RNA structure with functional annotation.

Figure 3 is a schematic showing the bacterial Class I RNase III cutting action of the hairpin precursor.

Figure 4 is a schematic showing the second step of maturation (first Dicer-cleavage step).

Figure 5 is a schematic showing the second Dicer cleavage step and maturation into active siRNA.

Figure 6, Panel A is a graph showing that CEQ 505 was able to silence mammalian β -catenin up to 90% in a dose-dependent manner in Cos-7 cells. Figure 6, Panel B is a graph showing that CEQ 221pNJSZc lamin (the equivalent strain targeting the *lamin* gene) was able to silence mammalian lamin up to 65% in a dose-dependent manner in SW480 cells.

Figure 7 is a schematic showing H3-shRNA with strand wobbles.

Figure 8, Panel A is a graph showing the invasive ability of the opa-expressing *E. Coli* strain at MOI 1. Figure 8, Panel B is a graph showing the invasive ability of the opa-expressing *E. Coli* strain at MOI 10.

Figure 9, Panel A is a graph showing the silencing of β -catenin mRNA using CEQ508 in human SW480 cells. Figure 9, Panel B is a photograph showing the silencing of β -catenin protein using CEQ508 in human SW480 cells.

Figure 10 is a photograph of an immunoblot showing the silencing of β -catenin protein using CEQ508 in human SW480 cells.

Figure 11 is a graph showing the silencing of β -catenin mRNA using CEQ509 BTPs in COS-7 cells.

DETAILED DESCRIPTION OF THE INVENTION

The invention pertains to compositions and methods of delivering small interfering RNAs (siRNAs) to eukaryotic cells using non-pathogenic or therapeutic strains of bacteria or bacterial therapeutic particles (BTPs). The bacteria or BTPs deliver DNA encoding siRNA, or siRNA itself, to effect RNA interference (RNAi) by invading into the eukaryotic host cells. Generally, to trigger RNA interference in a target cell, it is required to introduce siRNA into the cell. The siRNA is either introduced into the target cell directly or by transfection or can be transcribed within the target cell as hairpin-structured dsRNA (shRNA) from specific plasmids with RNA-polymerase III compatible promoters (e.g., U6, H1) (P. J. Paddison, A. A. Caudiy, G. J. Hannon, *PNAS* 99, 1443 (2002), T. R. Brummelkamp, R. Bernards, R. Agami, *Science* 296, 550 (2002)).

The interfering RNA of the invention regulates gene expression in eukaryotic cells. It silences or knocks down genes of interest inside target cells (e.g., decreases gene activity). The interfering RNA directs the cell-owned multienzyme-complex RISC (RNA-induced silencing complex) to the mRNA of the gene to be silenced. Interaction of RISC and mRNA results in degradation or sequestration of the mRNA. This leads to effective post-transcriptional silencing of the gene of interest. This method is referred to as Bacteria Mediated Gene Silencing (BMGS).

In the case of BMGS through delivery of siRNA expressing DNA plasmids, shRNA or siRNA are produced within the target cell after liberation of the eukaryotic transcription plasmid and trigger the highly specific process of mRNA degradation, which results in silencing of the targeted gene. Additionally, one or more cell-specific eukaryotic promoters may be used that limit the expression of siRNA or shRNA to specific target cells or tissues that are in particular metabolic states. In one embodiment of this method, the cell-specific promoter is albumin and the target cell or tissue is the liver. In another embodiment of this method, the cell-specific promoter is keratin and the specific target cell or tissue is the skin.

The non-virulent bacteria and BTPs of the invention have invasive properties (or are modified to have invasive properties) and may enter a mammalian host cell through various mechanisms. In contrast to uptake of bacteria or BTPs by professional phagocytes, which normally results in the destruction of the bacterium or BTP within a specialized lysosome, invasive bacteria or BTP strains have the ability to invade non-phagocytic host cells. Naturally occurring examples of such bacteria or BTPs are intracellular pathogens such as *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, and *Vibrio*, but this property can also be transferred to other bacteria or BTPs such as *E. coli*, *Lactobacillus*, *Lactococcus*, or *Bifidobacteriae*, including probiotics through transfer of invasion-related genes (P. Courvalin, S. Goussard, C. Grillot-Courvalin, *C.R.Acad.Sci.Paris* 318,1207 (1995)). In other embodiments of the invention, bacteria or BTPs used to deliver interfering RNAs to host cells include *Shigella flexneri* (D. R. Sizemore, A. A. Branstrom, J. C. Sadoff, *Science* 270, 299 (1995)), invasive *E. coli* (P. Courvalin,

S. Goussard, C. Grillot-Courvalin, *C.R.Acad.Sci.Paris* 318,1207 (1995), C. Grillot-Courvalin, S. Goussard, F. Huetz, D. M. Ojcius, P. Courvalin, *Nat Biotechnol* 16, 862 (1998)), *Yersinia enterocolitica* (A. Al-Mariri A, A. Tibor, P. Lestrade, P. Mertens, X. De Bolle, J. J. Letesson *Infect Immun* 70, 1915 (2002)) and *Listeria monocytogenes* (M. Hense, E. Domann, S. Krusch, P.

- 5 Wachholz, K. E. Dittmar, M. Rohde, J. Wehland, T. Chakraborty, S. Weiss, *Cell Microbiol* 3, 599 (2001), S. Pilgrim, J. Stritzker, C. Schoen, A. Kolb-Mäurer, G. Geginat, M. J. Loessner, I. Gentshev, W. Goebel, *Gene Therapy* 10, 2036 (2003)). Any invasive bacterium or BTP is useful for DNA transfer into eukaryotic cells (S. Weiss, T. Chakraborty, *Curr Opinion Biotechnol* 12, 467 (2001)).

BMGS is performed using the naturally invasive pathogen *Salmonella typhimurium*. In one aspect of this embodiment, the strains of *Salmonella typhimurium* include SL 7207 and VNP20009 (S. K. Hoiseth, B. A. D. Stocker, *Nature* 291, 238 (1981); Pawelek JM, Low KB, Bermudes D. *Cancer Res.* 57(20): 4537-44 (Oct. 15 1997)). In another embodiment of the invention, BMGS is performed using attenuated *E. coli*. In another aspect of this embodiment, the CEQ201 strain is engineered to possess cell-invading properties through an invasion plasmid. In one aspect of the invention, this plasmid is a TRIP (Transkingdom RNA interference plasmid) plasmid or pNJSZ.

A double “trojan horse” technique is also used with an invasive and auxotrophic bacterium or BTP carrying a eukaryotic transcription plasmid. This plasmid is, in turn, transcribed by the target cell to form one or more hairpin RNA structures that triggers the intracellular process of RNAi. This method of the invention induces significant gene silencing of a variety of genes. In certain aspects of this embodiment, the genes include a transgene (GFP), a mutated oncogene (*k-Ras*) and a cancer related gene (*β -catenin*) *in vitro*.

Another aspect of BMGS according to this invention is termed Transkingdom RNAi (tkRNAi). In this aspect of the invention, siRNA is directly produced by the invasive bacteria, or accumulated in the BTPs after production in the bacteria, as opposed to the target cell. A transcription plasmid controlled by a prokaryotic promoter (*e.g.*, T7) is inserted into the carrier bacteria through standard transformation protocols. siRNA is produced within the bacteria and is liberated within the mammalian target cell after bacterial lysis triggered either by auxotrophy or by timed addition of antibiotics.

Most bacteria contain a large number of RNA degrading enzymes, RNAses, which may degrade the siRNA causing a reduction in the activity of tkRNAi. In such cases where the RNAses of a specific bacterium will exhibit such degradation of siRNA, a targeted deletion of the gene encoding the RNase of interest (*e.g.*, the *rnc* gene encoding RNase III) is performed to yield higher levels of siRNA per tkRNAi bacterium, resulting in more siRNA being delivered to the target cells, as well as more efficient gene silencing of the gene of interest within the target cell.

The RNAi methods of the invention, including BMGS and tkRNAi are used to create transient “knockdown” genetic animal models as opposed to genetically engineered knockout models to discover gene functions. The methods are also used as *in vitro* transfection tool for research and

drug development

These methods use bacteria with desirable properties (invasiveness, attenuation, steerability) to perform BMGS and tkRNAi. Invasiveness as well as eukaryotic or prokaryotic transcription of one or several shRNA is conferred to a bacterium or BTP using plasmids (*e.g.*, TRIP) and vectors as described in greater detail herein.

1. Bacterium and/or Bacterial Therapeutic Particles (BTPs)

The present invention provides at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), including one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

According to the invention, any microorganism that is capable of delivering a molecule, *e.g.*, an RNA molecule or an RNA-encoding DNA molecule, into the cytoplasm of a target cell, such as by traversing the membrane and entering the cytoplasm of a cell, can be used to deliver RNA to such cells. In a preferred embodiment, the microorganism is a prokaryote. In an even more preferred embodiment, the prokaryote is a bacterium or BTP. Also within the scope of the invention are microorganisms other than bacteria that can be used for delivering RNA to a cell. For example, the microorganism can be a fungus, *e.g.*, *Cryptococcus neoformans*, protozoan, *e.g.*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania donovani*, and *plasmodia*.

In a preferred embodiment, the microorganism is a bacterium or BTP. A preferred invasive bacterium or BTP is capable of delivering at least one molecule, *e.g.*, an RNA or RNA-encoding DNA molecule, to a target cells, such as by entering the cytoplasm of a eukaryotic cell. Preferably, the RNA is siRNA or shRNA and the RNA-encoding DNA molecule encodes for siRNA or shRNA.

BTPs are fragments of bacteria used for therapeutic or preventive purposes. BTPs may include particles known in the art as minicells. Minicells are small cells produced by cell division that is faulty near the pole. They are devoid of nucleoid and, therefore, unable to grow and form colonies (Alder et al., (1967) Proc. Nat. Acad. Sci. U.S.A. 57, 321-326; for reviews see Sullivan and Maddock, (2000) Curr. Biol. 10:R249-R252; Margolin, (2001) Curr. Biol. 11, R395-R398; Howard and Kruse, (2005) J. Cell Biol. 168, 533-536). Minicell formation results due to mutations causing a defect in selection of the site for the septum formation for cell division. Such mutations include null alleles of minC, minD (Davie et al, (1984) J. Bacteriol. 158, 1202-1203; de Boer et al., 1988) J. Bacteriol. 170, 2106-2112) and certain alleles of ftsZ (Bi and Lutkenhaus, (1992) J. Bacteriol. 174, 5414-5423). Overexpression of FtsZ or MinC-MinD proteins has also been reported to cause the formation of minicells (Ward and Lutkenhaus, 1985; de Boer et al., 1988). Although minicells are devoid of nucleoid, they are capable of transcription and translation (Roozen et al., (1971) J. Bacteriol. 107, 21-33; Shepherd et al., (2001) J. Bacteriol. 183, 2527-34).

BTPs are distinct from bacteria in that they lack the bacterial genome and, therefore, provide a decreased risk of bacterial proliferation in patients. This is of particular value for immune-

compromised patients. Furthermore, the inability of BTPs to proliferate allows for their use in sensitive tissues, *e.g.*, the brain, and other areas of the body traditionally considered inaccessible to traditional siRNA. For example, the intraperitoneal delivery of bacteria can include the risk of adhesions and peritonitis, which is eliminated by utilizing BTPs. However, like the bacteria of this invention, BTPs contain the bacterial cell wall, some bacterial plasma contents and subcellular particles, one or more therapeutic components, *e.g.*, one or more siRNAs, one or more invasion factors, one or more phagosome degradation factors, and one or more factors for targeting specific tissues. The BTPs are produced from bacteria that have produced and accumulated siRNAs inside the bacteria, and then segregate the bacterial fragment (BTP) during cell division. In one embodiment of this invention, BTPs are obtained by fermenting the bacteria, during which the BTPs form abundantly, followed by isolation of the BTPs from live bacteria using differential size filtration, which will retain the bacteria but allow passage and collection of BTPs. In another embodiment of this invention, BTPs are separated from bacteria by centrifugation. In another embodiment of this invention, live bacterial cells are lysed through activation of a death signal. Once isolated, the BTPs can be lyophilized and formulated for use.

As used herein, the term "invasive" when referring to a microorganism, *e.g.*, a bacterium or BTP, refers to a microorganism that is capable of delivering at least one molecule, *e.g.*, an RNA or RNA-encoding DNA molecule, to a target cell. An invasive microorganism can be a microorganism that is capable of traversing a cell membrane, thereby entering the cytoplasm of said cell, and delivering at least some of its content, *e.g.*, RNA or RNA-encoding DNA, into the target cell. The process of delivery of the at least one molecule into the target cell preferably does not significantly modify the invasion apparatus.

Invasive microorganisms include microorganisms that are naturally capable of delivering at least one molecule to a target cell, such as by traversing the cell membrane, *e.g.*, a eukaryotic cell membrane, and entering the cytoplasm, as well as microorganisms which are not naturally invasive and which have been modified, *e.g.*, genetically modified, to be invasive. In another preferred embodiment, a microorganism that is not naturally invasive can be modified to become invasive by linking the bacterium or BTP to an "invasion factor", also termed "entry factor" or "cytoplasm-targeting factor". As used herein, an "invasion factor" is a factor, *e.g.*, a protein or a group of proteins which, when expressed by a non-invasive bacterium or BTP, render the bacterium or BTP invasive. As used herein, an "invasion factor" is encoded by a "cytoplasm-targeting gene".

In one embodiment of this invention, the microorganism is a naturally invasive bacterium or BTP selected from the group that includes, but is not limited to, *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, *Vibrio*, *E. coli*, and *Bifidobacteriae*. Optionally, the naturally invasive bacterium or BTP is *Yersinia* expressing an invasion factor selected from the group including, but not limited to, invasin and YadA

(*Yersinia enterocolitica* plasmid adhesion factor). Optionally, the naturally invasive bacterium or BTP is *Rickettsia* expressing the invasion factor RickA (actin polymerization protein). Optionally, the naturally invasive bacterium or BTP is *Legionella* expressing the invasion factor RaIF (guanine exchange factor). Optionally, the naturally invasive bacterium or BTP is *Neisseria* expressing an invasion factor selected from the group including, but not limited to, NadA (*Neisseria* adhesion/invasion factor), OpaA, OpaC and Opa52 (opacity-associated adhesions). Optionally, the naturally invasive bacterium or BTP is *Listeria* expressing an invasion factor selected from the group including, but not limited to, InlA (internalin factor), InlB (internalin factor), Hpt (hexose phosphate transporter), and ActA (actin polymerization protein). Optionally, the naturally invasive bacterium or BTP is *Shigella* expressing an invasion factor selected from the group including, but not limited to, the *Shigella* secreting factors IpaA (invasion plasmid antigen), IpaB, IpaC, IpgD, IpaB-IpaC complex, VirA, and IcsA. Optionally, the naturally invasive bacterium or BTP is *Salmonella* expressing an invasion factor selected from the group including, but not limited to, *Salmonella* secreting/exchange factors SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, and SptP. Optionally, the naturally invasive bacterium or BTP is *Staphylococcus* expressing an invasion factor selected from the group including, but not limited to, the fibronectin binding proteins FnBPA and FnBPB. Optionally, the naturally invasive bacterium or BTP is *Streptococcus* expressing an invasion factor selected from the group including, but not limited to, the fibronectin binding proteins ACP, Fba, F2, Sfb1, Sfb2, SOF, and PFBP. Optionally, the naturally invasive bacterium or BTP is *Porphyromonas gingivalis* expressing the invasion factor FimB (integrin binding protein fibriae).

In another embodiment of this invention, the microorganism is a bacterium or BTP that is not naturally invasive but has been modified, *e.g.*, genetically modified, to be invasive. Optionally, the bacterium or BTP that is not naturally invasive has been genetically modified to be invasive by expressing an invasion factor selected from the group including, but not limited to, invasin, YadA, RickA, RaIF, NadA, OpaA, OpaC, Opa52, InlA, InlB, Hpt, ActA, IpaA, IpaB, IpaC, IpgD, IpaB-IpaC complex, VirA, IcsA, SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, SptP, FnBPA, FnBPB, ACP, Fba, F2, Sfb1, Sfb2, SOF, PFBP, and FimB.

In another embodiment of this invention, the microorganism is a bacterium or BTP that may be naturally invasive but has been modified, *e.g.*, genetically modified, to express one or more additional invasion factors. Optionally, the invasion factor is selected from the group that includes, but is not limited to, invasin, YadA, RickA, RaIF, NadA, OpaA, OpaC, Opa52, InlA, InlB, Hpt, ActA, IpaA, IpaB, IpaC, IpgD, IpaB-IpaC complex, VirA, IcsA, SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, SptP, FnBPA, FnBPB, ACP, Fba, F2, Sfb1, Sfb2, SOF, PFBP, and FimB.

Naturally invasive microorganisms, *e.g.*, bacteria or BTPs, may have a certain tropism, *i.e.*, preferred target cells. Alternatively, microorganisms, *e.g.*, bacteria or BTPs can be modified, *e.g.*, genetically, to mimic the tropism of a second microorganism. Optionally, the bacterium or BTP is *Streptococcus* and the preferred target cells are selected from the group including, but not limited to,

pharyngeal epithelial cells, buccal epithelial cells of the tongue, and mucosal epithelial cells.

Optionally, the bacterium or BTP is *Porphyrromonas* and the preferred target cells are selected from the group including, but not limited to, oral epithelial cells. Optionally, the bacterium or BTP is

Staphylococcus and the preferred target cells are mucosal epithelial cells. Optionally, the bacterium

or BTP is *Neisseria* and the preferred target cells are selected from the group including, but not

limited to, urethral epithelial cells and cervical epithelial cells. Optionally, the bacterium or BTP is *E. coli* and the preferred target cells are selected from the group, including but not limited to, intestinal

epithelial cells, urethral epithelial cells, and the cells of the upper urinary tract. Optionally, the

bacterium or BTP is *Bordetella* and the preferred target cells are respiratory epithelial cells.

Optionally, the bacterium or BTP is *Vibrio* and the preferred target cells are intestinal epithelial cells.

Optionally, the bacterium or BTP is *Treponema* and the preferred target cells are mucosal epithelial

cells. Optionally, the bacterium or BTP is *Mycoplasma* and the preferred target cells are respiratory

epithelial cells. Optionally, the bacterium or BTP is *Helicobacter* and the preferred target cells are the

endothelial cells of the stomach. Optionally, the bacterium or BTP is *Chlamydia* and the preferred

target cells are selected from the group including, but not limited to, conjunctival cells and urethral

epithelial cells.

In another embodiment of this invention, the microorganism is a bacterium or BTP that has been modified, *e.g.*, genetically modified, to have a certain tropism. Optionally, the preferred target

cells are selected from the group including, but not limited to, pharyngeal epithelial cells, buccal

epithelial cells of the tongue, mucosal epithelial cells, oral epithelial cells, epithelial cells of the

urethra, cervical epithelial cells, intestinal epithelial cells, respiratory epithelial cells, cells of the

upper urinary tract, epithelial cells of the stomach, and conjunctival cells. Optionally, the preferred

target cells are dysplastic or cancerous epithelial cells. Optionally, the preferred target cells are

activated or resting immune cells.

Delivery of at least one molecule into a target cell can be determined according to methods

known in the art. For example, the presence of the molecule, by the decrease in expression of an

RNA or protein silenced thereby, can be detected by hybridization or PCR methods, or by

immunological methods that may include the use of an antibody.

Determining whether a microorganism is sufficiently invasive for use in the invention may

include determining whether sufficient siRNA was delivered to host cells, relative to the number of

microorganisms contacted with the host cells. If the amount of siRNA is low relative to the number of

microorganisms used, it may be desirable to further modify the microorganism to increase its invasive potential.

Bacterial or BTP entry into cells can be measured by various methods. Intracellular bacteria

or BTPs survive treatment by aminoglycoside antibiotics, whereas extracellular bacteria are rapidly

killed. A quantitative estimate of bacterial or BTP uptake can be achieved by treating cell monolayers

with the antibiotic gentamicin to inactivate extracellular bacteria or BTPs, then by removing said

antibiotic before liberating the surviving intracellular organisms with gentle detergent and determining viable counts on standard bacteriological medium. Furthermore, bacterial or BTP entry into cells can be directly observed, *e.g.*, by thin-section-transmission electron microscopy of cell layers or by immunofluorescent techniques (Falkow *et al.* (1992) Annual Rev. Cell Biol. 8:333).

- 5 Thus, various techniques can be used to determine whether a specific bacterium or BTP is capable of invading a specific type of cell or to confirm bacterial invasion following modification of the bacteria or BTP, such modification of the tropism of the bacteria to mimic that of a second bacterium.

Bacteria or BTPs that can be used for delivering RNA according to the method of the invention are preferably non-pathogenic. However, pathogenic bacteria or BTP s can also be used, so long as their pathogenicity has been attenuated, to thereby render the bacteria non-harmful to a subject to which it is administered. As used herein, the term "attenuated bacterium or BTP " refers to a bacterium or BTP that has been modified to significantly reduce or eliminate its harmfulness to a subject. A pathogenic bacterium or BTP can be attenuated by various methods, set forth below.

Without wanting to be limited to a specific mechanism of action, the bacterium or BTP delivering the RNA into the eukaryotic cell can enter various compartments of the cell, depending on the type of bacterium or BTP. For example, the bacterium or BTP can be in a vesicle, *e.g.*, a phagocytic vesicle. Once inside the cell, the bacterium or BTP can be destroyed or lysed and its contents delivered to the eukaryotic cell. A bacterium or BTP can also be engineered to express a phagosome degrading protein to allow leakage of RNA from the phagosome. In one embodiment of this invention, the bacterium or BTP expresses, either naturally or through modification, *e.g.*, genetic modification, a protein that contributes to pore-formation, breakage or degradation of the phagosome. Optionally, the protein is a cholesterol-dependent cytolysin. Optionally, the protein is selected from the group consisting of listeriolysin, ivanolysin, streptolysin, sphingomyelinase, perfringolysin, botulinolysin, leukocidin, anthrax toxin, phospholipase, IpaB (invasion plasmid antigen), IpaH, IcsB (intercellular spread), DOT/Icm (defect in organelle trafficking/intracellular multiplication defective), DOTU (stabilization factor for the DOT/Icm complex), IcmF, and PmrA (multidrug resistance efflux pump).

In some embodiments, the bacterium can stay alive for various times in the eukaryotic cell and may continue to produce RNA. The RNA or RNA-encoding DNA can then be released from the bacterium into the cell by, *e.g.*, leakage. In certain embodiments of the invention, the bacterium can also replicate in the eukaryotic cell. In a preferred embodiment, bacterial replication does not kill the host cell. The invention is not limited to delivery of RNA or RNA-encoding DNA by a specific mechanism and is intended to encompass methods and compositions permitting delivery of RNA or RNA-encoding DNA by a bacterium independently of the mechanism of delivery.

35 In one embodiment, the bacterium or BTP for use in the present invention is non-pathogenic or non-virulent. In another aspect of this embodiment, the bacterium or BTP is therapeutic. In another aspect of this embodiment, the bacterium or BTP is an attenuated strain or derivative thereof

selected from, but not limited to, *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Haemophilus*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, *Vibrio*, *E. coli*, and *Bifidobacteriae*. Optionally, the *Yersinia* strain is an attenuated strain of the *Yersinia pseudotuberculosis* species. Optionally, the *Yersinia* strain is an attenuated strain of the *Yersinia enterocolitica* species. Optionally, the *Rickettsia* strain is an attenuated strain of the *Rickettsia coronii* species. Optionally, the *Legionella* strain is an attenuated strain of the *Legionella pneumophila* species. Optionally, the *Mycobacterium* strain is an attenuated strain of the *Mycobacterium tuberculosis* species. Optionally, the *Mycobacterium* strain is an attenuated strain of the *Mycobacterium bovis* BCG species. Optionally, the *Helicobacter* strain is an attenuated strain of the *Helicobacter pylori* species. Optionally, the *Coxiella* strain is an attenuated strain of *Coxiella burnetti*. Optionally, the *Haemophilus* strain is an attenuated strain of the *Haemophilus influenza* species. Optionally, the *Chlamydia* strain is an attenuated strain of the *Chlamydia trachomatis* species. Optionally, the *Chlamydia* strain is an attenuated strain of the *Chlamydia pneumoniae* species. Optionally, the *Neisseria* strain is an attenuated strain of the *Neisseria gonorrhoeae* species. Optionally, the *Neisseria* strain is an attenuated strain of the *Neisseria meningitidis* species. Optionally, the *Burkholderia* strain is an attenuated strain of the *Burkholderia cepacia* species. Optionally, the *Bordetella* strain is an attenuated strain of the *Bordetella pertussis* species. Optionally, the *Borrelia* strain is an attenuated strain of the *Borrelia hermsii* species. Optionally, the *Listeria* strain is an attenuated strain of the *Listeria monocytogenes* species. Optionally, the *Listeria* strain is an attenuated strain of the *Listeria ivanovii* species. Optionally, the *Salmonella* strain is an attenuated strain of the *Salmonella enterica* species. Optionally, the *Salmonella* strain is an attenuated strain of the *Salmonella typhimurium* species. Optionally, the *Salmonella typhimurium* strain is SL 7207 or VNP20009. Optionally, the *Staphylococcus* strain is an attenuated strain of the *Staphylococcus aureus* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus pyogenes* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus mutans* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus salivarius* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus pneumoniae* species. Optionally, the *Porphyromonas* strain is an attenuated strain of the *Porphyromonas gingivalis* species. Optionally, the *Pseudomonas* strain is an attenuated strain of the *Pseudomonas aeruginosa* species. Optionally, the *Treponema* strain is an attenuated strain of the *Treponema pallidum* species. Optionally, the *Vibrio* strain is an attenuated strain of the *Vibrio cholerae* species. Optionally, the *E. coli* strain is MM294.

Set forth below are examples of bacteria that have been described in the literature as being naturally invasive (section 1.1), as well as bacteria which have been described in the literature as being naturally non-invasive bacteria (section 1.2), as well as bacteria which are naturally non-pathogenic or which are attenuated. Although some bacteria have been described as being non-

invasive (section 1.2), these may still be sufficiently invasive for use according to the invention. Whether traditionally described as naturally invasive or non-invasive, any bacterial strain can be modified to modulate, in particular to increase, its invasive characteristics (*e.g.*, as described in section 1.3).

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1.1 Naturally Invasive Bacteria

The particular naturally invasive bacteria employed in the present invention are not critical thereto. Examples of such naturally occurring invasive bacteria include, but are not limited to, *Shigella spp.*, *Salmonella spp.*, *Listeria spp.*, *Rickettsia spp.*, and enteroinvasive *Escherichia coli*.

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The particular *Shigella* strain employed is not critical to the present invention. Examples of *Shigella* strains that can be employed in the present invention include *Shigella flexneri* 2a (ATCC No. 29903), *Shigella sonnei* (ATCC No. 29930), and *Shigella dysenteriae* (ATCC No. 13313). An attenuated *Shigella* strain, such as *Shigella flexneri* 2a 2457T aroA virG mutant CVD 1203 (Noriega *et al. supra*), *Shigella flexneri* M90T icsA mutant (Goldberg *et al. Infect. Immun.*, 62:5664-5668 (1994)), *Shigella flexneri* Y SFL114 aroD mutant (Karnell *et al. Vacc.*, 10:167-174 (1992)), and *Shigella flexneri* aroA aroD mutant (Verma *et al. Vacc.*, 9:6-9 (1991)) are preferably employed in the present invention. Alternatively, new attenuated *Shigella spp.* strains can be constructed by introducing an attenuating mutation either singularly or in conjunction with one or more additional attenuating mutations.

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At least one advantage to *Shigella* bacteria as delivery vectors is their tropism for lymphoid tissue in the colonic mucosal surface. In addition, the primary site of *Shigella* replication is believed to be within dendritic cells and macrophages, which are commonly found at the basal lateral surface of M cells in mucosal lymphoid tissues (reviewed by McGhee, J. R. *et al. (1994) Reproduction, Fertility, & Development* 6:369; Pascual, D. W. *et al. (1994) Immunomethods* 5:56). As such, *Shigella* vectors may provide a means to target RNA interference or deliver therapeutic molecules to these professional antigen-presenting cells. Another advantage of *Shigella* vectors is that attenuated *Shigella* strains deliver nucleic acid reporter genes *in vitro* and *in vivo* (Sizemore, D. R. *et al. (1995) Science* 270:299; Courvalin, P. *et al. (1995) Comptes Rendus de l'Academie des Sciences Serie III-Sciences de la Vie-Life Sciences* 318:1207; Powell, R. J. *et al. (1996) In: Molecular approaches to the control of infectious diseases*. F. Brown, E. Norrby, D. Burton and J. Mekalanos, eds. Cold Spring Harbor Laboratory Press, New York. 183; Anderson, R. J. *et al. (1997) Abstracts for the 97th General Meeting of the American Society for Microbiology*:E.). On the practical side, the tightly restricted host specificity of *Shigella* stands to prevent the spread of *Shigella* vectors into the food chain via intermediate hosts. Furthermore, attenuated strains that are highly attenuated in rodents, primates and volunteers have been developed (Anderson *et al. (1997) supra*; Li, A. *et al. (1992) Vaccine* 10:395; Li, A. *et al. (1993) Vaccine* 11:180; Karnell, A. *et al. (1995) Vaccine* 13:88; Sansonetti, P. J. and J. Arondel (1989) *Vaccine* 7:443; Fontaine, A. *et al. (1990) Research in Microbiology* 141:907;

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Sansonetti, P. J. *et al.* (1991) *Vaccine* 9:416; Noriega, F. R. *et al.* (1994) *Infection & Immunity* 62:5168; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:3055; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:23; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:3055; Kotloff, K. L. *et al.* (1996) *Infection & Immunity* 64:4542). This latter knowledge will allow the development of well-tolerated *Shigella* vectors for use in humans.

Attenuating mutations can be introduced into bacterial pathogens using non-specific mutagenesis either chemically, using agents such as N-methyl-N'-nitro-N-nitrosoguanidine, or using recombinant DNA techniques; classic genetic techniques, such as Tn10 mutagenesis, P22-mediated transduction, λ phage mediated crossover, and conjugational transfer; or site-directed mutagenesis using recombinant DNA techniques. Recombinant DNA techniques are preferable since strains constructed by recombinant DNA techniques are far more defined. Examples of such attenuating mutations include, but are not limited to:

(i) auxotrophic mutations, such as *aro* (Hoiseth *et al.* *Nature*, 291:238-239 (1981)), *gua* (McFarland *et al.* *Microbiol. Path.*, 3:129-141 (1987)), *nad* (Park *et al.* *J. Bact.*, 170:3725-3730 (1988)), *thy* (Nnalue *et al.* *Infect. Immun.*, 55:955-962 (1987)), and *asd* (Curtiss, *supra*) mutations;

(ii) mutations that inactivate global regulatory functions, such as *cya* (Curtiss *et al.* *Infect. Immun.*, 55:3035-3043 (1987)), *crp* (Curtiss *et al.* (1987), *supra*), *phoP/phoQ* (Groisman *et al.* *Proc. Natl. Acad. Sci., USA*, 86:7077-7081 (1989); and Miller *et al.* *Proc. Natl. Acad. Sci., USA*, 86:5054-5058 (1989)), *phop^c* (Miller *et al.* *J. Bact.*, 172:2485-2490 (1990)) or *ompR* (Dorman *et al.* *Infect. Immun.*, 57:2136-2140 (1989)) mutations;

(iii) mutations that modify the stress response, such as *recA* (Buchmeier *et al.* *Mol. Micro.*, 7:933-936 (1993)), *htrA* (Johnson *et al.* *Mol. Micro.*, 5:401-407 (1991)), *htpR* (Neidhardt *et al.* *Biochem. Biophys. Res. Com.*, 100:894-900 (1981)), *hsp* (Neidhardt *et al.* *Ann. Rev. Genet.*, 18:295-329 (1984)) and *groEL* (Buchmeier *et al.* *Sci.*, 248:730-732 (1990)) mutations;

(iv) mutations in specific virulence factors, such as *IsyA* (Libby *et al.* *Proc. Natl. Acad. Sci., USA*, 91:489-493 (1994)), *pag* or *prg* (Miller *et al.* (1990), *supra*; and Miller *et al.* (1989), *supra*), *iscA* or *virG* (d'Hauteville *et al.* *Mol. Micro.*, 6:833-841 (1992)), *plcA* (Mengaud *et al.* *Mol. Microbiol.*, 5:367-72 (1991); Camilli *et al.* *J. Exp. Med.*, 173:751-754 (1991)), and *act* (Brundage *et al.* *Proc. Natl. Acad. Sci., USA*, 90:11890-11894 (1993)) mutations;

(v) mutations that affect DNA topology, such as *topA* (Galan *et al.* *Infect. Immun.*, 58:1879-1885 (1990));

(vi) mutations that disrupt or modify the cell cycle, such as *min* (de Boer *et al.* *Cell*, 56:641-649 (1989)).

(vii) introduction of a gene encoding a suicide system, such as *sacB* (Recorbet *et al.* *App. Environ. Micro.*, 59:1361-1366 (1993); Quandt *et al.* *Gene*, 127:15-21 (1993)), *nuc* (Ahrenholtz *et al.* *App. Environ. Micro.*, 60:3746-3751 (1994)), *hok*, *gef*, *kil*, or *phlA* (Molin *et al.* *Ann. Rev. Microbiol.*, 47:139-166 (1993));

(viii) mutations that alter the biogenesis of lipopolysaccharide and/or lipid A, such as rFb (Raetz in *Escherichia coli* and *Salmonella typhimurium*, Neidhardt *et al.*, Ed., ASM Press, Washington D.C. pp 1035-1063 (1996)), galE (Hone *et al.* J. Infect. Dis., 156:164-167 (1987)) and htrB (Raetz, *supra*), msbB (Raetz, *supra*)

(ix) introduction of a bacteriophage lysis system, such as lysogens encoded by P22 (Rennell *et al.* Virol, 143:280-289 (1985)), λ murein transglycosylase (Bienkowska-Szewczyk *et al.* Mol. Gen. Genet., 184:111-114 (1981)) or S-gene (Reader *et al.* Virol, 43:623-628 (1971)); and

The attenuating mutations can be either constitutively expressed or under the control of inducible promoters, such as the temperature sensitive heat shock family of promoters (Neidhardt *et al. supra*), or the anaerobically induced nirB promoter (Harborne *et al.* Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as uapA (Gorfinkiel *et al.* J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer *et al.* J. Bact., 176:6159-6164 (1994)).

The particular *Listeria* strain employed is not critical to the present invention. Examples of *Listeria* strains that can be employed in the present invention include *Listeria monocytogenes* (ATCC No. 15313). Attenuated *Listeria* strains, such as *L. monocytogenes* actA mutant (Brundage *et al. supra*) or *L. monocytogenes* plcA (Camilli *et al.* J. Exp. Med., 173:751-754 (1991)) are preferably used in the present invention. Alternatively, new attenuated *Listeria* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *Salmonella typhi* (ATCC No. 7251) and *S. typhimurium* (ATCC No. 13311). Attenuated *Salmonella* strains are preferably used in the present invention and include *S. typhi*-aroC-aroD (Hone *et al.* Vacc. 9:810 (1991) and *S. typhimurium*-aroA mutant (Mastroeni *et al.* Micro. Pathol. 13:477 (1992)). Alternatively, new attenuated *Salmonella* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella spp.* above.

The particular *Rickettsia* strain employed is not critical to the present invention. Examples of *Rickettsia* strains which can be employed in the present invention include *Rickettsia Rickettsiae* (ATCC Nos. VR149 and VR891), *Rickettsia prowaseckii* (ATCC No. VR233), *Rickettsia tsutsugamuchi* (ATCC Nos. VR312, VR150 and VR609), *Rickettsia mooseri* (ATCC No. VR144), *Rickettsia sibirica* (ATCC No. VR151), and *Rochalimaea quitana* (ATCC No. VR358). Attenuated *Rickettsia* strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular enteroinvasive *Escherichia* strain employed is not critical to the present invention. Examples of enteroinvasive *Escherichia* strains which can be employed in the present invention include *Escherichia coli* strains 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (Sansonetti *et al.* Ann. Microbiol. (Inst. Pasteur), 132A:351-355 (1982)). Attenuated enteroinvasive *Escherichia*

strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

Furthermore, since certain microorganisms other than bacteria can also interact with integrin molecules (which are receptors for certain invasion factors) for cellular uptake, such microorganisms can also be used for introducing RNA into target cells. For example, viruses, *e.g.*, foot-and-mouth disease virus, echovirus, and adenovirus, and eukaryotic pathogens, *e.g.*, *Histoplasma capsulatum* and *Leishmania major* interact with integrin molecules.

1.2 Less Invasive Bacteria

Examples of bacteria which can be used in the invention and which have been described in the literature as being non-invasive or at least less invasive than the bacteria listed in the previous section (1.1) include, but are not limited to, *Yersinia spp.*, *Escherichia spp.*, *Klebsiella spp.*, *Bordetella spp.*, *Neisseria spp.*, *Aeromonas spp.*, *Francisella spp.*, *Corynebacterium spp.*, *Citrobacter spp.*, *Chlamydia spp.*, *Hemophilus spp.*, *Brucella spp.*, *Mycobacterium spp.*, *Legionella spp.*, *Rhodococcus spp.*, *Pseudomonas spp.*, *Helicobacter spp.*, *Vibrio spp.*, *Bacillus spp.*, and *Erysipelothrix spp.* It may be necessary to modify these bacteria to increase their invasive potential.

The particular *Yersinia* strain employed is not critical to the present invention. Examples of *Yersinia* strains that can be employed in the present invention include *Y. enterocolitica* (ATCC No. 9610) or *Y. pestis* (ATCC No. 19428). Attenuated *Yersinia* strains, such as *Y. enterocolitica* Ye03-R2 (al-Hendy *et al.* Infect. Immun., 60:870-875 (1992)) or *Y. enterocolitica* aroA (O'Gaora *et al.* Micro. Path., 9:105-116 (1990)) are preferably used in the present invention. Alternatively, new attenuated *Yersinia* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Escherichia* strain employed is not critical to the present invention. Examples of *Escherichia* strains that can be employed in the present invention include *E. coli* Nissle 1917, MM294, H10407 (Elinghorst *et al.* Infect. Immun., 60:2409-2417 (1992)), and *E. coli* EFC4, CFT325 and CPZ005 (Donnenberg *et al.* J. Infect. Dis., 169:831-838 (1994)). Attenuated *Escherichia* strains, such as the attenuated turkey pathogen *E. coli* 02 carAB mutant (Kwaga *et al.* Infect. Immun., 62:3766-3772 (1994)) or CEQ201 are preferably used in the present invention. Alternatively, new attenuated *Escherichia* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Klebsiella* strain employed is not critical to the present invention. Examples of *Klebsiella* strains that can be employed in the present invention include *K. pneumoniae* (ATCC No. 13884). Attenuated *Klebsiella* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Bordetella* strain employed is not critical to the present invention. Examples of *Bordetella* strains that can be employed in the present invention include *B. bronchiseptica* (ATCC No. 19395). Attenuated *Bordetella* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Neisseria* strain employed is not critical to the present invention. Examples of *Neisseria* strains that can be employed in the present invention include *N. meningitidis* (ATCC No. 13077) and *N. gonorrhoeae* (ATCC No. 19424). Attenuated *Neisseria* strains, such as *N. gonorrhoeae* MS11 aro mutant (Chamberlain *et al.* Micro. Path., 15:51-63 (1993)) are preferably used in the present invention. Alternatively, new attenuated *Neisseria* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Aeromonas* strain employed is not critical to the present invention. Examples of *Aeromonas* strains that can be employed in the present invention include *A. eucrenophila* (ATCC No. 23309). Alternatively, new attenuated *Aeromonas* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Francisella* strain employed is not critical to the present invention. Examples of *Francisella* strains that can be employed in the present invention include *F. tularensis* (ATCC No. 15482). Attenuated *Francisella* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Corynebacterium* strain employed is not critical to the present invention. Examples of *Corynebacterium* strains that can be employed in the present invention include *C. pseudotuberculosis* (ATCC No. 19410). Attenuated *Corynebacterium* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Citrobacter* strain employed is not critical to the present invention. Examples of *Citrobacter* strains that can be employed in the present invention include *C. freundii* (ATCC No. 8090). Attenuated *Citrobacter* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Chlamydia* strain employed is not critical to the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (ATCC No. VR1310). Attenuated *Chlamydia* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Hemophilus* strain employed is not critical to the present invention. Examples of *Hemophilus* strains that can be employed in the present invention include *H. sordani* (ATCC No.

43625). Attenuated *Hemophilus* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Brucella* strain employed is not critical to the present invention. Examples of *Brucella* strains that can be employed in the present invention include *B. abortus* (ATCC No. 23448). Attenuated *Brucella* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Mycobacterium* strain employed is not critical to the present invention. Examples of *Mycobacterium* strains that can be employed in the present invention include *M. intracellulare* (ATCC No. 13950) and *M. tuberculosis* (ATCC No. 27294). Attenuated *Mycobacterium* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Legionella* strain employed is not critical to the present invention. Examples of *Legionella* strains that can be employed in the present invention include *L. pneumophila* (ATCC No. 33156). Attenuated *Legionella* strains, such as a *L. pneumophila* mip mutant (Ott, FEMS Micro. Rev., 14:161-176 (1994)) are preferably used in the present invention. Alternatively, new attenuated *Legionella* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Rhodococcus* strain employed is not critical to the present invention. Examples of *Rhodococcus* strains that can be employed in the present invention include *R. equi* (ATCC No. 6939). Attenuated *Rhodococcus* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Pseudomonas* strain employed is not critical to the present invention. Examples of *Pseudomonas* strains that can be employed in the present invention include *P. aeruginosa* (ATCC No. 23267). Attenuated *Pseudomonas* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that can be employed in the present invention include *H. mustelae* (ATCC No. 43772). Attenuated *Helicobacter* strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *Salmonella typhi* (ATCC No.

7251) and *S. typhimurium* (ATCC No. 13311). Attenuated *Salmonella* strains are preferably used in the present invention and include *S. typhi* aroC aroD (Hone *et al.* Vacc., 9:810-816 (1991)) and *S. typhimurium* aroA mutant (Mastroeni *et al.* Micro. Pathol, 13:477-491 (1992))). Alternatively, new attenuated *Salmonella* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Vibrio* strain employed is not critical to the present invention. Examples of *Vibrio* strains that can be employed in the present invention include *Vibrio cholerae* (ATCC No. 14035) and *Vibrio cincinnatiensis* (ATCC No. 35912). Attenuated *Vibrio* strains are preferably used in the present invention and include *V. cholerae* RSI virulence mutant (Taylor *et al.* J. Infect. Dis., 170:1518-1523 (1994)) and *V. cholerae* ctxA, ace, zot, cep mutant (Waldor *et al.* J. Infect. Dis., 170:278-283 (1994)). Alternatively, new attenuated *Vibrio* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Bacillus* strain employed is not critical to the present invention. Examples of *Bacillus* strains that can be employed in the present invention include *Bacillus subtilis* (ATCC No. 6051). Attenuated *Bacillus* strains are preferably used in the present invention and include *B. anthracis* mutant pX01 (Welkos *et al.* Micro. Pathol, 14:381-388 (1993)) and attenuated BCG strains (Stover *et al.* Nat., 351:456-460 (1991)). Alternatively, new attenuated *Bacillus* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Erysipelothrix* strain employed is not critical to the present invention. Examples of *Erysipelothrix* strains that can be employed in the present invention include *Erysipelothrix rhusiopathiae* (ATCC No. 19414) and *Erysipelothrix tonsillarum* (ATCC No. 43339). Attenuated *Erysipelothrix* strains are preferably used in the present invention and include *E. rhusiopathiae* Kg-1a and Kg-2 (Watarai *et al.* J. Vet. Med. Sci., 55:595-600 (1993)) and *E. rhusiopathiae* ORVAC mutant (Markowska-Daniel *et al.* Int. J. Med. Microb. Virol. Parasit. Infect. Dis., 277:547-553 (1992)). Alternatively, new attenuated *Erysipelothrix* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

1.3. Methods for Increasing the Invasive Properties of a Bacterial Strain

Whether organisms have been traditionally described as invasive or non-invasive, these organisms can be engineered to increase their invasive properties, *e.g.*, by mimicking the invasive properties of *Shigella spp.*, *Listeria spp.*, *Rickettsia spp.*, or enteroinvasive *E. coli spp.* For example, one or more genes that enable the microorganism to access the cytoplasm of a cell, *e.g.*, a cell in the natural host of said non-invasive bacteria, can be introduced into the microorganism.

Examples of such genes referred to herein as "cytoplasm-targeting genes" include genes encoding the proteins that enable invasion by *Shigella* or the analogous invasion genes of entero-

invasive *Escherichia*, or listeriolysin O of *Listeria*, as such techniques are known to result in rendering a wide array of invasive bacteria capable of invading and entering the cytoplasm of animal cells (Formal *et al.* Infect. Immun., 46:465 (1984); Bielecke *et al.* Nature, 345:175-176 (1990); Small *et al.* In: Microbiology-1986, pages 121-124, Levine *et al.* Eds., American Society for Microbiology, Washington, D.C. (1986); Zychlinsky *et al.* Molec. Micro., 11:619-627 (1994); Gentshev *et al.* (1995) Infection & Immunity 63:4202; Isberg, R. R. and S. Falkow (1985) Nature 317:262; and Isberg, R. R. *et al.* (1987) Cell 50:769). Methods for transferring the above cytoplasm-targeting genes into a bacterial strain are well known in the art. Another preferred gene that can be introduced into bacteria to increase their invasive character encodes the invasin protein from *Yersinia pseudotuberculosis*, (Leong *et al.* EMBO J., 9:1979 (1990)). Invasin can also be introduced in combination with listeriolysin, thereby further increasing the invasive character of the bacteria relative to the introduction of either of these genes. The above genes have been described for illustrative purposes; however, it will be obvious to those skilled in the art that any gene or combination of genes, from one or more sources, that participates in the delivery of a molecule, in particular an RNA or RNA-encoding DNA molecule, from a microorganism into the cytoplasm of a cell, *e.g.*, an animal cell, will suffice. Thus, such genes are not limited to bacterial genes, and include viral genes, such as influenza virus hemagglutinin HA-2 that promotes endosmolysis (Plank *et al.* J. Biol. Chem., 269:12918-12924 (1994)).

The above cytoplasm-targeting genes can be obtained by, *e.g.*, PCR amplification from DNA isolated from an invasive bacterium carrying the desired cytoplasm-targeting gene. Primers for PCR can be designed from the nucleotide sequences available in the art, *e.g.*, in the above-listed references and/or in GenBank, which is publicly available on the internet (www.ncbi.nlm.nih.gov/). The PCR primers can be designed to amplify a cytoplasm-targeting gene, a cytoplasm-targeting operon, a cluster of cytoplasm-targeting genes, or a regulon of cytoplasm-targeting genes. The PCR strategy employed will depend on the genetic organization of the cytoplasm-targeting gene or genes in the target invasive bacteria. The PCR primers are designed to contain a sequence that is homologous to DNA sequences at the beginning and end of the target DNA sequence. The cytoplasm-targeting genes can then be introduced into the target bacterial strain, *e.g.*, by using Hfr transfer or plasmid mobilization (Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); Bothwell *et al. supra*; and Ausubel *et al. supra*), bacteriophage-mediated transduction (de Boer, *supra*; Miller, *supra*; and Ausubel *et al. supra*), chemical transformation (Bothwell *et al. supra*; Ausubel *et al. supra*), electroporation (Bothwel *et al. supra*; Ausubel *et al. supra*; and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and physical transformation techniques (Johnston *et al. supra*; and Bothwell, *supra*). The cytoplasm-targeting genes can be incorporated into lysogenic bacteriophage (de Boer *et al.* Cell, 56:641-649 (1989)), plasmids vectors (Curtiss *et al. supra*) or spliced into the chromosome (Hone *et al. supra*) of the target strain.

In addition to genetically engineering bacteria and BTPs to increase their invasive properties, as set forth above, bacteria can also be modified by linking an invasion factor to the bacteria. Accordingly, in one embodiment, a bacterium is rendered more invasive by coating the bacterium, either covalently or non-covalently, with an invasion factor, *e.g.*, the protein invasin, invasin derivatives, or a fragment thereof sufficient for invasiveness. In fact, it has been shown that non-invasive bacterial cells coated with purified invasin from *Yersinia pseudotuberculosis* or the carboxyl-terminal 192 amino acids of invasin are able to enter mammalian cells (Leong *et al.* (1990) *EMBO J.* 9:1979). Furthermore, latex beads coated with the carboxyl terminal region of invasin are efficiently internalized by mammalian cells, as are strains of *Staphylococcus aureus* coated with antibody-immobilized invasin (reviewed in Isberg and Tran van Nhieu (1994) *Ann. Rev. Genet.* 27:395). Alternatively, a bacterium can also be coated with an antibody, variant thereof, or fragment thereof, which binds specifically to a surface molecule recognized by a bacterial entry factor. For example, it has been shown that bacteria are internalized if they are coated with a monoclonal antibody directed against an integrin molecule, *e.g.*, $\alpha 5\beta 1$, known to be the surface molecule with which the bacterial invasin protein interacts (Isberg and Tran van Nhieu, *supra*). Such antibodies can be prepared according to methods known in the art. The antibodies can be tested for efficacy in mediating bacterial invasiveness by, *e.g.*, coating bacteria with the antibody, contacting the bacteria with eukaryotic cells having a surface receptor recognized by the antibody, and monitoring the presence of intracellular bacteria, according to the methods described above. Methods for linking an invasion factor to the surface of a bacterium are known in the art and include cross-linking.

3. Plasmids and Vectors

The present invention also provides at least one vector or plasmid including at least one DNA molecule encoding one or more siRNAs and at least one promoter, wherein the expressed siRNAs interfere with at least one mRNA of a gene of interest. In one preferred embodiment, the present invention provides at least one prokaryotic vector including at least one DNA molecule encoding one or more siRNAs and at least one RNA-polymerase III compatible promoter or at least one prokaryotic promoter, wherein the expressed siRNAs interfere with at least one mRNA of a gene of interest.

The TRIP (transkingdom RNA interference plasmid) vectors and plasmids of the present invention include a multiple cloning site, a promoter sequence and a terminator sequence. The TRIP vectors and plasmids also include one or more sequences encoding for an invasion factor to permit the non-invasive bacterium or BTP to enter mammalian cells (*e.g.*, the *Inv* locus that encodes invasion that permits the bacterium or BTP to enter $\beta 1$ -integrin-positive mammalian cells) (Young *et al.*, *J. Cell Biol.* 116, 197-207 (1992)) and one or more sequences to permit the genetic material to escape from the entry vesicles (*e.g.*, *Hly A* gene that encodes listeriolysin O) (Mathew *et al.*, *Gene Ther.* 10, 1105-1115 (2003) and Grillot-Courvalin *et al.*, *Nat. Biotechnol.* 16, 862-866 (1998)). TRIP is further described (including a vector/plasmid schematic) in PCT Publication No. WO 06/066048. In

preferred embodiments, the TRIP vectors and plasmids will incorporate a hairpin RNA expression cassette encoding short hairpin RNA under the control of an appropriate promoter sequence and terminator sequence.

In the design of these constructs, an algorithm was utilized to take into account some known difficulties with the development of siRNA, namely: (1) Exclusion of disqualifying properties (SNPs, interferon motifs); (2) Exclusion of the sequence if there was homology in ref seq (19/21, >17 contiguous to any other genes) and (3) Exclusion of the sequence if there were significant miRNA seed type matches.

As described herein, the one or more DNA molecules encoding the one or more siRNAs are transcribed within the eukaryotic target cell or transcribed within the bacterium or BTP.

In embodiments where the DNA is transcribed within the eukaryotic cell, the one or more siRNAs are transcribed within the eukaryotic cells as shRNAs. The eukaryotic cell can be *in vivo*, *in vitro* or *ex vivo*. In one aspect of this embodiment, the one or more DNA molecules encoding the one or more siRNAs contain a eukaryotic promoter. Optionally, the eukaryotic promoter is a RNA-polymerase III promoter. Optionally, the RNA polymerase III promoter is a U6 promoter or an H1 promoter.

In embodiments where the DNA is transcribed within the bacterium or BTP, the one or more DNA molecules contain a prokaryotic promoter. Optionally, the prokaryotic promoter is an *E. coli* promoter. Preferably, the *E. coli* promoter can be a T7 promoter, lacUV5 promoter, modified lacUV5 promoter, RNA polymerase promoter, gapA promoter, pA1 promoter, lac regulated promoter, araC+ P_{araBAD} promoter, T5 promoter, P_{tac} promoter (Estrem et al, 1998, Proc. Natl. Acad. Sci. USA 95, 9761-9766; Meng et al., 2001, Nucleic Acids Res. 29, 4166-417; De Boer et al., 1983, Proc. Natl. Acad. Sci. USA 80, 21-25) or recA promoter.

Preferable, promoter sequences are recited in Table 1.

Table 1

Promoter	Sequence	SEQ ID NO:
T7 promoter	TAATACGACTCACTATAG	1
lacUV5 promoter	TAACCAGGCTTTACACTTTATGCTTCCGGCTC GTATAATGTGTGGAAGGATCC	2
RNA polymerase promoter	TAACCAGGCTTTACACTTTATGCTTCCGGCTC GTATAATGTGTGGAA	3
RNA polymerase promoter	TAAAATTCAAAAATTTATTTGCTTTCAGGAAA ATTTTCTGTATAATAGATTC	4
RNA polymerase promoter	TAATTGATACTTTATGCTTTTTTCTGTATAAT	5
gapA promoter	AAGCTTTCAGTCGCGTAATGCTTAGGCACAGG ATTGATTTGTGCGCAATGATTGACACGATTCCG CTTGACACTGCGTAAGTTTTGTGTTATAATGG ATCC	6
pA1 promoter	AAGCTTAAGGAGAGACAACCTTAAAGAGACTTA AAAGATTAATTTAAATTTATCAAAAAGAGTA TTGACTTAAAGTCTAACCTATAGGATACTTGG ATCC	7
lac regulated promoter	AAGCTTTGTGTGGAATTGTGAGCGGATAACAA	8

	TTCCACACATTGACACTTTTATGCTTCCGGCTC GTATAATGGATCC	
lac regulated promoter	AAGCTTGGAAAAATTTTTTTTAAAAAAGTCATG TGTGGAATTGTGAGCGGATAACAATTCCACAT ATAATGGATCC	9
araC+ P _{araBAD} promoter	GACTTCATATACCCAAGCTTTAAAAAAAAAAT CCTTAGCTTTTCGCTAAGGATCTCCGTCAAGCC GTCAATTGTCTGATTCTGTTACCAATTATGACA ACTTGACGGCTACATCATTCACTTTTTCTTCA CAACCGGCACGAACTCGCTCGGGCTGGCCCC GGTGCATTTTTTTAAATACTCGCGAGAAATAGA GTTGATCGTCAAAACCAACATTGCGACCGACG GTGGCGATAGGCATCCGGGTAGTGCTCAAAAG CAGCTTCGCCTGACTAATGCGTTGGTCCTCGC GCCAGCTTAAGACGCTAATCCCTAACTGCTGG CGGAAAAGATGTGACAGACGCGACGGCGACAA GCAAACATGCTGTGCGACGCTGGCGATATCAA AATTGCTGTCTGCCAGGTGATCGCTGATGTAC TGACAAGCCTCGCGTACCCGATTATCCATCGG TGGATGGAGCGACTCGTTAATCGCTTCCATGC GCCGCAGTAACAATTGCTCAAGCAGATTTATC GCCAGCAGCTCCGAATAGCGCCCTTCCCCTTG CCCGGCGTTAATGATTTGCCCAAAACAGGTCGC TGAAATGCGGCTGGTGCGCTTCATCCGGGCGA AAGAAACCCGTATTGGCAAATATTGACGGCCA GTTAAGCCATTTCATGCCAGTAGGCGCGCGGAC GAAAGTAAACCCACTGGTGATAACATTGCGGA GCCTCCGGATGACGACCGTAGTGATGAATCTC TCCTGGCGGGAACAGCAAAATATCACCCGGTC GGCAGACAAATTCTCGTCCCTGATTTTTCACC ACCCCTGACCGCGAATGGTGAGATTGAGAAT ATAACCTTTTCATTCCCAGCGGTCGGTCGATAA AAAAATCGAGATAACCGTTGGCCTCAATCGGC GTTAAACCCGCCACCAGATGGGCGTTAAACGA GTATCCCGGCAGCAGGGGATCATTTTGCGCTT CAGCCATACTTTTCATACTCCCACCATTCAGA GAAGAAACCAATTGTCCATATTGCATCAGACA TTGCCGTCACCTGCGTCTTTTACTGGCTCTTCT CGCTAACCCAACCGGTAACCCCGCTTATTAAA AGCATTCTGTAACAAAGCGGGACCAAAGCCAT GACAAAAACGCGTAACAAAAGTGTCTATAATC ACGGCAGAAAAGTCCACATTGATTATTTGCAC GGCGTCACACTTTGCTATGCCATAGCATTTTT ATCCATAAGATTAGCGGATCCTACCTGACGCT TTTTATCGCAACTCTCTACTGTAGATCTATCT GCGAT	10
T5 promoter	TAAAATTCAAAAATTTATTTGCTTTCAGGAAA ATTTTTCTGTATAATAGATTCCGATCC	11
recA promoter	TAATTGATACTTTATGCTTTTTTCTGTATAAT GGATCC	12
P _{tac} promoter	GACTTCATATACCCAAGCTTGGAAAAATTTTT TTAAAAAAGTCTTGACACTTTATGCTTCCGGC TCGTATAATGGATCC	13
P _{atac} promoter	GGAAAATTTTTTTTAAAAAAGTC	14

In embodiments where the DNA is transcribed within the bacterium or BTP, the *E. coli* promoter is associated with a terminator. Preferably, the *E. coli* terminator can be a T7 terminator,

lacUV5 terminator, Rho-independent terminator, Rho-dependent terminator, or RNA polymerase terminator.

Preferable, terminator sequences are recited in Table 2.

Table 2

Terminator	Sequence	SEQ ID NO:
T7 terminator	TAGCATAACCCCTTGGGGCCTCTAAACGGGTC TTGAGGGGTTTTTTT	15
lacUV5 terminator	TTGTCACGTGAGCGGATAACAATTTACACAG GAAACAGAATTCTTAAT	16
Rho-independent terminator	TTGTCACAAACCCCGCCACCGGCGGGGTTTTT TTCTGCTTAAT	17
Rho-dependent terminator	TTGTCACAATTCTATGGTGTATGCATTTATTT GCATACATTCAATCAATTGGATCCTGCATTAA T	18
RNA polymerase terminator	GTGAGCGGATAACAATTTACACAGGAAACAG AATTCCTTAAT	19
RNA polymerase terminator	AAACCCCGCCACCGGCGGGGTTTTTTCTGCT TAAT	20
RNA polymerase terminator	AATTCTATGGTGTATGCATTTATTTGCATACA TTCAATCAATTGGATCCTGCATTAAT	21

5

In additional embodiments, the vectors and plasmids of the present invention further include one or more enhancer sequences, selection markers, or lysis regulation system sequences.

In one aspect of the invention, the one or more DNA molecules contain a prokaryotic enhancer. Optionally, the prokaryotic enhancer is a T7 enhancer. Optionally, the T7 enhancer has the sequence GAGACAGG (SEQ ID NO: 22). In another aspect of this embodiment, the one or more DNA molecules contain a prokaryotic terminator.

In another aspect of the, the one or more DNA molecules are associated with one or more selection markers. In one aspect of this embodiment, the selection marker is an amber suppressor containing one or more mutations or a diamino pimelic acid (DAP) containing one or more mutations. Optionally, the dap gene is selected from, but not limited to, dapA and dapE.

Preferable, selection marker sequences are recited in Table 3.

Table 3

Selection Marker	Sequence	SEQ ID NO:
amber suppressor gene sequence	AATTCGGGGCTATAGCTCAGCTGGGAGAGCGCTT GCATCTAATGCAAGAGGTCAGCGGTTTCGATCCCG CTTAGCTCCACCACTGCA	23
amber suppressor sequence	AATTCGCCCCGATAGCTCAGTCGGTAGAGCAGGG GATTCTAAATCCCCGTGTCTTGGTTCGATTCCG AGTCCGGGCACTGCA	24
Rho- <i>lgt</i> with double amber mutation (<i>lgt am-am</i> allele of <i>lgt</i> gene) sequence	ATGACCAGTAGCTATCTGCATTAGCCGGAGTAGG ATCCGGTCATTTTCTCAATAGGACCCGTGGCGCT TCACTGGTACGGCCTGATGTATCTGGTGGGTTTC ATTTTTGCAATGTGGCTGGCAACACGACGGGCGA ATCGTCCGGGCAGCGGCTGGACCAAAAATGAAGT TGAAAACCTTACTCTATGCGGGCTTCCTCGGCGTC TTCCTCGGGGGACGTATTGGTTATGTTCTGTTCT ACAATTTCCCGCAGTTTATGGCCGATCCGCTGTA TCTGTTCCGTGTCTGGGACGGCGGCATGTCTTTC	25

	CACGGCGGCGCTGATTGGCGTTATCGTGGTGATGA TTATCTTCGCCCCGCGTACTAAACGTTCTTCTT CCAGGTCTCTGATTTTATCGCACCCTCATTCCG TTTGGTCTTGGTGCCGGGCGTCTGGGCAACTTTA TTAACGGTGAATTGTGGGGCCGCGTTGACCCGAA CTTCCCCTTTGCCATGCTGTTCCCTGGCTCCCGT ACAGAAGATATTTTGCTGCTGCAAACCAACCCGC AGTGGCAATCCATTTTCGACACTTACGGTGTGCT GCCGCGCCACCCATCACAGCTTTACGAGCTGCTG CTGGAAGGTGTGGTGTGTTTATTATCCTCAACC TGTATATTTCGTAAACCACGCCCAATGGGAGCTGT CTCAGGTTTGTTCCTGATTGGTTACGGCGCGTTT CGCATCATTGTTGAGTTTTTCCGCCAGCCCCGACG CGCAGTTTACCGGTGCCTGGGTGCAGTACATCAG CATGGGGCAAATTCTTTCCATCCCGATGATTGTC GCGGGTGTGATCATGATGGTCTGGGCATATCGTC GCAGCCCACAGCAACACGTTTCTCTGA	
<i>murA</i> with double amber mutation (<i>murA am-am</i> allele of <i>murA</i> gene) sequence	ATGGATAAATTTTCGTGTTTCAGGGGCCAACGAAGC TCCAGGGCGAAGTCACAATTTCCGGCGCTAAAAA TTAGTAGCTGCCTATCCTTTTGGCCGCACTACTG GCGGAAGAACCAGGTAGAGATCCAGAACGTCCCGA AACTGAAAGACGTGATACATCAATGAAGCTGCT AAGCCAGCTGGGTGCGAAAGTAGAACGTAATGGT TCTGTGCATATTGATGCCCCGCGACGTTAATGTAT TCTGCGCACCTTACGATCTGGTTAAAACCATGCG TGCTTCTATCTGGGCGCTGGGGCCGCTGGTAGCG CGCTTTGGTCAGGGGCAAGTTTCACTACCTGGCG GTTGTACGATCGGTGCGCGTCCGGTTGATCTACA CATTTCTGGCCTCGAACAATTAGGCGCGACCATC AAACTGGAAGAAGGTTACGTTAAAGCTTCCGTCG ATGGTCGTTTGAAAGGTGCACATATCGTGATGGA TAAAGTCAGCGTTGGCGCAACGGTGACCATCATG TGTGCTGCAACCCTGGCGGAAGGCACCACGATTA TTGAAAACGCAGCGCGTGAACCGGAAATCGTCGA TACCGCGAACTTCTTGATTACGCTGGGTGCGAAA ATTAGCGGTACAGGCACCGATCGTATCGTCATCG AAGGTGTGGAACGTTTAGGCGGCGGTGTCTATCG CGTTCTGCCGGATCGTATCGAAACCGGTACTTTC CTGGTGGCGGCGGCGATTTCTCGCGGCAAAATTA TCTGCCGTAACGCGCAGCCAGATACTCTCGACGC CGTGCTGGCGAAACTGCGTGACGCTGGAGCGGAC ATCGAAGTCGGCGAAGACTGGATTAGCCTGGATA TGCATGGCAAACGTCCGAAGGCTGTTAACGTACG TACCGCGCCGATCCGGCATTCCCGACCGATATG CAGGCCCAGTTCACGCTGTTGAACCTGGTGGCAG AAGGGACCGGGTTTATCACCGAAACGGTCTTTGA AAACCGCTTTATGCATGTGCCAGAGCTGAGCCGT ATGGGCGCGCACGCCGAAATCGAAAGCAATACCG TTATTTGTACGGTGTTGAAAACTTTCTGGCGC ACAGGTTATGGCAACCGATCTGCGTGATCAGCA AGCCTGGTGCTGGCTGGCTGTATTGCGGAAGGGA CGACGGTGGTTGATCGTATTTATCACATCGATCG TGGCTACGAACGCATTGAAGACAACTGCGCGCT TTAGGTGCAAATATTGAGCGTGTGAAAGGCGAAT AA	26
dapA sequence	GCCAGGCGACTGTCTTCAATATTACAGCCGCAAC TACTGACATGACGGGTGATGGTGTTTACAATTCC AGGGCGATCGGCACCCAACGCAGTGATCACCAGA TAATGTTGCGATGACAGTGTCAAACCTGGTTATTC CTTTAAGGGGTGAGTTGTTCTTAAGGAAAGCATA AAAAAACATGCATACAACAATCAGAACGGTTCT	27

	GTCTGCTTGCTTTTAAATGCCATACCAAACGTACC ATTGAGACACTTGTTTGCACAGAGGATGGCCCAT GTTCACGGGAAGTATTGTGCGGATTGTTACTCCG ATGGATGAAAAAGGTAATGTCTGTGCGGGCTAGCT TGAAAAAACTGATTGATTATCATGTGCGCCAGCGG TACTTTCGGCGATCGTTTCTGTTGGCACCCTGGC GAGTCCGCTACCTTAAATCATGACGAACATGCTG ATGTGGTGATGATGACGCTGGATCTGGCTGATGG GCGCATTCCGGTAATTGCCGGGACCGGCGCTAAC GCTACTGCGGAAGCCATTAGCCTGACGCAGCGCT TCAATGACAGTGGTATCGTTCGGCTGCCTGACGGT AACCCCTTACTACAATCGTCCGTGCAAGAAGGT TTGTATCAGCATTTCAAAGCCATCGCTGAGCATA CTGACCTGCCGCAAATTCTGTATAATGTGCCGTC CCGTACTGGCTGCGATCTGCTCCCGGAAACGGTG GGCCGTCTGGCGAAAGTAAAAAATATTATCGGAA TCAAAGAGGCAACAGGGAACTTAACGCGTGTA CCAGATCAAAGAGCTGGTTTCAGATGATTTTGT CTGCTGAGCGGCGATGATGCGAGCGCGCTGGACT TCATGCAATTGGGCGGTATGGGGTTATTTCCGT TACGGCTAACGTGCGCAGCGCGTGATATGGCCCAG ATGTGCAAACTGGCAGCAGAAGGGCATTTTGCCG AGGCACGCGTTATTAATCAGCGTCTGATGCCATT ACACAACAACTATTTGTGAAACCAATCCAATC CCGGTGAAATGGGCATGTAAGGAACTGGGTCTTG TGGCGACCGATACGCTGCGCCTGCCAATGACACC AATCACCGACAGTGGTCGTGAGACGGTCAGAGCG GCGCTTAAGCATGCCGGTTTGCTGTAAAGTTTAG GGAGATTTGATGGCTTACTCTGTTCAAAAGTCGC GCCTGGCAAAGGTTGCGGGTGTTTCGCTTGTTTT ATTACTCGCTGCCTGTAGTTCTGACTCACGCTAT AAGCGTCAGGTCAAGTGGTGATGAAGCCTACCTGG AAGCG	
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Optionally, the amber suppressor is associated with a promoter or a terminator. Optionally, the promoter is a lipoprotein promoter. Preferable, promoter sequences are recited in Table 4.

Table 4

Amber Suppressor Promoter Sequence	Sequence	SEQ ID NO:
lipoprotein promoter	CATGGCGCCGCTTCTTTGAGCGAACGATCAAAAA TAAGTGGCGCCCCATCAAAAAAATATTCTCAACA TAAAAAACTTTGTGTAATACTTGTAACGCTG	28
lipoprotein promoter	CATGGCGCCCCATCAAAAAAATATTCTCAACATA AAAAAATTTGTGTAATACTTGTAACGCTG	29

5

Optionally, the terminator is an rrnC terminator. Preferable, terminator sequences are recited in Table 5.

Table 5

Amber Suppressor Terminator Sequence	Sequence	SEQ ID NO:
rrnC terminator	GATCCTTAGCGAAAGCTAAGGATTTTTTTTAC	30
rrnC terminator	GATCCTTAGCGAAAGCTAAGGATTTTTTTTTT	31

Bacterial and BTP delivery is more attractive than viral delivery because they are more accessible to genetic manipulation, which allows the production of vector strains specifically tailored to certain applications. In one embodiment of the invention, the methods of the invention are used to create bacteria and BTPs that cause RNAi in a tissue specific manner.

5 Liberation of the siRNA encoding plasmid or the one or more siRNAs from the intracellular bacteria or BTPs occurs through active mechanisms. One mechanism involves the type III export system in *S. typhimurium*, a specialized multiprotein complex spanning the bacterial or BTP cell membrane whose functions include secretion of virulence factors to the outside of the cell to allow signaling towards the target cell, but which can also be used to deliver antigens into target cells
10 (Rüssmann H. *Int J Med Microbiol*, **293**:107-12 (2003)), or through bacterial lysis and liberation of bacterial or BTP contents into the cytoplasm. The lysis of intracellular bacteria or BTPs is triggered through various mechanisms, including addition of an intracellularly active antibiotic (tetracycline), naturally through bacterial metabolic attenuation (auxotrophy), or through a lysis regulation system or bacterial suicide system comprising a bacterial regulator, promoter and sensor that is sensitive to the
15 environment, *e.g.*, the pH, magnesium concentration, phosphate concentration, ferric ion concentration, osmolarity, anaerobic conditions, nutritional deficiency and general stress of the target cell or the host phagosome. When the bacteria or BTP lysis regulation system senses one or more of the above environmental conditions, bacterial or BTP lysis is triggered by one or more mechanisms including but not limited to antimicrobial proteins, bacteriophage lysins and autolysins expressed by
20 the bacteria or BTP, either naturally or through modification, or through pore-forming proteins expressed by the bacteria or BTPs, either naturally or through modification, *e.g.*, genetic modification, which break the phagosomes containing the bacteria or BTPs and liberate the siRNA-encoding plasmid or the one or more siRNAs.

The regulator of the lysis regulation system may be selected from the group that includes but
25 is not limited to OmpR, ArcA, PhoP, PhoB, Fur, RstA, EvgA and RpoS. Preferable, lysis regulator sequences are recited in Table 6.

Table 6

Lysis Regulation System Regulator Sequence	Sequence	SEQ ID NO:
OmpR regulator	ATGCAAGAGAACTACAAGATTCTGGTGGTCGATG ACGACATGCGCCTGCGTGCGCTGCTGGAACGTTA TCTCACCGAACAAGGCTTCCAGGTTCAAGCGTC GCTAATGCAGAACAGATGGATCGCCTGCTGACTC GTGAATCTTTCCATCTTATGGTACTGGATTTAAT GTTACCTGGTGAAGATGGCTTGTGCGATTGCCGA CGTCTTCGTAGTCAGAGCAACCCGATGCCGATCA TTATGGTGACGGCGAAAGGGGAAGAAGTGGACCG TATCGTAGGCCTGGAGATTGGCGCTGACGACTAC ATTCCAAAACCGTTTAACCCGCGTGAACTGCTGG CCCGTATCCGTGCGGTGCTGCGTCGTCAGGCGAA CGAACTGCCAGGCGCACCGTCACAGGAAGAGGCG GTAATTGCTTTCCGTAAGTTCAAACCTAACCTCG GTACGCGCGAAATGTTCCGCGAAGACGAGCCGAT	32

	GCCGCTCACCAGCGGTGAGTTTGCGGTACTGAAG GCACTGGTCAGCCATCCGCGTGAGCCGCTCTCCC GCGATAAGCTGATGAACCTTGCCCGTGGTTCGTGA ATATTCCGCAATGGAACGCTCCATCGACGTGCAG ATTTTCGCGTCTGCGCCGATGGTGGAAAGAAGATC CAGCGCATCCGCGTTACATTAGACCGTCTGGGG TCTGGGCTACGTCTTTGTACCGGACGGCTCTAAA GCATGA	
PhoP regulator	ATGCGCGTACTGGTTGTTGAAGACAATGCGTTGT TACGTCACCACCTTAAAGTTCAGATTAGGATGC TGGTCATCAGGTCGATGACGCAGAAGATGCCAAA GAAGCCGATTATTATCTCAATGAACATATACCGG ATATTGCGATTGTGATCTCGGATTGCCAGACGA GGACGGTCTGTCACTGATTGCGCGCTGGCGTAGC AACGATGTTTTCACTGCGGATTCTGGTATTAACCG CCCGTGAAAGCTGGCAGGACAAAGTCGAAGTATT AAGTGCCGGTGCTGATGATTATGTGACTAAACCG TTTCATATTGAAGAGGTGATGGCGCGAATGCAGG CATTAAATGCGGCGTAATAGCGGTCTGGCTTCACA GGTCATTTTCGCTCCCCCGTTTCAGGTTGATCTC TCTCGCCGTGAATTATCTATTAATGACGAAGTGA TCAAACTGACCGCGTTTCAATACACTATTATGGA AACGTTGATACGCAATAATGGCAAAGTGGTCAGC AAAGATTGTTAATGCTCCAACTCTATCCGGATG CGGAGCTGCGGGAAGCCATACCATGATGTAAT GATGGGACGTCTGCGCAAAAAAATTCAGGCACAA TATCCCCAAGAAGTGATTACCACCGTTGCGGGCC AGGGCTATCTGTTTCAATTGCGCTGA	33

The promoter of the lysis regulation system may be selected from the group that includes but is not limited to ompF, ompC, fadB, phoPQ, mgtA, mgrB, psiB, phnD, Ptp, sodA, sodB, sltA, sltB, asr, csgD, emrKY, yhiUV, acrAB, mdx and tolC. Preferable, lysis regulation system promoter sequences are recited in Table 7.

Table 7

Lysis Regulation System Promoter Sequence	Sequence	SEQ ID NO:
ompF promoter	GATCATCTGTACGGAATATTACATTGCAACAT TTACGCGCAAAAACTAATCCGCAATCTTATTGCG GATTAGTTTTTTCTTAGCTAATAGCACAATTTTC ATACTATTTTTTTGGCATTCTGGATGTCTGAAAGA AGATTTTGTGCCAGGTCGATAAAGTTTCCATCAG AAACAAAATTTCCGTTTAGTTAATTTAAATATAA GGAAATCATATAAATAGATTAAAAATTGCTGTAAA TATCATCACGTCTCTATGGAAATATGACGGTGTT CACAAAGTTCCTTAAATTTTACTTTTGGTTACAT ATTTTTTTCTTTTTGAAACCAATCTTTATCTTTG TAGCACTTTACGGTAGCGAAACGTTAGTTTGAA TGGAAGATGCCTGCA	34
ompC promoter	TTTAAAAAAGTTCCGTAAATTCATATTTTGAAA CATCTATGTAGATAACTGTAACATCTTAAAAGTT TTAGTATCATATTCGTGTTGGATTATTCTGTATT TTTGGCGAGAATGGACTTGCCGACTGGTTAATGA GGGTTAACCAGTAAGCAGTGGCATAAAAAAGCAA TAAAGGCATATAACAGAGGGTTAATAAC	35
fadB promoter	AGTGATTCCATTTTTTACCCTTCTGTTTTTTTGA CCTTAAGTCTCCGCATCTTAGCACATCGTTCATC CAGAGCGTGATTTCTGCCGAGCGTGATCAGATCG	36

	GCATTTCTTTAATCTTTTGTTCATATTTTAA CACAAAATACACACTTCGACTCATCTGGTACGAC CAGATCACCTTGCGGATTGAGGAGACTGAC	
phoPQ promoter	GAGCTATCACGATGGTTGATGAGCTGAAATAAAC CTCGTATCAGTGCCGGATGGCGATGCTGTCCGGC CTGCTTATTAAGATTATCCGCTTTTTATTTTTTC ACTTTACCTCCCCTCCCCGCTGGTTTATTTAATG TTTACCCCCATAACCACATAATCGCGTTACACTA TTTTAATAATTAAGACAGGGAGAAATAAAA	37
mgtA promoter	GCTTCAACACGCTCGCGGGTGAGCTGGCTCACGC CGCTTTCGTTATTCAGCACCCGGGAACTGTAGA TTTCCCCACGCCGCTTAAGCGCGGATATCTTTG ATGGTCAGCCGATTTTGCATCCTGTTGTCTGTGA ACGTGTTGTTTAATTATTTGAGCCTAACGTTACC CGTGCAATTCAGCAATGGGTAAAGTCTGGTTTATC GTTGGTTTAGTTGTGTCAGCAGGTATTATATCGCCA	38
Ptp promoter	GAGCTGTTGACAATTAATCATCGAACTAGTTAAC TAGTACGCAAGTTCACGTAAAAAGGGTATCTAGA ATTCT	39

The sensor of the lysis regulation system may be selected from the group that includes but is not limited to EnvZ, ArcB, PhoQ, PhoR, RstB and EvgS. Preferable, lysis regulation system sensor sequences are recited in Table 8.

5 Table 8

Lysis Regulation System Sensor Sequence	Sequence	SEQ ID NO:
EnvZ sensor	ATGAGGCGATTGCGCTTCTCGCCACGAAGTTCAT TTGCCCCGTACGTTATTGCTCATCGTCACCTTGCT GTTTCGCCAGCCTGGTGACGACTTATCTGGTGGTG CTGAACCTTCGCGATTTTGCCGAGCCTCCAGCAGT TTAATAAAAGTCCTCGCGTACGAAGTGCGTATGTT GATGACCGACAACTGCAACTGGAGGACGGCACG CAGTTGGTTGTGCCTCCCGCTTTCCGTCGGGAGA TCTACCGTGAGCTGGGGATCTCTCTACTCCAA CGAGGCTGCCGAAGAGGCAGGTCTGCGTTGGGCG CAACACTATGAATTCTTAAGCCATCAGATGGCGC AGCAACTGGGCGGCCCGACGGAAGTGCGCGTTGA GGTCAACAAAAGTTCGCCTGTCGTCTGGCTGAAA ACCTGGCTGTGCCCCAATATCTGGGTACGCGTGC CGCTGACCGAAATTCATCAGGGCGATTCTCTCC GCTGTTCCGCTATACGCTGGCGATTATGCTATTG GCGATAGGCGGGGCGTGGCTGTTTATTCGTATCC AGAACCGACCGTTGGTCGATCTCGAACACGCAGC CTTGCGAGGTTGGTAAAGGGATTATCCGCCGCCG CTGCGTGAGTATGGCGCTTCGGAGGTGCGTTCCG TTACCCGTGCCTTTAACCATATGGCGGCTGGTGT TAAGCAACTGGCGGATGACCGCACGCTGCTGATG GCGGGGGTAAGTCACGACTTGCGCACGCCGCTGA CGCGTATTGCGCTGGCGACTGAGATGATGAGCGA GCAGGATGGCTATCTGGCAGAATCGATCAATAAAA GATATCGAAGAGTGCAACGCCATCATTGAGCAGT TTATCGACTACCTGCGCACCGGGCAGGAGATGCC GATGGAAATGGCGGATCTTAATGCAGTACTCGGT GAGGTGATTGCTGCCGAAAGTGGCTATGAGCGGG AAATTGAAACCGCGCTTTACCCCGGCAGCATTGA AGTGAAAATGCACCCGCTGTGATCAAACGCGCG GTGGCGAATATGGTGGTCAACGCCGCCCGTTATG	40

	GCAATGGCTGGATCAAAGTCAGCAGCGGAACGGA GCCGAATCGCGCCTGGTTCCAGGTGGAAGATGAC GGTCCGGGAATTGCGCCGGAACAACGTAAGCACC TGTTCCAGCCGTTTGTCCGCGGCGACAGTGC GCG CACCATTAGCGGCACGGGATTAGGGCTGGCAATT GTGCAGCGTATCGTGGATAACCATAACGGGATGC TGGAGCTTGGCACCAGCGAGCGGGGCGGGCTTTC CATTGCGCCTGGCTGCCAGTGCCGGTAACGCGG GCGCAGGGCACGACAAAAGAAGGGTAA	
PhoQ sensor	ATGAAAAAATTACTGCGTCTTTTTTTCCCGCTCT CGCTGCGGGTACGTTTTCTGTTGGCAACGGCAGC GGTAGTACTGGTGCTTTCGCTTGCTTACGGAATG GTCGCGCTGATCGGTTATAGCGTCAGTTTCGATA AAACTACGTTTTCGGCTGTTACGTGGCGAGAGCAA TCTGTTCTATACCCTTGCGAAGTGGGAAAAACAAT AAGTTGCATGTGAGTTACCCGAAAAATATCGACA AGCAAAGCCCCACCATGACGCTAATTTATGATGA GAACGGGCAGCTTTTATGGGCGCAACGTGACGTG CCCTGGCTGATGAAGATGATCCAGCCTGACTGGC TGAAATCGAATGGTTTTTCATGAAATTGAAGCGGA TGTTAACGATAACCAGCCTCTTGCTGAGTGGAGAT CATTGATAACAGCAACAGTTGCAGGAAGTGC GGG AAGATGATGACGACGCGGAGATGACCCACTCGGT GGCAGTAAACGTCTACCCGGCAACATCGCGGATG CCAAAATTAACCATTGTGGTGGTGGATACCATT CGGTGGAGCTAAAAAGTTCCTATATGGTCTGGAG CTGGTTTATCTATGTGCTCTCAGCCAATCTGCTG TTAGTGATCCCGCTGCTGTGGGTGCGCCGCTGGT GGAGTTTACGCCCCATCGAAGCCCTGGCAAAAAGA AGTCCGCGAACTGGAAGAACATAACCGCGAATTG CTCAATCCAGCCACAACGCGAGAACTGACCAGTC TGGTACGAAACCTGAACCGATTGTTAAAAAGTGA ACGCGAACGTTACGACAAATACCGTACGACGCTC ACCGACCTGACCCATAGTCTGAAAAACGCCACTGG CGGTGCTGCAAAGTACGCTGCGTTCTCTGCGTAG TGAAAAGATGAGCGTCAGTGATGCTGAGCCGGTA ATGCTGGAGCAAATCAGCCGCATTTACAGCAAA TTGGCTACTACCTGCATCGTGCCAGTATGCGCGG CGGGACATTGCTCAGCCGCGAGCTGCATCCGGTC GCCCCACTGCTGGACAATCTCACCTCAGCGCTGA ACAAAGTGTATCAACGCAAAGGGGTCAATATCTC TCTCGATATTTGCGCCAGAGATCAGCTTTGTGCGT GAGCAGAACGATTTTGTGAGGTGATGGGCAACG TGCTGGATAATGCCTGTAAATATTGCCTCGAGTT TGTCGAAATTTCTGCAAGGCAAACCGACGAGCAT CTCTATATTGTGGTCGAGGATGATGGCCCCGTA TTCCATTAAGCAAGCGAGAGGTCATTTTCGACCG TGGTCAACGGGTTGATACTTTACGCCCTGGGCAA GGTGTAGGGCTGGCGGTAGCCCGCGAAATCACCG AGCAATATGAGGGTAAAAATCGTCGCCGAGAGAG CATGCTGGGCGGTGCGCGGATGGAGGTGATTTT GGTCGCCAGCATTCTGCGCCGAAAGATGAATAA	41

The lysis regulation system may comprise any combination of one or more of the above regulators, promoters and sensors.

In one example of this embodiment, the lysis regulation system comprises OmpR as the regulator, ompF as the promoter and EnvZ as the sensor and the stimulus is reduced osmolarity. In

another example of this embodiment, the lysis regulation system comprises OmpR as the regulator, ompC as the promoter and EnvZ as the sensor and the stimulus is reduced osmolarity.

In another example of this embodiment, the lysis regulation system comprises the ArcA as the regulator, fad as the promoter and Arc B as the sensor and the stimulus is anaerobic conditions.

5 In another example of this embodiment, the lysis regulation system comprises PhoP as the regulator, phoPQ as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration. In another example of this embodiment, the lysis regulation system comprises PhoP as the regulator, mgtA as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration. In another example of this embodiment, the lysis regulation system comprises PhoP as
10 the regulator, mgrB as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration.

In another example of this embodiment, the lysis regulation system comprises PhoB as the regulator, psiB as the promoter and PhoR as the sensor and the stimulus is reduced phosphate concentration. In another example of this embodiment, the lysis regulation system comprises PhoB as
15 the regulator, phnD as the promoter and PhoR as the sensor and the stimulus is reduced phosphate concentration. In another example of this embodiment, the lysis regulation system comprises RstA as the regulator, asr as the promoter and RstB as the sensor. In another example of this embodiment, the lysis regulation system comprises RstA as the regulator, csgD as the promoter and RstB as the sensor.

20 In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, emrKY as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, yhiUV as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, acrAB as the promoter and EvgS as the sensor. In another example of this embodiment, the
25 lysis regulation system comprises EvgA as the regulator, mdfA as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, tolC as the promoter and EvgS as the sensor.

In another example of this embodiment, the lysis regulation system comprises Fur as the regulator in combination with a promoter selected from the group comprising sodA, sodB, sltA or
30 sltB.

The antimicrobial protein may be selected from the group that includes but is not limited to α - and β -defensins, protegrins, cathelicidins (e.g., indolicidin and bactenecins), granulysin, lysozyme, lactoferrin, azurocidin, elastase, bactericidal permeability inducing peptide (BPI), adrenomedullin, brevinin, histatins and hepcidin. Additional antimicrobial proteins are disclosed in the following,
35 each of which is incorporated herein by reference in its entirety: Devine, D.A. et al., *Current Pharmaceutical Design*, 8, 703-714 (2002); Jack R.W., et al., *Microbiological Reviews*, 59 (2), 171-200 (June 1995).

Optionally, the antimicrobial protein is an α -defensin, β -defensin, or protegrin. Preferable, antimicrobial protein sequences are recited in Table 9.

Table 9

Antimicrobial Protein Sequence	Sequence	SEQ ID NO:
α -defensin-1 protein	CTATAGAAGACCTGGGACAGAGGACTGCTGTCTG CCCTCTCTGGTCACCCCTGCCTAGCTAGAGGATCT GTGACCCCAGCCATGAGGACCCCTCGCCATCCTTG CTGCCATTCTCCTGGTGGCCCTGCAGGCCCAGGC TGAGCCACTCCAGGCAAGAGCTGATGAGGTTGCT GCAGCCCCGGAGCAGATTGCAGCGGACATCCCAG AAGTGGTTGTTTCCCTTGCATGGGACGAAAGCTT GGCTCCAAAGCATCCAGGCTCAAGGAAAAACATG GCCTGCTATTGCAGAATACCAGCGTGCATTGCAG GAGAACGTCGCTATGGAACCTGCATCTACCAGGG AAGACTCTGGGCATTCTGCTGCTGAGCTTGCAGA AAAAGAAAAATGAGCTCAAAATTTGCTTTGAGAG CTACAGGGAATTGCTATTACTCCTGTACCTTCTG CTCAATTTCTTTTCTCATCCCAAATAAATGCCT TGGTACAAGAAAAG	42
α -defensin-3 protein	CCTTGCTATAGAAGACCTGGGACAGAGGACTGCT GTCTGCCCTCTCTGGTCACCCCTGCCTAGCTAGAG GATCTGTGACCCCAGCCATGAGGACCCCTCGCCAT CCTTGCTGCCATTCTCCTGGTGGCCCTGCAGGCC CAGGCTGAGCCACTCCAGGCAAGAGCTGATGAGG TTGCTGCAGCCCCGGAGCAGATTGCAGCGGACAT CCCAGAAGTGGTTGTTTCCCTTGCATGGGACGAA AGCTTGGCTCCAAAGCATCCAGGCTCAAGGAAAA ACATGGACTGCTATTGCAGAATACCAGCGTGCAT TGCAGGAGAACGTCGCTATGGAACCTGCATCTAC CAGGGAAGACTCTGGGCATTCTGCTGCTGAGCTT GCAGAAAAAGAAAAATGAGCTCAAAATTTGCTTT GAGAGCTACAGGGAATTGCTATTACTCCTGTACC TTCTGCTCAATTTCTTTTCTCATCTCAAAATAAA TGCCTTGTTAC	43
α -defensin-4 protein	GTCTGCCCTCTCTGCTCGCCCTGCCTAGCTTGAG GATCTGTACCCCAGCCATGAGGATTATCGCCCT CCTCGCTGCTATTCTCTTGGTAGCCCTCCAGGTC CGGGCAGGCCCCACTCCAGGCAAGAGGTGATGAGG CTCCAGGCCAGGAGCAGCGTGGGCCAGAAGACCA GGACATATCTATTTCTTTTGCATGGGATAAAAGC TCTGCTCTTCAGGTTTCAGGCTCAACAAGGGGCA TGGTCTGCTCTTGCAGATTAGTATTCTGCCGGCG AACAGAACTTCGTGTTGGGAACTGCCTCATTGGT GGTGTGAGTTTCACATACTGCTGCACGCGTGTCTG ATTAACGTTCTGCTGTCCAAGAGAATGTCATGCT GGGAACGCCATCATCGGTGGTGTAGCTTCACAT GCTTCTGCAGCTGAGCTTGCAGAATAGAGAAAAA TGAGCTCATAATTTGCTTTGAGAGCTACAGGAAA TGTTTGTCTCTCTATACTTTGTCTTAACATCT TTCTTGATCCTAAATATATATCTCGTAACAAG	44
α -defensin-5 protein	ATATCCACTCCTGCTCTCCCTCCTGCAGGTGACC CCAGCCATGAGGACCATCGCCATCCTTGCTGCCA TTCTCCTGGTGGCCCTGCAGGCCCAGGCTGAGTC ACTCCAGGAAAGAGCTGATGAGGCTACAACCCAG AAGCAGTCTGGGGAAGACAACCAGGACCTTGCTA TCTCCTTTGCAGGAAATGGACTCTCTGCTCTTAG AACCTCAGGTTCTCAGGCAAGAGCCACCTGCTAT TGCCGAACCGCCGTTGTGCTACCCGTGAGTCCC	45

	TCTCCGGGGTGTGTGAAATCAGTGGCCGCTCTA CAGACTCTGCTGTCGCTGAGCTTCCCTAGATAGAA ACCAAAGCAGTGCAAGATTCAAGGTCCTG AAAAAAGAAAAACATTTTACTCTGTGTACCTTGT GTCTTTCTAAATTTCTCTCTCCAAAATAAAGTTC AAGCATT	
α -defensin-6 protein	ACACATCTGCTCCTGCTCTCTCTCCTCCAGCGAC CCTAGCCATGAGAACCCTCACCATCCTCACTGCT GTTCTCCTCGTGGCCCTCCAGGCCAAGGCTGAGC CACTCCAAGCTGAGGATGATCCACTGCAGGCAAA AGCTTATGAGGCTGATGCCCAGGAGCAGCGTGGG GCAAATGACCAGGACTTTGCCGTCTCCTTTGCAG AGGATGCAAGCTCAAGTCTTAGAGCTTTGGGCTC AACAAGGGCTTTCACTTGCCATTGCAGAAGGTCC TGTTATTCAACAGAATATTCTATGGGACCTGCA CTGTCATGGGTATTAACCACAGATTCTGCTGCCT CTGAGGGATGAGAACAGAGAGAAATATATTCATA ATTTACTTTATGACCTAGAAGGAACTGTGCTGT GTCCCATACATTGCCATCAACTTTGTTTCTCAT CTCAAATAAAGTCTTTTCAGCAAAAAAAAAAAAA	46
β -defensin-1 protein	TCCCTTCAGTTCCGTGCGACGAGGTTGTGCAATCC ACCAGTCTTATAAATACAGTGACGCTCCAGCCTC TGGAAGCCTCTGTGAGCTCAGCCTCCAAAGGAGC CAGCGTCTCCCCAGTTCTGAAATCCTGGGTGTT GCCTGCCAGTCGCCATGAGAACTTCTACCTTCT GCTGTTTACTCTCTGCTTACTTTTGTCTGAGATG GCCTCAGGTGGTAACTTTCTCACAGGCCTTGGCC ACAGATCTGATCATTACAATTGCGTCAGCAGTGG AGGGCAATGTCTCTATTCTGCCTGCCCGATCTTT ACCAAATTCAGGCACCTGTTACAGAGGGAAGG CCAAGTGCTGCAAGTGAGCTGGGAGTGACCAGAA GAAATGACGCAGAAAGTGAATGAACTTTTTATAA GCATTCTTTTAATAAAGGAAAAATTGCTTTTGAAG TATACCTCCTTTGGGCCAAAAAAAAAAAAAAAAAA AAAAAAA	47
β -defensin-3 protein	TGAGTCTCAGCGTGGGGTGAAGCCTAGCAGCTAT GAGGATCCATTATCTTCTGTTTGCTTTGCTCTTC CTGTTTTTGGTGCCTGTCCCAGGTCATGGAGGAA TCATAAACACATTACAGAAATATTATTGCAGAGT CAGAGGCGGCCGGTGTGCTGTGCTCAGCTGCCTT CCAAAGGAGGAACAGATCGGCAAGTGCTCGACGC GTGGCCGAAAATGCTGCCGAAGAAAAGAAATAAAA ACCCTGAAACATGACGAGAGTGTGTAAAGTGTG GAAATGCCTTCTTAAAGTTTATAAAAGTAAATC AAATTACATTTTTTTTTTCAAAAAAAAAAAAAA	48
β -defensin-4 protein	AGACTCAGCTCCTGGTGAAGCTCCAGCCATCAG CCATGAGGGTCTTGATCTCCTCTTCTCGTTCCT CTTCATATTCCTGATGCCTCTTCCAGGTGTTTTT GGTGGTATAGGCGATCCTGTTACCTGCCTTAAGA GTGGAGCCATATGTCATCCAGTCTTTTGCCCTAG AAGGTATAAAACAAATTGGCACCTGTGGTCTCCCT GGAACAAAATGCTGCAAAAAGCCATGAGGAGGCC AAGAAGCTGCTGTGGCTGATGCGGATTAGAAAAG GGCTCCCTCATCAGAGACGTGCGACATGTAAACC AAATTAAACTATGGTGTCCAAAGATACGCA	49
protegrin-1 protein	ATGGAGACCCAGAGAGCCAGCCTGTGCCTGGGGC GCTGGTCACTGTGGCTTCTGCTGCTGGCACTCGT GGTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTAC AGGGAGGCCGTGCTTCTGTGCTGTGGATCGCCTCA ACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCT CCTGGAGCTGGACCAGCCGCCCAAGGCCGACGAG	50

	GACCCGGGCACCCCGAAACCTGTGAGCTTCACGG TGAAGGAGACTGTGTGTCCCAGGCCGACCCGGCA GCCCCGGAGCTGTGTGACTTCAAGGAGAACGGG CGGGTGAAACAGTGTGTGGGGACAGTCACCCCTGG ATCAGATCAAGGACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTGAGGGGAGGTGCGCTGTGC TATTGTAGGCGTAGGTTCTGCGTCTGTGTGCGAC GAGGATGACGGTTGCGACGGCAGGCTTTCCTCC CCCAATTTTCCCGGGGCCAGGTTTCCGTCCCCCA ATTTTTCCGCCTCCACCTTTCGGCCCCGACCAT TCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAG GATTTGCAGGCAACTCACCCAGAAGGCCTTTTCGG TACATTAAAATCCCAGCAAGGAGACCTAAGCATC TGCTTTGCCCAGGCCCGCATCTGTCAAATAAAATT CTTGTGAAACC	
protegrin-3 protein	ATGGAGACCCAGAGAGCCAGCCTGTGCCTGGGGC GCTGGTCACTGTGGCTTCTGCTGCTGGCACTCGT GGTGCCCTCGGCCAGCGCCAGGCCCTCAGCTAC AGGGAGGCCGTGCTTCTGTGCTGTGGATCGCCTCA ACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCT CCTGGAGCTGGACCAGCCGCCCAAGGCCGACGAG GACCCGGGCACCCCGAAACCTGTGAGCTTCACGG TGAAGGAGACTGTGTGTCCCAGGCCGACCCGGCA GCCCCGGAGCTGTGTGACTTCAAGGAGAACGGG CGGGTGAAACAGTGTGTGGGGACAGTCACCCCTGG ATCAGATCAAGGACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTGAGGGGAGGTGGCCTGTGC TATTGTAGGCGTAGGTTCTGCGTCTGTGTGCGAC GAGGATGACGGTTGCGACGGCAGGCTTTCCTCC CCCAATTTTCCCGGGGCCAGGTTTCCGTCCCCCA ATTTTTCCGCCTCCACCTTTCGGCCCCGACCAT TCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAG GATTTGCAGGCAACTCACCCAGAAGGCCTTTTCGG TACATTAAAATCCCAGCAAGGAGACCTAAGCATC TGCTTTGCCCAGGCCCGCATCTGTCAAATAAAATT CTTGTGAAACC	51
protegrin-4 protein	ATGGAGACCCAGAGAGCCAGCCTGTGCCTGGGGC GCTGGTCACTGTGGCTTCTGCTGCTGGCACTCGT GGTGCCCTCGGCCAGCGCCAGGCCCTCAGCTAC AGGGAGGCCGTGCTTCTGTGCTGTGGATCGCCTCA ACGAGCAGTCCTCGGAAGCTAATCTCTACCGCCT CCTGGAGCTGGACCAGCCGCCCAAGGCCGACGAG GACCCGGGCACCCCGAAACCTGTGAGCTTCACGG TGAAGGAGACTGTGTGTCCCAGGCCGACCCGGCA GCCCCGGAGCTGTGTGACTTCAAGGAGAACGGG CGGGTGAAACAGTGTGTGGGGACAGTCACCCCTGG ATCAGATCAAGGACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTGAGGGGAGGTGCGCTGTGC TATTGTAGGGGTTGGATCTGCTTCTGTGTGCGAC GAGGATGACGGTTGCGACGGCAGGCTTTCCTCC CCCAATTTTCCCGGGGCCAGGTTTCCGTCCCCCA ATTTTTCCGCCTCCACCTTTCGGCCCCGACCAT TCGGTCCACCAAGGTTCCCTGGTAGACGGTGAAG GATTTGCAGGCAACTCACCCAGAAGGCCTTTTCGG CACATTAAAATCCCAGCAAGGAGACCTAAGCATC TGCTTTGCCCAGGCCCGCATCTGTCAAATAAAATT CTTGTGAAACC	52

The bacteriophage lysin may be selected from the group that includes but is not limited to holins and endolysins or lysins (e.g., lysozyme, amidase and transglycosylase). Additional lysins are

disclosed in the following, each of which is incorporated herein by reference in its entirety: Kloos D.-U., et al., *Journal of Bacteriology*, 176 (23), 7352-7361 (December 1994); Jain V., et al., *Infection and Immunity*, 68 (2), 986-989 (February 2000); Srividhya K.V., et al., *J. Biosci.*, 32, 979-990 (2007); Young R.V., *Microbiological Reviews*, 56 (3), 430-481 (September 1992).

5 The autolysin may be selected from the group that includes but is not limited to peptidoglycan hydrolases, amidases (e.g., N-acetylmuramyl-L-alanine amidases), transglycosylases, endopeptidases and glucosaminidases. Additional autolysins are disclosed in the following, each of which is incorporated herein by reference in its entirety: Heidrich C., et al., *Molecular Microbiology*, 41 (1), 167-178 (2001); Kitano K., et al., *Journal of Bacteriology*, 167 (3), 759-765 (September 1986);
 10 Lommatzsch J., et al., *Journal of Bacteriology*, 179 (17), 5465-5470 (September 1997); Oshida T., et al., *PNAS*, 92, 285-289 (January 1995); Lenz L.L., et al., *PNAS*, 100 (21), 12432-12437 (October 14, 2003); Ramadurai L., et al., *Journal of Bacteriology*, 179 (11), 3625-3631 (June 1997); Kraft A.R., et al., *Journal of Bacteriology*, 180 (12), 3441-3447 (July 1998); Dijkstra A.J., et al., *FEBS Letters*, 366, 115-118 (1995); Huard C., et al., *Microbiology*, 149, 695-705 (2003).

15 In one aspect of the invention, the control exerted by the lysis regulation system may further be enhanced by bacterial or BTP strain-specific regulation. In one aspect of this embodiment, the strain-specific regulation is attenuation caused by deletion of a nutritional gene. The nutritional gene may be selected from the group that includes but is not limited to *dapA*, *aroA* and *guaBA*. In one example of this embodiment, *dapA* attenuation results in deficiency in the biosynthesis of lysine and
 20 peptidoglycan. In this particular embodiment, transcription of genes including but not limited to *lysC* may be activated by mechanisms such as transcriptional induction, antitermination and riboswitch. In another example of this embodiment, *aroA* attenuation results in deficiency in aromatic amino acids and derepression of one or more genes including but not limited to *aroF*, *aroG* and *aroH* by regulators such as *TrpR* and *TyrR*. In another example of this embodiment, *guaBA* attenuation results in
 25 derepression of one or more genes that are repressed by *PurR*.

In addition to the lysis regulation system and strain-specific regulation, the bacteria or BTP may further contain an inducible system that includes but is not limited to a Tet-on expression system to facilitate bacterial or BTP lysis at a time desired by the clinician. Upon administration of tetracycline, which activates the Tet-on promoter, the bacteria or BTP express a protein that triggers
 30 lysis of the bacteria or BTP. In one example of this embodiment, the protein expressed under the Tet-on expression system is selected from the group that includes but is not limited to defensins and protegrins.

The present invention also provides a lysis regulation system in combination with strain-specific attenuation (e.g., nutritional attenuation). As shown in PCT Publication No.

35 WO2008/156702 at Figure 30, a global regulator can sense an extracellular condition and regulate transcription, starvation for specific nutrient such as an amino acid *in vivo*, in contrast to laboratory growth in the presence of excess of the nutrient and a positive or negative regulator in response to

starvation. In the schematic shown in PCT Publication No. WO2008/156702 at Figure 31 there can be three cassettes, any of which may be place on either the bacterial chromosome or on a plasmid.

As described, the present invention provides a plasmid containing a lysis regulation system comprising OmpR as the regulator, ompF or ompC as the promoter and protegrin or β -defensin as the antimicrobial protein, in combination with a Tet-on expression system, which provides two levels of control of bacterial lysis. This embodiment is illustrated in PCT Publication No. WO2008/156702 at Figure 32.

In another aspect of the invention, the DNA insert comprises one or more of the following constructs, each of which contains an HPV target sequence, a hairpin sequence and BamHI and SalI restriction sites to facilitate incorporation into the hairpin RNA expression cassette of the TRIP plasmid as shown in Table 10.

Table 10

HPV Target Sequence Construct				
BamHI	sense (19bp)	loop	antisense (21bp)	SalI
5' - GATCC	TAGGTATTTGAATTTGCAT	TTCAAGAGA	ATGCAAATTCAAATACCTTTT	G-3' (SEQ ID NO:53)
3' - G	ATCCATAAACTTAAACGTA	AAGTTCTCT	TACGTTTAAGTTTATGGAAAA	CAGCT-5' (SEQ ID NO:54)

4. Cell and Gene Targets

The present invention also provides methods of using the various bacterium, BTP and vectors provided in the invention. For example, the present invention provides methods of delivering one or more siRNAs to mammalian cells. The methods include introducing at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells.

The present invention also provides methods of regulating gene expression in mammalian cells. The method includes introducing at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells, where the expressed siRNAs interfere with at least one mRNA of a gene of interest thereby regulating gene expression.

The invention provides a method for delivering RNA to any type of target cell. As used herein, the term "target cell" refers to a cell that can be invaded by a bacterium, *i.e.*, a cell that has the necessary surface receptor for recognition by the bacterium.

Preferred target cells are eukaryotic cells. Even more preferred target cells are animal cells. "Animal cells" are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxonomic position lies within the kingdom *animalia*. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular tissue source of the cells is not critical to the present invention.

The recipient animal cells employed in the present invention are not critical thereto and include cells present in or derived from all organisms within the kingdom *animalia*, such as those of the families *mammalia*, *pisces*, *avian*, *reptilia*.

Preferred animal cells are mammalian cells, such as humans, bovine, ovine, porcine, feline, canine, goat, equine, and primate cells. The most preferred mammalian cells are human cells. The cells can be *in vivo*, *in vitro* or *ex vivo*.

In some embodiments of the invention, the cell is a cervical epithelial cell, a rectal epithelial cell or a pharyngeal epithelial cell, macrophage, gastrointestinal epithelial cell, skin cell, melanocyte, keratinocyte, hair follicle, colon cancer cell, an ovarian cancer cell, a bladder cancer cell, a pharyngeal cancer cell, a rectal cancer cell, a prostate cancer cell, a breast cancer cell, a lung cancer cell, a renal cancer cell, a pancreatic cancer cell, a hepatocyte, a hepatocellular carcinoma (HCC) cell, a neural cell, or a hematologic cancer cell such as a lymphoma or leukemia cell. In one aspect of this embodiment, the colon cancer cell is an SW 480 cell. In another aspect of this embodiment, the pancreatic cancer cell is a CAPAN-1 cell.

In a preferred embodiment, the target cell is in a mucosal surface. Certain enteric pathogens, *e.g.*, *E. coli*, *Shigella*, *Listeria*, and *Salmonella*, are naturally adapted for this application, as these organisms possess the ability to attach to and invade host mucosal surfaces (Kreig *et al. supra*). Therefore, in the present invention, such bacteria can deliver RNA molecules or RNA-encoding DNA to cells in the host mucosal compartment.

Although certain types of bacteria may have a certain tropism, *i.e.*, preferred target cells, delivery of RNA or RNA-encoding DNA to a certain type of cell can be achieved by choosing a bacterium which has a tropism for the desired cell type or which is modified such as to be able to invade the desired cell type. Thus, *e.g.*, a bacterium could be genetically engineered to mimic mucosal tissue tropism and invasive properties, as discussed above, to thereby allow said bacteria to invade mucosal tissue, and deliver RNA or RNA-encoding DNA to cells in those sites.

Bacteria can also be targeted to other types of cells. For example, bacteria can be targeted to erythrocytes of humans and primates by modifying bacteria to express on their surface either, or both of, the *Plasmodium vivax* reticulocyte binding proteins-1 and -2, which bind specifically to erythrocytes in humans and primates (Galinski *et al. Cell*, 69:1213-1226 (1992)). In another embodiment, bacteria are modified to have on their surface asialoorosomucoid, which is a ligand for the asialoglycoprotein receptor on hepatocytes (Wu *et al. J. Biol. Chem.*, 263:14621-14624 (1988)). In yet another embodiment, bacteria are coated with insulin-poly-L-lysine, which has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz *et al. Expt. Cell Res.*, 199:323-329 (1992)). Also within the scope of the invention are bacteria modified to have on their surface p60 of *Listeria monocytogenes*, which allows for tropism for hepatocytes (Hess *et al. Infect. Immun.*, 63:2047-2053 (1995)), or a 60 kD surface protein from *Trypanosoma cruzi* which causes specific

binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria *et al.* Cell, 67:411-421 (1991)).

Yet in another embodiment, a cell can be modified to become a target cell of a bacterium for delivery of RNA. Accordingly, a cell can be modified to express a surface antigen that is recognized by a bacterium for its entry into the cell, *i.e.*, a receptor of an invasion factor. The cell can be modified either by introducing into the cell a nucleic acid encoding a receptor of an invasion factor, such that the surface antigen is expressed in the desired conditions. Alternatively, the cell can be coated with a receptor of an invasion factor. Receptors of invasion factors include proteins belonging to the integrin receptor superfamily. A list of the type of integrin receptors recognized by various bacteria and other microorganisms can be found, *e.g.*, in Isberg and Tran Van Nhieu (1994) Ann. Rev. Genet. 27:395. Nucleotide sequences for the integrin subunits can be found, *e.g.*, in GenBank, publicly available on the internet.

As set forth above, yet other target cells include fish, avian, and reptilian cells. Examples of bacteria that are naturally invasive for fish, avian, and reptilian cells are set forth below.

Examples of bacteria that can naturally access the cytoplasm of fish cells include, but are not limited to, *Aeromonas salminocida* (ATCC No. 33658) and *Aeromonas schubertii* (ATCC No. 43700). Attenuated bacteria are preferably used in the invention, and include *A. salmonicida* vapA (Gustafson *et al.* J. Mol. Biol., 237:452-463 (1994)) or *A. salmonicida* aromatic-dependent mutant (Vaughan *et al.* Infect. Immun., 61:2172-2181 (1993)).

Examples of bacteria that can naturally access the cytoplasm of avian cells include, but are not restricted to, *Salmonella galinarum* (ATCC No. 9184), *Salmonella enteritidis* (ATCC No. 4931) and *Salmonella typhimurium* (ATCC No. 6994). Attenuated bacteria are preferred to the invention and include attenuated *Salmonella* strains such as *S. galinarum* cya crp mutant (Curtiss *et al.* (1987) *supra*) or *S. enteritidis* aroA aromatic-dependent mutant CVL30 (Cooper *et al.* Infect. Immun., 62:4739-4746 (1994)).

Examples of bacteria that can naturally access the cytoplasm of reptilian cells include, but are not restricted to, *Salmonella typhimurium* (ATCC No. 6994). Attenuated bacteria are preferable to the invention and include, attenuated strains such as *S. typhimurium* aromatic-dependent mutant (Hormaeche *et al. supra*).

The invention also provides for delivery of RNA to other eukaryotic cells, *e.g.*, plant cells, so long as there are microorganisms that are capable of invading such cells, either naturally or after having been modified to become invasive. Examples of microorganisms which can invade plant cells include *Agrobacterium tumefaciens*, which uses a pilus-like structure which binds to the plant cell via specific receptors, and then through a process that resembles bacterial conjugation, delivers at least some of its content to the plant cell.

Set forth below are examples of cell lines to which RNA can be delivered according to the method of this invention.

Examples of human cell lines include but are not limited to ATCC Nos. CCL 62, CCL 159, HTB 151, HTB 22, CCL 2, CRL 1634, CRL 8155, HTB 61, and HTB104.

Examples of bovine cell lines include ATCC Nos. CRL 6021, CRL 1733, CRL 6033, CRL 6023, CCL 44 and CRL 1390.

5 Examples of ovine cells lines include ATCC Nos. CRL 6540, CRL 6538, CRL 6548 and CRL 6546.

Examples of porcine cell lines include ATCC Nos. CL 184, CRL 6492, and CRL 1746.

Examples of feline cell lines include CRL 6077, CRL 6113, CRL 6140, CRL 6164, CCL 94, CCL 150, CRL 6075 and CRL 6123.

10 Examples of buffalo cell lines include CCL 40 and CRL 6072.

Examples of canine cells include ATCC Nos. CRL 6213, CCL 34, CRL 6202, CRL 6225, CRL 6215, CRL 6203 and CRL 6575.

Examples of goat derived cell lines include ATCC No. CCL 73 and ATCC No. CRL 6270.

Examples of horse derived cell lines include ATCC Nos. CCL 57 and CRL 6583.

15 Examples of deer cell lines include ATCC Nos. CRL 6193-6196.

Examples of primate derived cell lines include those from chimpanzee's such as ATCC Nos. CRL 6312, CRL 6304, and CRL 1868; monkey cell lines such as ATCC Nos. CRL 1576, CCL 26, and CCL 161; orangutan cell line ATCC No. CRL 1850; and gorilla cell line ATCC No. CRL 1854.

The invention also provides methods of regulating the expression of one or more genes.

20 Preferably, regulating the expression of one or more genes means decreasing or lessening the expression of the gene and/or decreasing or lessening the activity of the gene and its corresponding gene product.

In one embodiment, the expressed siRNAs direct the multienzyme complex RISC (RNA-induced silencing complex) of the cell to interact with the mRNAs to be regulated. This complex
25 degrades or sequesters the mRNA. This causes the expression of the gene to be decreased or inhibited.

In some embodiments, the gene is an animal gene. Preferred animal genes are mammalian genes, such as humans, bovine, ovine, porcine, feline, canine, goat, equine, and primate genes. The most preferred mammalian genes are human cells.

30 The gene to be regulated can be a viral gene, anti-inflammatory gene, obesity gene or autoimmune disease or disorder gene. In some embodiments, more than one gene can be regulated from a single plasmid or vector.

In preferred embodiments, the gene can be, but is not limited to, ras, *β-catenin*, one or more HPV oncogenes, APC, eotaxin-1 (CCL11), HER-2, MCP-1 (CCL2), MDR-1, MRP-2, FATP4,
35 SGLUT-1, GLUT-2, GLUT-5, apobec-1, MTP, IL-6, IL-6R, IL-7, IL-12, IL-13, IL-13 Ra-1, IL-18, IL-21R, IL-32α, the p19 subunit of IL-23, LY6C, p38/JNK MAP kinase, p65/NF-κB, CCL20 (MIP-

3α), Claudin-2, Chitinase 3-like 1, apoA-IV, MHC class I and MHC class II. In one aspect of this embodiment, the ras is *k-Ras*. In another aspect of this embodiment, the HPV oncogene is E6 or E7.

Preferable *β-catenin* target gene sequences are recited in Table 11. The sequences in Table 11 are cross-species target sequences as they are capable of silencing the beta-catenin gene

5 (CTNNB1) in human, mouse, rat, dog and monkey.

Table 11.

<i>β-catenin</i> target gene sequences	SEQ ID NO:
AGCCAATGGCTTGGAATGAGA	55
ATCAGCTGGCCTGGTTTGATA	56
CTGTGAACTTGCTCAGGACAA	57
AGCAATCAGCTGGCCTGGTTT	58
CCTCTGTGAACTTGCTCAGGA	59
TTCCGAATGTCTGAGGACAAG	60
CCAATGGCTTGGAATGAGACT	61
GGTGCTGACTATCCAGTTGAT	62
CAATCAGCTGGCCTGGTTTGA	63
CACCCTGGTGCTGACTATCCA	64
CACCACCCTGGTGCTGACTAT	65
TGCTTTATTCTCCATTGAAA	66
CTGGTGCTGACTATCCAGTTG	67
TCTGTGCTCTTCGTCATCTGA	68
TGCCATCTGTGCTCTTCGTCA	69
TGGTGCTGACTATCCAGTTGA	70
CCTGGTGCTGACTATCCAGTT	71
ACCCTGGTGCTGACTATCCAG	72
GAGCCTGCCATCTGTGCTCTT	73
CTGGTTTGATACTGACCTGTA	74
TGGTTTGATACTGACCTGTAA	75
TCGAGGAGTAACAATACAAAT	76
ACCATGCAGAATACAAATGAT	77
AGGAGTAACAATACAAATGGA	78
GTGAGGAGTAACAATACAAA	79
TTGTTGTAACCTGCTGTGATA	80
GAGTAATGGTGTAGAACACTA	81
AGTAATGGTGTAGAACACTAA	82
CACACTAACCAAGCTGAGTTT	83
TTTGGTCGAGGAGTAACAATA	84
TACCATTCCATTGTTTGTGCA	85
TAGGGTAAATCAGTAAGAGGT	86
CTAACCAAGCTGAGTTTCCTA	87
TGGTCGAGGAGTAACAATACA	88
CTGGCCTGGTTTGATACTGAC	89
TAACCTCACTTGCAATAATTA	90
ATCCCCTGGCCTCTGATAAA	91
GACCACAAGCAGAGTGCTGAA	92
CACAAGCAGAGTGCTGAAGGT	93
CTAACCTCACTTGCAATAATT	94
AGCTGATATTGATGGACAG	95

Preferable HPV target gene sequences are recited in Table 12. The sequences in Table 12 are target sequences as they are capable of silencing the HPV E6 oncogene.

Table 12

HPV target gene sequences	SEQ ID NO:
CGGTGCCAGAAACCGTTGAATCC	96
CACTGCAAGACATAGAAATAACC	97
AGGTGCCCTGCGGTGCCAGAAACC	98
GCGGTGCCAGAAACCGTTGAATC	99
TCACTGCAAGACATAGAAATAAC	100
CCCATGCTGCATGCCATAAATGT	101
ATGCTGCATGCCATAAATGTATA	102
GTGGTGTATAGAGACAGTATACC	103
GCGCGCTTTGAGGATCCAACACG	104
CTGCGGTGCCAGAAACCGTTGAA	105
CCCCATGCTGCATGCCATAAATG	106
ACCCCATGCTGCATGCCATAAAT	107
AACACTGGGTTATACAATTTATT	108
ACGACGCAGAGAAACACAAGTAT	109
AAGGTGCCCTGCGGTGCCAGAAAC	110
GGTGCCCTGCGGTGCCAGAAACCG	111
CATGCTGCATGCCATAAATGTAT	112
GACGCAGAGAAACACAAGTATAA	113
TTCACGCAAGACATAGAAATAA	114
GGTGCCAGAAACCGTTGAATCCA	115
TGGCGCGCTTTGAGGATCCAACA	116
TGTGGTGTATAGAGACAGTATAC	117
GTGCCTGCGGTGCCAGAAACCGT	118
CTGCATGCCATAAATGTATAGAT	119
GACTCCAACGACGCAGAGAAACA	120
CTGGGCACTATAGAGGCCAGTGC	121
TGCTGCATGCCATAAATGTATAG	122
GTGCCAGAAACCGTTGAATCCAG	123
TTACAGAGGTATTTGAATTTGCA	124
GAGGCCAGTGCCATTCGTGCTGC	125

5

Additional preferable HPV target gene sequences are recited in Table 13. The sequences in Table 13 are target sequences as they are capable of silencing the HPV E7 oncogene.

Table 13

HPV target gene sequences	SEQ ID NO:
ATTCCGGTTGACCTTCTATGTCA	126
GATGGAGTTAATCATCAACATTT	127
AAGCCAGAATTGAGCTAGTAGTA	128
CATGGACCTAAGGCAACATTGCA	129
AACCACAACGTCACACAATGTTG	130
ATGGACCTAAGGCAACATTGCAA	131
TAAGCGACTCAGAGGAAGAAAAC	132
GAAGCCAGAATTGAGCTAGTAGT	133
GAGCCGAACCACAACGTCACACA	134
ACGTCACACAATGTTGTGTATGT	135
GAACCACAACGTCACACAATGTT	136
AGGCAACATTGCAAGACATTGTA	137
AAGACATTGTATTGCATTTAGAG	138
TAAGGCAACATTGCAAGACATTG	139
CCAGCCCGACGAGCCGAACCACA	140
AAGCTCAGCAGACGACCTTCGAG	141
GCCCCGACGAGCCGAACCACAACG	142
TTCCGGTTGACCTTCTATGTCAC	143
TGCATGGACCTAAGGCAACATTG	144
TTCCAGCAGCTGTTTCTGAACAC	145
AACACCCTGTCCTTTGTGTGTCC	146
CTTCTATGTCACGAGCAATTAAG	147
ACGAGCCGAACCACAACGTCACA	148
TTGAGCTAGTAGTAGAAAGCTCA	149
CAGCAGACGACCTTCGAGCATTC	150
AGCCAGAATTGAGCTAGTAGTAG	151
GTCACACAATGTTGTGTATGTGT	152
CCGACGAGCCGAACCACAACGTC	153
AATTCCGGTTGACCTTCTATGTC	154
ATTCCAGCAGCTGTTTCTGAACA	155

Additional preferable HPV target gene sequences are recited in Table 14. The sequences in Table 14 are target sequences shared by both HPV E6 and E6.

Table 14

HPV target gene sequences	SEQ ID NO:
TAGGTATTTGAATTTGCAT	156
GAGGTATTTGAATTTGCAT	157

A preferable MDR-1 target gene sequence is recited in Table 15. The sequence in Table 15 is capable of silencing the MDR-1 gene in human.

5

Table 15

MDR-1 target gene sequences	SEQ ID NO:
ATGTTGTCTGGACAAGCACT	158

A preferable *k-Ras* target gene sequence is recited in Table 16. The sequence in Table 16 is capable of silencing the *k-Ras* gene in human.

Table 16

<i>k-Ras</i> target gene sequences	SEQ ID NO:
GTTGGAGCTGTTGGCGTAG	159

10

Preferable IL-6R target gene sequences are recited in Table 17. The sequences in Table 17 are capable of silencing IL-6R in human.

Table 17

IL-6R target gene sequences	SEQ ID NO:
CTCCTGGAACATCTTTCTA	160
GCTCTCCTGCTTCCGGAAGAG	161
CTCCACGACTCTGGAACTAT	162
CAGAAAGTTCTCCTGCCAGTTA	163
CCGGAAGACAATGCCACTGTT	164
CTGAACGGTCAAAGACATTCA	165
CACAACATGGATGGTCAAGGA	166
ATGCAGGCACTTACTACTAAT	167
ATCGGGCTGAACGGTCAAAGA	168
AGCTCTCCTGCTTCCGGAAGA	169
CAGCTCTCCTGCTTCCGGAAG	170
CAGGCACTTACTACTAATAAA	171
CACTTGCTGGTGGATGTTCCC	172
AACGGTCAAAGACATTCACAA	173
TGCACAAGCTGCACCCCTCAGG	174

15

Additional referable IL-6R target gene sequences are recited in Table 18. The sequences in Table 18 are capable of silencing the IL-6R gene in mouse.

20

Table 18

IL-6R target gene sequences	SEQ ID NO:
ATCCTGGAGGGTGACAAAGTA	175
TGGGTCTGACAATACCGTAAA	176
AACGAAGCGTTTCACAGCTTA	177
CCGCTGTTTCCTATAACAGAA	178
ACGAAGCGTTTCACAGCTTAA	179
CTGCTGTGAAAGGGAAATTTA	180
AACCTTGTGGTATCAGCCATA	181
CACAGTGTGGTGCTTAGATTA	182
CAGCTTCGATACCGACCTGTA	183
CAGTGTGGTGCTTAGATTAAA	184
CCCGGCAGGAATCCTCTGGAA	185
CCCGCTGTTTCCTATAACAGA	186
AACCACGAGGATCAGTACGAA	187
ACCTGCCGTCTTACTGAACTA	188
ACCACGAGGATCAGTACGAAA	189
ACAGCTTGTGATGACTGAATA	190
AGGATCAGTACGAAAGTTCTA	191
AACCCGCTGTTTCCTATAACA	192
CAGTACGAAAGTTCTACAGAA	193
TACGCGAGTGACAATTTCTCA	194
ACGAAAGTTCTACAGAAGCAA	195
CAGGCACTTACTACTAATAAA	196
CACTTGCTGGTGGATGTTCCC	197
AACGGTCAAAGACATTCACAA	198
TGCACAAGCTGCACCCTCAGG	199

Preferable IL-7 target gene sequences are recited in Table 19. The sequences in Table 19 are capable of silencing the IL-7 gene in human.

5

Table 19

IL-7 target gene sequences	SEQ ID NO:
TAAGAGAGTCATAAACCTTAA	200
AACAAGGTCCAAGATACCTAA	201
AAGATTGAACCTGCAGACCAA	202
AAGAGATTTCAAGAGATTTAA	203
AAGCGCAAAGTAGAAACTGAA	204
TAGCATCATCTGATTGTGATA	205
TAAGATAATAATATATGTTTA	206
ATGGTCAGCATCGATCAATTA	207
TTGCCTGAATAATGAATTTAA	208
ATCTGTGATGCTAATAAGGAA	209
AACAAACTATTTCTTATATAT	210
AACATTTATCAATCAGTATAA	211
ATCAATCAGTATAATTTCTGTA	212
AAGGTATCAGTTGCAATAATA	213

Additional preferable IL-7 target gene sequences are recited in Table 20. The sequences in Table 20 are capable of silencing the IL-7 gene in mouse.

Table 20

IL-7 target gene sequences	SEQ ID NO:
CGGATCCTACGGAAGTTATGG	214
GACCATGTTCCATGTTTCTTT	215
AACCTAAATGACCTTTATTAA	216
CAGGAGACTAGGACCCATATA	217
TAGGGTCTTATTCGTATCTAA	218
ATGAGCCAATATGCTTAATTA	219
GCCAATATGCTTAATTAGAAA	220
CAGCATCGATGAATTGGACAA	221
TTGCCTGAATAATGAATTTAA	222
CTGATAGTAATTGCCCGAATA	223
AAGGGTTTGCTTGTACTGAAT	224
AACATGTATGTGATGATACAA	225
TTGCAACATGTAATAATTTAA	226
AAGAGACTACTGAGAGAAATA	227
AAGAATCTACTGGTTCATATA	228
TGCCGTCAGCATATACATATA	229
AGGGCTCACGGTGATGGATAA	230

Additional preferable IL-7 target gene sequences are recited in Table 21. The sequences in Table 21 are cross species sequences as they are capable of silencing the IL-7 gene in human and mouse.

Table 21

IL-7 target gene sequences	SEQ ID NO:
CGCCTCCCGCAGACCATGTTTC	231
TCCGTGCTGCTCGCAAGTTGA	232
GCCTCCCGCAGACCATGTTCC	233
CCTCCCGCAGACCATGTTCCA	234
CTCCCGCAGACCATGTTCCAT	235
TCCCGCAGACCATGTTCCATG	236
CCCGCAGACCATGTTCCATGT	237
CCGCAGACCATGTTCCATGTT	238
CGCAGACCATGTTCCATGTTT	239
GCAGACCATGTTCCATGTTTC	240
CAGACCATGTTCCATGTTTCT	241
AGACCATGTTCCATGTTTCTT	242

Preferable IL-13Ra-1 target gene sequences are recited in Table 22. The sequences in Table 22 are capable of silencing the IL-13Ra-1 gene in human.

Table 22

IL-13Ra-1 target gene sequences	SEQ ID NO:
AACCTGATCCTCCACATATTA	243
CCTGATCCTCCACATATTA	244
AGAAATGTTTGGAGACCAGAA	245
CAAATAATGGTCAAGGATAAT	246
TTCCTGATCCTGGCAAGATTT	247
TAAAGAAATGTTTGGAGACCA	248

ATGTTTGGAGACCAGAATGAT	249
CTCCAATTCCTGATCCTGGCA	250

Additional preferable IL-13Ra-1 target gene sequences are recited in Table 23. The sequences in Table 23 are capable of silencing the IL-13Ra-1 gene in mouse.

Table 23

IL-13Ra-1 target gene sequences	SEQ ID NO:
CAAGAAGACTCTAATGATGTA	251
CACAGTCAGAGTAAGAGTCAA	252
ACCCAGGGTATCATAGTTCTA	253
CTGCTTTGAAATTTCCAGAAA	254
ATCATAGTTCTAAGAATGAAA	255
AAGGCTTAAGATCATTATATT	256
AACTACTTATAAGAAAGTAAA	257
CACAGAACATCTAGCAAACAA	258
CTCGTTCCTTGTTCAATCCTAA	259
AAC TTGTAGGTTACATATTA	260
AACCATTTCTGCAAATTTAAA	261
CTCAGTGTAGTGCCAATGAAA	262
CAGGCCTTAGGGACTCATAAA	263
AAGTATGACATCTATGAGAAA	264
GTGGAGGTCAATAATACTCAA	265
CAGAGTATAGGTAAGGAGCAA	266

5

A preferable IL-18 target gene sequence is recited in Table 24. The sequence in Table 24 is capable of silencing the IL-18 gene in human.

Table 24

IL-18 target gene sequences	SEQ ID NO:
TTGAATGACCAAGTTCTCTTC	267

10

Additional preferable IL-18 target gene sequences are recited in Table 25. The sequences in Table 25 are capable of silencing the IL-18 gene in mouse.

Table 25

IL-18 target gene sequences	SEQ ID NO:
CTCTCTGTGAAGGATAGTAAA	268
CCGCAGTAATACGGAATATAA	269
CAAGGAAATGATGTTTATTGA	270
CAGACTGATAATATACATGTA	271
TTGGCCGACTTCACTGTACAA	272
CCAGACCAGACTGATAATATA	273
AAGATGGAGTTTGAATCTTCA	274
ACGCTTTACTTTTATACCTGAA	275
TACAACCGCAGTAATACGGAA	276
CTGCATGATTTATAGAGTAAA	277
CCCGAGGCTGCATGATTTATA	278
CACGCTTTACTTTTATACCTGA	279
CGCCTGTATTTCCATAACAGA	280

CGCAGTAATACGGAATATAAA	281
TACATGTACAAAGACAGTGAA	282
CAGGCCCTGACATCTTCTGCAA	283
TTCGAGGATATGACTGATATT	284
CTGTATTTCCATAACAGAATA	285
GAGGATATGACTGATATTGAT	286
CAAGTTCTCTTCGTTGACAAA	287
CACTAACTTACATCAAAGTTA	288
ACCGCAGTAATACGGAATATA	289
CTCTCACTAACTTACATCAAA	290

Preferable CCL20 target gene sequences are recited in Table 26. The sequences in Table 26 are capable of silencing the CCL20 gene in human.

Table 26

CCL20 target gene sequences	SEQ ID NO:
ATCATCTTTCACACAAAGAAA	291
AACAGACTTGGGTGAAATATA	292
ATGGAATTGGACATAGCCCAA	293
GAGGGTTTGTGCTTATCTAA	294
CTCACTGGACTTGTCCAATTA	295
ATCATAGTTTGCTTTGTTTAA	296
TTGTTTAAGCATCACATTAAA	297
AAGCATCACATTAAAGTTAAA	298
CCCAAAGAACTGGGTACTCAA	299
CACATTAAAGTTAACTGTAT	300
CAGATCTGTTCTTTGAGCTAA	301
TTGGTTTAGTGCAAAGTATAA	302
CAGACCGTATTCTTCATCCTA	303
AACATTAATAAGACAAATATT	304
GACCGTATTCTTCATCCTAAA	305

5

Additional referable CCL20 target gene sequences are recited in Table 27. The sequences in Table 27 are capable of silencing the CCL20 gene in mouse.

Table 27

CCL20 target gene sequences	SEQ ID NO:
AAGCTTGTGACATTAATGCTA	306
CAATAAGCTATTGTAAAGATA	307
ATCATCTTTCACACGAAGAAA	308
AGCTATTGTAAAGATATTTAA	309
CAGCCTAAGAGTCAAGAAGAT	310
CCCAGTGGACTTGTCAATGGA	311
ATGAAGTTGATTCATATTGCA	312
AAGTTGATTCATATTGCATCA	313
TCACATTAGAGTTAAGTTGTA	314
CACATTAGAGTTAAGTTGTAT	315
TATGTTATTTATAGATCTGAA	316
ATGTTTAGCTATTTAATGTTA	317
TTAGTGGAAGGATTAATATTA	318
ACCCAGCACTGAGTACATCAA	319

TATGTTTAAGGGAATAGTTTA	320
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Additional preferable CCL20 target gene sequences are recited in Table 28. The sequences in Table 28 are cross-species target sequences as they are capable of silencing the CCL20 gene in human and mouse.

5 Table 28.

CCL20 target gene sequences	SEQ ID NO:
ATGAAGTTGATTCATATTGCA	321
TGAAGTTGATTCATATTGCAT	322
GAAGTTGATTCATATTGCATC	323
AAGTTGATTCATATTGCATCA	324
AGTTGATTCATATTGCATCAT	325
GTTGATTCATATTGCATCATA	326
TTGATTCATATTGCATCATAG	327
TGATTCATATTGCATCATAGT	328
TCAATGCTATCATCTTTCACA	329
CAATGCTATCATCTTTCACAC	330
TAATGAAGTTGATTCATATTG	331
AATGAAGTTGATTCATATTGC	332

Preferable CCL20 target gene sequences are recited in Table 29. The sequences in Table 29 are capable of silencing the CCL20 gene in human.

Table 29

Claudin-2 target gene sequences	SEQ ID NO:
AGCATGAAATTTGAGATTGGA	333
TACAGAGCCTCTGAAAGACCA	334
CACTACAGAGCCTCTGAAAGA	335
CTGACAGCATGAAATTTGAGA	336
ATCTCTGTGGTGGGCATGAGA	337
CATGAAATTTGAGATTGGAGA	338
TCTGGCTGAGGTTGGCTCTTA	339
GTGGGCTACATCCTAGGCCTT	340

10

Additional preferable CCL20 target gene sequences are recited in Table 30. The sequences in Table 30 are capable of silencing the CCL20 gene in mouse.

Table 30

Claudin-2 target gene sequences	SEQ ID NO:
CAGCTTCCTGCTAAACCACAA	341
CAAGAGTGAGTTCAACTCATA	342
CTGGTTCCTGACAGCATGAAA	343
TGGCTGGGACTATATATATAA	344
GAGGGCAATTGCTATATCTTA	345
CAGCAGCCAAACGACAAGCAA	346
CAAGGGTTTCCTTAAGGACAA	347
CAGATACTTGTAAGGAGGAAA	348
AAGAAATGGATTAGTCAGTAA	349
AAGGAAAGCACAAGAAGCCAA	350

CTGGCTGAGGTTGGCTCTTAA	351
AACCTGGGATCTAAAGAAACA	352
AAGGGCTTGGGTATCAAAGAA	353
CAGGCTCCGAAGATACTTCTA	354
CCCAATATATAAATTGCCTAA	355
CTGACCCAGCTTCCTGCTAAA	356

Preferable Chitinase-3 target gene sequences are recited in Table 31. The sequences in Table 31 are capable of silencing the Chitinase-3 gene in human.

Table 31

Chitinase-3 target gene sequences	SEQ ID NO:
ACCCACATCATCTACAGCTTT	357
CATCATCTACAGCTTTGCCAA	358
CAGCTGGTCCCAGTACCGGGA	359
CACCAAGGAGGCAGGGACCCCT	360
CCGGTTCACCAAGGAGGCAGG	361
AGCTGGTCCCAGTACCGGGAA	362
CAGGCCGGTTCACCAAGGAGG	363
GGCCGGTTCACCAAGGAGGCA	364

5

Additional preferable Chitinase-3 target gene sequences are recited in Table 32. The sequences in Table 32 are capable of silencing the Chitinase-3 gene in mouse.

Table 32

Chitinase-3 target gene sequences	SEQ ID NO:
TAGGTTTGACAGATACAGCAA	365
AACCCCTGTTAAGGAATGCAAA	366
ATCAAGTAGGCAAATATCTTA	367
CGCAGCTTTGTCAGCAGGAAA	368
TTGGATCAAGTAGGCAAATAT	369
TTGAGGGACCATACTAATTAT	370
GAGGACAAGGAGAGTGTCAAA	371
TGCGTACAAGCTGGTCTGCTA	372
CAGGAGTTTAATCTCTTGCAA	373
ATCAAGGAACTGAATGCGGAA	374
CACCCCTGATCAAGGAACTGAA	375
CACTTGGATCAAGTAGGCAAA	376
CAGGATTGAGGGACCATACTA	377
AACTATGACAAGCTGAATAAA	378
ATGCAAATTCTCAGACTCTAA	379
ATCCTTCCCTTAGGAACTTAA	380

10 **5. Treatment of Diseases and Disorders**

The present invention also provides methods of treating or preventing a disease or disorder in a mammal. The methods include regulating the expression of at least one gene in a cell known to cause a disease or disorder by introducing to the cells of the mammal at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more

DNA molecules encoding one or more siRNAs, where the expressed siRNAs interfere with the mRNA of the gene known to cause the disease or disorder of interest.

The RNAi methods of the invention, including BMGS and tkRNAi are used to treat any disease or disorder for which gene expression regulation would be beneficial. This method is effected by silencing or knocking down (decreasing) genes involved with one or more diseases and disorders.

The gene to be regulated to treat or prevent a disease or disorder of interest, can be, but is not limited to, *ras*, *β -catenin*, one or more HPV oncogenes, APC, eotaxin-1 (CCL11), HER-2, MCP-1 (CCL2), MDR-1, MRP-2, FATP4, SGLUT-1, GLUT-2, GLUT-5, apobec-1, MTP, IL-6, IL-6R, IL-7, IL-12, IL-13, IL-13 Ra-1, IL-18, IL-21R, IL-32 α , the p19 subunit of IL-23, LY6C, p38/JNK MAP kinase, p65/NF- κ B, CCL20 (MIP-3 α), Claudin-2, Chitinase 3-like 1, apoA-IV, MHC class I and MHC class II. In one aspect of this embodiment, the *ras* is *k-Ras*. In another aspect of this embodiment, the HPV oncogene is E6 or E7.

The present invention provides methods of treating or preventing a disease or disorder associated with the over-expression of a gene including, but not limited to, *ras*, *β -catenin*, one or more HPV oncogenes, APC, eotaxin-1 (CCL11), HER-2, MCP-1 (CCL2), MDR-1, MRP-2, FATP4, SGLUT-1, GLUT-2, GLUT-5, apobec-1, MTP, IL-6, IL-6R, IL-7, IL-12, IL-13, IL-13 Ra-1, IL-18, IL-21R, IL-32 α , the p19 subunit of IL-23, LY6C, p38/JNK MAP kinase, p65/NF- κ B, CCL20 (MIP-3 α), Claudin-2, Chitinase 3-like 1, apoA-IV, MHC class I and MHC class II. Preferably, the gene is *β -catenin* and the disease disorder to be treated is one associated with the over-expression of *β -catenin*. The term "over-expression" as used herein refers to an increased expression (DNA, RNA or protein) when compared to normal or wild-type expression. Preferably, the disease or disorder to be treated is selected from the group consisting of colon cancer, rectal cancer, colorectal cancer, Crohn's disease, ulcerative colitis, familial adenomatous polyposis (FAP), Gardner's syndrome, hepatocellular carcinoma (HCC), basal cell carcinoma, pilomatricoma, medulloblastoma, and ovarian cancer.

Preferably, the present invention provides methods of treating or preventing cancer or a cell proliferation disorder, viral disease, an inflammatory disease or disorder, a metabolic disease or disorder, an autoimmune disease or disorder, or a disease, disorder or cosmetic concern in the skin or hair in a mammal by regulating the expression of a gene or several genes known to be associated with the onset, propagation or prolongation of the disease or disorder by introducing a bacterium or BTP to the cell. The bacterium or BTP contain one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, where the expressed siRNAs interfere with the mRNA of the gene known to cause, propagate or prolong the disease or disorder of interest.

In some preferred embodiments, the viral disease can be, but is not limited to, hepatitis B, hepatitis C, Human Papilloma Virus (HPV) infection or epithelial dysplasia or cancer caused by HPV infection or HPV induced transformation, including cervical cancer, rectal cancer and pharyngeal cancer.

In some preferred embodiments, the inflammatory disease or disorder can be, but is not limited to, inflammatory bowel disease, Crohn's disease, ulcerative colitis, an allergy, rheumatoid arthritis or airway disease.

In some preferred embodiments, the automimmune disease or disorder can be, but is not limited to, celiac disease, rheumatoid arthritis, systemic lupus erythematosus or encephalomyelitis.

In some preferred embodiments, the disease, disorder or cosmetic concern can be, but is not limited to, psoriasis, eczema, albinism, balding or gray hair.

The mammal can be any mammal including, but not limited to, human, bovine, ovine, porcine, feline, canine, goat, equine, or primate. Preferably, the mammal is a human.

The terms "treating" and "treatment" as used herein refer to the administration of an agent or formulation (*e.g.*, bacterium and/or BTP containing an siRNA or a DNA encoding for an siRNA) to a clinically symptomatic individual afflicted with an adverse condition, disorder, or disease, so as to effect a reduction in severity and/or frequency of symptoms, eliminate the symptoms and/or their underlying cause, and/or facilitate improvement or remediation of damage.

The terms "preventing" and "prevention" refer to the administration of an agent or composition to a clinically asymptomatic individual who is susceptible to a particular adverse condition, disorder, or disease, and thus relates to the prevention of the occurrence of symptoms and/or their underlying cause.

6. Pharmaceutical Compositions and Modes of Administration

In a preferred embodiment of the invention, the invasive bacteria or BTPs containing the RNA molecules, and/or DNA encoding such, are introduced into an animal by intravenous, intramuscular, intradermal, intraperitoneally, peroral, intranasal, intraocular, intrarectal, intravaginal, intraosseous, oral, immersion and intraurethral inoculation routes.

The amount of the invasive bacteria or BTPs of the present invention to be administered to a subject will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed will be about 10^3 to 10^{11} viable organisms, preferably about 10^5 to 10^9 viable organisms per subject.

The invasive bacteria or BTPs of the present invention are generally administered along with a pharmaceutically acceptable carrier and/or diluent. The particular pharmaceutically acceptable carrier and/or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine *et al.* J. Clin. Invest., 79:888-902 (1987); and Black *et al.* J. Infect. Dis., 155:1260-1265 (1987)), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine *et al.* Lancet, II:467-470 (1988)). Examples of carriers include proteins, *e.g.*, as found in skim milk, sugars, *e.g.*, sucrose, or

polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-30% (w/v) but preferably at a range of 1-10% (w/v).

Set forth below are other pharmaceutically acceptable carriers or diluents which may be used for delivery specific routes. Any such carrier or diluent can be used for administration of the bacteria of the invention, so long as the bacteria or BTPs are still capable of invading a target cell. *In vitro* or *in vivo* tests for invasiveness can be performed to determine appropriate diluents and carriers. The compositions of the invention can be formulated for a variety of types of administration, including systemic and topical or localized administration. Lyophilized forms are also included, so long as the bacteria are invasive upon contact with a target cell or upon administration to the subject. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the composition, *e.g.*, bacteria or BTPs, of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the pharmaceutical compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated

containing a powder mix of the composition, *e.g.*, bacteria, and a suitable powder base such as lactose or starch.

The pharmaceutical compositions may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The pharmaceutical compositions may also be formulated in rectal, intravaginal or intraurethral compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the bacteria of the invention are formulated into ointments, salves, gels, or creams as generally known in the art, so long as the bacteria are still invasive upon contact with a target cell.

The compositions may, if desired, be presented in a pack or dispenser device and/or a kit that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The invasive bacteria or BTPs containing the RNA or RNA-encoding DNA to be introduced can be used to infect animal cells that are cultured *in vitro*, such as cells obtained from a subject. These *in vitro*-infected cells can then be introduced into animals, *e.g.*, the subject from which the cells were obtained initially, intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host tissue. When delivering RNA to individual cells, the dosage of viable organisms administered will be at a multiplicity of infection ranging from about 0.1 to 10^6 , preferably about 10^2 to 10^4 bacteria per cell.

In yet another embodiment of the present invention, bacteria can also deliver RNA molecules encoding proteins to cells, *e.g.*, animal cells, from which the proteins can later be harvested or purified. For example, a protein can be produced in a tissue culture cell.

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is

defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The present invention is further illustrated by the following examples that should not be construed as limiting in any way. The contents of all cited references including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis *et al.* U.S. Pat. No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu *et al.* eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

The following non-limiting examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention.

EXAMPLES

Example 1: Knockdown of β -catenin and *k-Ras*

Previous studies have demonstrated the powerful nature of the siRNA knockdown technology disclosed herein. For example, *in vitro* and *in vivo* knockdown of beta catenin and k-ras utilizing bacterial delivery is described in PCT Publication No. WO 06/066048, which is incorporated herein by reference in its entirety.

Example 2: TRIP with Multiple shRNA Expression Cassettes

The TRIP described herein, and described in further detail in PCT Publication No. WO 06/066048, can be modified to produce a plasmid which allows targeting of multiple genes simultaneously or multiple sequences within one gene simultaneously. For example, TRIP with multiple hairpin expression cassettes to produce shRNA can target different sequences in a given gene, or target multiple genes through a simultaneous bacterial treatment.

The TRIP plasmid can incorporate multiple (up to ten) cloning sites to express different shRNA constructs (as shown in PCT Publication No. WO2008/156702 at Figure 1). The purpose of such a plasmid will be to allow silencing of various genes through a single therapeutic bacterium which will be empowered by the Multiple-expression cassette-TRIP (mec-TRIP) to synthesize short hairpin RNA against a variety of targets simultaneously.

These different hairpins can either be expressed competitively at high levels through the use of an identical high level promoter (such as T7 promoter or a different high level bacterial promoter), or they can be expressed at different levels through the use of promoters with different levels of activity, this will depend on the intended use of the plasmid and the desired relative silencing levels of the target gene.

This mec-TRIP could be useful to treat complex diseases as described herein (e.g. inflammatory diseases, or cancer), through the simultaneous silencing(targeting) of multiple targets as described herein (e.g. multiple oncogenes, such as k-ras and beta-catenin in the case of colon cancer, or HER-2 and MDR-1 in breast cancer, or other combinations).

Example 3: Operator Repressor Titration System

The TRIP system (bacteria and plasmid) have been modified to include the ORT (Operator Repressor Titration) system from Cobra Biomanufacturing (Keele, UK). This adaptation helps to maintain the plasmid in suitable strains in the absence of selective antibiotics. The bacterial carrier strain has been modified accordingly to allow for the ORT system to function (deletion of the DAP gene and replacement with an ORT-controlled DAP gene expression system). The plasmid has been modified to remove the antibiotic selection sequences to support the ORT system. Further changes have been introduced to the bacterial genome, including for example, (a) deletion of the aroA gene (in some CEQ strains) to make the bacteria more susceptible to nutrient shortage, particularly in the intracellular compartment where they will die due to lack of nutrients; (b) insertion of T7RNAPolymerase gene into the chromosome and or (c) integration of a shRNA expression cassette under T7 promoter into the chromosome.

PCT Publication No. WO2008/156702 at Figure 2 shows development examples of bacterial strains. Further strains developed include, but are not limited to, CEQ922 (CEQ919 without aroA deletion), CEQ923 (CEQ920 without aroA deletion), CEQ924 (CEQ921 without aroA deletion).

Example 4: Intestinal Tract Gene Delivery

S. typhimurium was investigated to determine if it could be used as a vector for RNAi delivery into the epithelial cells lining the intestinal tract. Mice were treated orally with a single dose of 10^8 SL 7207 and sacrificed at various time points after administration. SL7207 were then stained using the Salmonella specific antibody. 2h after treatment, numerous SL7207 could be seen invading the intestinal epithelial layer (Salmonella stained red), suggesting that oral administration of SL7207

may be a useful tool to deliver payloads to the intestinal and colonic mucosa. In a follow up experiment, mice were treated with SL7207 harboring a GFP expression plasmid (pEGFPC1, Invitrogen). At 24h after a single treatment, a small percentage (approximately 1%) of cells was clearly found to express GFP.

PCT Publication No. WO2008/156702 at Figure 3 shows the efficient invasion and plasmid delivery into the intestinal mucosa by *S. typhimurium*. SL7207 were stained using red fluorescent antibody 6h after oral administration. Intact SL7207 and fragments of SL7207 were seen in epithelial cells as well as underlying cells of the lamina propria (top left/right). SL7207 successfully deliver expressed DNA into the intestinal mucosa: intestinal mucosal cells expressing GFP after treatment with SL7207 carrying a eukaryotic expression plasmid for GFP (pEGFP-C1)(lower left). For fluorescence microscopy, SL7207 were stained with red fluorescent antibody and nuclei were counterstained with Hoechst 37111.

To test whether SL7207 could be used for the delivery of RNAi to target genes in the intestinal tract, GFP transgenic mice (4 per group) were treated with *S. typhimurium* harboring a shRNA expression plasmid directed against GFP (SL-siGFP) or a shRNA expression plasmid directed against k-RAS (SL-siRAS). 10^8 c.f.u. was given three times weekly for two weeks by oral gavage. Colonic tissues were subsequently reviewed with fluorescent microscopy (data not shown) and stained analyzed after immunohistochemistry staining for GFP expression using a specific antibody (Living Colors®, Invitrogen). There was a significant reduction in the overall GFP expression level and significant reduction in the number of GFP expressing crypts in the SL-siGFP treated animals compared with the SL-siRAS treated animals (33.9% vs 50%, $p < 0.05$), suggesting that this method could be useful to deliver therapeutic RNAi into the colonic epithelium.

PCT Publication No. WO2008/156702 at Figure 4 shows that bacteria-mediated RNA interference reduces target gene expression in the gastrointestinal epithelium. After treatment with SL7207 carrying expression plasmids targeting GFP (SL-siGFP, right bottom panel), colon tissues showed lower levels of GFP expression, and fewer colonic crypts were stained positive for GFP compared with animals treated with SL-siRAS (left bottom panel). Slides were stained with GFP-specific antibody.

Example 5: Construction of CEQ503 Bacterial Strain

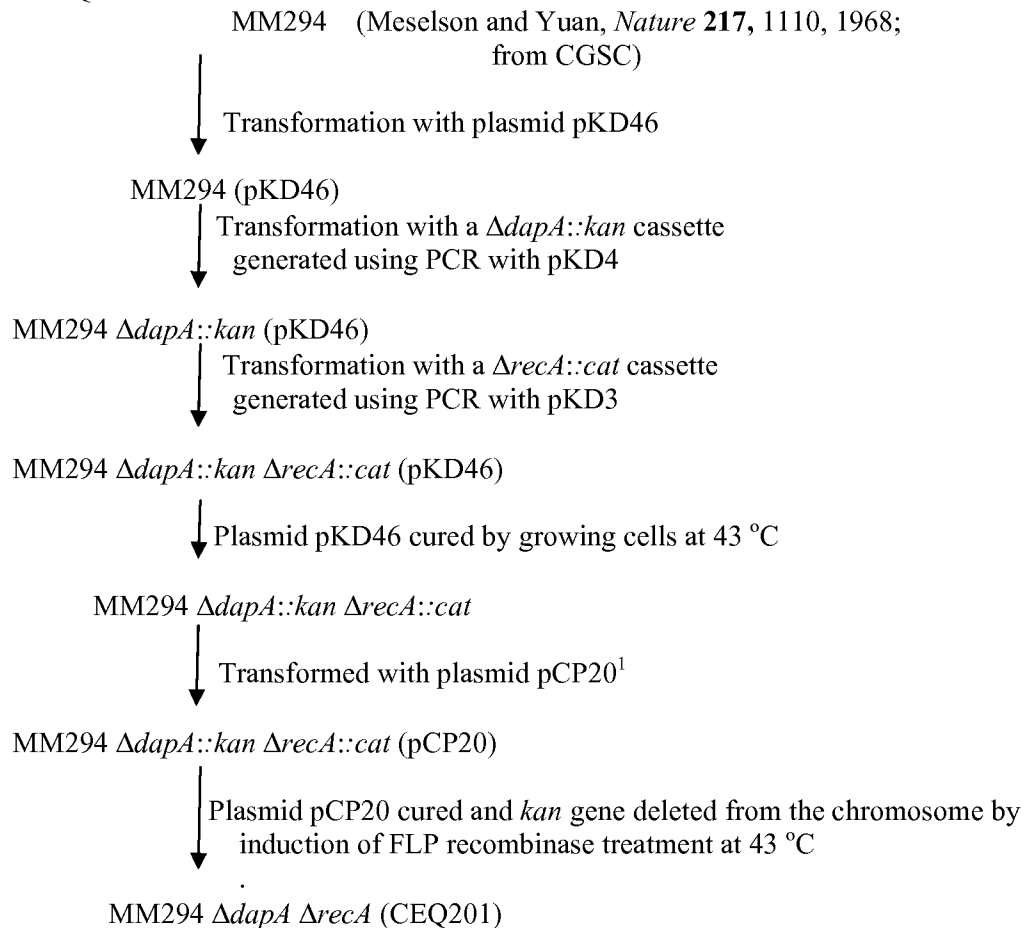
Derivation and Description of CEQ 503 (Strain CEQ201 (pNJSZ))

CEQ503 consists of a combination of an attenuated *E.coli* strain (CEQ201) with a specially engineered TRIP plasmid (pNJSZ). The plasmid confers the abilities required to induce tkRNAi (in this case: invasiveness, escape from the entry vesicle, expression of short hairpin RNA). Strain

Description of CEQ503 (pNJSZ):

1. Genotype: *Escherichia coli* CEQ201 [*glnV44(AS)*, *LAM*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻*), *creC510* *ΔdapA*, *ΔrecA*].

2. Derivation of CEQ201



3. Plasmid: pNJSZ, shown schematically in PCT Publication No. WO2008/156702 at Figure 5, is a 10.4 kb plasmid that confers kanamycin resistance to our bacterial strain (CEQ503). This plasmid contains two genes, *hly* and *inv*, and the H3 hairpin sequence:

ggatccAGGAGTAACAATACAAATGGATTCAAGAGATCCATTTGTATTGTTACTCCTTTgtcgac (SEQ ID NO:383), which includes BamHI and SalI restriction sites. To verify the presence of this plasmid, PCRs are performed to verify chromosomal deletion of *dapA*, and minipreps and/or PCR are performed to confirm *inv*, *hly* and 341-H3 on the plasmid.

4. Nutritional Requirements: Althea Media Broth or LB, Miller (Luria-Bertani) broth (Amresco; cat. no.: J106-2KG) and 50 μg/ml of DL-Δ;ε-Diaminopemilic acid (DAP) (SIGMA; cat. no.: D1377-10G).

5. Growth Conditions: 37 °C

Example 6: BTP Production

BTPs or minicells containing a suitable plasmid such as TRIP have been engineered for delivery of tkRNAi. These cells will express invasins or Opa to enable entry into mammalian cells and listeriolysin will allow lysis of phagosome following minicell degradation/ lysis. Additionally, a method for manufacturing minicells has been developed that utilizes a suicide construct for killing intact cells to aid in the purification of minicells. Such suicide plasmids have been described in the literature (Kloos et al., (1994) J. Bacteriol. 176, 7352-61; Jain and Mekalanos, (2000) Infect. Immun. 68, 986-989). Summarily, the lambda S and R genes that code for holing and lysozyme are placed under regulation of an inducible promoter on the bacterial chromosome. When induced, they will lyse intact cells but not minicells since minicells lack chromosomes. A number of different types of regulators such as lacI, araC, lambda cI857 and rhaS-rhaR can be used for development of an inducible suicide gene construct. Similarly, a number of different types of suicide genes, including E. coli autolysis genes and antimicrobial small peptides, can be used in a similar scheme. Purification is enhanced by treatments or mutations that induce filamentation (see, for example, Ward and Lutkenhaus, (1985) Cell 42, 941-949; Bi and Lutkenhaus, 1992). Initial purification involves low speed centrifugation to separate intact cells and retain minicells in the supernatant. This can be followed by density gradient purification or filtration (for example, Shull et al., (1971) J. Bacteriol. 106, 626-633).

Any cell death-triggering gene, also known as a suicide gene, including but not limited to genes encoding antimicrobial proteins, bacteriophage lysins or autolysins can be used in this method for obtaining BTPs from a mixture containing BTPs and bacteria. Suicide genes can kill live bacteria by mechanisms that include but are not limited to cell lysis, or by the destruction, degradation or poisoning of cellular components such as chromosomal DNA or filament components. Any inducible promoter may be used in conjunction with this system. In one embodiment of this invention, the suicide genes are integrated within the chromosome, thereby limiting their presence only in intact bacterial cells as BTPs or minicells will not incorporate these genes because they do not harbor chromosomal DNA.

As shown in PCT Publication No. WO2008/156702 at Figure 6, induction of suicide genes will lyse intact bacterial cells. The lambda S and R genes (suicide genes) are put under the control of P_{lacUV5} (inducible promoter). The leaky basal activity is repressed by a “super-repressor” coded by $lacI^q$ gene on a P_{gapA} (strong promoter). This cassette is put at the *minCD* locus.

Example 7: siRNA Inhibition of Human Papillomavirus (HPV) Ongogenes

Cell Culture: Hela cells were cultured in Minimum Essential Medium (MEM, ATCC No. 30-2003) with 10% FBS supplemented with antibiotics: 100 U/ml penicillin G, 10 µg/ml streptomycin (Sigma).

Bacterial Culture: Plasmids were transformed into BL21(DE3) strain (Invitrogen). Bacteria were grown at 37°C in LB Broth containing 100 µg/ml ampicillin. Bacterial cell density (in CFU/ml) was calculated using OD₆₀₀ measurement. For cell infection, overnight cultures were inoculated into fresh medium for another 2-3 h growth until the optical density at 600 nm [OD₆₀₀] reached 0.6.

5 Invasion Assay: For bacterial invasion, Hela cells were plated in 6-well dishes at 200,000 cells/well and allowed to incubate overnight in 2 ml complete growth medium. The bacterial cells were grown to mid-exponential phase with optical density at 600 nm [OD₆₀₀] 0.6 in LB Broth with Ampicillin, and then centrifuged at 3,400 rpm for 10 minutes at 4°C. Bacterial pellets were resuspended in MEM without serum or the antibiotics and the bacteria were added to the cells at an
10 MOI of 1:1000, 1:500, 1:250, 1:125, or 1:62.5 and allowed to invade the Hela cells for 2 hours at 37°C in 5% CO₂. The cells were washed 4 times with MEM containing 10% FBS and penicillin-streptomycin (100 IU of penicillin and 100 µg of streptomycin per ml). Cells were incubated in fresh complete medium for further 48 hours at 37°C in 5% CO₂ and total RNA was then isolated by the Qiagen RNeasy system with on-column DNase digestion or by TRIZOL extraction method.

15 siRNA Transfection: One day before the transfection, cells were plated in complete growth medium without antibiotics so that the cells will be 30-50% confluent at the time of transfection. Diluted various concentrations of siRNA from a stock of 20µM in 175 µl of Opti-MEM. Mixed 4 µl of Oligofectamine separately in 15 µl of Opti-MEM. Mixed gently and incubated for 5-10 min at room temperature. Combined the diluted siRNA with diluted oligofectamine and incubated for 15-20
20 min at room temperature. While the complexes were being formed, removed the growth medium from the cells and added 800 µl of medium without serum to each well containing cells. Added the 200 µl of siRNA/oligofectamine complexes to the cells and incubated at 37°C for 4h. Added 1ml of growth medium containing 3X the normal concentration of serum without removing the transfection mixture. Gene silencing was assayed at 48 h.

25 RT-PCR: Quantitative real-time reverse transcription PCR (RT-PCR) was performed with the TaqMan RT-PCR master Mix Reagents Kit (Applied Biosystems) using the following primers and a probe set for detection of HPV18E6E7 transcripts:

Forward Primer: 5'-CTGATCTGTGCACGGAAGTGA-3' (148-168) (SEQ ID NO:384)

Reverse Primer: 5'-TGTCTAAGTTTTTCTGCTGGATTCA-3'(439-463) (SEQ ID NO:385)

30 Probe: 5'-TTGGAAGTTACAGAGGTGCCTGCGC-3' (219-233 and 416-425) (SEQ ID NO:386)

The probe was labeled at the 5' end with a reporter fluorescent dye, FAM and at the 3' end with fluorescent dye quencher TAMRA. GAPDH was used to detect human GAPDH transcripts for the normalization.

HPV shRNA sequences:

35 H1 (working sequence)

5' - **gg**ATCCTAGGTATTTGAATTTGCATTTCAAGAGAATGCAAATTCAAATACCTTTT**gTCgAC** (SEQ ID NO:387)

5' - **GTCGAC**AAAAGGTATTTGAATTTGCATTTCTCTTGAATGCAAATTCAAATACCTAG**GATCC** (SEQ ID NO:388)

H2 (ineffective sequence)

5' - **ggATCCTCAGAAAACTTAGACACCTTCAAGAGAGGTGTCTAAGTTTTCTGTTTgTCgAC** (SEQ ID NO:389)

5' - **GTCGACAAACAGAAAACTTAGACACCTCTCTTGAAGGTGTCTAAGTTTTCTGAGGATCC** (SEQ ID NO:390)

- 5 Western Blot: Hela cells were lysed using 1X Cell lysis Buffer (Cell Signaling Technology, Cat No. 9803). For electrophoresis, 50 µg of total protein in 2X loading buffer was loaded to each well of a 12% SDS-PAGE gel. After transferring the blot was blocked and probed with primary antibody at 2h followed by incubation with HRP-conjugated secondary antibody before detection by ECL. All primary antibodies were used at 1/1000 dilution except HPV18E7 antibody at 1/250.
- 10 Anti-Human pRb antibody: BD Pharmingen (Cat No. 554136), Sec Ab: HRP-anti Mouse HPV18E7: Santa Cruz (Cat No. sc-1590), Sec Ab: donkey anti-goat IgG-HRP Cat no. sc 2020 p53: Santa Cruz (Cat No. sc-126), Sec Ab: HRP-anti Mouse p21: Santa Cruz (Cat No. sc-397), Sec Ab: HRP-anti Rabbit c-Myc: Cell Signaling Technology (Cat No. 9402), Sec Ab: HRP-anti Rabbit
- 15 Colony Formation Assay: Hela cells were harvested after bacterial invasion for 2h. The cells in either control treated or HPV shRNA treated cells were washed 3x times with complete MEM and one time with PBS. The cells were then trypsinized and counted. 500 cells from each treatment were added to a single well of a six well plate containing 2 ml of complete growth medium. The cells were allowed to grow for 10 days following which the colonies were fixed with GEIMSA stain.
- 20 MTT Assay: Hela cells were harvested after bacterial invasion for 2h. The cells in either control treated or HPV shRNA treated cells were washed 3x times with complete MEM and one time with PBS. The cells were then trypsinized and counted. 5000 cells from each treatment were added to a single well of a 96 well plate in 100 µl of complete growth medium in triplicates. The cells were incubated at 37°C for 48-72h following which 10 µl of 0.5 mg/ml MTT was added to each well. The
- 25 plate was further incubated at 37°C for 3h, the medium was aspirated off from the wells and after incubation, 100 µl of MTT solubilization solution [10% Triton X-100 in acidic isopropanol (0.1 N HCl)] was added to each well to stop the reaction. The absorbance was read at 570 nm on the plate reader.

In this example, the suppressive effect of a short hairpin RNA directed towards HPV 18 E6 and E7 oncogenes was investigated. The short hairpin RNA was delivered by infecting human

30 cervical cancer cells (Hela) with bacterial strains that produce the short hairpin RNA. The shRNA expression cassette contained 19 nucleotide (nt) of the target sequence followed by the loop sequence (TTCAAGAGA) (SEQ ID NO:391) and the reverse complement to the 19nt. For the 19 nt, two shRNA sequences published in Cancer Gene Therapy (2006) 13, 1023-1032, were used to measure

35 siRNA delivery and gene silencing efficiency, oligofectamine reagent in a 6 well format was used. Briefly, Hela cells were plated at a cell density of about 40% confluence in antibiotic free medium.

On the next day, siRNA was added to 6 well plates at varying concentrations of 50, 100, 200 nM. The control siRNA was added at a single concentration of 100 nM.

As shown in PCT Publication No. WO2008/156702 at Figure 7, the oligofectamine transfection method resulted in a decrease in E6 mRNA in HeLa cells with respect to the control siRNA. The siRNA (H1) showed up to about 40% of reduction in E6 mRNA. The knockdown response was not dose dependent.

Next, the hairpin of the siRNA (H1) was cloned into the TRIP vector. In order to determine if gene silencing could be achieved through the transkingdom system, the shRNA in human cervical cancer cells (HeLa) was tested in an invasion assay. Briefly, HeLa cells were plated in a six-well plate at 2×10^5 cells/well, allowed to grow overnight and incubated the next day for 2h at different MOIs with bacteria (*E.coli*) engineered to produce the hairpin RNA. The bacteria were washed off with medium containing 10% FBS and Pen Strep four times and the mammalian cells were further incubated for an additional 48h in the complete medium. RNA or protein was isolated from the bacteria.

PCT Publication No. WO2008/156702 at Figures 8 and 9 demonstrate that siRNA downregulates HPV E6 expression in HeLa cells. Cells were plated in six well plates and allowed to grow to a confluence of 40% (about 40,000 cells). Oligofectamine/siRNA transfection complexes were prepared in Opti-MEM serum-free medium by mixing 4 μ l of oligofectamine with siRNAs (final concentration in 185 μ l of medium is 50, 100, 200 nM). 48 hours post-transfection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. Two different negative control siRNAs were used at a single concentration of 200 nM.

PCT Publication No. WO2008/156702 at Figure 10, Panels A - C show real time PCR results following invasion assay of HeLa cells. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection the cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

PCT Publication No. WO2008/156702 at Figure 11 shows the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. 50 μ g of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, p53, actin, p110Rb, p21 and c-myc as indicated.

PCT Publication No. WO2008/156702 at Figures 12 and 13 show a colony formation and MTT assay, respectively. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at

different multiplicities of infection (MOI). 2h post-infection cells were washed trypsinized and counted and an equal number of cells for each MOI was added to a well of a six well plate (For CFA: added 500 cells to each well of a 6 well plate, for MTT added 5000 cells in each well of a 96 well plate). For colony formation, the cells were allowed to grow for 10 days and stained with Geimsa, MTT assay was analyzed at 72h post plating.

PCT Publication No. WO2008/156702 at Figures 14 and 15 show real time PCR results following invasion assay of Hela cells. Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

PCT Publication No. WO2008/156702 at Figure 16 shows the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets. Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, p53, actin, p110Rb as indicated.

PCT Publication No. WO2008/156702 at Figure 17 shows real time PCR results following invasion assay of Hela cells with a frozen aliquot of negative sHRNA control and HPV sHRNA in BL21 (DE3). Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

PCT Publication No. WO2008/156702 at Figure 18 shows the plating efficiency of frozen aliquots of negative sHRNA control and HPV sHRNA in BL21 (DE3). The frozen bacteria were thawed and resuspended to a final concentration of 3.38×10^8 cells/ml. Invasion assay was performed with this concentration taking 2 mls of 3.38×10^8 cells/ml as an MOI of 1000. Some stock control bacteria or HPV bacteria were serially diluted (1:100) and plated on LB plates to assess for the number and viability of bacteria treated cells at 48h. Gene silencing was analyzed either by quantitative real-time PCR using the $\Delta\Delta C_t$ relative quantitation method or by western blot analysis. HPV E6 mRNA levels were normalized to an endogenous control, GAPDH. The final data were further normalized to the RNA from the untreated cells. For Protein analysis, cell lysates were prepared in Cell Lysis Buffer (Cell Signaling Technology) and the protein concentration was determined using a BCA kit from BioRad. For electrophoresis, the protein expression was normalized to Actin loading control.

Example 8: Knockdown of HPV E6 gene assessed by western blotting with HPV 18 E7 antibody:

Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) (HPVH1 construct below) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. The HPV E6 specific knockdown was compared with a negative shRNA control. Briefly, 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, and actin as indicated.

HPVH1

5' -GATCC TAGGTATTTGAATTTGCAT TTCAAGAGA ATGCAAATTCAAATACCTTTT G-3' (SEQ ID NO:392)
 3' -G ATCCATAAACTTAAACGTA AAGTTCTCT TACGTTTAAGTTTATGGAAAA CAGCT-5' (SEQ ID NO:393)

PCT Publication No. WO2008/156702 at Figure 19 shows the knockdown of HPV E6 gene assessed by western blotting with HPV 18 E7 antibody. Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. The HPV E6 specific knockdown was compared with a negative shRNA control. Briefly, 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7 and actin as indicated.

Example 9: Inhibition of CCL20 Expression in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method.

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) were diluted to 47uL with serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes.

50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 hours at 37C at which time the media was removed and replaced with 400uLs of DMEM/10% FCS containing 100ng/mL of LPS for 2 hours. Following stimulation, the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 50 cycles.

PCT Publication No. WO2008/156702 at Figure 20 shows the knockdown of CCL20 expression with the various siRNA sequences in CMT93 cells. The siRNA sequences tested are listed in Table 33.

Table 33

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
G1	GCUUGUGACAUUAAUGCUAAtt	394	UAGCAUUAUUGUCACAAGCtt	395
H1	AUAAGCUAUUGUAAAGAUAtt	396	UAUCUUUACAAUAGCUUAUtg	397
G2	CAUCUUUCACACGAAGAAAtt	398	UUUCUUCGUGUGAAAGAUGat	399
H2	CUAUUGUAAAGAUUUUAAtt	400	UUAAAUAUCUUUACAAUAGct	401
G3	GCCUAAGAGUCAAGAAGAUtt	402	AUCUUCUUGACUCUUAGGctg	403
G4	CAGUGGACUUGUCA AUGGAtt	404	UCCAUUGACAAGUCCACUGgg	405
G5	GAAGUUGAUUCAUUAUGCAtt	406	UGCAAUAUGAAUCAACUUCat	407
G6	GUUGAUUCAUUAUGCAUCAtt	408	UGAUGCAAUAUGAAUCAACtt	409
G7	ACAUUAGAGUUAAGUUGUAAtt	410	UACAACUUAACUCUAAUGUga	411
G8	CAUUAGAGUUAAGUUGUAUtt	412	AUACAACUUAACUCUAAUGtg	413
G9	UGUUAUUUAUAGAUCUGAAtt	414	UUCAGAUUCUAUAAAUAACAta	415
G10	GUUUAGCUAUUUAAUGUUAAtt	416	UAACAUAUAAAUAGCUAAACat	417
G11	AGUGGAAGGAUUAUUAUUAAtt	418	UAAUAUUAUCCUUCACUaa	419
F12	CCAGCACUGAGUACAUCAAtt	420	UUGAUGUACUCAGUGCUGGgt	421
G12	UGUUUAAGGGAAUAGUUUAAtt	422	UAAACUAUUCUUAACAta	423

Example 10: Inhibition of Expression of Claudin-2 in CMT93 cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uL added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in

transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 or 48 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 50 cycles.

PCT Publication No. WO2008/156702 at Figure 21 shows the knockdown of Claudin-2 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 34.

Table 34

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
D4	GCUGGGACUAUAUAUAUAAtt	424	UUUAUAUAUAUAGUCCCAGCca	425
D5	GGGCAAUUGCUAUAUCUUAAtt	426	UAAGAUAUAGCAAUUGCCCtc	427
D6	GCAGCCAAACGACAAGCAAAtt	428	UUGCUUGUCGUUUGGCUGCtg	429
D7	AGGGUUUCCUUAAGGACAAtt	430	UUGUCCUUAAGGAAACCCUtg	431
C9	GAAAUUGGAUUAGUCAGUAAtt	432	UUACUGACUAAUCCAUUUCtt	433
D11	GGCUCCGAAGAUACUUCUAAtt	434	UAGAAGUAUCUUCGGAGCCtg	435

Example 11: Inhibition of Expression of IL6-Ra in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells.

5 Transfection was for 24, 48 or 72 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

PCT Publication No. WO2008/156702 at Figure 22 shows the knockdown of IL6-RA expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 35.

Table 35

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
E1	CCUGGAGGGUGACAAAGUA _{tt}	436	UACUUUGUCACCCUCCAGG _{at}	437
F1	GGUCUGACAAUACCGUAAA _{tt}	438	UUUACGGUAUUGUCAGACC _{ca}	439
F2	GCUGUUUCCUAUAACAGAA _{tt}	440	UUCUGUUAUAGGAAAACAGC _{gg}	441
F3	GCUGUGAAAGGGAAAUUUA _{tt}	442	UAAAUUUCCCUUUCACAGC _{ag}	443
E4	CCUUGUGGUUAUCAGCCAUA _{tt}	444	UAUGGCUGAUACCACAAGG _{tt}	445
E5	GCUUCGAUACCGACCUGUA _{tt}	446	UACAGGUCGGUAUCGAAGC _{tg}	447
E6	CGGCAGGAAUCCUCUGGA _{tt}	448	UUCCAGAGGAUCCUGCCG _{gg}	449
E7	CCACGAGGAUCAGUACGA _{tt}	450	UUCGUACUGAUCCUCGUGG _{tt}	451
E8	CACGAGGAUCAGUACGAA _{tt}	452	UUUCGUACUGAUCCUCGUG _{gt}	453
E9	GAUCAGUACGAAAGUUCUA _{tt}	454	UAGAACUUUCGUACUGAUC _{ct}	455
E10	GUACGAAAGUUCUACAGAA _{tt}	456	UUCUGUAGAACUUUCGUAC _{tg}	457
E11	GAAAGUUCUACAGAAGCA _{tt}	458	UUGCUUCUGUAGAACUUUC _{gt}	459
E12	GGGUCUGACAAUACCGUA _{tt}	460	UUACGGUAUUGUCAGACCC _{ag}	461

Example 12: Inhibition of Expression of IL13-Ra1 in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well

plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 or 72 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

PCT Publication No. WO2008/156702 at Figure 23 shows the knockdown of IL13-RA1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 36.

Table 36

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
F1	AGAAGACUCUAAUGAUGUAAtt	462	UACAUCAUUAGAGUCUUCUtg	463
F2	CAGUCAGAGUAAGAGUCAAtt	464	UUGACUCUUACUCUGACUGtg	465
F8	CAGAACAUUCUAGCAAACAAtt	466	UUGUUUGCUAGAUGUUCUGtg	467
F9	CUUGUAGGUUCACAUUUUAAtt	468	UAAUAUGUGAACCUACAAGtt	469
F10	CAGUGUAGUGCCAAUGAAAAtt	470	UUUCAUUGGCACUACACUGag	471
F11	GUAUGACAUCUAUGAGAAAAtt	472	UUUCUCAUAGAUGUCAUACtt	473

Example 13: Inhibition of Expression of IL-18 in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of Lipofectamine RNAiMAX transfection

reagent (Invitrogen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

PCT Publication No. WO2008/156702 at Figure 24 shows the knockdown of IL18 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 37.

Table 37

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
B2	AGGAAAUGAUGUUUAUUGAtt	474	UCAAUAAACAUCAUUUCUtg	475
B3	GGCCGACUUCACUGUACAAtt	476	UUGUACAGUGAAGUCGCCaa	477
B4	GAUGGAGUUUGAAUCUUCAtt	478	UGAAGAUUCAAACUCCAUCtt	479
B5	CAACCGCAGUAAUACGGAAtt	480	UUCCGUAUUACUGCGGUUGta	481
B6	CGAGGCUGCAUGAUUUUAUAtt	482	UAUAAAUCAUGCAGCCUCGgg	483
B7	CCUGUAUUUCCAUAACAGAtt	484	UCUGUUAUGGAAAUAACAGGcg	485
B8	CAUGUACAAAGACAGUGAAtt	486	UUCACUGUCUUUGUACAUGta	487
B9	CGAGGAUAUGACUGAUUUUtt	488	AAUAUCAGUCAUAUCCUCGaa	489
B10	GGAUAUGACUGAUUUUGAUtt	490	AUCAUAUCAGUCAUAUCCctc	491
B11	CUAACUUACAUCAAAGUUAtt	492	UAACUUUGAUGUAAGUUAGtg	493
B12	CUCACUAACUUACAUCAAAtt	494	UUUGAUGUAAGUUAGUGAGag	495

Example 14: Inhibition of Expression of IL-7 in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of Lipofectamine RNAiMAX transfection

reagent (Invitrogen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

PCT Publication No. WO2008/156702 at Figure 25 shows the knockdown of IL-7 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 38.

Table 38

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
A1	GAUCCUACGGAAGUUAUGGtt	496	CCAUAACUCCGUAGGAUCcg	497
B1	CCAUGUCCAUGUUUCUUUtt	498	AAAGAAACAUGGAACAUGGtc	499
A2	CCUCCCGCAGACCAUGUUCtt	500	GAACAUGGUCUGCGGGAGGcg	501
A3	CUCCCGCAGACCAUGUUCtt	502	GGAACAUGGUCUGCGGGAGgc	503
A4	UCCCGCAGACCAUGUUCAtt	504	UGGAACAUGGUCUGCGGGAgg	505
A5	CCCGCAGACCAUGUUCCAUtt	506	AUGGAACAUGGUCUGCGGGag	507
A6	CCGCAGACCAUGUUCCAUGtt	508	CAUGGAACAUGGUCUGCGGga	509
A7	CGCAGACCAUGUUCCAUGUtt	510	ACAUGGAACAUGGUCUGCGgg	511
A10	AGACCAUGUUCCAUGUUUctt	512	GAAACAUGGAACAUGGUCUgc	513
A12	ACCAUGUUCCAUGUUUCUUtt	514	AAGAAACAUGGAACAUGGUct	515

Example 15: Inhibition of Expression of Chitinase3-like-1 (CH13L1) Expression in CMT93 Cells

In a 1.7ml microcentrifuge tube, 2.4 µl of 20 µM double-stranded RNA solution (from Qiagen) was diluted into 394 µl Opti-MEM serum-free medium (Invitrogen) containing 1 µl Lipofectamine RNAiMAX (Invitrogen), mixed, and incubated 10 min at room temperature to enable the formation of transfection complexes. 100 µl of this mixture was added to each of three wells of a 24-well tissue culture dish, on top of which CMT-93 cells were plated in a 500 µl volume, resulting in a final volume of 600 µl per well and a final RNA concentration of 20 nM. After 24 h transfection, 0.1 µg/ml lipopolysaccharide (LPS) (Sigma) was added to each well and cells were incubated for a further 24 h to stimulate CHI3L1 production, after which cells were washed in PBS and harvested for RNA extraction. CMT-93 cells were prepared for transfection as follows. 1 confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FBS) and the cells thoroughly mixed by pipetting. From this solution, 10mls was transferred into a sterile 50ml tube and 40mLs of DMEM 10% FBS added. Cells were well mixed and 500 µLs added to each well of a 24-well plate. This concentration of cells resulted in approximately 70% confluency after 24 h of growth.

Figure 26 in WO2008/156702 shows the knockdown of CH13L1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed in Table 39.

Table 39

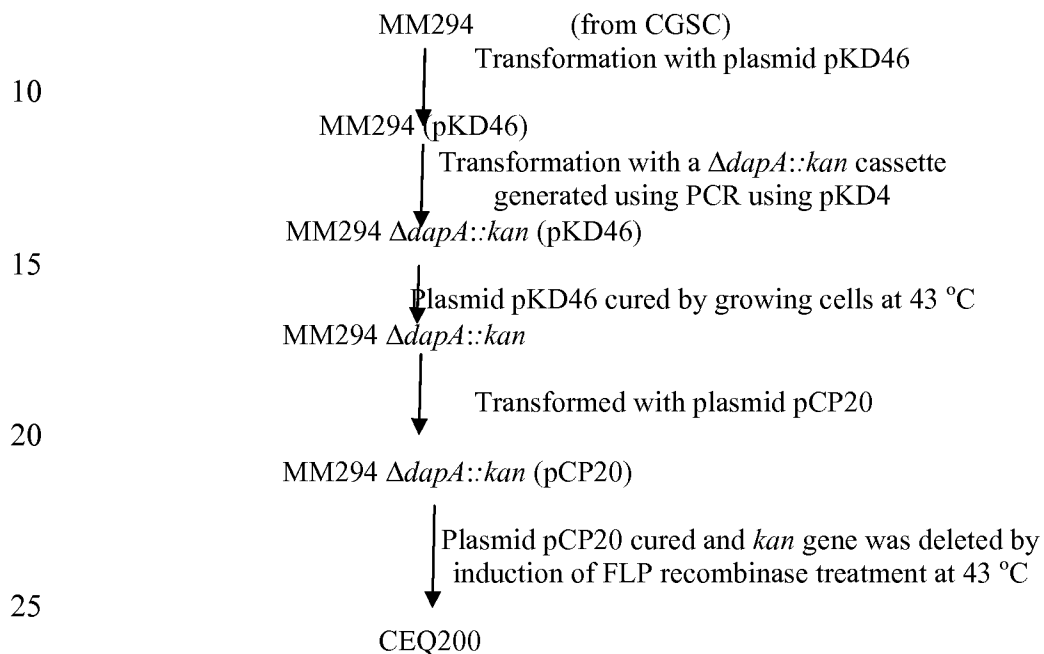
Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
G1	CCACAUCAUCUACAGCUUUtt	516	AAAGCUGUAGAUGAUGUGGgt	517
H1	GGUUUGACAGAUACAGCAAtt	518	UUGCUGUAUCUGUCAAAACtca	519
G2	UCAUCUACAGCUUUGCCAAtt	520	UUGGCAAAGCUGUAGAUGAtg	521
H2	CCCUGUUAAGGAAUGCAAAtt	522	UUUGCAUUCUUAACAGGGtt	523
H3	CAAGUAGGCAAAUAUCUUAAtt	524	UAAGAUAAUUGCCUACUUGAt	525
H4	CAGCUUUGUCAGCAGGAAAtt	526	UUUCCUGCUGACAAAGCUGcg	527
G5	GGUUCACCAAGGAGGCAGGtt	528	CCUGCCUCCUUGGUGAACCGg	529
H5	GGAUCAAGUAGGCAAAUAUtt	530	AUAUUUGCCUACUUGAUCCaa	531
H6	GAGGGACCAUACUAAUUAUtt	532	AUAAUUAGUAUGGUCCCUc	533
G7	GGCCGGUUCACCAAGGAGGtt	534	CCUCCUUGGUGAACCGGCctg	535
H7	GGACAAGGAGAGUGUCAAAtt	536	UUUGACACUCUCCUUGUCctc	537
G8	CCGGUUCACCAAGGAGGCAtt	538	UGCCUCCUUGGUGAACCGGcc	539
H8	CGUACAAGCUGGUCUGCUAtt	540	UAGCAGACCAGCUUGUACGca	541
G9	GGAGUUUAAUCUCUUGCAAtt	542	UUGCAAGAGAUUAAACUCctg	543
H9	CAAGGAACUGAAUGCGGAAtt	544	UUCCGCAUUCAGUCCUUGAt	545
G10	CCCUGAUCAAGGAACUGAAtt	546	UUCAGUUCUUGAUCAGGGtg	547
H10	CUUGGAUCAAGUAGGCAAAtt	548	UUUGCCUACUUGAUCCAAGtg	549
G11	GGAUUGAGGGACCAUACUAtt	550	UAGUAUGGUCCCUCAAUCctg	551
G12	GCAAAUUCUCAGACUCUAAtt	552	UUAGAGUCUGAGAAUUUGCat	553
H12	CCUCCCCUAGGAACUUAAtt	554	UUAAGUCCUAAGGGAAGGat	555

Example 16: Construction of CEQ200

CEQ200 has the following genotype: *glnV44(AS)*, *LAM⁻*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, *(r_k⁻m_k⁺)*, *creC510 ΔdapA*. The MM294 has the following genotype: *glnV44(AS)*, *LAM⁻*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, *(r_k⁻m_k⁺)*, *creC510*. We purchased the plasmids from CGSC (see

5 Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA **97**,6640-6645).

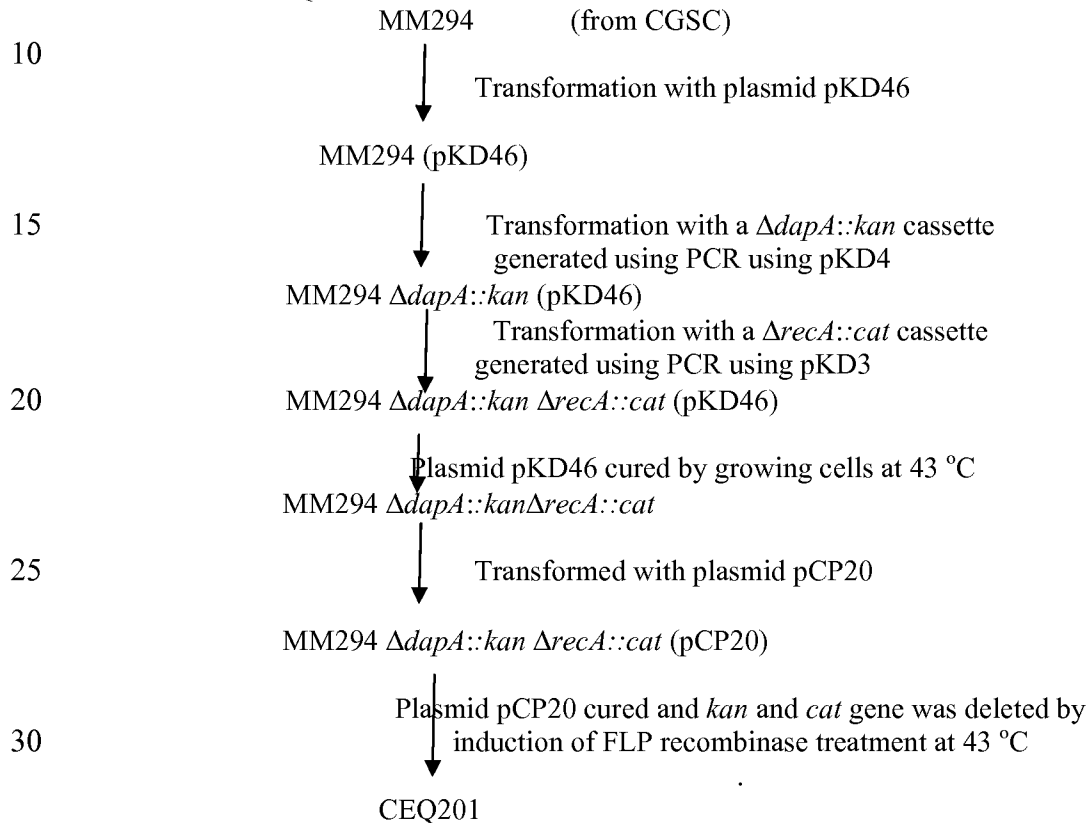
Derivation of CEQ200

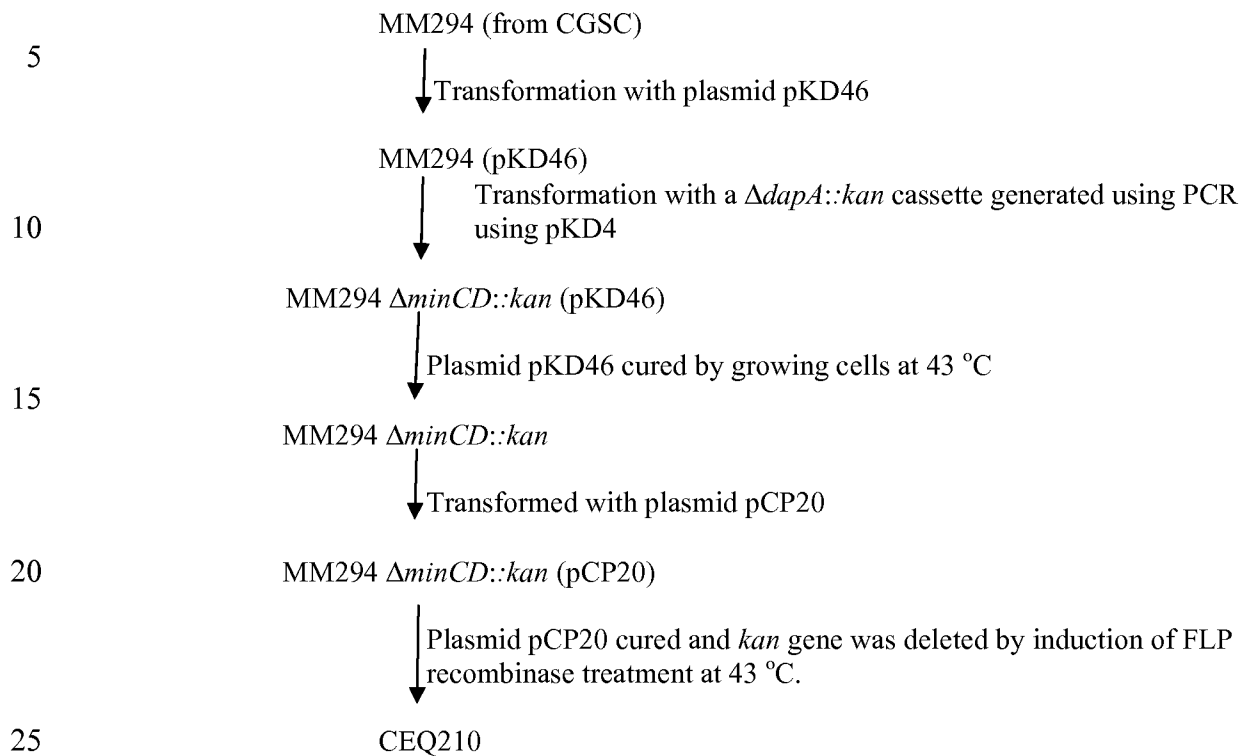


Example 17: Construction of CEQ201

CEQ201 has the following genotype: CEQ200 [*glnV44(AS)*, *LAM⁻*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510* Δ *dapA* Δ *recA*]. The MM294 has the following genotype: *glnV44(AS)*, *LAM⁻*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510*. We purchased the plasmids from

5 CGSC (see Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA **97**,6640-6645).

Derivation of CEQ200

Example 18: Construction of BTPs (CEQ210) by deletion of minC and/or minD genes from MM294**Example 19: Illustration of the pMBV40, pMBV43 and pMBV44 plasmids**

30 The pMBV40, pMBV43 and pMBV44 plasmids may be used as final or intermediary plasmid in the tkRNA system and may be constructed as follows: pUC19 digested with restriction enzyme *Pvu*II. Resultant ~ 2.4 kb fragment was ligated with a ~ 200 bp DNA fragment generated by annealing 5 oligonucleotides with each other. The oligonucleotides have the following names and sequences:

35 **OHTOP1**: GACTTCATATACCCAAGCTTGGAAAATTTTTTTTAAAAAAGTCTTGACACTTTATGCTTCCGGCTCGTATAATGG
ATCCAGGAGTAACAATACAAATGGA (SEQ ID NO:556)

OHTOP2: TTCAAGAGATCCATTTGTATTGTTACTCCTTTTTTTTTTGTGACGATCCTTAGCGAAAGCTAAGGATTTTTTT
TTTACTCGAGCGGATTACTACATAC (SEQ ID NO:557)

40 **OHBOT1**: GTATGTAGTAATCCGCTCGAGTAAAAAATCCTTAGCTTTCGCTAAGGATCGTCGACAAAAA
(SEQ ID NO:558)

OHBOT2: AGGAGTAACAATACAAATGGATCTCTTGAATCCATTTGTATTGTTACTCCTGGATCCATT (SEQ ID
NO:559)

45 **OHBOT3**: ATACGAGCCGGAAGCATAAAGTGTCAAGACTTTTTTAAAAAATTTTCCAAGCTTGGGTATATGAAGTC
(SEQ ID NO:560)

50 ↓
Ligation mix was transformed in *E. coli* and Ampicillin resistant transformants were selected. Plasmid DNA from a transformant that had the expected DNA sequence of the insert and restriction map was named pMBV38.

↓
pMBV38 was digested with *Nde*I and blunt end ligated with a ~ 6
kb fragment generated by *Bam*HI-*Sal*I digestion of the plasmid
pKSII-inv-hly

5

The predicted sequence of is shown in Table 40.

Table 40.

pKSII-inv-hly (SEQ ID NO:561)
CTAAATTGTAAGCGTTAATATTTTGTAAATTCGCGTTAAATTTTTGTAAATCAGCTCATTTTTTAACCAAT AGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTGTTCCAGTTTGG ACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCC ACTACGTGAACCATCACCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTAAAG GGAGCCCCCGATTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGA GCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCC GCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGC TATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAAACGCCAGGGTTTTCCAGTCA CGACGTTGTAAACGACGGCCAGTGAGCGCGCTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGG TGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGCTTGGGCCGTAAGATCGGCAGCAATTAATCGCGACAAT CCTTTTAAAAAACAGCGCCGCTCAATTAACCTGAGCGCGTGTCTTCTTGACGCTTTGCTACTTATGGGGCG AGTCTAGGATTGCCGACTCCCATTCGCGCCCCAAATAATCAGCTCATTAACCTGTTCTTATTGCTATCTGTTA TCTGGTTATATTGACAGCGCACAGAGCGGGAACGCCAAGTATGCAGGCCCTGGTTGCAGTGCGCCTGTGTCCAT ATTCATGGTTTTCAAAATCCGTGCTGGTCTTTTTGACCCAATATTCACCAGATTGCCAATCAGAACTATACGCGG TCAAGCTCCCCCACTCGCCCCACAATGTCCCGTCAGGCGCACGCGTTCCGTGGTTGCAGTGAGGATTCAAGA ACCGCAGACATATCTGAACCTTGGCATTGTCTGCTGGCCTCGAGACTGGATACCAGCGATCTGCCGCCATCGTA TATCCACCGATTTGGGTAGAACCGATAACTCACCGAATAACTTGGGAATTTTTTACTTTTCGCCGTCACAGCCA CTTCGCTATAGGTTTGGTAGGTAATCGTCACCTGACCCTGATCGTTAACCGATACATTGGGTGTGAATGACGAC GACCACTCATACTGAGTATTATTAGCAACATCGTTATCCATCTGTAACCTGGAATGTGGCGTTTTTAAAGATCGT TTTCGGGAACCCCTTATCCGTAGCGAAATTTTGCCCGTTAACCAGAATACCGGTGAGCGTAGGTACCGGGAATA GGGATATTTTTTCTGCAATGTACTCAGTATCAGGGTATCAACCTGCGGCGTGATTGTGACATACCGGACACTA TTCCCAACCCACCGTCGCGGTATAGCTATCTGGCTGCTCGGTAATGGGGCTAATACTCACCGGCACACCGTTTTG AGTAAAACTCAAGCCCTGCATCCCACTGATAAAATGGCCATTCTTATCGACAGGGACAAAGGATAATGTGGAAC TCATCGTGCCATCAGCCAAGATATCCGGTGTGGAGACGGTGAAACTGGAGCGGCCAGCATCTGGAATAGGATCT GCCGTGAAATTAACCGTCACACTCGGCACACTGAACGCAGCCCCATCCACTTTCACCGTTACTGTTGCTACCCC CAACGTGGTACTGGTCAATGGTGCCTATAAGTGCCGTCATTGTGATCCGTGATAACGCCCATATTGCCTAAGG TTGTGTCAAAAGCCACATTCGCGCCAGCCTGCGGGTCCCATAGGTATCCTTCAACTCCAACGTGATGGTTGAA GCCATTAGACCATCAGCGATGATAGATGTGCTACCGCAGCCAGAGTGGATTTATCCGCCGCGCATAGTACCCCTT AACAAAGTGGGTATCAACACTTTGCGGTTGCCCTCCACTTCTGCTGTGACTACCGTCACGCCATCTGTGCTAT TGGTTAATGCAATGCGCGCGACGCCATTTGCATCTGTCTTTTCCGTGATTTTATTCGGTAGCGCACCATTATTG GTGGTTATCACCACTCCTGCCCGGCTAAGGGTTTTCCCTCAAAATCAGCAACGGTGAACCTCAACGGTGATTGC AGTTTTCCCATTAGCCGGTGCGCCATCACCAATGACGGCGCGCGTTAATGTCAACTGAGGCTGCTGAACGGTGA CGCTCAATGTGAATGAGTTAGATCGGTTTCTTGGTGATCAACCGCGAGCGCACTAAGCGAATAAAAGTTGGCT GTCAGGTGTCGTTACCCGACTCACTTGTGCTGTGCGTTTTATAAGGCGGTAAAACCAAGTTGAATTGTGTGGT ACTCAGTGGTGTAAATGTGCCGCCAGCGCAATCAGTTCGGCATCACTCCAGACAATTTCCCTTACAGCAGATG CCCCTGTACTTGTGCGTTACCTGATAAACCTGACCCGGCAGGCCGAGATAGTTGCTGGCGATAATGTCAGT TTAACCACCTGCTGTTTCTGATACTCCAACAGATATTATTGTTACGATCGACAAGGTTATAGCGGCTCTCCGC CAGTAGACGTGTTCTGCCACCGCTGAAGGGCTAAGTTGCGACTGAAAACCTCTCGCCCAGGCGATAGTTCATTT GGAGGTTCCACTGTGTTTCATGCTTACTGCTTTTCCCCATACGCTGATCTACCCCGACAGTGAGTAGAGGCACG GGGGTGTAATTGATCCCGGCAGTCACGGCATAAGGGTTGCGTTGCAGATTATCTTTACCAAATAAAGCAACACG CTCACCGGTGATTGCTCATACATCAACTTCCCCCCCAGTTGTGGGAGTGCAGGTAAATAAGCATTCGCGCGCA AATCCCCCCCAGTGGCTGGGCGCTCTTTATAGTCGGAGAAATCACGCGACGAGTGCCATCCATTGAGGCGAAAA TACCCATTGGCAGCCAACCTGTAAATAATCGGTCCAGGCCCTCGGCACCAAGACCGATACGGTGGTTGTGGCCGGT CAAATCATTATCATAAAAAGTATTAAGTCCGTACAGCCAACCGTTCTCCAATGTACGTATCCCGACGCCAAGGT TAAGTGTGTTGCGGCTGTCTTTATTGCGAATACCTAAGTACTGATAAAAAAGAGGAATGAAGCAGAGTCATACCA GAGGCCAGCCAATCAAGAGAGCTTTCTTTTAGCGAAAAATTTTTGTCAAAATTCAGATTAACTTGGCCGTACC GAATCGATTTAACCACCTGTTTGATTTCTTGATTAAACCGCATCGCCACCATTTAGTGAGCAACATCAGATGCC TGCCTGATGCAGCTAACCTGGCCCCGGTGCTTATCATCTTATTCACCGCTTCAGTCTCCTGCTCCTTATTGGCG CGATCTATTATTGCAGCATTTCTTTCTGTATCCGATGCGGAAAAGGGATTGATTGAACTCTCCATTTTATTATT AGGATGGAGATTTTCAAATGCAGATGAAGAGACAGAATAAGGCTGGACCTGTTGCGGTGCGTTAGCATCATATT TTTCTGAAGCCCCAGCCATGAACATTCACATATCAAAAAGATACAAATAACTATTCGTGAAATAATATTAAAT

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 TGAGTTATTTGAGGTATAACTGGCCACAAACGAGCATTTGAAATCACCTTGACCATTAATTAAGATGCAATAG
 TTGAAAGTGAACTTGTCTTCTAATTTAGTAAAGACATTAAGAGGATAGCACTTTTTTAAAAAACAGACTGGG
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 AGCAGGTGAGAATATCAAAAAAACAATAACAAATGAACATTATCATATAAATAATATCAATTAGAATAA
 GCCCCCTTCATTTGATGTTGTGAGTTGTCTGCTGCGGTTTTTATTTCTACTTTTCACTGTTACTCCGC
 AATATCCGCATTAATCCTGATGGTTGCCCTTGATGACTGCAGGAATTCGATCCCTCCTTTGATTAGTATATTCT
 ATCTTAAAGTGACTTTTATGTTGAGGCATTAACATTTGTAAACGACGATAAAGGGACAGCAGGACTAGAATAAA
 GCTATAAAGCAAGCATATAATATTGCGTTTCATCTTTAGAAAGCGAATTTCCGCAATATTATAATTATCAAAAG
 GAGGGGTGGCAAACGGTATTTGGCATTAATTAGGTTAAAAAATGTAGAAGGAGAGTGAAACCCATGAAAAAATA
 ATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCAATTGCGCAACAACTGAAGCAAAGGATGCATCTGC
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 AACTCAGCATTGATTTGCCAGGTATGACTAATCAAGACAATAAAATCGTTGTAAAAATGCCCAATAAATCAAA
 CGTTAAACAACGAGTAAATACATTAGTGGAAAGATGGAATGAAAAATATGCTCAAGCTTATCCAAATGTAAGTG
 CAAAAATTGATTATGATGACGAAATGGCTTACAGTGAATCACAATTAATTGCGAAATTTGGTACAGCATTTAAA
 GCTGTAAATAATAGCTTGAATGTAAACTTCGGCGCAATCAGTGAAGGGAAAAATGCAAGAAGAAGTCATTAGTTT
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 CTGGAGGATACGTTGCTCAATTCACATTTCTTGGGATGAAGTAAATTTATGATCCTGAAGGTAACGAAATTTGT
 CAACATAAAAACTGGAGCGAAAAAATAAAAGCAAGCTAGCTCATTTTACATCGTCCATCTATTTGCCAGGTAA
 CGCGAGAAATATTAATGTTTACGCTAAAGAAATGCAGTGGTTTAGCTTGGGAATGGTGGAGAACGGTAATTGATG
 ACCGGAACCTTACCACTTGTGAAAAATAGAAATATCTCCATCTGGGGCACCACGCTTTATCCGAAATATAGTAAT
 AAAGTAGATAATCCAATCGAATAATTTGTAAGTAATAAAAAATTAAGAATAAAACCGCTTAACACACACGAAA
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 GTCAGATTGATTGTAGGTGGCTTAAACTTAGTTTTACGAATTAATAAAGGAGCGGTGAAATGAAAAGTAAACTTA
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 ACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATG
 GTCATAGCTGTTTCTGTGTGAAATTTGTTATCCGCTCACAATTCACACACATACGAGCCGGAAGCATAAAGT
 GTAAAGCCTGGGGTGCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCGCTTTCCAGTCG
 GGAAACCTGTGCTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGCTATTGGGCGCTC
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 CGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAATGTGAGCAAAAGGCCAGCAAAAGGCC
 AGGAACCGTAAAAAGGCCGCGTGTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCG
 ACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCCCTGGAAGCTCCCTCG
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 TCTCATAGCTCACGCTGTAGGTATCTCAGTTCCGTTGAGTTCGTTCCGCTCCAAGCTGGGCTGTGTGCACGAACC
 CCCCCTTCAGCCCGACCGCTGCGCTTATCCGTAACATATCGTCTTGAGTCCAACCCGTAAGACACGACTTAT
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 GAAAATCAGCTTAAGGGATTTTGGTCAAGAGATTATCAAAAAGGATCTTACCTAGATCCTTTTAAATTAATA
 ATGAAGTTTTTAAATCAATCTAAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGG
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 CGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCAGGCTCCAGATTTATC
 AGCAATAAACACAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCGCAACTTTATCCGCCCTCCATCCAGTCTA
 TTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCCGCGATTAAAGTTTGGCGAACGTTGTTGCCATTGCTACA
 GGCATCGTGGTGTACGCTCGTCTTGGTATGGCTTCATTAGCTCCGTTCCCAACGATCAAGGCGAGTTAC

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ATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTCAGAAGTAAGTTGGCCG
CAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT
GTGACTGGTGAGTACTCAACCAAGTCATTTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTC
AATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAAGTGCTCATCATTTGGAAAACGTTCTTCGGGGCGAA
AACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCA
TCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGC
GACACGGAAATGTTGAATACTCATACTCTTCCTTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCA
TGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTG
CCAC

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↓
 Ligation mix was transformed in *E. coli* and Ampicillin resistant transformants were selected. Plasmid DNA from a transformant that had insertion of *inv* and *hly* genes was named **pMBV40**.

↓
 pMBV40 was digested with *Bsp*HI and the resultant 7.4 kb DNA fragment was ligated with a PCR fragment containing *kan* gene generated using plasmid pKD4 (purchased from CGSC (see Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA **97**,6640-6645) as the template.

↓
 Ligation mix was transformed in *E. coli* and Kanamycin resistant transformants were selected. They were screened restriction mapping. They two different orientation of *kan* gene. The plasmids having clockwise and anticlockwise orientation of open reading frame of *kan* gene were called pMBV43 and pMBV44, respectively

As shown in PCT Publication No. WO2008/156702 at Figure 27, the pMBV40 (*amp* selected having H3 hairpin) or pMBV43 and pMBV44 (*kan* selected having H3 hairpin) plasmids, are followed by the respective sequences.

Table 41.

pMBV40 (SEQ ID NO:562)

```

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG
CGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGGCTGGCTTAACTATG
CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATCGACGGTATCGATAAGCTTGATAAGCTTTTAAATCAGC
AGGGGTCTTTTTGGCTTGTGTATTATTTTGAAGTTTTTCTTCCCCGACAGAATCTGCTTTTACCGTCATAGTGAA
ATGAGCCTGAAAAGCTATTACCATGATGATACAAATAAGTTTACTTTTCAATTCACCGCTCCTTTTTAATTCGTA
AACTAAGTTTAAAGCCACCTACAATAATCTGACAGAGAGAGTTAAGGACACGTTTTTTAGTATATGTGGAACT
AAATTATACGTTTTGCAGTAGAACTATAGGTGGCTTAAACCTTTGGGATATGCTTATATTATATGGATAAACAGT
CAGATATTCTTTTACATTTGTTAATTTCTTCTAAAAAATAAAAAATAAGCCTGTTTCTACATTTCTCACAAAAT
AATTTACGAAGAGTGCAAAACAAGCTTATTTTTTTCGTGTGTGTTAAGCGGTTTTATTCTTAATTTTTTATTACTT
TTACAATTATTCGATTGGATTATCTACTTTATTACTATATTTTCGGATAAAGCGTGGTGCCCCAGATGGAGATATT
TCTATTTTTTCAACAAGTGTAAGTTCCGGTCATCAATTACCGTTCTCCACCATTCCCAAGCTAAACCAGTGCATTCT
TTTAGCGTAAACATTAATATTTCTCGCGTTACCTGGCAAAATAGATGGACGATGTGAAATGAGCTAGCTTGCTTTT
ATTGTTTTTCGCTCCAGTTTTTTATGTTGAACAATTTTCGTTACCTTCAGGATCATAATTTACTTCATCCCAAGAAAT
GTTGAATTGAGCAACGTATCCTCCAGAGTGATCGATGTTAATTTTTTCCATCTGTATAAGCTTTTGAAGTTGTTTC
AATATATTCTGAGTTGTTTTTAATAACAGCTAATTCATTGTCTTTTAGGAAGTTTGTGTATAAGCAATGGGAAC
TCCTGGTGTCTCTGATTAAAAGTAGCGCCTTTTTTCAAATATCGCGTAAGTCTCCGAGGTTGCCGTCGATGAT
TTGAACTTCATCTTTGCGGAACCTCCGTAAATTACGGCTTTGAAGGAAGAATTTTTGATGATATTTGTTAGTTC
TACATCACCTGAGACAGATTTTCCGCTTACGGCAGCATCAAAGCAGCTTTTACTTTAGTACTATGGGAATTAGT
TGATAATTTCAAATAAACTTGACGGCCATACGCCACACTTGAGATATATGCAGGAGGATTTTCTGCATTCACTCC
AAGCGCTTGCAACTGCTCTTTAGTAACAGCTTTGCCGAAAAATCTGGAAGGTCTGTAGGTTCAATTAACATTCAC
GTTATAGTAAATTTGTTTAAAACTAATGACTTCTTCTTGCAATTTTCCCTTCACCTGATTGCGCCGAAGTTTACATT
CAAGCTATTATTTACAGCTTTAAATGCTGTACCAAATTTTCGCAATTAATTGTGATTCACCTGTAAGCCATTTCTGTC

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ATCATAATCAATTTTTGCACTTACATTTGGATAAGCTTGAGCATATTTTTTCATTCCATCTTTCCACTAATGTATT
 TACTGCGTTGTTAACGTTTGAATTTAGTGGCATTTTTTTACAACGATTTTATTGTCTTGATTAGTCATACCTGGCAA
 ATCAATGCTGAGTGTTAATGAATCACGTTTTTACAGGGAGAACATCTGGTTGATTTTCTACTAATTCGAATTCGC
 TTTTACGAGAGCACCTGGATAGGTTAGGCTCGAAATTGCATTCACTTGAATGTCTGCATTATTTTGATTGAT
 GGATTTCTTCTTTTTCTCCACAACAATATATTCATTTCCATCTTTGTAACCTTTTCTTGGCGGCACATTTGTCAC
 TGCATCTCCGTGGTATACTAATACATTGTTTTTATTGTAATCCAATCCTTGTATATACTTATCGATTTTCATCCGC
 GTGTTTCTTTTCGATTGGCGTCTTAGGACTTGCAGGCGGAGATGCTGGTGGTGCCATGGATGAAATTGAATTTTC
 TTTATTGAATGCAGATGCATCCTTTGCTTCAGTTTGTGCGCAATTGGTAGACTAACTAATATAAGTGAATAAA
 AACTAGCATTATTTTTTTCATGGGTTTCACTCTCCTTCTACATTTTTTAAACCTAATAATGCCAAATACCGTTTGC
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 AGTTGTTGAGCACAGCCAACGCACATGCAGTTTGACGTATGACAGGTATGCTTTATTTTCAATTAATTAATGATGG
 TTTTCCAGCCAATCAGTGAGTTTCTCTTGATAAGGAATCGGGAATGTCTATGTATTTTAAATAAAATAAATTCAT
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 TCGGGATACGTACATTGGAGAACGGTTGGCTGTACGGACTTAATACTTTTTTATGATAATGATTTGACCGGCCACA
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 TGGGCGAGAGTTTTCAGTCGCAACTTAGCCCTTCAGCGGTGGCAGGAACACGTCTACTGGCGGAGAGCCGCTATA
 ACCTTGTGATCGTAACAATAATATCGTGTGGAGTATCAGAAACAGCAGGTGGTTAAACTGACATTATCGCCAG
 CAACTATCTCCGGCTGCCGGGTGAGGTTTATCAGGTGAACGCACAAGTACAAGGGGACATCTGCTGTAAGGGAAA
 TTGTCTGGAGTGATGCCGAACCTGATTGCCGCTGGCGGCACATTAAACCACTGAGTACACACAATTAACCTTGG
 TTTTACCGCCTTATAAACGCACAGCACAAGTGAGTCGGGTAAACGACGACCTGACAGCCAACCTTTTATTCGCTTA
 GTGCGCTCGCGGTTGATCACCAAGGAAACCGATCTAACTCATTACATTGAGCGTCACCGTTTCAGCAGCCTCAGT
 TGACATTAAACGGCGGCCGTCATTGGTGATGGCGCACCGGCTAATGGGAAAACCTGCAATCACCGTTGAGTTACCG
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 TCACAGCAGAAGTGAGGGGCAACGGCAAAGTGTTGATACCCACTTTGTAAAGGGTACTATCGCGGCGGATAAAT
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 ATCACAATGACGGCACTTATAGCGCACCATTTGACCAGTACCACGTTGGGGGTAGCAACAGTAACGGTGAAAGTGG
 ATGGGGCTGCGTTAGTGTCGCGAGTGTCGCGTTAATTTACGGCAGATCCTATTCCAGATGCTGGCCGCTCCA
 GTTTCACCGTCTCCACACCGGATATCTTGGCTGATGGCACGATGAGTTCCACATTATCCTTTGTCCCTGTGCGATA
 AGAATGGCCATTTTATCAGTGGGATGCAGGGCTTGAATTTTACTCAAAACGGTGTGCCGGTGAGTATTAGCCCCA
 TTACCGAGCAGCCAGATAGCTATACCGCGACGGTGGTTGGGAATAGTGTGCGTGATGTCAATCACGCCGCGAGG
 TTGATACCTGATACTGAGTACATTGCAGAAAAAATATCCCTATTCCCGGTACCTACGCTGACCGGTATTCTGG
 TTAACGGGCAAAATTTTCGTACGGATAAAGGTTCCCGAAAACGATCTTTAAAAACGCCACATTCCAGTTACAGA
 TGGATAACGATGTTGCTAATAATACTCAGTATGAGTGGTCTGTCGTCATTACACCCAATGTATCGGTTAACGATC
 AGGGTCAGGTGACGATTACCTACCAAACCTATAGCGAAGTGGCTGTGACGGCGAAGTAAAAAATTTCCCAAGTT
 ATTCGGTGAGTTATCGGTTCTACCCAAATCGTGGATACGATGGCGGCGAGATCGCTGGTATCCAGTCTCGAGG
 CTAGCAGACAATGCCAAGGTTTCAAGATATGTCGCGGTTCTTGAATCCTCACGTGCAACCAACGGAACGCGTGCGC
 CTGACGGGACATTGTGGGGCGAGTGGGGGAGCTTGACCGCGTATAGTTCTGATTGGCAATCTGGTGAATATTGGG
 TCAAAAAGACCAGCACGGATTTTGAACCATGAATATGGACACAGGCGCACTGCAACCAGGGCCTGCATACTTGG
 CGTTCGCGCTCTGTGCGCTGTCAATATAACAGATAACAGATAGCAATAAGAACAGTTTAATGAGCTGATTATTT
 GGGGCGCAATGGGAGTCCGGCAATCCTAGACTCGCCCCATAAGTAGCAAACGTCCAGAAGAACAACGCCGCTCA
 GGTTAATTGAGCGGCGCTGTTTTTTTAAAAGGATTGTGCGGATTAAATGCCGATCTTACGGCCCAGCTGCAGCCC
 GGGGGATCTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCCGCCATT

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AGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGGACTTCATATACCCAAGC
TTGGAAAATTTTTTTTAAAAAAGTCTTGACACTTTATGCTTCCGGCTCGTATAATGGATCCAGGAGTAACAATAC
AAATGGATTCAAGAGATCCATTTGTATTGTACTCCTTTTTTTTTTTTGTGCGACGATCCTTAGCGAAAGCTAAGGA
TTTTTTTTTTTACTCGAGCGGATTACTACATACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGC
GTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTTCGCTTCGGCTGCGGCGAGCGGTATCAG
CTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCC
AGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGTGCGCTTTTTTCCATAGGCTCCGCCCCCTGACGAGCATC
ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCCCTGGAA
GCTCCCTCGTGCGCTCTCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGAAGCG
TGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTTCGGTGTAGGTTCGCTCCAAGCTGGGCTGTGTGC
ACGAACCCCCCGTTAGCCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
TGAAGTGGTGGCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCT
TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGT'TTTTTTGT'TTGCAAGC
AGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA
ACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTA
AATGAAGTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGG
CACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCCTGACTCCCCGTCGTGTAGATAAATCAGTAC
GGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAG
CAATAAACCCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCTCCATCCAGTCTATTA
ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCA
TCGTGGTGTACGCTCGTCTGTTGGTATGGCTTCATTCAGCTCCGGT'TCCCAACGATCAAGGCGAGTTACATGAT
CCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGAGTGT
TATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTTCATGCCATCCGTAAGATGCTTTTCTGTGACTG
GTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGG
ATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTTGGAACCGTTCTTCGGGGCGAAAACCTCTCAA
GGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACCTGATCTTCAGCATCTTTTACTT
TCACCAGCGTTTCTGGGTGAGCAAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAAT
GTTGAATACTCATACTCTTCTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA
TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCT
AAGAAACCATTATTATCATGACATTAACTATAAAAAATAGGCGTATCACGAGGCCCT'TTTCGTC

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Table 42 contains the 8427 base pair sequence of a predicted pMBV43 plasmid. The sequence contains the following regions: *hly* orf (682-2271 bp); *inv* orf (2994-5954 bp) (6212-6282 bp) (6483-6534 bp); shRNA promoter (6303-6361 bp); Sense strand (6362-6383 bp); Loop (6384-6390 bp); Antisense strand (6391-6412 bp); Terminator I (6413-6422 bp); Terminator II (6423-6460 bp); Origin of replication (6720-7307 bp); and *kan* orf (7498-8292 bp).

Table 42

Predicted pMBV43 (SEQ ID NO: 563)

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TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG
CGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGT'TGGCGGGTGT'CGGGGCTGGCTTA'ACTATG
CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATCGACGGTATCGATAAGCTTGATAAGCTTTTAAATCAGC
AGGGGTCTTTTTGGCTTGTGTATTATTTTGAAGTTTTTCTTCCCCGACAGAATCTGCTTTTACCGTCATAGTGAA
ATGAGCCTGAAAAGCTATTACCATGATGATACAAATAAGTTTACTTTTCA'TTTCACCGCTCCTTTTAAATTCGTA
AAACTAAGTTTAAAGCCACTACAATAATCTGACAGAGAGAGTTAAGGACACGTTTTT'TAGTATATGTGGAACT
AAATTATACGTTTTCAGTAGAACTATAGGTGGCTTAACTTTGGGATATGCTTATATTATATGGATAAACAGT
CAGATATTCTTTTACATTTGTTAATTCTTCTAAAAAATTA'AAAAATAAGCCTGTTTCTACATTCTTCA'AAAAT
AATTTACGAAGAGTGCAAAACAAGCTTATTTTTTTCGTGTGTGTTAAGCGGTTTTATTTCTTAATTTTTTATTACTT
TTACAATTATTCGATTGGATTATCTACTTTATTACTATATTTTCGGATAAAGCGTGGTGCCCCAGATGGAGATATT
TCTATTTTTTACAAGTGTTAAGTTCGGGTCATCAATTACCGTCTCCACCATTCCCAAGCTAAACCAGTGCATTC
TTTAGCGTAAACATTAAATATTTCTCGCGTTACCTGGCAATAGATGGACGATGTGAAATGAGCTAGCTTGCTTTT
ATTGTTTTTCGCTCCAGTTTTTTATGTTGAACAATTTTCGTTACCTTCAGGATCATAATTTACTTTCATCCCAAGAAAT
GTTGAATTGAGCAACGTATCCTCCAGAGTGATCGATGTTAATTTTTCATCTGTATAAGCTTTTGAAGTTGTTTC
AATATATTCTGAGTTGTTTTTAATAACAGCTAATTCATTGTCTTTTAGGAAGTTTGTGTATAAGCAATGGGAAC
TCCTGGTGT'TTCTCGATTAAAGTAGCGCTTTTTTCAAATATCGCGTAAGTCTCCGAGGTTGCCGTCGATGAT

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TTGAACTTCATCTTTTGCAGAACCTCCGTAAATTACGGCTTTGAAGGAAGAATTTTTGATGATATTTGTTAGTTC
 TACATCACCTGAGACAGATTTTCCGCTTACGGCAGCATCAAAAGCAGCTTTTACTTTAGTACTATGGGAATTAGT
 TGATAATTTCAAATAAACTTGACGGCCATACGCCACACTTGAGATATATGCAGGAGGATTTTCTGCATTCCTCC
 AAGCGCTTGCAACTGCTCTTTAGTAACAGCTTTGCCGAAAAATCTGGAAGGTCTTGTAGGTTCAATTAACATTCAC
 GTTATAGTAAATTTGTTTAAAACTAATGACTTCTTCTTGCAATTTTCCCTTCAC'TGATTGCGCCGAAGTTTACATT
 CAAGCTATTATTTACAGCTTTAAATGCTGTACCAAATTTGCAATTAATTGTGATTCACTGTAAGCCATTTTCGTC
 ATCATAATCAATTTTGCACCTTACATTTGGATAAGCTTGAGCATATTTTTCATTCCATCTTTCCACTAATGTATT
 TACTGCGTTGTTAACGTTTGAATTTAGTGGCATTTTTTTACAACGATTTTATTGTCTTGATTAGTCATACCTGGCAA
 ATCAATGCTGAGTGTAAATGAATCACGTTTTACAGGGAGAACATCTGGTTGATTTTCTACTAATTCCGAATTCGC
 TTTTACGAGAGCACCTGGATAGGTTAGGCTCGAAATTGCATTACAACTTGAATGTCTGCATTATTTTGAATTGAT
 GGATTTCTTCTTTTCTCCACAACAATATATTCAATTTCCATCTTTGTAACCTTTTCTTGGCGGCACATTTGTCAC
 TGCATCTCCGTGGTATACTAATAACATTTGTTTTATTGTAATCCAATCCTTGTATATACTTATCGATTTTCATCCGC
 GTGTTTCTTTTCGATTGGCGTCTTAGGACTTGCCAGGCGGAGATGCTGGTGGTGCCATGGATGAAATTGAATTTTC
 TTTATTGAATGCAGATGCATCCTTTGCTTCAGTTTGTGCGCAATTTGGTAGACTAACTAATAAGTGAATAAA
 AACTAGCATTATTTTTTTCATGGGTTTCACTCTCCTTCTACATTTTTTAACTAATAATGCCAAATACCGTTTGC
 CACCCCTCTCTTTTGATAATTATAATATTGGCGAAATTCGCTTCTAAAGATGAAACGCAATATTATATGCTTGCT
 TTATAGCTTTATTCTAGTCCTGCTGTCCCTTTATCGTCGTTAACAAATGTTAATGCCCTCAACATAAAAGTCACTT
 TAAGATAGGAATATACTAATCAAAGGAGGGATCGAATTCCTGCGCATCAAGGCAACCATCAGGATTAAATGCGG
 ATATTGCGGAGTAACACTTCAGACTGAAAGTAGAAAATAAAACCGCAGCAGACAACCTGACAACATCAAATGAAGG
 GGGCTTATTCTAATTGATATTATTTATATGATAAATAGTTTCATTTTGTATTTTTGTTTTTTTTGATATTCTCACCT
 GCTTAGTTACAATAAAATCAATTTCTATCGCTGTATGGTATAGACTGTTTTATTATATATTTTTGAATATTTTTAATC
 TGCCAGTCTGGTTTTTTTTAAAAAAGTGCTATCCTCTTAATGTCTTTACTAAATTAGAAAACAAGTTTCACTTTCA
 ACTATTGCATCTTTAATTAATGGTCAAGGTGATTTCAAATGCTCGTTTGTGGCCAGTTATACCTCAAATAACTCA
 AGTTGTTGAGCACAGCCAACGCACATGCAGTTTGACGTATGACAGGTATGCTTTATTTCAATTTAAATTATGATGG
 TTTTCCAGCCAATCAGTGAGTTTCTCTTGATAAGGAATGCGGGAATGTCTATGTATTTTAATAAAATAATTTTCAT
 TTAATATTATTTACGAATAGTTATTTGTATCTTTTTGATATGTGGAATGTTTCATGGCTGGGGCTTCAGAAAAAT
 ATGATGCTAACGCACCGCAACAGGTCCAGCCTTATTCTGTCTCTTCATCTGCATTTGAAAATCTCCATCCTAATA
 ATGAAATGGAGAGTTCAATCAATCCCTTTTCCGCATCGGATACAGAAAGAAATGCTGCAATAATAGATCGCGCCA
 ATAAGGAGCAGGAGACTGAAGCGGTGAATAAGATGATAAGCACCGGGGCCAGGTTAGCTGCATCAGGCAGGGCAT
 CTGATGTTGCTCACTCAATGGTGGGCGATGCGGTTAATCAAGAAATCAAACAGTGGTTAAATCGATTCCGTACGG
 CTCAAGTTAATCTGAATTTTGACAAAAATTTTTCGCTAAAAGAAAGCTCTCTTGATTGGCTGGCTCCTTGGTATG
 ACTCTGCTTCATTCTCTTTTTTAGTCACTTAGGTATTTCGCAATAAAGACAGCCGCAACACACTTAACCTTGGCG
 TCGGGATACGTACATTGGAGAACGGTTGGCTGTACGGACTTAATACTTTTTTATGATAATGATTTGACCGGCCACA
 ACCACCGTATCGGTCTTGGTGCCGAGGCCCTGGACCGATTATTTACAGTTGGCTGCCAATGGGTATTTTCGCCTCA
 ATGGATGGCACTCGTCGCGTGATTTCTCCGACTATAAAGAGCGCCAGCCACTGGGGGGGATTTGCGCGCGAATG
 CTTATTTACCTGCACTCCCACAACCTGGGGGGGAAGTTGATGTATGAGCAATACACCGGTGAGCGTGTTGCTTTAT
 TTGGTAAAGATAAATCTGCAACGCAACCCTTATGCCGTGACTGCCGGGATCAATTACACCCCGTGCCTCTACTCA
 CTGTGCGGGTAGATCAGCGTATGGGGAAAAGCAGTAAGCATGAAACACAGTGGAACCTCCAAATGAACATATCGCC
 TGGGCGAGAGTTTTTCACTCGCAACTTAGCCCTTCAGCGGTGGCAGGAACACGTCTACTGGCGGAGAGCCGCTATA
 ACCTTGTGATCGTAACAATAATATCGTGTTGGAGTATCAGAAACAGCAGGTGGTTAAACTGACATTATCGCCAG
 CAACTATCTCCGGCTGCCGGTTCAGGTTTATCAGGTGAACGCACAAGTACAAGGGGCATCTGCTGTAAGGGAAA
 TTGTCTGGAGTGATGCCGAACGATTGCGGCTGGCGGCACATTAACACCCTGAGTACCACACAATTCAACTTGG
 TTTTACCGCCTTATAAACGCACAGCACAAAGTGAGTCGGGTAACGGACGACCTGACAGCCAACCTTTTATTTCGCTTA
 GTGCGCTCGCGTTGATCACCAAGGAAACCGATCTAACTCATTACATTGAGCGTCACCGTTTCAGCAGCCTCAGT
 TGACATTAACGGCGGCGCGTCATTGGTGATGGCGCACCGGCTAATGGGAAAACCTGCAATCACCGTTGAGTTACCG
 TTGCTGATTTTGAGGGGAAACCTTAGCCGGGCAGGAGGTGGTGATAACCACCAATAATGGTGCGCTACCGAATA
 AAATCACGGAAAAAGACAGATGCAATGGCGTTCGCGCGCATTGCATTAAACCAATACGACAGATGGCGTGACGGTAG
 TCACAGCAGAAAGTGAGGGGCAACGGCAAAGTGTTGATACCCACTTTTGTAAAGGGTACTATCGCGGCGGATAAAT
 CCACTCTGGCTGCGGTACCGACATCTATCATCGCTGATGGTCTAATGGCTTCAACCATCACGTTGGAGTTGAAGG
 ATACCTATGGGGACCCGAGGCTGGCGCGAATGTGGCTTTTGACACAACCTTAGGCAATATGGGCGTTATCACGG
 ATCACAATGACGGCACTTATAGCGCACCATTGACCAGTACCACGTTGGGGGTAGCAACAGTAACGGTGAAAGTGG
 ATGGGGCTGCGTTCACTGTGCCGAGTGACGGTTAATTTACGGCAGATCCTATTCCAGATGCTGGCCGCTCCA
 GTTTACCGCTCTCCACACCGGATATCTTGGCTGATGGCAGCATGAGTTCCACATTATCTTTGCTCCCTGTGCGATA
 AGAATGGCCATTTTATCAGTGGGATGCAGGGCTGAGTTTACTCAAAACGGTGTGCCGTTGAGTATTAGCCCCA
 TTACCGGACGCCAGATAGCTATACCGCGACGGTGTGTTGGGAATAGTGTGCGGTGATGTCACAATACGCCGCGAGG
 TTGATAACCTGATACTGAGTACATTGCAGAAAAAATATCCCTATTCCCGGTACCTACGCTGACCGGTATTCTGG
 TTAACGGGCAAAAATTTTCGCTACGGATAAAGGGTTCCCGAAAACGATCTTTAAAAACGCCACATTTCCAGTTACAGA
 TGGATAACGATGTTGCTAATAATACTCAGTATGAGTGGTCTGTCGTCATTACACCCCAATGTATCGGTTAACGATC
 AGGGTCAGGTGACGATTACCTACCAAACCTATAGCGAAGTGGCTGTGACGGCGAAAAGTAAAAAATTTCCCAAGTT
 ATTCGGTGAGTTATCGGTTCTACCCAAATCGGTGGATATACGATGGCGGCAGATCGCTGGTATCCAGTCTCGAGG
 CCAGCAGACAATGCCAAGGTTGAGATATGTCTGCGGTTCTTGAATCCTCACGTGCAACCAACGGAACGCGTGCGC

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CTGACGGGACATTGTGGGGCGAGTGGGGGAGCTTGACCGCGTATAGTTCTGATTGGCAATCTGGTGAATATTGGG
TCAAAAAGACCAGCACGGATTTTGAACCATGAATATGGACACAGGCGCACTGCAACCAGGGCCTGCATACTTGG
CGTTCCCGCTCTGTGCGCTGTCAATATAACCAGATAACAGATAGCAATAAGAACAGTTTAAATGAGCTGATTATTT
GGGGCGCGAATGGGAGTCCGGCAATCCTAGACTCGCCCCATAAGTAGCAAACGTCCAGAAGAACAACGCCGCTCA
GGTTAATTGAGCGGCGCTGTTTTTTTTTAAAAGGATTGTGCGGATTAAATGCCGATCTTACGGCCCAGCTGCAGCCC
GGGGGATCTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCATTGCGCATTC
AGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGGACTTCATATACCCAAGC
TTGGAAAATTTTTTTTAAAAAAGTCTTGACACTTTATGCTTCCGGCTCGTATAATGGATCCAGGAGTAACAATAC
AAATGGATTCAAGAGATCCATTTGTATTGTTACTCCTTTTTTTTTTTTTTGTGCGACGATCCTTAGCGAAAGCTAAGGA
TTTTTTTTTTTACTCGAGCGGATTACTACATACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGC
GTATTGGGCGCTCTTCCGCTTCCCTCGCTCACTGACTCGCTGCGCTCGGTTCGGCTGCGGCGAGCGGTATCAG
CTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCC
AGCAAAAAGGCCAGGAACCGTAAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATC
ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAA
GCTCCCTCGTGCGCTCTCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGAAGCG
TGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTTCGCTCCAAGCTGGGCTGTGTGC
ACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACATATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCTGGGTACAGAGTTCT
TGAAGTGGTGGCTAACTACGGCTACACTAGAAGGACAGTATTTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCT
TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTTGTGCAAGC
AGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGA
ACGAAAACTCACGTTAAGGGATTTTGGTCATGATCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGATGGCT
TTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATG
ATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAA
CAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTGACGCGAGGGGCGCCCGGTTCTTTTTGTCAAGACC
GACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCGAGCGCGCTATCGTGGCTGGCCACGACGGGCGTTCTCT
TGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGAT
CTCCTGTCTATCTACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTT
GATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGT
CTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACGTGTTCCGCCAGGCTCAAGGCG
CGCATGCCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCGTGCTTGCCGAATATCATGGTGGAAAATGGC
CGCTTTTCTGGATTATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGT
GATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCTCGTGCTTTACGGTATCGCCGCTCCCGATTCTG
CAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAG
CGACGCCCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCTTCTATGAAATCATGACATTAACCTATAAAA
ATAGGCGTATCAGGAGGCCCTTTCGTC

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Table 43 contains the 8443 base pair sequence of a verified pMBV43 plasmid. The sequence contains the following regions: *hly* orf (682-2271 bp); *inv* orf (2992-5952 bp); shRNA promoter (6317-6375 bp); Sense strand (6376-6397 bp); Loop (6398-6404 bp); Antisense strand (6405-6426 bp); Terminator I (6427-6437 bp); Terminator II (6438-6475 bp); Origin of replication (6735-7322 bp); and *kan* orf (7513-8307 bp).

Table 43

Verified pMBV43 (SEQ ID NO:564)

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TCGCGCGTTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG
CGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGCTGGCTTAACCTATG
CGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATCGACGGTATCGATAAGCTTGATAAGCTTTTAAATCAGC
AGGGGTCTTTTTGGCTTGTGTATTATTTGAAGTTTTTCTTCCCCGACAGAATCTGCTTTTACCGTCATAGTGAA
ATGAGCCTGAAAAGCTATTACCATGATGATACAAATAAGTTTACTTTTTCATTTACCGCTCCTTTTTTAATTCGTA
AACTAAGTTTAAGCCACCTACAATAATCTGACAGAGAGAGTTAAGGACACGTTTTTTAGTATATGTGGGAACT
AAATTATACGTTTTGCGTAGAACTATAGGTGGCTTAAACTTTGGGATATGCTTATATTATATGGATAAACAGT
CAGATATTCTTTTACATTTGTAAATTTCTTCTAAAAAATTAATAAGCCTGTTTCTACATTCTTCACAAAAT
AATTTACGAAGAGTGCAAAAACAGCTTATTTTTTCGTGTGTGTTAAGCGGTTTTATTTCTTAATTTTTTATTACTT
TTACAATTATTCGATTGGATTATCTACTTTATTACTATATTTTCGGATAAAGCGTGGTGCCCCAGATGGAGATATT
TCTATTTTTTCAAGTGGAAGTTCCGGTCATCAATTACCGTTCTCCACCATTCCCAAGCTAAACCAGTGCATTC

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TTTAGCGTAAACATTAATATTTCTCGCGTTACCTGGCAAATAGATGGACGATGTGAAATGAGCTAGCTTGCTTTT
 ATTGTTTTTCGCTCCAGTTTTTATGTTGAACAATTTTCGTTACCTTCAGGATCATAATTTACTTCATCCCAAGAAAT
 GTTGAATTGAGCAACGTATCCTCCAGAGTGATCGATGTTAATTTTTCCATCTGTATAAGCTTTTGAAGTTGTTTC
 AATATATTCTGAGTTGTTTTTAATAACAGCTAATTCATTGTCTTTTAGGAAGTTTGTGTATAAGCAATGGGAAC
 TCCTGGTGTCTCGATTAAAAAGTAGCGCTTTTTTTCAAATATCGCGTAAGTCTCCGAGGTTGCCGTCGATGAT
 TTGAACTTCATCTTTGCGGAACCTCCGTAAATTACGGCTTTGAAGGAAGAATTTTTGATGATATTTGTTAGTTC
 TACATCACCTGAGACAGATTTTCCGCTTACGGCAGCATCAAAGCAGCTTTTACTTTAGTACTATGGGAATTAGT
 TGATAATTTCAAATAAACTTGACGGCCATACGCCACACTTGAGATATATGCAGGAGGATTTTCTGCATTCACTCC
 AAGCGCTTGCAACTGCTCTTTAGTAACAGCTTTGCCGAAAAATCTGGAAGGTCTGTAGGTTCAATTAACATTCAC
 GTTATAGTAAATTTGTTTAAAACTAATGACTTCTTCTTGCAATTTTCCCTTCAGTATTGCGCCGAAGTTTACATT
 CAAGCTATTATTTACAGCTTTAAATGCTGTACCAAATTTTCGCAATTAATTTGTGATTCACTGTAAGCCATTTTCGTC
 ATCATAATCAATTTTTGCACTTACATTTGGATAAGCTTGAGCATATTTTTTCATTCCATCTTTCCACTAATGTATT
 TACTGCGTTGTTAACGTTTTGATTTAGTGGCATTTTTTTACAACGATTTTATTGTCTTGATTAGTCATACCTGGCAA
 ATCAATGCTGAGTGTTAATGAATCACGTTTTACAGGGAGAACATCTGGTTGATTTTCTACTAATTCGAATTCGC
 TTTTACGAGAGCACCTGGATAGGTTAGGCTCGAAATTGCATTCACAACCTTGAATGTCTGCATTATTTTGATTGAT
 GGATTTCTTCTTTTTCTCCACAACAATATATTCATTTCCATCTTTGTAACCTTTTCTTGGCGGCACATTTGTCAC
 TGCATCTCCGTGGTATACTAATACATTGTTTTTATTGTAATCCAATCCTTGTATATACTTATCGATTTTCATCCGC
 GTGTTTCTTTTCGATTGGCGTCTTAGGACTTGCCAGGCGAGATGCTGGTGGTGCCATGGATGAAATGAATTTTCT
 TTTATTGAATGCAGATGCATCCTTTGCTTCAGTTTGTTCGCAATTTGGTAGACTAATAATATAAGTGAATATAAA
 AACTAGCATTATTTTTTTCATGGGTTTCACTCTCTTCTACATTTTTTAACTAATAATGCCAAATACCGTTTGC
 CACCCCTCTCTTTTGATAATTATAATATTGGCGAAATTGCTTCTAAAGATGAAACGCAATATTATATGCTTGCT
 TTATAGCTTTTATTCTAGTCCTGCTGCTCCCTTTATCGTCGTTAACAAATGTTAATGCCTCAACATAAAAGTCACCT
 TAAGATAGGAATATACTAATCAAAGGAGGGATCGAATTCCTGCAGTCATCAAGGCAACCATCAGGATTAATGCGG
 ATATTGCGGAGTAACACTTCAGACTGAAAGTAGAAATAAAACCCGAGCAGACAACCTGACAACATCAAATGAAGG
 GGGCTTATTCTAATTGATATTATTTATATGATAATAGTTTCAATTTGTATTTTGTTTTTTTGATATTCTCACCTGC
 TTAGTTACAATAAATCAATTCTATCGCTGTATGGTATAGACTGTTTTATTATATATTTTGAATATTTTTAATCTG
 CCCAGTCTGGTTTTTTAAAAAAGTGCTATCCTCTTAATGTCTTTACTAAATTAGAAAACAAGTTTCACTTTCAAC
 TATTGCATCTTTAATTAATGGTCAAGGTGATTTCAAATGCTCGTTTTGTGGCCAGTTATACCTCAAATAACTCAAG
 TTGTTGAGCACAGCCAACGCACATGCAGTTTGACGTATGACAGGTATGCTTTATTTTCAATTAATATGATGGTT
 TTCCAGCCAATCAGTGAGTTTCTCTTGATAAAGGAATGCGGGAATGTCTATGTATTTTAAATAAAATAATTTCAATTT
 AATATTATTTTACGAATAGTTATTTGTATCTTTTTGATATGTGGAATGTTTATGGCTGGGGCTTCAGAAAAATAT
 GATGCTAACGCACCGCAACAGGTCCAGCCTTATTTCTGCTCTTTCATCTGCATTTGAAAATCTCCATCCTAATAAT
 GAAATGGAGAGTTCAATCAATCCCTTTTCCGCATCGGATACAGAAAGAAATGCTGCAATAATAGATCGCGCCAAT
 AAGGAGCAGGAGACTGAAGCGGTGAATAAGATGATAAGCACCGGGGCCAGGTTAGCTGCATCAGGCAGGGCATCT
 GATGTTGCTCACTCAATGGTGGGCGATGCGGTTAATCAAGAAATCAAACAGTGGTTAAATCGATTGGTACGGCT
 CAAGTTAATCTGAATTTTGACAAAAATTTTCGCTAAAAGAAAGCTCTCTTGATTGGCTGGCTCCTTGGTATGAC
 TCTGCTTCATTCTCTTTTTTAGTCAGTTAGGTATTTCGCAATAAAGACAGCCGCAACACTTAACCTTGGCGTC
 GGGATACGTACATTGGAGAACCGGTTGGCTGTACGGACTTAATACTTTTTTATGATAATGATTTGACCGGCCACAAC
 CACCGTATCGGTCTTGGTGCCGAGGCCCTGGACCGATTATTTACAGTTGGCTGCCAATGGGTATTTTCGCCTCAAT
 GGATGGCACTCGTCGCGTGATTTCTCCGACTATAAAGAGCGCCAGCCACTGGGGGGGATTTGCGCGCGAATGCT
 TATTTACCTGCACTCCCACTGAGGGGGGAAGTTGATGTATGAGCAATACACCGGTGAGCGTGTGCTTTATTT
 GGTAAAGATAATCTGCAACGCAACCTTATGCCGTGACTGCCGGGATCAATTACACCCCGTGCCTCTACTCACT
 GTCGGGGTAGATCAGCGTATGGGAAAAGCAGTAAGCATGAAACACAGTGAACCTCCAAATGAATATCGCCTG
 GCGGAGAGTTTTAGTCGCAACTTAGCCCTTCAGCGGTGGCAGGAACACGTCTACTGGCGGAGAGCCGCTATAAC
 CTTGTGATCGTAACAATAATATCGTGTGGAGTATCAGAAACAGCAGGTGGTTAAACTGACATTATCGCCAGCA
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Table 44

pMBV44 (SEQ ID NO:565)

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ATAGGCGTATCAGGAGGCCCTTTCGTC

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Example 23: Construction of pNJSZc Plasmid

pNJSZ is a 10.4 kb plasmid that confers the abilities required to induce tkRNAi. It contains two genes, *inv* and *hly*, that allow bacteria to invade mammalian cells and to escape from the entry vacuole. Expression of the short hairpin RNA is different between the original Trip plasmid and pNJSZ. In pNJSZ, expression of shRNA is under the control of a constitutive bacterial promoter, which allows for continuous expression. This is different from the original Trip plasmid, which has an ITPG inducible promoter, which controls the expression of the shRNA. Moreover, pNJSZ and the original Trip plasmid contain different antibiotic resistant genes. pNJSZ has the kanamycin resistance gene, whereas the original Trip plasmid has the ampicillin resistance gene. pNJSZc was constructed from pNJSZ by removing any regions of pNJSZ that were not required for its maintenance or abilities to induce tkRNAi.

Step 1 as shown in PCT Publication No. WO2008/156702 at Figure 28: Removed an extra BamH1 site at 9778 by digesting pNJSZ with both SpeI (9784) and XmaI (9772), T4 DNA

polymerase filled-in these two sites and then allowed the plasmid to self ligate, creating pNJSZ ΔBamH1.

Step 2 as shown in PCT Publication No. WO2008/156702 at Figure 29: Removed both an extra SalI site at 972 and the fl origin of replication by digesting pNJSZ ΔBamH1 with BglII (208)

5 and PmeI (982), T4 DNA polymerase filled-in these two sites and allowed the plasmid to self ligate, creating pNJSZc.

The pNJSZc DNA sequence is shown in Table 45.

Table 45

pNJSZc (SEQ ID NO:566)
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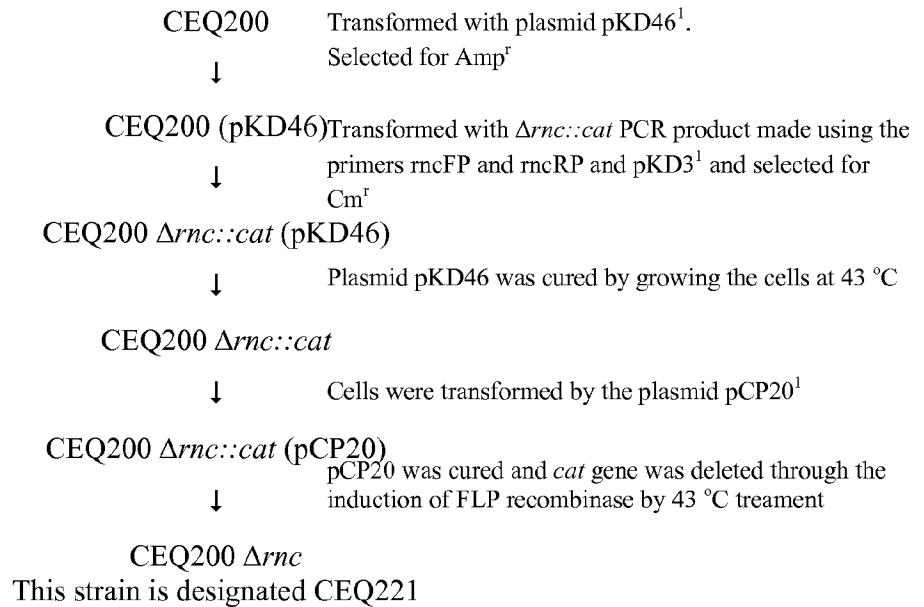
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 CTTATAAACGCACAGCACAAGTGAGTCGGGTAACGAGCAGCTGACAGCCAACCTTTTATTCTAGTTCGCGCTCG
 CGGTGATCACCAAGGAAACCGATCTAATCTTACATTTAGCGCTCACCGTTGACGAGCTCAGTTGACATTAA
 CGGCGGCGCTCATTGGTGATGGCGCACCGGCTAATGGGAAAACTGCAATCACCGTTGAGTTACCGTTGCTGATT
 TTGAGGGGAAAACCTTAGCCGGGCGAGGAGGTGGTGATAACCACCAATAATGGTGCGCTACCGAATAAAATCACGG
 AAAAGACAGATGCAAAATGGCGTCGCGCGCATTTGCATTAACCAATACGACAGATGGCGTGACGGTAGTCACAGCAG
 AAGTGAGGGGGCAACGGCAAAGTGTGATACCCACTTTGTTAAGGGTACTATCGCGGCGGATAAATCCACTCTGG
 CTGCGGTACCGACATCTATCATCGCTGATGGTCTAATGGCTTCAACCATCACGTTGGAGTTGAAGGATACCTATG
 GGGACCCGCGAGGCTGGCGCGAATGTGGCTTTTGACACAACCTTAGGCAATATGGGCGTTATCACGGATCACAATG
 ACGGCACTTATAGCGCACCATTTGACCAGTACCAGTTGGGGGTAGCAACAGTAACGGTGAAAGTGGATGGGGCTG

CGTTCAGTGTGCCGAGTGTGACGGTTAATTTACGGCAGATCCTATTCCAGATGCTGGCCGCTCCAGTTTACCG
TCTCCACACCGGATATCTTGGCTGATGGCACGATGAGTTCCACATTATCCTTTGTCCCTGTCGATAAGAATGGCC
ATTTTATCAGTGGGATGCAGGGCTTGAGTTTTACTCAAAACGGTGTGCCGGTGAGTATTAGCCCCATTACCGAGC
AGCCAGATAGCTATACCGCGACGGTGGTTGGGAATAGTGTGCGTGATGTCACAATCACGCCGCAGGTTGATACCC
TGATACTGAGTACATTGCAGAAAAAATATCCCTATTCCCGGTACCTACGCTGACCGGTATTCTGGTTAACGGGC
AAAATTTGCTACGGATAAAGGGTTCCCGAAAACGATCTTTAAAAACGCCACATTCCAGTTACAGATGGATAACG
ATGTTGCTAATAATACTCAGTATGAGTGGTCGTGTCATTACACCCCAATGTATCGGTTAACGATCAGGGTCAGG
TGACGATTACCTACCAAACCTATAGCGAAGTGGCTGTGACGGCGAAAAGTAAAAAATCCCAAGTTATTCCGTGA
GTTATCGGTTCTACCCAAATCGGTGGATATACGATGGCGGCAGATCGCTGGTATCCAGTCTCGAGGCCAGCAGAC
AATGCCAAGGTTAGATATGTCTGCGGTTCTTGAATCCTCACGTGCAACCAACGGAACGCGTGCGCCTGACGGGA
CATTGTGGGGCGAGTGGGGGAGCTTGACCGCGTATAGTTCTGATTGGCAATCTGGTGAATATTGGGTCAAAAAGA
CCAGCACGGATTTTGAAACCATGAATATGGACACAGGCGCACTGCAACCAGGGCCTGCATACTTGGCGTTCCCGC
TCTGTGCGCTGTCAATATAACCAGATAACAGATAGCAATAAGAACAGTTTAAATGAGCTGATTATTTGGGGCGCGA
ATGGGAGTCCGGCAATCCTAGACTCGCCCCATAAGTAGCAAACGTCCAGAGAACAACGCCGCTCAGGTTAATTGA
GCGGCGTTGTTTTTTTAAAAGGATTTGTGCGGATAAGCGTGAGCTGGCGTTAAATGCCGATCTTACGGCCCAGCT
GCAGCCCGGCTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTAATCGGCATCATTACCAAGCTTGCCAGGC
GACTGTCTTCAATATTACAGCCGCAACTACTGACATGGCGGGTGATGGTGTTCACTATTCCAGGGCGATCGGCAC
CCAACGCAGTGATCACCAGATAATGTTGCGATGACAGTGTCAAAC TGTTATTCTTCAAGGGGTGAGTTGTTCT
TAAGCATGCCGTTTGTGCTGTAAAGTTTAGGGAGATTTGATGGCTTACTCTGTTCAAAAGTCGCGCCTGGCAAAGG
TTGCGGGTGTTTCGCTTGTTTTATTACTCGCTGCCTGTAGTTCTGACTCACGCTATAAGCGTCAGGTCAGTGGTG
ATGAAGCCTACCTGGAAGCGCCATGGCATGCAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCCTAGAC
CAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAAGGATCCAGGAGTAACAATACAAATGGATTCAA
GAGATCCATTTGTATTGTTACTCCTTTGTGCTGACTGGACAGTTCAAGAGACTGTCCATCAATATCAGCTTTGTCAC
AAACCCCGCCACCGGCGGGGTTTTTTTCTGCTCTAG

Example 24: Deletion of *rnc* encoding RNase III in CEQ200 (CEQ221 Δrnc)

Most bacteria contain a large number of RNA degrading enzymes, RNases, which may degrade the siRNA causing a reduction in the activity of tkRNAi. In such cases where the RNases of a specific bacterium will exhibit such degradation of siRNA, a targeted deletion of the gene encoding the RNase of interest (*e.g.*, the *rnc* gene encoding RNase III) is performed to yield higher levels of siRNA per tkRNAi bacterium, resulting in more siRNA being delivered to the target cells, as well as more efficient gene silencing of the gene of interest within the target cell.

Construction of Δrnc 

¹From Datsenko and Wanner (2000) *Proc. Natl. Acad. Sci. USA* **97**,6640-6645. These plasmids were purchased from CGSC.

A gel analysis showed confirmed the construction of CEQ221 and Δrnc Genotype verification. Gel analysis confirmed the expression of Proteus 23S rRNA in Δrnc strain and the inability to process 23S dsDNA. Total cellular RNA from CEQ200 and CEQ221 bearing pPM2 was extracted and run on the 1.5% agarose gel. 23S rRNA transcribed from the pPM2 has an intervening sequence in helix 25, which is processed by RNase III during maturation, resulting in a 23S rRNA fragmentation: 23S' and 23S''. RNase III deficient strain CEQ221 cannot process such a helix, therefore, a Proteus 23S rRNA appears to be intact. This is a sign that the Δrnc strain CEQ221 has lost RNase III activity.

Δrnc strain CEQ221 demonstrated increased production of shRNA and increased delivery of higher amounts of shRNA into target cells. When transformed with the same plasmid, pNJSZc-H3, Δrnc bacteria (CEQ221) contain significantly more shRNA compared with wt-*rnc* bacteria (CEQ200)

transformed with the same plasmid. Δrnc bacteria deliver larger amounts of shRNA into cells during an in vitro invasion assay experiment.

Cells treated with CEQ221 (H3- Δrnc) contain higher levels of shRNA compared with cells treated with equal amounts of CEQ200 (H3). SW480 cells were treated with *E. coli*- Δrnc carrying a tkRNAi plasmid against a gene target (beta-catenin H3) or with *E. coli* with a wild-type *rnc* gene carrying the same tkRNAi plasmid (H3) against beta-catenin. Cells were harvested at the indicated time points and cell extracts were analyzed to measure the amount of shRNA that had been deposited into the cells by the carrier bacteria. The Δrnc strain (CEQ221-red columns) was able to deposit a significantly larger amount of shRNA into target cells compared with its wild-type *rnc* counterpart (blue columns).

Figure 1 shows increased gene silencing potency (Maximum effect) and efficacy (Ic50). Treatment with CEQ221(Δrnc) achieves significantly higher levels of gene suppression compared with treatment with CEQ200 (wt *rnc*). As shown in Figure 1, Cos-7 cells were treated with rising doses of bacteria carrying the plasmids pNJSZc-H3 (or control plasmid pNJSZc-HPVb) and analyzed after 48h for expression of the target gene beta-catenin. Beta-catenin gene expression (mRNA) levels are shown in relation to cells that had been treated with the control bacteria (containing plasmid pNJSZc-HPVb and producing shRNA against the virus HPV) at the same bacterial dose. Results show that there is a dose-dependent decrease ("knock-down") of beta-catenin gene expression observed. The potency of the - Δrnc strain (CEQ221) is significantly greater than the one of the wt-*rnc* strain (CEQ200) with maximum levels of gene silencing of 76% compared to 57%. These results show that the efficacy of CEQ221 is approximately 10-fold greater compared with CEQ200: IC₅₀ for CEQ221 is 10⁶cfu/ml as compared to IC₅₀for CEQ200 is 10⁷cfu/ml.

Example 25: Design of RNase III Substrates as Precursors of Functional shRNA for Use in tkRNAi

In the preceeding example, it was demonstrated that limiting RNase activity within the delivery bacteria is beneficially to protect unwanted processing and degradation of shRNA. In an alternative embodiment, it is beneficial to design a hairpin RNA molecule that permits improved, more accurate and efficient Dicer processing that is essential for effective RNA interference. Dicer is an RNase enzyme having activity specific for dsRNAs, whereby the RNase III cleavage product contains 5' phosphate and 3' hydroxyl termini and a 2-nt overhang at the 3' end. The dicer products are further characterized by a discrete size of approximately 21 nt. Thus, the present example provides a hairpin RNA molecule that provides a substrate for processing by RNase III in the bacterial (tkRNAi) carrier, resulting in a substrate for Dicer processing within the host target cell.

RNase III enzymes can be divided into three classes. Class I enzymes, found in bacteria, bacteriophage and fungi contain a single RNase III domain and a dsRNA binding domain (dsRBD). Class II and III enzymes are characterized by Drosha and Dicer, respectively. Dicer is the most

complicated RNase III enzyme that typically contains a DExD/H-box helicase domain, a small domain of unknown function (DUF283), a PAZ (Piwi Argonaute Zwillie) domain, two tandem RNase III domains (RNase IIIa and IIIb), and a dsRBD. Some Dicer or Dicer-like proteins from lower eukaryotes have a simpler domain structure; for example, the Dicer protein from *Giardia intestinalis* contains only a PAZ and two RNase III domains. Previous mutational and enzymatic studies on *Escherichia coli* RNase III and human Dicer had led to the "single processing center model" for RNase III cleavage. This model centers on two RNase III domains forming a catalytic dimer: intermolecular homodimer for class I enzymes and intramolecular pseudodimer between RNase IIIa and IIIb domains for Dicer and Drosha. This dimerization creates a single processing center for dsRNA cleavage, with each RNase III domain cleaving one strand of the dsRNA. The distance between the two cleavage sites dictates the generation of the characteristic 2-nt 3' overhang. For Dicer, the distance between the terminus-binding PAZ domain and the RNase III domains determines the length of the cleavage product (Du, Lee, Tjhen et al in PNAS 105(7) 2008).

Bacteria contain a class I RNase III enzyme that cuts dsRNA. There is evidence that this class I RNase III recognizes specific motifs that determine where the dsRNA will be cleaved. The enzyme performs said cleavage in such a way that leaves a 2nt 3' overhang (see Pertzev and Nicholson Nucleic Acid Research vol. 34(13) 2006 and reviewed by Nicholson in FEMS Micro Reviews 23 1999). In addition, sequences have been described that exclude binding and cleavage by RNase III; so called anti-determinants.

The following example makes use of bacterial Class I RNase III processing of the hairpin RNA within the tkRNAi bacteria prior to release into the mammalian cytoplasm. The defined proximal and distal box sequences required by bacterial RNase III were placed "below" a pseudo-tetraloop structure, which is optional as variants of this design may be constructed with and without the loop, and a "spacer" sequence "above" the pseudo-tetraloop to extend the hairpin sequence by ~21 nucleotides. The proximal/distal box motif will encompass only ~10nt, therefore the remaining 11nt stretch adjacent to the silencing sequence should be composed of all anti-determinant base pairings. Bacterial RNase III will recognize the distal and proximal box sequences and cut the dsRNA at or 2nt below the proximal box (Figure 2) leaving a longer (*i.e.* more stable) hairpin structure. Furthermore, the presence of anti-determinant base pairings "above" the proximal/distal box motif protects the hairpin from further processing/degradation and maintains the appropriate length of the hairpin such that when Dicer processes the hairpin inside the target cell, a 21nt silencing siRNA will be produced.

Figure 2 shows a schematic illustration of the RNase III substrate hairpin RNA structure with functional annotation.

Figure 3 shows a schematic illustration of the bacterial Class I RNase III cutting action of the hairpin precursor. The cleavage is positively directed to occur at approximately 10nt distal of the pseudo tetraloop structure to result in an ideal Dicer-substrate precursor. This step will occur within the bacteria before delivery to the target cell. Cleavage by Class I RNase III will result in a hairpin of

approximately 100 nucleotides containing a 2-nucleotide overhang at the 3' end, which directs the next enzymatic processing step (see Figure 4).

Figure 4 shows a functional annotation of the second step of maturation (first Dicer-cleavage step). This step occurs after release of the RNA hairpin molecule into the cytoplasm of the target cell.

5 The 2-nucleotide overhang at the 3' end of the hairpin RNA structure left by the Class I RNase III processing helps direct and trigger the cleavage of the RNA structure by Dicer 21 nucleotides upstream (cleavage site is indicated by arrows designating "1st Dicer cut site").

Figure 5 shows the second Dicer cleavage step and maturation into active siRNA. This second Dicer cleavage occurs in the cytoplasm of the host cell and removes the hairpin loop, leaving a
10 functional siRNA for loading into the RISC complex. Again, the 2-nt overhang left by the first Dicer cleavage at the 3' end of the RNA helps direct Dicer.

A timecourse experiment bacterial Class I RNase III cleavage of hairpin RNA resulting in a decrease from a 150 nt RNA to a 100 nt RNA. Single-stranded RNA containing a hairpin sequence was synthesized from a plasmid template using the MEGAshortscript Kit (Ambion). RNA was then
15 exposed to purified bacterial RNase III for the indicated amounts of time, run on a 10% TBE-Urea gel, and visualized by ethidium bromide staining. The appearance of an approximately 100 nt RNA species appeared after 4 minutes of digestion.

Example 26: Construction of CEQ505

20 Drug candidate CEQ505 consists of an *E. coli* strain derived from MM294 through deletion of the *dapA* gene and the *rnc* gene. The internal designation of this E.Coli strain is CEQ221 transformed with the plasmid pNJSZc-H3, which is an expression plasmid encoding for the expression of invasin through the *inv* gene, listeriolysin O through the *hly* gene and short hairpin RNA to target the beta-catenin mRNA through the shRNA expression cassette including the hairpin
25 sequence H3.

FACS analysis showed surface expression of *Yersinia* invasin is required by CEQ 200 Δ*rnc* pNJSZc H3 for mammalian cell entry. Both *Yersinia* and CEQ 200 Δ*rnc* pNJSZc H3 have surface expression of invasin.

LLO activity is required by CEQ200 Δ*rnc* pNJSZc H3 for shRNA to escape mammalian cell
30 endosome. LLO activity was detected by hemolysin assay, which demonstrated that CEQ505 has hemolysin activity whereas CEQ 221 without plasmid does not.

shRNA H3 is required to silence β-catenin in mammalian cells. Relative H3 hairpin expression was determined by PK, which showed that CEQ505 expressed H3 shRNA, while the untransformed strain CEQ221 does not.

35 Figure 6 shows silencing of genes using CEQ 505. Panel A shows that CEQ 505 was able to silence mammalian β-catenin up to 90% in a dose-dependent manner in Cos-7 cells. Panel B shows

that CEQ 221pNJSZc lamin (the equivalent strain targeting the *lamin* gene) was able to silence mammalian lamin up to 65% in a dose-dependent manner in SW480 cells.

Example 26: Modification of pMBV40, 43 and 44 to Produce Hairpins without 5' or 3' Tails

The original TRIP plasmid expressed shRNA under the control of the T7 RNA polymerase promoter, enhancer and terminator. In this format, transcription begins inside the T7 RNA polymerase promoter sequence. As a consequence, the T7 enhancer, *Bam*HI site, *Sal*I site and most of the terminator are transcribed. Whereas the shRNA hairpin is about 55nt in length, the resultant transcript is predicted to be about 115 bases in length. The enhancer and restriction site *Bam*HI used for cloning form a 5' tail and the T7 RNA polymerase terminator form the 3' tail.

Thus, new promoter-terminator constructs were designed for use in pMBV40, 43 and 44 (see Example 19) to make hairpins without 5' or 3' tails. The *Bam*HI site used for cloning the hairpin was included in the promoter element shortly after the -10 consensus sequence (Lisser and Margalit, 1993, *Nucleic Acids Res.*, 21, 1507-1516). The promoter was made stronger by including an UP element (Estrem *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95, 9761-9766; Meng *et al.*, 2001, *Nucleic Acids Res.* 29, 4166-4178). For efficient termination, a run of Ts was added at the end of the hairpin prior to the *Sal*I site used for cloning the hairpin (terminator I). Rho-independent terminators include an A-rich sequence followed by a stem loop of 4 to 18 bp followed by a run of Ts (for example, Lesnik *et al.*, 2001, *Nucleic Acids Res.* 29, 3583-3594). Since there is no A-rich sequence, the shRNA stem loop is 19-21 bp long and since the gene is unusually small, efficiency of this terminator was hard to predict. Therefore, another rho-dependent terminator from the flagellin genes was also included (terminator II). Since there are two terminators, two transcripts were predicted. Transcripts I and II are terminated by terminator I and terminator II, respectively.

Example 27: Cloning of an Arabinose-Inducible Invasin Gene for Use in tkRNAi

In tkRNAi, intracellular delivery of therapeutic shRNA is achieved by equipping the carrier bacteria with invasive proteins that allow the bacteria to enter the host target cell through interaction with host cell surface receptors. The invasin protein encoded by the *inv* gene of *Yersinia* is one example of an invasive protein that triggers uptake of the bacteria into the host cell after interaction with host cell surface proteins called beta-1-integrins. However, high levels of invasin protein expression can be toxic to the bacterial carrier strains. Therefore, bacterial strains were constructed capable of inducible invasin expression through the addition of arabinose in the bacterial growth medium for the purpose of increasing efficacy and potency of tkRNAi-mediated gene silencing.

A plasmid was constructed having an arabinose-inducible invasin cassette containing the *araC* gene encoding the AraC protein, which is the Arabinose operon repressor-activator; the P_{araBAD}, arabinose promoter, which is under the regulation of the catabolite repressor protein (CRP) and the AraC protein; and the *inv* gene, which is cloned under the P_{araBAD} promoter.

There are different states of expression of invasin: (1) in the presence of glucose and absence of arabinose, the promoter is repressed by both catabolite repression and AraC-mediated repression; (2) an uninduced state occurs in the absence of any sugar (no glucose and no arabinose) as there is no catabolite repression and no AraC-mediated induction; and (3) an induced state occurs in the absence of glucose but in the presence of arabinose as there is AraC-mediated induction but no catabolite repression.

These states were tested for invasin according to the following protocol. The cells were grown overnight in the presence of glucose and then diluted and grown for 4 hours in the presence of glucose (repressed) or absence of any sugar (two cultures). One of the cultures grown in the absence of arabinose was induced with arabinose (10mM) for 2 hr. The cells were harvested by centrifugation and the expression of invasin was measured by FACS. *E. coli* cells lacking any plasmid were used as a negative control and *Yersinia* grown at 26 °C was used as a positive control.

In the presence of arabinose, high level expression of invasin was found, as determined by FACS, to be comparable with the levels of invasin expression observed in the positive control (*Yersinia*). During the two-hour induction of invasin with arabinose, bacterial growth appeared to stop as measured by OD. There was also a 100-fold loss in viability within two hours consistent with our initial hypothesis. We then optimized the assay conditions to give good invasin expression as well as good viability. We found that 0.3 to 1 mM arabinose causes no detectable loss in viability although there is a measurable decrease in growth rate. These conditions also showed induction of invasin indistinguishable from that of 10 mM arabinose by FACS. The results demonstrate that bacterial growth (as determined by OD) is a function of arabinose-induction of invasion.

Example 28: Alternative shRNA Structures for tkRNAi

Transkingdom RNA interference (tkRNAi) uses vector bacteria to synthesize and deliver short hairpin RNA (shRNA), which are deposited into the cytoplasm of the target cell. To achieve this, bacteria are equipped with expression plasmids or chromosomal integrations that allow them to express at least three novel properties, a surface-expressed invasion marker (e.g. *Yersinia* invasin protein encoded by the *inv* gene), an endosomal release function (e.g. Listeriolysin O protein – LLO, encoded by the *hly* gene) and the therapeutic payload- a shRNA that triggers RNA interference once it is delivered into the host cell cytoplasm. Experiments have shown that the expression of large amounts of hairpin RNA presents a burden on the bacteria and leads slower growth and/or plasmid or hairpin modification by the bacteria. shRNA designs having higher structural energy make it harder for the bacterial transcription machinery to separate the two strands, however, such shRNAs are more difficult to clone than those with lower structural energy. In this example, we disclose a method of expressing alternative hairpin RNA with lower energy coefficients to allow for easier maintenance of the shRNA expressing plasmid inside the bacteria while maintaining the ability to induce gene silencing through RNA interference.

The advantage of the design shown in Figure 7 over the existing shRNA structure primarily lies in significant lower structural energy, allowing for ease of cloning the tkRNAi plasmids, more stable maintenance of the plasmid within the bacteria, and facilitated sequencing of the plasmids. Introduction of the wobbles into the 3' end of the sense strand [3'(S) wobbles] are tolerated and do not change the silencing ability of the construct, whereas introduction of 5'(S) wobbles might reduce silencing ability. RNAi can be triggered through shRNA that does not have the traditional double strand structure. Additionally, half-overlapping structures can induce gene silencing as long as the antisense (AS) strand is of full length (19nt) and is covered on the 5' end with a sense strand.

Example 29: Derepression of *inv* Expression in TRIP and pNJSZc Plasmids

The *Yersinia pseudotuberculosis* surface-expressed invasin protein mediates entry into human cells by binding to members of the beta-1 integrin family. For this reason, we have cloned the invasin gene (*inv*), complete with its native promoter, into pTRIP and pNJSZc plasmids to mediate internalization of *E. coli* into human intestinal epithelial cells. Expression of invasin in *Y. pseudotuberculosis* is repressed when H-NS (a histon-like protein) binds to the *inv* promoter region in complex with YmoA. Together, the two proteins form a repressive complex that reduces the expression of *inv* to basal levels. Up regulation of *inv* expression occurs when temperature-regulated RovA (Regulatory of virulence A) binds within the same promoter region of *inv*, displacing H-NS/YmoA. Homologues of H-NS and YmoA are present in *E. coli*. However, RovA is not present in *E. coli*, which results in constant basal level expression of invasin. It was reported that removing the regulatory binding region within *inv*'s promoter region results in the constant up regulation of *inv*. In this example, we describe a method to remove the regulatory binding region from the *inv* promoter, as cloned in pTRIP and pNJSZc, to allow for constant up regulation of *inv*.

The primers shown in Table 46 were designed to delete 153 nucleotides located within *Y. pseudotuberculosis* *inv* promoter region, believed to be associated with the repression of *inv* in *E. coli*. The primers were used in combination with pTRIP (template) and QuikChange Lightning Site-Directed Mutagenesis kit (Stratagen) to delete the regulatory binding region shown in Table 46 within the *inv* promoter region cloned in pTRIP containing either the H3 or lamin hairpin.

Table 46

Description	Sequence	SEQ ID NO:
Forward Primer	CTGAAAGTAGAAATAAAAAACCGCAGCATATTTTGAATATTTTAAATCTGCCAG	567
Forward Primer	CTGGGCAGATTAAAAATATTCAAAATATGCTGCGGTTTTTATTTCTACTTTCAG	568
Regulatory Binding Region	GACAACTGACAACATCAAATGAAGGGGGCTTATTCTAATTGATATTATTTATATG ATAATAGTTTCATTTTGTATTTTGTATTTTGTATATTCTCACCTGCTTAGTTACAA TAAATCAATTCTATCGCTGTATGGTATAGACTGTTTTATTATA	569

The resulting *inv*-derepressed gene was sequenced verified and cloned into pNJSZc within the NruI and ScaI sites.

Invasin expression was tested by FACS where CEQ221/pTRIP and CEQ221/pNJSZc were grown overnight at 37°C, washed with PBS and probed with the anti-*inv* monoclonal antibody 3A2. The positive control was *Y. pseudotuberculosis* grown at 26°C for optimal expression of invasin.

FACS analysis showed that removing the *inv* regulatory region resulted in an increase of invasion expression in *E. coli* transformed with both pTRIP (de-repressed mutant is called pGB60) and pNJSZc (de-repressed mutant is called pGB69). The original and de-repressed plasmids were tested for their abilities to induce internalization of *E. coli* within Vero cells by the standard Gentamycin protection assay. The data showed that de-repression of *inv* resulted in an increase of internalization of *E. coli* within Vero cells.

Example 30: Opa52 Mediated Invasion of *E. coli* into T84 Human Intestinal Epithelial Cells

Opa52 was engineered for use in apical, broad range targeting of intestinal epithelial cells to deliver tkRNAi. Opa52 will bind to CEACAMs 1, 3, 5 and 6 of which CEACAM1, -5 and -6 are expressed by epithelial cells. This will allow delivery of tkRNAi into healthy and polarized epithelial cells. The following example describes the construction and use of an Opa52 bacterial expression vector for invasion of *E. coli* into highly polarized T84 human intestinal epithelial cells.

The *opa52* gene sequence was obtained from GenBank (accession # Z18929) and modified to include a start codon, signal sequence and stop codon. The signal sequence is identical to the natural secretion signals of several other Opa proteins and was codon-optimized for expression in *E. coli*.

This gene was then placed under control of a modified lacUV5 promoter that contains a second *lacO* site to enable enhanced transcriptional repression. The lambda t0 terminator sequence was included downstream of the *opa52* stop codon. This entire DNA fragment was synthesized by Blue Heron Biotechnology (Bothell, WA), cloned on pUC19 and confirmed. In order to facilitate expression of Opa52 on a low copy, ColE1-compatible plasmid, we subcloned the entire synthetic cassette from Blue Heron into a modified version of pACYC177 called pJS15. This is a small (2 kb), low copy (10-12 copies per cell), Kanamycin resistant vector that is compatible with ColE1 plasmids. The resultant *opa52* construct has been designated pJS34 is 1040 basepairs in length and is shown in Table 47. The sequence includes: restriction sites for KpnI (1-6), SpeI (101-106), NdeI (922-927), NotI (1023-1030), and PmeI (1033-1040); *lacO* sites (for LacI binding) (7-25 and 70-88); RBS (95-100); ptac -35 (32-37) and -10 (56-62); the leader sequence (109-177) and the lambda t0 terminator sequence (928-1022).

Table 47

Final sequence with lacUV5 promoter (SEQ ID NO:570)
GGTACCTTGTGAGCGGATAACAATTCCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAGGACTAGTCTatgaacccggcgccgaaaaaacgctccctgctgttttccctccctgctgttttccctccgcggcgagggcgaggtgaagaccatggcgcgcccgatgtgacggcgagatctggcttacgcctacgagcacattacccgcgattatcccgatgcagccgggtgcaacaaaggcaaaataagcacggtaagcgattatttcagaaacatccgtacgcattccatccaccccgagggtgtcggtcggtacgacttcggcggtggcgcatcgccgcgggattatgcccgttacaggaaatggcacaacaataaatattccgtgaacataaaaagagttggaaga

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aagaataataaaaacttctggcggcgaccagcttaacataaaaataccaaaagacggaacatcaggaaaacggcaca
ttccacgcggtttcttctctcggcttgtcaaccggtttacgatttcagagtcacagataaattcaaaccctatc
ggtgtgctgctcggctacggacacgtcagacacggtatcgattcgactaaaaaaacgaaaaatactcttaccgcc
taccatgggtgctggcacaaaaacctacgtattatgatgatagattcgggaaaaaaacaaaaaaacacttatcgc
caaaaccgcagcagccgccgcttgggcttcggcgcgatggcggcgctgggcatagacgtcgcgccccggcctgacc
ttggacgcggctaccgctaccactattggggacgcctggaaaacacccgcttcaaaaccacgaagcctcattg
ggcgtgctgctaccgcttcTGACATATGGACTCCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATT
TGTTTCAGAACGCTCGGTTGCCGCCGGCGCTTTTTTATTGGTGAGAATGCGGCCGCTTGTTTAAAC

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Table 48 shows the translated 270 amino acid sequence with a leader 23 amino acid peptide added. The leader peptide is amino acids 1-23.

Table 48

Translated Sequence (SEQ ID NO: 571)
MNPAPKKPSLLFSSLLFSSAAQAAGEDHGRGPYVQADLAYAYEHITRDYPDAAGANKGKISTVSDYFRNIRTHSI HPRVSVGYDFGGWRIAADYARYRKWHNNKYSVNIKELERKNNKTSGGDQLNIKYQKTEHQENGTFHAVSSLGLST VYDFRVNDKFKPYIGVRVGYGHRHGIDSTKKTNTLTAYHGAGTKPTYDDIDSGKNQKNTYRQNRSSRRLGFG AMAGVGIDVAPGLTLTDAGYRYHYWGRLENTFRKTHEASLGVRYSF

Plating and culture of bacteria released from highly polarized T84 cells demonstrated significantly higher invasive ability of the opa-expressing E.Coli strain compared with the invasion-expressing E.Coli strain. Figure 8 shows the data from a repeat experiment:

To allow targeting of healthy (non-dysplastic) epithelium through tkRNAi, we have developed 2 alternate invasion strategies both based on single proteins, from entero-invasive pathogens that target epithelial cell surface receptors.

The first is a member of the Opa family of proteins expressed by *Neisseria* species and, depending on the Opa variant used, differentially targets members of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family expressed on the apical aspect of epithelial cells. Primary attachment is mediated by the pilus, followed by a more intimate interaction via the opacity (Opa) proteins present in the bacterial outer membrane. The Opa proteins recognize distinct receptors present on epithelial cells. Certain Opa proteins bind to the cell surface heparan sulfate proteoglycans (HSPG) syndecan-1 and -4, while other Opa proteins bind to members of the carcinoembryonic antigen (CEA) or CD66 family, recently renamed the CEACAM family. CEACAMs can be found on epithelial cells and neutrophils, two cell types that are targeted by neisserial strains during natural infection. The interaction between Opa proteins and the CEACAM family members is highly specific; *i.e.*, each Opa variant demonstrates a particular tropism for only certain members of the CEACAM receptor family.

The second of these targeting proteins is derived from *Listeria monocytogenes* and is termed Internalin A or InlA and targets the E-cadherin protein component of the adherens junction. InlA, in conjunction with InlB, enable *L. monocytogenes* to invade a wide range of nonphagocytic cells in the susceptible host. InlA promotes entry of *L. monocytogenes* into intestinal epithelial cells by targeting the N-terminal domain of the E-cadherin, the dominant molecule in the adherens

junctional complex. Recent work has shown that native InlA utilizes a temporal window during intestinal epithelial maturation and shedding at the villus tip to gain entry into the host. In other words, intestinal epithelial cells are produced by self-renewing stem-cell like cells located at the base of the villus crypts and progressively mature as they move from the crypts toward the villus tips.

5 Once intestinal epithelial cells reach the villus tip they are shed in a normal turnover process or through injury-induced apoptosis. In either case, there is then a transient exposure of the adherens junction proteins (*i.e.*, E-cadherin) that allows InlA binding and entry of *Listeria* into the cells. Furthermore, in pathologic conditions such as IBD, the integrity of the epithelial barrier is compromised not only at the site of a lesion but also in surrounding uninvolved areas. In this case, the
10 compromised barrier will expose the adherens junctions and, thus, E-cadherin such that an InlA-expressing delivery strain could gain access to cells in the inflammatory foci as well as the surrounding epithelium. This is advantageous in that during active flares of inflammation the delivery strain would preferentially invade and silence the target of interest at the site of
15 inflammation/compromised barrier while leaving areas of normal epithelium untouched. As such, any tkRNAi delivery platform relying on InlA for targeting will be useful as a therapeutic agent for treatment during active inflammation.

Results have shown significantly increased invasive ability for *E. Coli* carrying the Opa expressing plasmids compared with the invasin expressing plasmids.

20 **Example 31: CEQ508 for the Treatment of Disorders Mediated by Upregulation of Beta Catenin**

The drug candidate CEQ508 consists of *E. coli* strain CEQ221 containing the pMBV43-H3 plasmid. Strain CEQ221 is derived from *E. coli* strain MM294 through sequential deletion of *dapA* and *rnc* genes using the bacteriophage lambda Red recombination system with the help of 5-strain
25 gene-disruption set of Datsenko and Wanner, 2000 (*Proc. Natl. Acad. Sci. USA* **97**, 6640) purchased from CGSC. The plasmid pMBV43 in this example encodes shRNA hairpin to target the *beta-catenin* mRNA through the shRNA expression cassette including the hairpin sequence "H3" (disclosed previously), *Yersinia pseudotuberculosis* invasin (coded by the *inv* gene) and *Listeria monocytogenes* Listeriolysin O (LLO) (coded by the *hly* gene). The pMBV43 plasmid is derived from pUC19 with
30 the following alterations:

1. The hairpin cassette has a modified P_{lacUV5} promoter linked to an UP element and a set of two terminators.
2. Cloning of a fragment containing *inv* and *hly* genes from another pre-existing plasmid pKSII-
35 *inv-hly*.
3. Replacement of *amp* as antibiotic resistance marker with kanamycin (*kan*).

The modified P_{lacUV5} promoter used in the pMBV43 plasmid in this example contains at least three important distinctions (as shown in Table 49) from P_{lacUV5} promoter used in the pNJSZ plasmid used previously:

1. -35 consensus element (shaded is mutated from TTTACA to TTGACA;
2. An UP element is added upstream of -35 element;
3. The *lacO* (lac operon operator) element from P_{lacUV5} is deleted and replaced with a *Bam*HI site.

Table 49

Promoter	Sequence	SEQ ID NO:
P_{lacUV5}	CCAGGCTTTACTACTTTATGCTTCCGGCTCGTATAATGTGTGGAA TTGTGAGCGGATAACAATTTCACACAGGAAACAGAATTCCTATG	572
P_{pMBV43} or Modified P_{lacUV5}	AAGCTTGGAAAAATTTTTTTTAAAAAAGTCTTGACACTTTATGCT TCCGGCTCGTATAATGGATCC	573

The P_{lacUV5} promoter is 87 basepairs in length and is shown in Table 49. The -35 and -10 consensus elements of the P_{lacUV5} promoter are basepairs 7-12 and 31-37, respectively. The *lacO* (lac operon operator) is shown as basepairs 43-64.

The modified P_{lacUV5} promoter is 65 basepairs in length and is shown in Table 49. The -35 and -10 consensus elements of the P_{lacUV5} promoter are basepairs 30-35 and 54-60, respectively. The UP element is shown as basepairs 7-26.

An additional distinction of the pMBV43 plasmid is that it has two sets of terminators:

1. Terminator 1: is a run of Ts that functions as a terminator when it immediately follows the shRNA hairpin.
2. Terminator 2 is the same as the *E. coli rrnC* terminator and has the following sequence: GATCCTTAGCGAAAGCTAAGGATTTTTTTT (SEQ ID NO: 574).

Moreover, the pNJSZ plasmid produces a shRNA comprising a total length of about 131 to 135 bases, consisting of a 5' overhang of about 8 bases, 51 base pairs of shRNA, and a 3' overhang consisting of about 72 to 76 bases. In contrast, the pMBV43 plasmid does not produce a 5' overhang, produces a significantly smaller 3' overhang consisting of 2-5 bases, and produces 51 base pairs of shRNA with the total length of shRNA in the range of 53 to 70 bases. For proper functioning, the 3' overhang requires at least 2 bases. Accordingly, the total length of the shRNA ranges from 53 to 70 nucleotides in length, preferably 53 to 65 nucleotides in length, more preferably, 53 to 58 nucleotides in length, and most preferably 53 to 55 nucleotides in length.

FACS analysis showed surface expression of *Yersinia* invasin is required by CEQ 221 pMBV43-H3 for mammalian cell entry. Both *Yersinia* and CEQ 221 pMBV43-H3 (CEQ508) have

surface expression of invasion. CEQ221 without the pMBV43 plasmid shows no invasin expression. Negative control: *Yersinia* with no antibody.

Listeriolysin (LLO) activity is required by CEQ508 to allow escape of the therapeutic payload (shRNA) from the mammalian cell endosome after invasion. LLO activity is detected by the hemolytic assay depicted above. CEQ508 shows clear hemolytic activity whereas CEQ 221 without plasmid or PBS does not.

Quantitative real-time PCR of the relative H3 hairpin RNA expression demonstrated that CEQ508 contains approximately 20 times more H3 shRNA compared to its predecessor, CEQ505. As disclosed previously, CEQ505 consists of an *E. coli* strain derived from MM294 through deletion of the *dapA* gene and the *rnc* gene, resulting in the *E. coli* strain designated as CEQ221, subsequently transformed with the plasmid pNJSZc-H3 encoding for the expression of invasin through the *inv* gene, listeriolysin O through the *hly* gene and short hairpin RNA to target the beta-catenin mRNA through the shRNA expression cassette including the hairpin sequence H3. In contrast, as previously disclosed, CEQ508 consists of *E. coli* strain CEQ221 containing the pMBV43-H3 plasmid, which encodes *Yersinia pseudotuberculosis* invasin (coded by the *inv* gene), *Listeria monocytogenes* Listeriolysin O (LLO) (coded by the *hly* gene) and the shRNA hairpin sequence H3.

Figure 9 shows silencing of genes using CEQ508 in human cells (SW480). Panel A shows that CEQ 508 was able to silence mammalian β -catenin mRNA by as much as 90% in a dose-dependent manner in SW480 cells. Controls were treated with CEQ221-pMBV43-lamin and CEQ221-pMBV43-luciferase. Panel B shows that CEQ508 was able to silence mammalian β -catenin protein by as much as 72% in a dose-dependent manner in SW480 cells. Controls were treated with CEQ221-pMBV43-luciferase. Similar experiments conducted in DLD-1 cells showed that CEQ508 was able to silence β -catenin mRNA by over 50%. Additional experiments showed β -catenin silencing in HeLa cells with CEQ508-H3. HeLa cells were cultured in standard DMEM (10% FBS, 1% Pen-Strep) at 37°C, and CEQ508-H3 was added to the culture when cells were 80% confluent. Controls were treated with *E. coli* bacterial strains of identical genetic background, but expressing hairpin RNA against human lamin (hlam) instead of β -catenin. Cells were treated with various multiplicities of infection (MOIs) ranging from 1:6.5 to 1:51 for 2 hours, followed by four washes. Fresh medium containing antibiotics tetracycline and ofloxacin was then added, and the cells were harvested at 48 hours after invasion. RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA), and gene expression analysis was performed using quantitative real-time PCR. Dose dependent silencing of β -catenin was observed in HeLa Cells treated with CEQ508-H3, but not in cells treated with control strain. At the highest MOI, β -catenin expression was reduced to 45% of baseline.

Figure 10 shows reduction of β -catenin gene expression in SW480 cells, as seen at the protein level (via Western Blot), after a single treatment with CEQ508 that is not observed with any of the control strains. SW480 cells were cultured in standard DMEM (10% FBS, 1% Pen-Strep) at 37°C. CEQ508 was added when the cells were 70% confluent. Controls were treated with *E. coli* bacterial

strains of identical genetic background, but expressing either: (a) hairpin RNA against luciferase (*luc*), (b) bacteria with invasive properties *invasin* and *listeriolysin*, but not expressing hairpin RNA, or (c) empty *E. coli* CEQ221 not carrying the pMBV43 plasmid (non-invasive). Cells were treated with various multiplicities of infection (MOIs) ranging from 1:50 to 1:150 for 2 hours, followed by four washes. Fresh medium containing antibiotics tetracycline and ofloxacin was added, and the cells were harvested at 48h after invasion followed by whole protein extraction using standard protocols. Dose dependent silencing of β -catenin was observed in SW480 cells treated with CEQ508, but not in cells treated with control strains. At the highest MOI, β -catenin expression was reduced to nearly undetectable levels.

Moreover, a time course experiment was performed and showed as least 50%, preferably at least 60%, more preferably at least 70%, and most preferably at least 80 % long-term silencing for at least 1 day, preferably at least 2 days, more preferably at least 3 days, and most preferably at least 4 day following administration, of β -catenin expression in SW480 cells following a single treatment with CEQ508. SW480 cells were cultured in standard DMEM (10% FBS, 1% Pen-Strep) at 37°C. CEQ508 was added when the cells were 70% confluent. Controls were treated with bacterial strains of identical genetic background (*E. coli* strain CEQ221) not carrying the pMBV43 plasmid (i.e., which do not carry the gene for invasion and are thus non-invasive). Cells were treated with MOI 1:200 for 2 hrs, followed by four washes. Fresh medium containing antibiotics tetracycline and ofloxacin was added, and the cells were allowed to grow and passaged when 90% confluence was reached. Cells from parallel wells were harvested at the indicated time points after invasion. RNA was extracted using TRIZOL, and gene expression analysis was performed using quantitative real-time PCR. The results show robust (>80%) gene silencing after a single treatment with CEQ508, which persists for at least four days before β -catenin levels slowly return to normal. An apparent “overshoot” around day 10 is interpreted to be an artifact caused by passaging of the cell lines.

Treatment with CEQ508 (CEQ221/pMBV43H3) at two different doses (low- 10^7 /ml and high- 10^8 /ml) did not lead to significant changes in the gene expression (as measured using quantitative real-time PCR) of *lamin* in human cells (SW480), confirming no deleterious effects on other genes. Control treatment was performed with CEQ221 bacteria without plasmid (no plasmid high) at 10^8 /ml, CEQ221-pMBV43-luciferase (pMBV43luc high and low) at 10^7 /ml and 10^8 /ml respectively, and with CEQ221-pMBV43 expressing no short hairpin RNA (pMBV43 no hairpin high/low) at 10^8 /ml and 10^7 /ml respectively. These data demonstrate that CEQ508 treatment does not cause problems for other genes.

Timecourse profiles were performed to evaluate the presence or absence of inflammatory cytokines from 0h to 12h after a single oral feeding with CEQ508 in wildtype mice and polyp-bearing APCmin mice. Animals (wild type as well as APCmin mice, 3-7 per group per time point) were treated with a single dose of CEQ508 and serum was taken at the indicated time points. Animals used for positive control were i.v. injected with 400 μ g LPS. The inflammatory cytokines TNF α , IL6, IL12,

IFN γ , MCP-1, and IL-10 were analyzed as indicators of an inflammatory response to orally administered CEQ508. Overall, none of the tested inflammatory cytokines showed an increase after oral treatment with CEQ508, demonstrating that there is no systemic inflammatory cytokine response after CEQ508 treatment in either wild type or polyp-bearing APCmin mice.

- 5 Table 50 shows the pharmacokinetics of shRNA in gastrointestinal mucosa after oral feeding with CEQ508 or CEQ501.

Table 50

Treatment Groups	Daily Dose (cfu)	No. of Treatments	Time of sacrifice after the last treatment	Amount of H3 shRNA (pg per μ g total RNA)
PBS+15% glycerol		1	5	n.d.
CEQ508	5×10^9	1	5	6.8 ± 8.9
PBS		1	24	n.d.
CEQ508	5×10^9	1	24	n.d.
CEQ508	5×10^9	3	5	3.6 ± 2.6
CEQ508	5×10^9	3	24	1.2 ± 0.4
CEQ501	5×10^9	1	5	1.5 ± 0.2
CEQ501	5×10^9	1	24	1.5 ± 0.3
CEQ501	5×10^9	3	5	1.4 ± 0.3
CEQ501	5×10^9	3	24	1.1 ± 0.3

- Mice were dosed with one or repeated treatment of CEQ508 or CEQ501 and gastrointestinal mucosal tissue was analyzed to quantify the amount of shRNA deposited within the GI mucosa at various time points after treatment. H3 shRNA was detectable in the intestinal mucosa of mice dosed with either CEQ501 or CEQ508 whereas PBS/glycerol treated mice were devoid of H3 shRNA. Furthermore, the amount of H3 shRNA detected in the mucosa was significantly higher after treatment with CEQ508 compared to CEQ501, which is consistent with the Δ rne mutation conferring higher yields and greater stability of the H3 shRNA. When assayed at 24 hours post last dose (multiple once daily regimen), both CEQ501 and CEQ508 show comparable levels of H3 shRNA in the intestinal mucosa suggesting that both delivery platforms achieve equivalent steady-state levels of delivered hairpin.

Table 51 shows the experimental groups for the evaluation of pharmacokinetics for live CEQ508 bacteria after oral treatment in mice.

Table 51

Daily Treatment Dose (cfu)	Route of Administration	No. of Treatments	Analysis Point (hrs post last treatment)	Males	Females	Total No. of animals Wt/APC ^{min}
5.0×10^9	PO	1	24 hrs	2	2	2/2
5.0×10^9	PO	3	24 hrs	2	2	2/2
5.0×10^9	PO	7	24 hrs	2	2	2/2
1.0×10^7	IV	1	2 hr	1	1	2
1.0×10^7	IV	1	24 hrs	1	1	2
1.0×10^7	IV	1	48 hrs	1	1	2
1.0×10^7	IV	1	72 hrs	1	1	2
1.0×10^7	IV	1	96 hrs	1	1	2

5 Mice were treated 1, 3 or 7 times by oral gavage with CEQ508. Each dose contained 5×10^9 cfu of CEQ508. Tissues were analyzed 24h after the last dosing. Tissues were extracted sterilely and examined for the presence of live therapeutic CEQ508 bacteria. Positive control animals were treated with intravenous injection of CEQ508.

10 Table 52 shows the pharmacokinetics demonstrating that after these single or multiple (once daily up to 7 days) oral treatments, viable CEQ508 was not recovered from any of the examined organs.

Table 52

Route	No. of Treatments	Analysis point (hrs post last treatment)	Dose (cfu)	Mouse #	Gender	Blood		Liver		Spleen		Kidney		Lung		Heart		Brain	
						DAP	Kan/DAP	DAP	Kan/DAP	DAP	Kan/DAP	DAP	Kan/DAP	DAP	Kan/DAP	DAP	Kan/DAP	DAP	Kan/DAP
IV	1	2	1x10 ⁷	2357	F	58	69	>1000	>1000	>1000	>1000	0	0	280	260	40	39	0	2
				2299	M	23	17	>1000	>1000	>1000	>1000	0	0	200	160	27	51	0	0
	1	24	1x10 ⁷	2481	F	0	0	14	15	41	40	0	0	75	108	6	1	0	0
				2478	M	0	0	57	47	38	41	0	0	81	90	10	6	0	0
	1	48	1x10 ⁷	2442	F	0	0	2	5	4	6	2	0	47	31	6	7	0	0
				2448	M	0	0	1	1	7	1	0	0	56	62	6	7	0	0
	1	72	1x10 ⁷	2443	F	0	0	2	0	2	0	2	0	6	6	5	4	0	0
				2449	M	1	0	3	1	5	0	4	4	65	82	5	4	0	0
	1	96	1x10 ⁷	2368	F	0	0	1	0	0	2	0	0	10	14	2	1	0	0
				2450	M	10	0	15	0	37	1	4	0	15	15	0	1	0	0
PO	1	24	5x10 ⁹	2192	F wt	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				2295	F min	0	0	0	0	0	0	2	0	0	0	0	0	0	0
				2296	M wt	0	0	0	0	0	0	0	0	50*	36*	0	0	0	0
				2139	M min	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	24	5x10 ⁹	2302	F wt	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				2193	F min	0	0	0	0	0	0	0	0	0	0	0	0	2	0
				2297	M wt	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				2138	M min	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	24	5x10 ⁹	2311	F wt	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				2483	F min	57	2	0	0	0	0	0	0	0	0	0	0	0	0
				2298	M wt	0	0	0	0	0	0	0	0	0	1	0	0	0	0
				2141	M min	0	0	0	0	0	0	0	0	0	0	0	0	0	0

These results confirm that CEQ508 is incapable of escaping the gastrointestinal tract in mice (with the exception of two animals showing false positive bacteria after gavage injuries). This was observed for both wild type mice (normal, healthy mice with an intact gut barrier) as well as in APC^{min} mice that have compromised epithelial barrier integrity due to dysplasia. CEQ508 was, however, recovered from stool samples taken five hours post dose, confirming transit of viable bacteria throughout the length of the intestine. As expected, the number of viable CEQ508 recovered in the stool rapidly diminished by 24 hours post dose. Viable CEQ508 was recovered from mice given a single intravenous injection via the tail vein. In these animals, CEQ508 was recovered in the blood and organs examined at two hours post injection. The number of viable bacterial subsequently declined but was recoverable in the liver for up to 96 hours post dose (iv).

Stool samples were collected from animals after receiving a single oral dose of CEQ508; 5.0x10⁹cfu via gavage in the total volume of 0.2 mL. Stool samples were collected hourly for the first six hours and then every two hours up to 24 hrs, and then every 12 hrs up to 108 hrs. To be able to collect stool samples according to the schedule total number of 24, the mice were subdivided into two cohorts of 12 mice each and received a single oral dose of CEQ508 with 12 hrs shift. Stool samples were resuspended in 1mL of sterile PBS, diluted up to 10mL with sterile PBS and 50 µL of this suspension was subsequently plated on nonselective LB/DAP plates, where all bacteria from the stool sample is expected to grow, as well as on selective LB/Kan/DAP containing plates, where only therapeutic CEQ508 bacteria from the stool samples is expected to grow. Bacterial colonies were then counted. In accordance with the abundance of CEQ508 in stool samples, mice were subdivided

into the following groups: 0 CFUs, <100 CFUs, <1000 CFUs, >1000 CFUs. Finally, the percentage of mice having a certain defined amount of bacteria was calculated, as illustrated in Table 53.

Table 53

Time points	% of mice with determined CFUs of CEQ508 in stool samples			
	0 CFUs	<100 CFUs	<1000 CFUs	>1000 CFUs
0	100.0%	0.0%	0.0%	0.0%
1	100.0%	0.0%	0.0%	0.0%
2	41.7%	25.0%	0.0%	33.3%
3	8.3%	16.7%	8.3%	66.7%
4	0.0%	0.0%	16.7%	83.3%
5	0.0%	0.0%	0.0%	100.0%
6	0.0%	0.0%	0.0%	100.0%
8	0.0%	0.0%	0.0%	100.0%
10	0.0%	0.0%	16.7%	83.3%
12	0.0%	0.0%	16.7%	83.3%
14	0.0%	0.0%	75.0%	25.0%
16	0.0%	25.0%	33.3%	41.7%
18	0.0%	8.3%	66.7%	25.0%
20	0.0%	41.7%	58.3%	0.0%
22	0.0%	58.3%	41.7%	0.0%
24	4.2%	62.5%	33.3%	0.0%
36	66.7%	33.3%	0.0%	0.0%
48	100.0%	0.0%	0.0%	0.0%
60	100.0%	0.0%	0.0%	0.0%
72	100.0%	0.0%	0.0%	0.0%
84	100.0%	0.0%	0.0%	0.0%
96	100.0%	0.0%	0.0%	0.0%
108	100.0%	0.0%	0.0%	0.0%

5

Table 53 shows that after a single oral treatment with CEQ508, mice begin to shed viable bacteria as early as two hours. The amount of CEQ508 in stool samples peaks by 5 hrs (i.e. all mice shed >1000 CFUs of viable CEQ508), remains elevated for up to 8 hours post dose and gradually declines thereafter. At 24 hours post dose, the majority of treated mice shed only low numbers of CEQ508 (i.e. 33% <1000 CFUs and 63% <100 CFUs). Only one third of the total number of treated mice (33.3%) continue to shed viable CEQ508 (<100 CFUs) in stool samples 36 hrs after the treatment while none of the animals shed viable CEQ508 at 48, 60, 72, 84, 96 or 108 hrs after the treatment. Taken together, these data demonstrate that CEQ508 not only transits the gastrointestinal tract intact but is also rapidly eliminated and is incapable of residing and proliferating in the gut.

15

CEQ508 peaks by 5h post dosing in stool samples, remains elevated for up to 8 hours post dose and

gradually declines thereafter, while none of the animals shed viable CEQ508 at 48, 60, 72, 84, 96 or 108 hrs post dose.

Example 32: CEQ509 for the Treatment of Disorders Mediated by the Upregulation of Beta Catenin

The drug candidate CEQ509 consists of Bacterial Therapeutic Particles (BTPs), which are minicells derived from *E. coli* strain CEQ210 containing the pNJSZc plasmid. Strain CEQ210 is derived from *E. coli* strain MM294 through deletion of *minC* gene using the bacteriophage lambda Red recombination system with the help of 5-strain gene-disruption set of Datsenko and Wanner, 2000 (*Proc. Natl. Acad. Sci. USA* **97**, 6640) purchased from CGSC. The pNJSZc plasmid encodes shRNA hairpin, *Yersinia pseudotuberculosis* invasin encoded by the *inv* gene, and *Listeria monocytogenes* Listeriolysin O (LLO), encoded by the *hly* gene. The BTPs were purified by low speed centrifugation yielding > 99.9% purity based on their ability to form colonies.

A number of assays have been performed to assess the tkRNAi activity of CEQ509, including an assay for LLO protein encoded by the *hly* gene of the pNJSZc plasmid. CEQ509 BTPs demonstrate as much LLO activity as CEQ501 when an equal amount of BTPs (equal biomass) were used.

Listeria monocytogenes Listeriolysin O (LLO) activity is required by CEQ509 to escape the phagosome and release the hairpin in the cytoplasm. LLO activity was assayed by hemolysis at pH5.5. Hemolysis was observed visually and quantified by measuring absorbance at 540 nm. For the measurement of absorbance, the spectrophotometer was blanked using the PBS-treated samples. CEQ509-H3 and CEQ509-HPV are BTPs that contain the H3 shRNA against β -catenin and HPV E6 protein (used here as a control).

FACS analysis demonstrated the presence of invasin on the surface of CEQ509 BTPs.

Surface expression of *Yersinia pseudotuberculosis* invasin by CEQ509 is required for mammalian cell entry by CEQ509 BTPs.

Quantitative real-time PCR (qPCR) analysis demonstrated that CEQ509 BTPs do not contain any H3 shRNA and generate only a background signal.

Finally, the BTPs were tested in a tkRNAi assay as illustrated in Figure 11. Figure 11 shows silencing of beta catenin using CEQ509. COS-7 cells were infected by CEQ509-H3 or CEQ509-HPV BTPs at the indicated multiplicity of infection (MOI). RNA from the cells was harvested after 48 hr and subjected to qPCR-based quantitation of β -catenin mRNA. The ratio of relative quantitation (RQ) of cells infected with CEQ509-H3 to that of CEQ509-HPV at the corresponding MOI is plotted above. This and other data show 30-50% silencing of β -catenin by CEQ509. The experiments demonstrate that the level of invasin expression was sufficient to induce tkRNAi mediated gene silencing.

CLAIMS

What is claimed is:

1. An invasive *E. coli* bacterium comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one Inv locus and at least one HlyA gene, wherein said siRNAs interfere with the mRNA of β -catenin and wherein said invasive *E. coli* bacterium having reduced RNase III activity when compared to wild-type *E. coli* bacterium.
2. A prokaryotic vector comprising one or more DNA molecules encoding one or more siRNAs, a modified P_{lacUV5} promoter, at least one Inv locus and at least one HlyA gene, wherein said siRNAs interfere with the mRNA of β -catenin.
3. A method of delivering one or more siRNAs to mammalian cells, the method comprising introducing to said mammalian cells at least one invasive *E. coli* bacterium of claim 1.
4. A method of regulating gene expression in mammalian cells, the method comprising introducing to said mammalian cells at least one invasive *E. coli* bacterium of claim 1.
5. A method of treating or preventing a disease or disorder associated with the over expression of β -catenin in a mammal in need thereof, the method comprising regulating the expression of β -catenin in said mammal comprising introducing to the cells of said mammal at least one invasive *E. coli* bacterium of claim 1.
6. The invasive *E. coli* bacterium of claim 1, wherein said bacterium comprises a deletion of an *rnc* gene encoding RNase III.
7. The invasive *E. coli* bacterium of claim 1, wherein said RNase III activity is reduced at least 90% when compared to wild-type *E. coli* bacterium.
8. The invasive *E. coli* bacterium of claim 1, wherein said RNase III activity is reduced at least 95% when compared to wild-type *E. coli* bacterium.
9. The invasive *E. coli* bacterium of claim 1, wherein said RNase III activity is reduced at least 99% when compared to wild-type *E. coli* bacterium.

10. The invasive *E. coli* bacterium of claim 1, wherein said one or more DNA molecules are transcribed into one or more shRNAs within the invasive bacterium.
11. The invasive *E. coli* bacterium of claim 10, wherein said one or more shRNAs comprise a 3' overhang or a blunt end.
12. The invasive *E. coli* bacterium of claim 11, wherein said 3' overhang is 2-5 base pairs.
13. The invasive *E. coli* bacterium of claim 11, wherein said 3' overhang is no more than 2 base pairs.
14. The invasive *E. coli* bacterium of claim 10, wherein said one or more shRNAs are processed into one or more siRNAs.
15. The invasive *E. coli* bacterium of claim 1, wherein said prokaryotic vector further comprises at least one terminator sequence.
16. The invasive *E. coli* bacterium of claim 15, wherein said at least one terminator sequence comprises at least 5 consecutive thymidine base pairs.
17. The invasive *E. coli* bacterium of claim 15, wherein said bacterium further comprises a second terminator sequence.
18. The invasive *E. coli* bacterium of claim 17, wherein said second terminator sequence is an *rrnC* terminator sequence.
19. The invasive *E. coli* bacterium of claim 1, wherein said prokaryotic promoter further comprises at least one UP element.
20. The invasive bacterium of claim 1, wherein said invasive bacterium is an attenuated, non-pathogenic or non-virulent bacterium.
21. A composition comprising the invasive bacterium of claim 1 and a pharmaceutically acceptable carrier.

22. The method of claim 5, wherein said mammalian cells are infected with about 10^3 to 10^{11} invasive bacteria.
23. The method of claim 22, wherein said mammalian cells are infected with about 10^5 to 10^9 invasive bacteria.
24. The method of claim 5, wherein said mammalian cells are infected at a multiplicity of infection ranging from about 0.1 to 10^6 .
25. The method of claim 25, wherein said mammalian cells are infected at a multiplicity of infection ranging from about 10^2 to 10^4 .
26. The method of claim 5, wherein said expression of β -catenin is reduced as compared to wild-type β -catenin expression or as compared to β -catenin expression prior to introducing said invasive bacterium to said cell.
27. The method of claim 26, wherein said reduced expression of β -catenin is reduced expression of β -catenin mRNA.
28. The method of claim 26, wherein said reduced expression of β -catenin is reduced expression of β -catenin protein.
29. The method of claim 26, wherein said expression of β -catenin is reduced at least 50% as compared to wild-type β -catenin expression or as compared to β -catenin expression prior to introducing said invasive bacterium to said cell.
30. The method of claim 26, wherein said expression of β -catenin is reduced at least 75% as compared to wild-type β -catenin expression or as compared to β -catenin expression prior to introducing said invasive bacterium to said cell.
31. The method of claim 26, wherein said expression of β -catenin is reduced at least 90% as compared to wild-type β -catenin expression or as compared to β -catenin expression prior to introducing said invasive bacterium to said cell.
32. The method of claim 5, wherein the disease or disorder associated with the over expression of β -catenin in a mammal is selected from the group consisting of colon cancer, rectal cancer, colorectal cancer, Crohn's disease, ulcerative colitis, familial adenomatous polyposis (FAP), Gardner's

syndrome, hepatocellular carcinoma (HCC), basal cell carcinoma, pilomatricoma, medulloblastoma, and ovarian cancer.

33. The method of claim 5, wherein the mammalian cells are selected from the group consisting of a colon epithelial cell, a rectal epithelial cell, an intestinal epithelial cell, a hepatocyte, a skin epithelial cell, a hair cell, a neural cell, and an ovarian cell.

34. The method of claim 5, wherein said mammalian is selected from the group consisting of human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, and primate.

35. The method of claim 34, wherein said mammal is a human.