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Title: COMPOSITIONS AND METHODS FOR DOWNREGULATING PROKARYOTIC GENES

FIG. 19

Spacer designed from an endogenous gene

Transformation of engineered CRISPR into studied bacteria

Endogenous gene normally expressed

Bacterial DNA

CRISPR

Bacterial DNA

CRISPR

Spacer

Abstract: An isolated polynucleotide is disclosed. The polynucleotide comprises a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of the CRISPR is sufficiently complementary to a portion of at least one prokaryotic gene so as to down-regulate expression of the prokaryotic gene. Uses of the polynucleotides and pharmaceutical compositions comprising the polynucleotides are also disclosed.
COMPOSITIONS AND METHODS FOR DOWNREGULATING PROKARYOTIC GENES

CROSS-REFERENCE TO RELATED APPLICATIONS
This application claims priority to U.S. Provisional Patent Application, 61/193,742, filed on December 22, 2008, which is hereby incorporated by reference in its entirety.

STATEMENT OF GOVERNMENTAL SUPPORT
This invention was supported by Contract No. DE-AC02-05CH11231 awarded to Lawrence Berkeley National Laboratory by the Department of Energy. The government has certain rights in the invention.

REFERENCE TO SEQUENCE LISTING
This application hereby incorporates the sequence listing, attached as a .txt file, entitled, "2814PCT_sequencelisting.txt".

FIELD OF THE INVENTION
The present invention, in some embodiments thereof, relates to methods of downregulating prokaryotic genes and, more particularly, but not exclusively, to methods of downregulating bacterial genes.

BACKGROUND OF THE INVENTION
One of the most successful ways for understanding the function of a gene within an organism is to silence its expression. This is usually done by modifying, interrupting or deleting the DNA of the gene, and is referred to as gene 'knockout' (Austin et al., 2004, Nature Genetics 36:921-4). Gene knockouts are mostly carried out through homologous recombination, and this method was used in multiple studies and in multiple organisms from bacteria to mammals.

Despite the fact that gene targeting by knockout is a powerful tool, it is a complex, labor intensive and time consuming procedure. It is therefore hard to scale up cost-effectively, and most studies are generally limited to a knockout of a single gene rather than a complete pathway. Moreover, knockout is mostly limited to organisms in which homologous
recombination is relatively efficient, such as mouse [Austin et al., 2004, Nature Genetics 36:921-4] and yeast [Deutscher et al., 2006, Nature Genetics 38:993-8].

A breakthrough in the search for efficient alternative to gene knockouts in eukaryotes was achieved when it was realized that RNA-interference (RNAi) could be used to silence the expression of specific genes without interruption of their DNA. RNAi is a conserved biological mechanism first discovered in the nematode Caenorhabditis elegans, where it was demonstrated that injection of long dsRNA into this nematode led to sequence-specific degradation of the corresponding mRNAs. This silencing response has been subsequently found in other eukaryotes including fungi, plants and mammals [Fire et al., 1998, Nature 391(6669):806-11; Hannon, 2002, Nature 418(6894):244-51].

While the role of RNAi is, at least in part, to protect against viral infections and mobile element infestations, it was also shown that artificial transfection of short dsRNA duplexes, which target specific endogenous mRNAs, into mammalian cells can trigger gene specific silencing [Elbashir et al., 2001, Nature. 2001 May 24;411(6836):494-8]. These short dsRNAs (called siRNAs) are converted into single strands by the RNA-induced silencing protein complex (RISC), and the RISC-siRNA complex identifies target mRNAs by base pairing, leading to their degradation by an RNA nuclease. Researchers are now using this RNAi gene silencing technology and its derivatives to understand the biological function of endogenous genes in many eukaryotic organisms, and usage of this technology has led to important scientific breakthroughs.

The huge advantages of RNAi in genetic studies have so far not been reproduced in prokaryotes (bacteria and archaea), because the RNAi system seems to be limited to the eukaryotic lineage.

U.S. Patent Application 20040053289 teaches the use of sihybrids to down-regulate prokaryotic genes.

CRISPR is a genetic system comprised of a cluster of short repeats (24-47bp long), interspersed by similarly sized non repetitive sequences (called spacers). Additional components of the system include CRISPR-associated (CAS) genes and a leader sequence (Figure 15A). This system is abundant among prokaryotes, and computational analyses show that CRISPRs are found in ~40 % of bacterial and ~90 % of archaeal genomes sequenced to date [Grissa et al., 2007, BMC Bioinformatics 8: 17].

CRISPR arrays and CAS genes vary greatly among microbial species. The direct repeat sequences frequently diverge between species, and extreme sequence divergence is
also observed in the CAS genes. The size of the repeat can vary between 24 and 47 bp, with spacer sizes of 26-72 bp. The number of repeats per array can vary from 2 to the current record holder, *Verminephrobacter eiseniae*, which has 249 repeats per array and, although many genomes contain a single CRISPR locus, *M.jannaschii* has 18 loci. Finally, although in some CRISPR systems only 6, or fewer, CAS genes have been identified, others involve more than 20. Despite this diversity, most CRISPR systems have some conserved characteristics (Figure 15A).

It was recently demonstrated experimentally that in response to phage infection bacteria integrate new spacers that are derived from phage genomic sequences, resulting in CRISPR-mediated phage resistance. The new repeat-spacer units were added at the leader-proximal end of the array, and had to match the phage sequence exactly (100 % identity), to provide complete resistance. When such phage-derived spacers were artificially introduced into the CRISPR array of a phage-sensitive *S. thermophilus* strain, it became phage-resistant [Barrangou et al, 2007, Science 315(5819): 1709-12]. Indeed, spacers found in naturally occurring CRISPR arrays are frequently derived from phages and other extrachromosomal elements [Boletin et al., 2005, Microbiology 151(Pt 8): 2551-61].


**SUMMARY OF THE INVENTION**

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide, comprising a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of the CRISPR is sufficiently complementary to a portion of at least one prokaryotic gene so as to down-regulate expression of the prokaryotic gene.

According to some embodiments of the invention, the at least one spacer comprises at least two spacers, each being sufficiently complementary to a portion of different prokaryotic genes so as to down-regulate expression of the different prokaryotic genes.

According to some embodiments of the invention, the at least one spacer comprises 26-72 base pairs.

According to some embodiments of the invention, the prokaryotic gene is a bacterial gene.
According to some embodiments of the invention, the bacterial gene is associated with down-regulation of biofuel production.

According to some embodiments of the invention, the bacterial gene is selected from the group consisting of acetate kinase, phosphate acetyltransferase and L-lactate dehydrogenase.

According to some embodiments of the invention, the bacterial gene is a genetic repressor CcpN.

According to some embodiments of the invention, the bacterial gene is an antibiotic resistance gene.

According to some embodiments of the invention, the antibiotic resistance gene is a methicillin resistance gene or a vancomycin resistance gene.

According to some embodiments of the invention, the bacterial gene is a bacterial virulence gene.

According to some embodiments of the invention, the bacterial gene is a ribosomal RNA gene, a ribosomal protein gene or a tRNA synthetase gene.

According to some embodiments of the invention, the bacterial gene is selected from the group consisting of dnaB, fabl, folA, gyrB, murA,pytH, metG, and tufA(B).

According to some embodiments of the invention, the prokaryotic gene is an archaenal gene.

According to some embodiments of the invention, the isolated polynucleotide further comprises a nucleic acid sequence encoding a CRISPR leader sequence.

According to some embodiments of the invention, the isolated polynucleotide further comprises a nucleic acid sequence encoding at least one CRISPR associated (CAS) polypeptide.

According to some embodiments of the invention, the isolated polynucleotide is non-naturally occurring.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide of the present invention.

According to some embodiments of the invention, the nucleic acid construct comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 6 and 11.
According to some embodiments of the invention, the nucleic acid construct comprises a cis regulatory element.

According to some embodiments of the invention, the cis regulatory element is a promoter.

According to some embodiments of the invention, the promoter is an inducible promoter.

According to an aspect of some embodiments of the present invention there is provided a method of down-regulating expression of a gene of a prokaryotic cell, the method comprising introducing into the cell a CRISPR array polynucleotide, wherein a spacer of the CRISPR array is sufficiently complementary with a portion of the gene to down-regulate expression of the gene, and wherein the gene is not introduced into the cell by a bacteriophage, thereby down-regulating expression of gene of a prokaryotic cell.

According to some embodiments of the invention, the gene is integrated into a chromosome of the cell.

According to some embodiments of the invention, the gene is endogenous to the prokaryotic cell.

According to some embodiments of the invention, the gene is epichromosomal.

According to some embodiments of the invention, the method further comprises introducing into the cell a naïve CRISPR array system.

According to an aspect of some embodiments of the present invention there is provided a method of treating a bacterial infection in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an isolated polynucleotide, comprising a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of the CRISPR is sufficiently complementary to a portion of at least one bacterial gene so as to down-regulate expression of the bacterial gene, the bacterial gene being a vital bacterial gene or a bacterial virulence gene, thereby treating the bacterial infection.

According to an aspect of some embodiments of the present invention there is provided a method of treating an antibiotic-resistant bacterial infection in a subject in need thereof, the method comprising administering to the subject the isolated polynucleotide of claim 8, thereby treating the antibiotic-resistant bacterial infection.

According to some embodiments of the invention, the bacterial infection is induced by methicillin resistant Staphylococcus aureus or vancomycin resistant Staphylococcus aureus.
According to an aspect of some embodiments of the present invention there is provided a method of annotating a prokaryotic gene, the method comprising:

(a) introducing the isolated polynucleotide of claim 1 into a prokaryote under conditions that allow downregulation of the prokaryotic gene; and

(b) assaying a phenotype of the prokaryote, wherein a change in phenotype following the introducing is indicative of the prokaryotic gene being associated with the phenotype.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a polynucleotide sequence of a CRISPR construct for silencing of GFP expression in E. coli (SEQ ID NO: 1). The sequence in red (SEQ ID NO: 2) is a sequence of a spacer which targets the antisense strand of GFP. The sequence in blue (SEQ ID NO: 3) is a sequence of a spacer which targets the sense strand of GFP. The sequence in green (SEQ ID NO: 4) is a sequence of a spacer which targets the antisense strand of GFP. The sequence in purple (SEQ ID NO: 5) is a sequence of a spacer which targets the sense strand of GFP. The highlighted yellow region shows the repeat sequence of this construct.

FIG. 2 is a polynucleotide sequence of a CRISPR construct for silencing of malF expression in E. coli (SEQ ID NO: 6). The sequence in red (SEQ ID NO: 7) is a sequence of a spacer which targets the antisense strand of malF. The sequence in blue (SEQ ID NO: 8) is a sequence of a spacer which targets the sense strand of malF.
8) is a sequence of a spacer which targets the sense strand of malF. The sequence in green (SEQ ID NO: 9) is a sequence of a spacer which targets the antisense strand of malF. The sequence in purple (SEQ ID NO: 10) is a sequence of a spacer which targets the sense strand of malF. The highlighted yellow region shows the repeat sequence of this construct.

FIG. 3 is a polynucleotide sequence of a CRISPR construct for silencing of GFP and malF expression, together, in E. coli (SEQ ID NO: 11). The sequence in red (SEQ ID NO: 2) is a sequence of a spacer which targets the antisense strand of GFP. The sequence in blue (SEQ ID NO: 8) is a sequence of a spacer which targets the sense strand of malF. The sequence in green (SEQ ID NO: 4) is a sequence of a spacer which targets the antisense strand of GFP. The sequence in purple (SEQ ID NO: 10) is a sequence of a spacer which targets the sense strand of malF. The highlighted yellow region shows the repeat sequence of this construct.

FIG. 4 is a polynucleotide sequence of a control CRISPR construct which does not target any gene of interest (SEQ ID NO: 12). The highlighted yellow region shows the repeat sequence of this construct.

FIG. 5 is a polynucleotide sequence of GFP showing the positions of the sequences targeted by an exemplary CRISPR construct (SEQ ID NO: 92).

FIG. 6 is a polynucleotide sequence of malF showing the positions of the sequences targeted by an exemplary CRISPR construct (SEQ ID NO: 93).

FIG. 7 is a schematic drawing illustrating the K12W31 10 CRISPR system with the GFP spacers.

FIG. 8 is a schematic drawing of GFPuv5 BL21(DE3) kan+ illustrating the positions of the sequences targeted by C1 (SEQ ID NO: 3), T1 (SEQ ID NO: 2), C2 (SEQ ID NO: 5) and T2 (SEQ ID NO: 4).

FIG. 9 is a schematic drawing illustrating the K12W31 10 CRISPR system with the malF spacers.

FIG. 10 is a schematic drawing of malF in the GFPuv5 BL21(DE3) kan+ strain illustrating the positions of the sequences targeted by C1 (SEQ ID NO: 8), T1 (SEQ ID NO: 7), C2 (SEQ ID NO: 10) and T2 (SEQ ID NO: 9).

FIG. 11 is a schematic drawing illustrating the organization of the Alkalilimnicola ehrlichei MLHE-1 CRISPR system (Genbank NC_008340).

FIG. 12 is a schematic drawing illustrating the organization of the Deinococcus geothermalis DSM 11300 plasmid 1 CRISPR system (Genbank: NC_008010).
FIG. 13 is a schematic drawing illustrating the organization of the Clostridium thermocellum CRISPR system (ATCC 27405: Genbank NC_009012).

FIG. 14 is a schematic drawing illustrating the organization of the Shewanella baltica OS185 CRISPR system (Genbank NC_009665).

FIG. 15A is a typical structure of a CRISPR locus (system).

FIG. 15B is a model illustrating how CRISPR acquires phage-derived spacers which provide immunity (adapted from Sorek et al. Nature Reviews Microbiology, 6, 181, 2007). Following an attack by phage, phage nucleic acids proliferate in the cell and new particles are produced leading to death of the majority of sensitive bacteria. A small number of bacteria acquire phage derived spacers (blue spacer, marked by asterix) leading to survival, presumably via CRISPR-mediated degradation of phage mRNA or DNA.

FIG. 16 is a model teaching an exemplary method of silencing of self genes with an engineered CRISPR system. The CRISPR system (containing the CRISPR array as well as Cas proteins) is cloned into a plasmid. Fragments from a chromosome-encoded gene (green) that is expressed to RNA the cell (green waves) are engineered into the CRISPR array as new spacers. When the engineered CRISPR system is inserted inside the prokaryotic cell, the system silences the expression of the RNA of the self gene (silenced RNA is depicted in the figure as dashed green waves). The plasmid carrying the CRISPR can contain an inducible promoter, that turns the expression of the system on only in a certain condition. Thus, a conditional silencing of self genes could be achieved.

FIG. 17 is a model teaching an exemplary method of silencing of self genes with an engineered CRISPR array. In bacteria that already naturally carry a CRISPR system, their natural system could be used to silence their own genes. In this case a plasmid that carries only a CRISPR array is inserted into the bacterial cell. Fragments from a chromosome-encoded gene (green) that is expressed to RNA in the cell (green waves) are engineered into the CRISPR array as new spacers. When the engineered CRISPR array is inserted inside the prokaryotic cell, the system naturally carried by the cell uses the inserted array to silence the expression of the RNA of the self gene (silenced RNA is depicted in the figure as dashed green waves). The plasmid carrying the CRISPR contains a promoter that leads to the expression of the CRISPR array. The promoter could be either constitutively active or inducible. Thus, a conditional silencing of self genes could be achieved.

FIG. 18 is a schematic drawing illustrating the organization of the Escherichia coli W3110 Genbank AC_000091 CRISPR system.
FIG. 19 is a cartoon showing silencing of endogenous genes as an alternative to knockout methods. Fragments from a chromosome-encoded gene (arrow pointing to spacer) are engineered into a CRISPR array as spacers. If, as suggested, the CRISPR system indeed functions by the silencing of RNA 16, this might lead to silencing of the endogenous gene.

FIG. 20 shows a cartoon of a construct assembled from a plasmid containing a spacer from the relevant CRISPR species and a green florescent protein (GFP) under the control of an inducable promoter that will be introduced into the CRISPR containing strains.

FIG. 21 shows a flow chart for an experimental study as described in Example 3.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of downregulating prokaryotic genes and, more particularly, but not exclusively, to methods of downregulating bacterial genes.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

The clusters of regularly interspaced short palindromic repeat (CRISPR) system is associated with defense of bacteria and archaea providing protection thereto against invading phages. Resistance is acquired by incorporating short stretches of invading DNA sequences in genomic CRISPR loci. These integrated sequences are thought to function as a genetic memory that prevents the host from being infected by viruses containing this recognition sequence.

In one embodiment, the present invention provides methods to manipulate a CRISPR ability to down-regulate extrachromosomal DNA such that it can also down-regulate genes that are integrated into the chromosome of a cell. In one embodiment, the methods are used to selectively down-regulate a gene of interest by transforming a CRISPR-bearing plasmid into a prokaryotic cell of choice (e.g. bacterial cell) with one of the spacers being changed to match the gene of interest. Moreover, the present methods using the array nature of CRISPRs could allow the simultaneous knockdown of multiple genes.

Thus, according to one aspect of the present invention there is provided a method of down-regulating expression of a gene of a prokaryotic cell. The method comprises introducing into the cell a CRISPR array polynucleotide, wherein a spacer of the CRISPR
array is sufficiently complementary with a portion of the gene to down-regulate expression of the gene. In an exemplary embodiment, the gene is not introduced into the cell by a bacteriophage.

As used herein, the phrase "down-regulating" refers to reducing or inhibiting. According to one embodiment, the gene is down-regulated by at least 10 %. According to another embodiment, the gene is down-regulated by at least 20 %. According to another embodiment, the gene is down-regulated by at least 30 %. According to another embodiment, the gene is down-regulated by at least 40 %. According to another embodiment, the gene is down-regulated by at least 50 %. According to another embodiment, the gene is down-regulated by at least 60 %. According to another embodiment, the gene is down-regulated by at least 70 %. According to another embodiment, the gene is down-regulated by at least 80 %. According to another embodiment, the gene is down-regulated by at least 90 %. According to another embodiment, the gene is down-regulated by 100 % (i.e. inhibiting gene expression).

As used herein, the term "gene" refers to a DNA sequence which encodes a polypeptide or a non-coding, functional RNA.

The phrase "gene of a prokaryotic cell" refers to a gene that is present in the prokaryotic cell but not necessarily integrated into the chromosome of the prokaryotic cell.

According to one embodiment, the genes which are down-regulated are those that are integrated into the chromosome of the prokaryote.

According to another embodiment, the genes which are down-regulated are those that remain outside the chromosome i.e. remain epichromosomal.

According to another embodiment, the gene is endogenous to the cell. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism.

Examples of contemplated genes that may be downregulated according to the method of this aspect of the present invention, include, but are not limited to genes associated with down-regulation of organic material production in bacteria.

Thus, for example, the present invention contemplates the down-regulation of genes whose knockout enhanced the production of ethanol as a biofuel. Shaw et al., (PNAS 2008, Sep 16; 105(37): 1769-74) teaches the knock-out of a number of genes (namely acetate kinase, phosphate acetyltransferase and L-lactate dehydrogenase, examples of sequences of each can be found in refseq accession no: NC_009012, Clostridium thermocellum ATCC 27405, complete genome) that resulted in the production of ethanol at high yields.
Tannler S et al [Metab Eng. 2008 Sep;10(5):216-26], teaches enhanced ethanol production in bacteria by down-regulating expression of the gluconeogenic genes gapB and pckA (examples of sequences of each can be found in refseq accession no: NC_00964, Bacillus subtilis subsp. subtilis str. 168, complete genome) through knockout of their genetic repressor CcpN.

The present invention also contemplates the down-regulation of genes whose knockout enhanced the production of hydrogen as a biofuel.

Vardar-Schara et al [Microbial Biotechnology VoII, Issue 2, Pages 107-125] incorporated herein by reference, teaches that a number of strains of genetically engineered bacteria which generate hydrogen. Vardar-Schara et al states therein, that the hydrogen yield is suboptimal in a number of those strains due to the presence of uptake hydrogenases. Accordingly, the present invention contemplates downregulation of these uptake hydrogenases, Hyd-1 and -2 (hyaB and hybC respectively) for the enhancement of hydrogen production. Examples of sequences of each can be found in refseq accession no: NC_01 1742, Escherichia coli S88, complete genome.

In addition, the present invention contemplates down-regulation of genes of metabolic pathways that compete for hydrogen production.

Exemplary genes that may be down-regulated to increase hydrogen production in bacteria include, but are not limited to lactate dehydrogenase (idhA), the FHL repressor (hyeA), fumarate reductase (frdBQ, the Tat system (tatA-E), the alpha subunit of the fumarate dehydrogenase-N and -O (fdnG and fdoG respectively), the alpha subunit of nitrate reductase A ( narG), pyruvate dehydrogenase (aceE), pyruvate oxidase (poxB) and proteins that transport formate (foeA and foeB) - see Vardar-Schara et al [Microbial Biotechnology VoII, Issue 2, Pages 107-125]. Examples of sequences of each can be found in refseq accession no: NC_01 1742, Escherichia coli S88, complete genome.

Lactic Acid Bacteria (LAB) play an essential role in the preservation, taste and texture of cheese, yogurt, sausage, sauerkraut and a large variety of traditional indigenous fermented foods. Down-regulation of such genes would ensure for example that lactic acid bacteria used in the food industry would have a better taste or smell. According to another embodiment, the genes that are down-regulated in bacteria are those which are involved in taste or odor.

For example, the buttermilk aroma diacetyl is formed from the carbon metabolism of dairy Lactococcus bacteria during buttermilk fermentation. Lactococcal strains that have low
levels of a diacetyl reductase, acetoin reductase and butanediol dehydrogenase have been found to produce more diacetyl. Down-regulation of such enzymes would therefore be beneficial. Examples of sequences of each can be found in refseq accession no: NC_009004, Lactococcus lactis subsp. cremoris MG1363.

For example, down-regulation of those Lactobacillus bulgaricus genes associated with lactic acid production would be beneficial for the generation of mild forms of yoghurt.

As used herein, the expression "lactic acid bacterium" refers to a group of gram-positive, microaerophilic or anaerobic bacteria having in common the ability to ferment sugars and citrate with the production of acids including lactic acid as the predominantly produced acid, acetic acid, formic acid and propionic acid. The industrially most useful lactic acid bacteria are found among Lactococcus species, Streptococcus species, Lactobacillus species, Leuconostoc species, Oenococcus species and Pediococcus species. In the dairy industry, the strict anaerobes belonging to the genus Bifidobacterium is generally included in the group of lactic acid bacteria as these organisms also produce lactic acid and are used as starter cultures in the production of dairy products.

It will be appreciated that the present invention may be used to enhance production of any industrial, agricultural, pharmaceutical (e.g. recombinant protein production) product in bacteria by suppressing genes associated with lower levels of expression of that industrial product.

Other examples of bacterial genes that may be downregulated according to the method of this aspect of the present invention are genes that if down-regulated would aid in the treatment of a bacterial infection. Such genes include for example, antibiotic resistance genes, bacterial virulence genes and genes that are essential for the growth of bacteria.

The phrase "antibiotic resistance genes" as used herein refers to genes that confer resistance to antibiotics, for example by coding for enzymes which destroy it, by coding for surface proteins which prevent it from entering the microorganism, or by being a mutant form of the antibiotic's target so that it can ignore it.

Example of antibiotic resistance genes may be found on the ARDB - Antibiotic Resistance Genes Database - http://ardbdotcbsdotumddotedu/. Particular examples of antibiotic resistance genes include, but are not limited to methicillin resistance gene or a vancomycin resistance gene.

The phrase "virulence gene" as used herein refers to a nucleic acid sequence of a microorganism, the presence and/or expression of which correlates with the pathogenicity of
the microorganism. In the case of bacteria, such virulence genes may in an embodiment comprise chromosomal genes (i.e. derived from a bacterial chromosome), or in a further embodiment comprise a non-chromosomal gene (i.e. derived from a bacterial non-chromosomal nucleic acid source, such as a plasmid). In the case of E. coli, examples of virulence genes and classes of polypeptides encoded by such genes are described below. Virulence genes for a variety of pathogenic microorganisms are known in the art.

Examples of virulence genes include, but are not limited to genes encoding toxins, hemolysins, fimbrial and afimbrial adhesins, cytotoxic factors, microcins and colicins and also those identified in Sun et al., Nature medicine, 2000; 6(11): 1269-1273.

According to one embodiment of the invention, the bacterial virulence gene may be selected from the group consisting of actA (example is given in genebank accession no: NC_003210.1), Tem (example is given in genebank accession no: NC_009980), Shv (example is given in genebank accession no: NC_009648), oxa-1 (example is given in genebank accession no: NW_139440), oxa-7 (example is given in genebank accession no: X75562), pse-4 (example is given in genebank accession no: J05162), ctx-m (example is given in genebank accession no: NC_010870), ant(3")-Ia (aadAl) (example is given in genebank accession no: DQ489717), ant(2")-Ia (aadB)b (example is given in genebank accession no: DQ1 76450), aac(3)-IIa (aacC2) (example is given in genebank accession no: NC_010886), aac(3)-IV (example is given in genebank accession no: DQ241380), aph(3')-Ia (aphAl) (example is given in genebank accession no: NC_007682), aph(3')-Ia (aphA2) (example is given in genebank accession no: NC_010170), tet(A) (example is given in genebank accession no: NC_005327), tet(B) (example is given in genebank accession no: FJ41 1076), tet(C) (example is given in genebank accession no: NC_010558), tet(D) (example is given in genebank accession no: NC_010558), tet(E) (example is given in genebank accession no: M34933), tet(Y) (example is given in genebank accession no: AB089608), catl (example is given in genebank accession no: NC_005773), catl II NC_01019, catlIII (example is given in genebank accession no: X07848), floR (example is given in genebank accession no: NC_009140), dhfrI (example is given in genebank accession no: NC_002525), dhfrV (example is given in genebank accession no: NC_010488), dhfrVII (example is given in genebank accession no: DQ388126), dhfrIX (example is given in genebank accession no: NC_010410), dhfrXII (example is given in genebank accession no: NC_000962), dhfrXV (example is given in genebank accession no: Z83311), sull (example is given in genebank accession no: NC_000913), sulll (example is given in genebank accession no: NC_000913),
integron class 1 3'-CS (example is given in genebank accession no: AJ867812), vat (example is given in genebank accession no: NC_01_1742), vatC (example is given in genebank accession no: AFOI5628), vatD (example is given in genebank accession no: AF368302), vatE (example is given in genebank accession no: NC_004566), vga (example is given in genebank accession no: AFI 17259), vgb (example is given in genebank accession no: AFI 17258), and vgbB (example is given in genebank accession no: AFO15628).

As mentioned, in order to kill various pathogenic bacteria, CRISPR could be used in order to silence essential genes (i.e., compatible with life) in the bacteria. Essential genes could be identified by their conservation among several pathogens (Payne et al, Nature Reviews Drug Discovery 6, 29-40 (January 2007)). Such genes include ribosomal RNA genes (16S and 23S), ribosomal protein genes, tRNA-synthetases, as well as additional genes shown to be essential such as dnaB, fabl, folA, gyrB, murA, pytH, metG, and tufA(B) NC_009641 for Staphylococcus aureus subsp. aureus str. Newman and NC_003485 for Streptococcus pyogenes MGAS8232 (DeVito et al, Nature Biotechnology 20, 478-483 (2002)).

As mentioned, the present invention teaches a method of down-regulating a gene (or genes) in a prokaryotic cell.

Examples of prokaryotic cells include, but are not limited to bacterial cells and archaeal cells (e.g. those belonging to the two main phyla, the Euryarchaeota and Crenarchaeota).

The bacteria whose genes may be down-regulated may be gram positive or gram negative bacteria. The bacteria may also be photosynthetic bacteria (e.g. cyanobacteria).

The term "Gram-positive bacteria" as used herein refers to bacteria characterized by having as part of their cell wall structure peptidoglycan as well as polysaccharides and/or teichoic acids and are characterized by their blue-violet color reaction in the Gram-staining procedure. Representative Gram-positive bacteria include: Actinomyces spp., Bacillus anthracis, Bifidobacterium spp., Clostridium botulinum, Clostridium perfringens, Clostridium spp., Clostridium tetani, Corynebacterium diphtheriae, Corynebacterium jeikeium, Enterococcus faecalis, Enterococcus faecium, Erysipelothrix rhusiopathiae, Eubacterium spp., Gardnerella vaginalis, Gemella morbillorum, Leuconostoc spp., Mycobacterium abscessus, Mycobacterium avium complex, Mycobacterium cheloneae, Mycobacterium fortuitum, Mycobacterium haemophilum, Mycobacterium kansasi, Mycobacterium leprae, Mycobacterium marinum, Mycobacterium scrofulaceum, Mycobacterium smegmatis,
Mycobacterium terrae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Nocardiaceae spp., Peptococcus niger, Peptostreptococcus spp., Propionibacterium spp., Staphylococcus aureus, Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus saccharolyticus, Staphylococcus saprophyticus, Staphylococcus schleiferi, Staphylococcus similans, Staphylococcus warneri, Staphylococcus xylosus, Streptococcus agalactiae (group B streptococcus), Streptococcus anginosus, Streptococcus bovis, Streptococcus canis, Streptococcus equi, Streptococcus milleri, Streptococcus mitior, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus pyogenes (group A streptococcus), Streptococcus salivarius, Streptococcus anginosus, Streptococcus bovis, Streptococcus canis, Streptococcus equi, Streptococcus milleri, Streptococcus mitior, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus pyogenes (group A streptococcus), Streptococcus salivarius, Streptococcus mitior.


As mentioned, the method of the present invention is effected by introducing into the cell a CRISPR array polynucleotide, wherein a spacer of the CRISPR array is sufficiently complementary with a portion of the gene to down-regulate expression of the gene.

Below is a short summary of CRISPR arrays.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) arrays together with the CAS genes form the CRISPR system.
CRISPR arrays also known as SPIDRs (SPacer Interspersed Direct Repeats) constitute a family of recently described DNA loci that are usually specific to a particular bacterial species. The CRISPR array is a distinct class of interspersed short sequence repeats (SSRs) that were first recognized in *E. coli* (Ishino et al, J. Bacterid., 169:5429-5433). In subsequent years, similar CRISPR arrays were found in *Mycobacterium tuberculosis, Haloferax mediterranei, Methanocaldococcus jannaschii, Thermotoga maritima* and other bacteria and archaea. The repeats of CRISPR arrays are short elements that occur in clusters that are always regularly spaced by unique intervening sequences with a constant length. Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions differ from strain to strain. The repeat sequences are partially palindromic DNA repeats typically of 24 to 47 bp, containing inner and terminal inverted repeats of up to 11 bp. These repeats have been reported to occur from 1 to 249 times. Although isolated elements have been detected, they are generally arranged in clusters (up to about 20 or more per genome) of repeated units spaced by unique intervening 26-72 bp sequences.

As used herein, the phrase "CRISPR array polynucleotide" refers to a DNA or RNA segment which comprises sufficient CRISPR repeats such that it is capable of downregulating a complementary gene.

According to one embodiment, the CRISPR array polynucleotide comprises at least 2 repeats with 1 spacer between them.

In an exemplary embodiment, the CRISPR array polynucleotide comprises all of the CRISPR repeats, starting with the first nucleotide of the first CRISPR repeat and ending with the last nucleotide of the last (terminal) repeat.

Various computer software and web resources are available for the analysis of and identification of CRISPR systems and therefore CRISPR arrays. These tools include software for CRISPR detection, such as PILERCR, CRISPR Recognition Tool and CRISPRFinder; online repositories of pre-analyzed CRISPRs, such as CRISPRdb; and tools for browsing CRISPRs in microbial genomes, such as Pygram. Databases for CRISPR systems include: httpV/crisprdot-psuddotfr/crispr/CRISPRHomePagedotphp.

It has been revealed that CRISPR systems are found in approximately 40% and 90% of sequenced bacterial and archaeal genomes, respectively, and the use of CRISPR arrays from all such CRISPR systems is contemplated.
According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate the gene of interest, is 100% homologous to the naturally occurring (wild-type) sequence.

According to another embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 99% homologous to the naturally occurring (wild-type) sequence.

According to another embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 98% homologous to the naturally occurring (wild-type) sequence.

According to another embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 97% homologous to the naturally occurring (wild-type) sequence.

According to another embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 96% homologous to the naturally occurring (wild-type) sequence.

According to another embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 95% homologous to the naturally occurring (wild-type) sequence.

According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 90% homologous to the naturally occurring (wild-type) sequence.

According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 80% homologous to the naturally occurring (wild-type) sequence.
According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 75% homologous to the naturally occurring (wild-type) sequence.

According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 70% homologous to the naturally occurring (wild-type) sequence.

According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 65% homologous to the naturally occurring (wild-type) sequence.

According to one embodiment, the CRISPR array polynucleotide comprises a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate a gene of interest, is 60% homologous to the naturally occurring (wild-type) sequence.

Particular examples of CRISPR arrays that may be used according to this aspect of the present invention include, but are not limited to the CRISPR array from the E.coli strain K12W31 10 (SEQ ID NO: 15,16); the CRISPR array from the Alkalilimnicola ehrlichei MLHE-I (SEQ ID NO: 17, 18); the CRISPR array from the Deinococcus geothermalis DSM 11300 plasmid 1 (SEQ ID NO: 19, 20) the CRISPR array from Clostridium thermocellum (SEQ ID NO: 21, 22) the CRISPR array from Shewanella baltica OS185 (SEQ ID NO: 23, 24).

Typically, once a CRISPR array is identified it may be amplified and isolated. Amplification of the CRISPR may be achieved by any method known in the art, including polymerase chain reaction (PCR). In the present invention, oligonucleotide primers may be designed for use in PCR reactions to amplify all or part of a CRISPR array.

The term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced (i.e., in the presence of nucleotides and an inducing agent - such as DNA polymerase and at a suitable temperature and pH). In some embodiments, the primer is single stranded for maximum efficiency in
amplification, although in other embodiments, the primer is double stranded. In some embodiments, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact length of the primers depends on many factors, including temperature, source of primer, and the use of the method. PCR primers are typically at least about 10 nucleotides in length, and most typically at least about 20 nucleotides in length. Methods for designing and conducting PCR are well known in the art, and include, but are not limited to methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially mismatched primers, etc.

Exemplary primers that can amplify the E.coli strain K12-W31 10 CRISPR array are set forth in SEQ ID NO: 13 and 14.

As mentioned, in order to down-regulate the prokaryotic gene of interest, a spacer of the CRISPR array is replaced with a nucleic acid sequence, the nucleic acid sequence being sufficiently complementary to a portion of the prokaryotic gene.

As used herein, the term "spacer" refers to a non-repetitive spacer sequence that is found between multiple short direct repeats (i.e., CRISPR repeats) of CRISPR arrays. In some preferred embodiments, CRISPR spacers are located in between two identical CRISPR repeats. In some embodiments, CRISPR spacers are identified by sequence analysis at the DNA stretches located in between two CRISPR repeats.

In some preferred embodiments, CRISPR spacer is naturally present in between two identical, short direct repeats that are palindromic.

The phrase "portion of a gene" relates to a portion from the coding or non-coding region of the gene.

The phrase "sufficiently complementary" as used herein, refers to the sequence of the spacer being adequately complementary such that it is capable of downregulating expression of the gene.

According to one embodiment of this aspect of the present invention, a sequence which is sufficiently complementary to a portion of the prokaryotic gene is one which is at least about 70, about 75, about 80, about 85, or about 90 % identical, or at least about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, or about 99 % identical to the prokaryotic gene. In some preferred embodiments, the sequence is 100 % complementary to the prokaryotic gene.
Assays to test down-regulation of expression are known in the art and may be effected on the RNA or protein level.

**Methods of detecting the expression level of RNA**

The expression level of the RNA in prokaryotic cells can be determined using methods known in the arts.

**Northern Blot analysis:** This method involves the detection of a particular RNA in a mixture of RNAs. An RNA sample is denatured by treatment with an agent (e.g., formaldehyde) that prevents hydrogen bonding between base pairs, ensuring that all the RNA molecules have an unfolded, linear conformation. The individual RNA molecules are then separated according to size by gel electrophoresis and transferred to a nitrocellulose or a nylon-based membrane to which the denatured RNAs adhere. The membrane is then exposed to labeled DNA probes. Probes may be labeled using radio-isotopes or enzyme linked nucleotides. Detection may be using autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of particular RNA molecules and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the gel during electrophoresis.

**RT-PCR analysis:** This method uses PCR amplification of relatively rare RNAs molecules. First, RNA molecules are purified from the cells and converted into complementary DNA (cDNA) using a reverse transcriptase enzyme (such as an MMLV-RT) and primers such as, oligo dT, random hexamers or gene specific primers. Then by applying gene specific primers and Taq DNA polymerase, a PCR amplification reaction is carried out in a PCR machine. Those of skills in the art are capable of selecting the length and sequence of the gene specific primers and the PCR conditions (i.e., annealing temperatures, number of cycles and the like) which are suitable for detecting specific RNA molecules. It will be appreciated that a semi-quantitative RT-PCR reaction can be employed by adjusting the number of PCR cycles and comparing the amplification product to known controls.

**RNA in situ hybridization stain:** In this method DNA or RNA probes are attached to the RNA molecules present in the cells. Generally, the cells are first fixed to microscopic slides to preserve the cellular structure and to prevent the RNA molecules from being degraded and then are subjected to hybridization buffer containing the labeled probe. The hybridization buffer includes reagents such as formamide and salts (e.g., sodium chloride and sodium citrate) which enable specific hybridization of the DNA or RNA probes with their target mRNA molecules *in situ* while avoiding non-specific binding of probe. Those of skills
in the art are capable of adjusting the hybridization conditions (i.e., temperature, concentration of salts and formamide and the like) to specific probes and types of cells. Following hybridization, any unbound probe is washed off and the slide is subjected to either a photographic emulsion which reveals signals generated using radio-labeled probes or to a colorimetric reaction which reveals signals generated using enzyme-linked labeled probes.

**In situ RT-PCR stain:** This method is described in Nuovo GJ, et al. [Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am J Surg Pathol. 1993, 17: 683-90] and Komminoth P, et al. [Evaluation of methods for hepatitis C virus detection in archival liver biopsies. Comparison of histology, immunohistochemistry, in situ hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and in situ RT-PCR. Pathol Res Pract. 1994, 190: 1017-25]. Briefly, the RT-PCR reaction is performed on fixed cells by incorporating labeled nucleotides to the PCR reaction. The reaction is carried on using a specific in situ RT-PCR apparatus such as the laser-capture microdissection PixCell 1 LCM system available from Arcturus Engineering (Mountainview, CA).

**DNA microarrays/DNA chips:**

The expression of thousands of genes may be analyzed simultaneously using DNA microarrays, allowing analysis of the complete transcriptional program of an organism during specific developmental processes or physiological responses. DNA microarrays consist of thousands of individual gene sequences attached to closely packed areas on the surface of a support such as a glass microscope slide. Various methods have been developed for preparing DNA microarrays. In one method, an approximately 1 kilobase segment of the coding region of each gene for analysis is individually PCR amplified. A robotic apparatus is employed to apply each amplified DNA sample to closely spaced zones on the surface of a glass microscope slide, which is subsequently processed by thermal and chemical treatment to bind the DNA sequences to the surface of the support and denature them. Typically, such arrays are about 2 x 2 cm and contain about individual nucleic acids 6000 spots. In a variant of the technique, multiple DNA oligonucleotides, usually 20 nucleotides in length, are synthesized from an initial nucleotide that is covalently bound to the surface of a support, such that tens of thousands of identical oligonucleotides are synthesized in a small square zone on the surface of the support. Multiple oligonucleotide sequences from a single gene are synthesized in neighboring regions of the slide for analysis of expression of that gene. Hence, thousands of genes can be represented on one glass slide. Such arrays of synthetic oligonucleotides may be referred to in the art as "DNA chips", as opposed to "DNA

Oligonucleotide microarray - In this method oligonucleotide probes capable of specifically hybridizing with the polynucleotides of the present invention are attached to a solid surface (e.g., a glass wafer). Each oligonucleotide probe is of approximately 20-25 nucleic acids in length. To detect the expression pattern of the polynucleotides of the present invention in a specific cell sample (e.g., blood cells), RNA is extracted from the cell sample using methods known in the art (using e.g., a TRIZOL solution, Gibco BRL, USA). Hybridization can take place using either labeled oligonucleotide probes (e.g., 5'-biotinylated probes) or labeled fragments of complementary DNA (cDNA) or RNA (cRNA). Briefly, double stranded cDNA is prepared from the RNA using reverse transcriptase (RT) (e.g., Superscript II RT), DNA ligase and DNA polymerase I, all according to manufacturer's instructions (Invitrogen Life Technologies, Frederick, MD, USA). To prepare labeled cRNA, the double stranded cDNA is subjected to an in vitro transcription reaction in the presence of biotinylated nucleotides using e.g., the BioArray High Yield RNA Transcript Labeling Kit (Enzo, Diagnostics, Affymetix Santa Clara CA). For efficient hybridization the labeled cRNA can be fragmented by incubating the RNA in 40 mM Tris Acetate (pH 8.1), 100 mM potassium acetate and 30 mM magnesium acetate for 35 minutes at 94 °C. Following hybridization, the microarray is washed and the hybridization signal is scanned using a confocal laser fluorescence scanner which measures fluorescence intensity emitted by the labeled cRNA bound to the probe arrays.

For example, in the Affymetrix microarray (Affymetrix®, Santa Clara, CA) each gene on the array is represented by a series of different oligonucleotide probes, of which, each probe pair consists of a perfect match oligonucleotide and a mismatch oligonucleotide. While the perfect match probe has a sequence exactly complimentary to the particular gene, thus enabling the measurement of the level of expression of the particular gene, the mismatch probe differs from the perfect match probe by a single base substitution at the center base position. The hybridization signal is scanned using the Agilent scanner, and the Microarray Suite software subtracts the non-specific signal resulting from the mismatch probe from the signal resulting from the perfect match probe.

Methods of detecting expression and/or activity of proteins

22
Expression and/or activity level of proteins expressed in prokaryotic cells can be determined using methods known in the arts.

**Enzyme linked immunosorbent assay (ELISA):** This method involves fixation of a sample (e.g., fixed cells or a proteinaceous solution) containing a protein substrate to a surface such as a well of a microtiter plate. A substrate specific antibody coupled to an enzyme is applied and allowed to bind to the substrate. Presence of the antibody is then detected and quantitated by a colorimetric reaction employing the enzyme coupled to the antibody. Enzymes commonly employed in this method include horseradish peroxidase and alkaline phosphatase. If well calibrated and within the linear range of response, the amount of substrate present in the sample is proportional to the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

**Western blot:** This method involves separation of a substrate from other protein by means of an acrylamide gel followed by transfer of the substrate to a membrane (e.g., nylon or PVDF). Presence of the substrate is then detected by antibodies specific to the substrate, which are in turn detected by antibody binding reagents. Antibody binding reagents may be, for example, protein A, or other antibodies. Antibody binding reagents may be radiolabeled or enzyme linked as described hereinabove. Detection may be by autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of substrate and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

**Radio-immunoassay (RIA):** In one version, this method involves precipitation of the desired protein (i.e., the substrate) with a specific antibody and radiolabeled antibody binding protein (e.g., protein A labeled with I$^{125}$) immobilized on a precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of substrate.

In an alternate version of the RIA, a labeled substrate and an unlabelled antibody binding protein are employed. A sample containing an unknown amount of substrate is added in varying amounts. The decrease in precipitated counts from the labeled substrate is proportional to the amount of substrate in the added sample.

**Fluorescence activated cell sorting (FACS):** This method involves detection of a substrate in situ in cells by substrate specific antibodies. The substrate specific antibodies are linked to fluorophores. Detection is by means of a cell sorting machine which reads the
wavelength of light emitted from each cell as it passes through a light beam. This method may employ two or more antibodies simultaneously.

**Immunohistochemical analysis:** This method involves detection of a substrate in situ in fixed cells by substrate specific antibodies. The substrate specific antibodies may be enzyme linked or linked to fluorophores. Detection is by microscopy and subjective or automatic evaluation. If enzyme linked antibodies are employed, a colorimetric reaction may be required. It will be appreciated that immunohistochemistry is often followed by counterstaining of the cell nuclei using for example Hematoxyline or Giemsa stain.

**In situ activity assay:** According to this method, a chromogenic substrate is applied on the cells containing an active enzyme and the enzyme catalyzes a reaction in which the substrate is decomposed to produce a chromogenic product visible by a light or a fluorescent microscope.

**In vitro activity assays:** In these methods the activity of a particular enzyme is measured in a protein mixture extracted from the cells. The activity can be measured in a spectrophotometer well using colorimetric methods or can be measured in a non-denaturing acrylamide gel (i.e., activity gel). Following electrophoresis the gel is soaked in a solution containing a substrate and colorimetric reagents. The resulting stained band corresponds to the enzymatic activity of the protein of interest. If well calibrated and within the linear range of response, the amount of enzyme present in the sample is proportional to the amount of color produced. An enzyme standard is generally employed to improve quantitative accuracy.

According to one embodiment, the spacer which is replaced has the same number of base pairs as the "replacing spacer" i.e. the one that is complementary to the prokaryotic gene.

According to another embodiment, at least two spacers of the CRISPR are replaced with a nucleic acid sequence, each nucleic acid sequence being sufficiently complementary to opposite strands of the gene.

It is contemplated that it is possible to replace any number of the spacers of the wild-type CRISPR.

The replacement spacers may target the same gene or a number of different genes. According to one embodiment, at least about 10% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 20% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 30% of
the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 40% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 50% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 60% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 70% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 80% of the spacers are exchanged for a replacing spacer. According to another embodiment, at least about 90% of the spacers are exchanged for a replacing spacer. According to still another embodiment, about 100% of the spacers are exchanged for a replacing spacer.

According to one embodiment, at least one of the replaced spacers in the CRISPR array of the present invention is the one which is at the most 5’ end of the array.

It will be appreciated that the polynucleotide which comprises the modified CRISPR array of the present invention may also comprise other sequences.

Thus, for example, the modified CRISPR array of the present invention may also comprise a leader sequence 5’ to the array.

The CRISPR leader is a conserved DNA segment of defined size which is located immediately upstream of the first repeat.

The leader sequence can be of a different length in different bacteria. In some embodiments, the leader sequence is at least about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95, about 100, about 200, about 300, about 400, or about 500 or more nucleotides in length.

For example, the leader sequence of S. thermophilicis CRISPR1 is the DNA segment starting immediately after the stop codon of gene str0660, and ending just before the first repeat. The CRISPR leader in the CRISPR1 locus of Streptococcus thermophilus strain CNRZ1 066 is: 5’-

CAAGGACAGTTATTTTATAATCACTATGIGGTATAAAAAACGTCAAAATTT
CATTGTA G-3’ (SEQ ID NO: 25). The CRISPR leader in the CRISPR1 locus of E. coli W3110 CRISPR system is: 5’-

AAACAAAGAATTAGCTGATCTTTTATAATAAGAAATGTACATTACATATGTTGTA GGGTTTTTTATGGGAAAAATGCCTTT AAGAAACAAATGT TGTTTTAGA-3’ (SEQ ID NO: 26).
According to one embodiment, the leader sequence is directly 5' to the array with no intervening base pairs.

According to another embodiment the leader sequence is the same leader sequence found in the wild type CRISPR system.

Thus, for example if the CRISPR array that is introduced into the prokaryotic cell is derived from the E. coli W3110 CRISPR system, then the leader sequence is that leader sequence that is found in the wild-type E. coli W3110 CRISPR system.

The modified CRISPR array of the present invention may also comprise a nucleic acid sequence encoding one or more CAS proteins (i.e. cas genes).

As used herein, the term "cas gene" refers to the genes that are generally coupled, associated or close to or in the vicinity of flanking CRISPR arrays that encode CAS proteins.

CRISPR arrays are typically found in the vicinity of four genes named casl to cas4. The most common arrangement of these genes is cas3-cas4-cas 1-cas2. The Cas3 protein appears to be a helicase, whereas Cas4 resembles the RecB family of exonucleases and contains a cysteine-rich motif, suggestive of DNA binding. The casl gene (NCBI COGs database code: COGl 518) is especially noteworthy, as it serves as a universal marker of the CRISPR system (linked to all CRISPR systems except for that of *Pyrococcus abyssi*). Cas2 remains to be characterized casl-4 are typically characterized by their close proximity to the CRISPR loci and their broad distribution across bacterial and archaeal species. Although not all casl-4 genes associate with all CRISPR loci, they are all found in multiple subtypes.

In addition, there is another cluster of three genes associated with CRISPR structures in many bacterial species, referred to herein as cas B, cas5 and casó; (See, Barrangou et al, 2007, Science 315(5819): 1709-12). In some embodiments, the cas gene is selected from casl, cas2, cas3, cas4, casIB, cas5 and/or casó, fragments, variants, homologues and/or derivatives thereof. In some additional embodiments, a combination of two or more cas genes find use, including any suitable combinations, including those provided in WO 07/025097, incorporated herein by reference.

In some embodiments, the cas genes comprise DNA, while in other embodiments, the cas comprise RNA. In some embodiments, the nucleic acid is of genomic origin, while in other embodiments, it is of synthetic or recombinant origin. In some embodiments, the cas genes are double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof.
In some embodiments it is preferred that the cas gene is the cas gene that is closest to
the leader sequence or the first CRISPR repeat at the 5’ end of the CRISPR locus- such as
cas4 or cas6.

It will be appreciated that a given set of cas genes or proteins is typically associated
with a given repeated sequence within a particular CRISPR array. Thus, cas genes appear to
be specific for a given DNA repeat (i.e., cas genes and the repeated sequence form a
functional pair).

Thus, for example if the CRISPR array which is used to downregulate a gene
comprises the same repeat sequences as the CRISPR array from W3110 CRISPR system,
then the cas genes that may also be comprised in the polynucleotide may be those from the
W3110 CRISPR system. According to one embodiment, the same number of cas genes are
added to the polynucleotide as the number of cas genes that appear in the original system.
According to another embodiment, at least one of the cas genes that appears in the original
system is added to the polynucleotide.

Thus for example, cas genes that appear in the W3110 CRISPR system include Cas3
(SEQ ID NO: 27), CasA SEQ ID NO: 28, CasB SEQ ID NO: 29, CasC SEQ ID NO: 30,
CasD SEQ ID NO: 31, CasE SEQ ID NO: 32, Casl SEQ ID NO: 33 and Cas2 (SEQ ID NO:
34). Polynucleotides comprising repeat sequences from the W3110 CRISPR system may
therefore preferably comprise at least one of these sequences.

Preferably, a cas gene comprises at least 50%, more preferably at least 65%, more
preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more
preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more
preferably at least 98%, most preferably at least 99% of the wild-type sequence. Preferably, a
cas gene retains 50%, more preferably 60%, more preferably 70%, more preferably 80%,
more preferably 85%, more preferably 90%, more preferably 95%, more preferably 96%,
more preferably 97%, more preferably 98%, or most preferably 99% activity of the wild-type
polypeptide or nucleotide sequence.

Other exemplary cas gene sequences are provided in SEQ ID NOs: 35-91.

According to an exemplary embodiment, where a prokaryotic cell already comprises a
CRISPR system, the CRISPR array polynucleotide which is introduced into the cell
comprises the identical CRISPR array repeat sequence which is endogenous to that bacteria
(it does not necessarily have to have an array of the same size). Accordingly, the choice of
CRISPR array that is introduced into a cell is mainly dependent on the prokaryote whose gene or genes are being down-regulated.

In the case where the prokaryotic cell does not comprise a CRISPR system it will be appreciated that any CRISPR array may be introduced into the cell. According to this embodiment, the other components which make up the CRISPR system are also introduced into the cell. Such components typically match the CRISPR array (i.e. originate from the same CRISPR system). The other components may be introduced into the cell (together with a non-modified, naive spacer, or on their own) prior to administration of the CRISPR array with the modified spacer. Alternatively, the other components may be introduced into the cell concomitant with (on the same or on a separate vector) the CRISPR array with the modified spacer.

Typically, the polynucleotides of the present invention are inserted into nucleic acid constructs so that they are capable of being expressed and propagated in bacterial cells.

Such nucleic acid constructs typically comprise a prokaryotic origin of replication and other elements which drive the expression of the CRISPR array and associated cas genes.

Preferably, the promoter utilized by the nucleic acid construct of the present invention is active in the specific cell population transformed.

Constitutive promoters suitable for use with the present invention are promoter sequences which are active under most environmental conditions and most types of cells such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV).

According to one embodiment, the promoter is an inducible promoter, i.e., a promoter that induces the CRISPR expression only in a certain condition (e.g. heat-induced promoter) or in the presence of a certain substance (e.g., promoters induced by Arabinose, Lactose, IPTG, etc).

Examples of bacterial constructs include the pET series of E. coli expression vectors (Studier et al. (1990) Methods in Enzymol. 185:60-89).

Methods of introducing the polynucleotides of the present invention into prokaryotic cells are well known in the art - these include, but are not limited to, transforming with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the CRISPR array sequence.

It will be appreciated that downregulating bacterial genes that are essential or vital to bacterial functioning may be used as a method for treating a bacterial infection. Similarly,
downregulating bacterial genes that are associated with bacterial virulence may also be used as a method for treating a bacterial infection.

Thus, according to another aspect of the present invention, there is provided a method of treating a bacterial infection in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an isolated polynucleotide, comprising a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of the CRISPR is sufficiently complementary to a portion of at least one bacterial gene so as to down-regulate expression of the bacterial gene, the bacterial gene being a vital bacterial gene or a bacterial virulence gene, thereby treating the bacterial infection.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

The phrase "bacterial infection" as used herein, refers the invasion and colonization of bacteria in a bodily tissue producing subsequent tissue injury and disease.

The bacterial infection may be on the body surface, localized (e.g., contained within an organ, at a site of a surgical wound or other wound, within an abscess), or may be systemic (e.g., the subject is bacteremic, e.g., suffers from sepsis). Of particular interest is the treatment of bacterial infections that are amenable to therapy by topical application of the phage of the invention. Also of particular interest is the treatment of bacterial infections that are present in an abscess or are otherwise contained at a site to which the phage of the invention can be administered directly.

The present invention also contemplates coating of surfaces other than body surfaces with the constructs of the present invention. U.S. Pat. No. 6627215 teaches coating of wound dressings with nucleic acids that comprise anti-bacterial activity. U.S. Pat. No. 6,617,142 teaches methods for attaching DNA or RNA to medical device surfaces.

Contacting a surface with the constructs can be effected using any method known in the art including spraying, spreading, wetting, immersing, dipping, painting, ultrasonic welding, welding, bonding or adhering. The peptides of the present invention may be attached as monolayers or multiple layers.
The present invention coating a wide variety of surfaces with the constructs of the present invention including fabrics, fibers, foams, films, concretes, masonries, glass, metals, plastics, polymers, and like.

An exemplary solid surface that may be coated with the peptides of the present invention is an intracorporial or extra-corporeal medical device or implant.

An "implant" as used herein refers to any object intended for placement in a human body that is not a living tissue. The implant may be temporary or permanent. Implants include naturally derived objects that have been processed so that their living tissues have been devitalized. As an example, bone grafts can be processed so that their living cells are removed (acellularized), but so that their shape is retained to serve as a template for ingrowth of bone from a host. As another example, naturally occurring coral can be processed to yield hydroxyapatite preparations that can be applied to the body for certain orthopedic and dental therapies. An implant can also be an article comprising artificial components.

Thus, for example, the present invention therefore envisions coating vascular stents with the peptides of the present invention. Another possible application of the peptides of the present invention is the coating of surfaces found in the medical and dental environment.

Surfaces found in medical environments include the inner and outer aspects of various instruments and devices, whether disposable or intended for repeated uses. Examples include the entire spectrum of articles adapted for medical use, including scalpels, needles, scissors and other devices used in invasive surgical, therapeutic or diagnostic procedures; blood filters, implantable medical devices, including artificial blood vessels, catheters and other devices for the removal or delivery of fluids to patients, artificial hearts, artificial kidneys, orthopedic pins, plates and implants; catheters and other tubes (including urological and biliary tubes, endotracheal tubes, peripherally insertable central venous catheters, dialysis catheters, long term tunneled central venous catheters peripheral venous catheters, short term central venous catheters, arterial catheters, pulmonary catheters, Swan-Ganz catheters, urinary catheters, peritoneal catheters), urinary devices (including long term urinary devices, tissue bonding urinary devices, artificial urinary sphincters, urinary dilators), shunts (including ventricular or arterio-venous shunts); prostheses (including breast implants, penile prostheses, vascular grafting prostheses, aneurysm repair devices, heart valves, artificial joints, artificial larynxes, otological implants), anastomotic devices, vascular catheter ports, clamps, embolic devices, wound drain tubes, hydrocephalus shunts, pacemakers and
implantable defibrillators, and the like. Other examples will be readily apparent to practitioners in these arts.

Surfaces found in the medical environment include also the inner and outer aspects of pieces of medical equipment, medical gear worn or carried by personnel in the health care setting. Such surfaces can include counter tops and fixtures in areas used for medical procedures or for preparing medical apparatus, tubes and canisters used in respiratory treatments, including the administration of oxygen, of solubilized drugs in nebulizers and of anesthetic agents. Also included are those surfaces intended as biological barriers to infectious organisms in medical settings, such as gloves, aprons and face shields. Commonly used materials for biological barriers may be latex-based or non-latex based. Vinyl is commonly used as a material for non-latex surgical gloves. Other such surfaces can include handles and cables for medical or dental equipment not intended to be sterile. Additionally, such surfaces can include those non-sterile external surfaces of tubes and other apparatus found in areas where blood or body fluids or other hazardous biomaterials are commonly encountered. Other surfaces include medical gauzes and plasters such as band-aids.

Other surfaces related to health include the inner and outer aspects of those articles involved in water purification, water storage and water delivery, and those articles involved in food processing. Thus the present invention envisions coating a solid surface of a food or beverage container to extend the shelf life of its contents.

Surfaces related to health can also include the inner and outer aspects of those household articles involved in providing for nutrition, sanitation or disease prevention. Examples can include food processing equipment for home use, materials for infant care, tampons and toilet bowls.

Typically, the subject being treated is a mammalian subject - e.g. human, fowl, rodent or primate.

According to one embodiment, the above-mentioned nucleic acid construct is administered as naked DNA or in a carrier - such as a liposome. According to another embodiment of this aspect of the present invention the polynucleotides are delivered to the bacteria using a targeting moiety (see for example Yacoby and Benhar, Infect Disord Drug Targets. 2007 Sep;7(3):221-9).

Thus, according to another embodiment of this aspect of the present invention, the subject is administered with the polynucleotides of the present invention using bacteriophages. According to one embodiment, the bacteriophages are lytic phages.
Treatment of bacterial infections using bacteriophages is well known in the art.

Bacteriophage(s) suitable for use in treatment of a subject can be selected based upon the suspected bacterial pathogen infecting the subject. Methods for diagnosis of bacterial infections and determination of their sensitivities are well known in the art. Where such diagnosis involves culturing a biological sample from the subject, the clinician can at the same time test the susceptibility of the infecting pathogen to growth inhibition by one or more therapeutic phages that are candidates for subsequent therapy.

In order to address the problem of rapid clearance by the spleen, liver and the reticuloendothelial system of bacteriophages, long-circulating variants of wild type phages (see for example Merrill et al (Proc. Natl. Acad. Sci. USA 93, 3188 (1996) and U.S. Pat. No. 5,688,501) or holing modified bacteriophages - see for example U.S. Pat. Appl. 2004015683 l - can be used.

Selection of the gene to be downregulated will depend on the bacterial infection being treated. According to one embodiment, the spacers of the modified CRISPRs of the present invention are designed such that they target a gene that is highly conserved among bacteria; such spacers will lead to broad-spectrum killing. According to one embodiment, the spacers of the modified CRISPRs of the present invention are designed such that they target a gene that is unique to a specific bacteria; such spacers will lead to narrow-spectrum killing.

According to another embodiment, the bacterial infection being treated is an antibiotic resistant bacterial infection - e.g. infections induced by methicillin resistant Staphylococcus aureus or vancomycin resistant Staphylococcus aureus. In this case, the spacers of the modified CRISPRs of the present invention are designed such that they target an antibiotic resistance gene.

The bacteriophages comprising the modified CRISPRs of the present invention may be administered per se, or as part of a pharmaceutical composition.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of the active agent to an organism.

Herein the term "active ingredient" refers to the modified CRISPR accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does
not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the
art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. 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 a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water biased solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of bacterial infection or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.
Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.l).

Dosage amount and interval may be adjusted individually to provide tissue or blood levels of the active ingredient are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which 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 pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier.
may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

As mentioned, the modified bacteria of the present invention may be present in food products as well as in food additives.

The phrase "food additive" [defined by the FDA in 21 C.F.R. 170.3(e)(1)] includes any liquid or solid material intended to be added to a food product. This material can, for example, include an agent having a distinct taste and/or flavor or a physiological effect (e.g., vitamins).

The food additive composition of the present invention can be added to a variety of food products.

As used herein, the phrase "food product" describes a material consisting essentially of protein, carbohydrate and/or fat, which is used in the body of an organism to sustain growth, repair and vital processes and to furnish energy. Food products may also contain supplementary substances such as minerals, vitamins and condiments. See Merriam-Webster's Collegiate Dictionary, 10th Edition, 1993. The phrase "food product" as used herein further includes a beverage adapted for human or animal consumption. A food product containing the food additive of the present invention can also include additional additives such as, for example, antioxidants, sweeteners, flavorings, colors, preservatives, nutritive additives such as vitamins and minerals, amino acids (i.e. essential amino acids), emulsifiers, pH control agents such as acidulants, hydrocolloids, antifoams and release agents, flour improving or strengthening agents, raising or leavening agents, gases and chelating agents, the utility and effects of which are well-known in the art.

It will be appreciated that the CRISPR polynucleotides of the present invention may also be used to identify a function of a particular prokaryotic gene.

According to this aspect of the present invention, the modified CRISPR polynucleotides of the present invention are introduced into prokaryotic cells, wherein a spacer of the CRISPR is directed against a prokaryotic gene of unknown function. A phenotype of the prokaryote is then assayed. Depending on the outcome of the assay, the function of the gene can be determined (i.e. annotated).

According to one embodiment, the phenotype is examined using "phenotype microarray analysis" - see for example Zhou et al Journal of Bacteriology, August 2003, p. 4956-4972, Vol. 185, No. 16.
The modified CRISPR arrays of the present invention may also be used to generate a library of clones, each of which containing a different down-regulated gene. Such libraries have been prepared for Escherichia coli K-12, [Baba et al., Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection" Molecular Systems Biology 2 Article number: 2006.0008]. However, construction of this library was extremely labor intensive and expensive. Organisms of interest for production of such libraries include bio-energy relevant organisms such as Synechocystis sp. PCC 6803 or in which it is needed to determine which genes are needed to be silenced in order to enhance the production of the desired output biofuel. This could also be done for organisms generating a biotechnologically relevant product (other than a bio-fuel).

As mentioned, the constructs of the present invention may be useful in the generation of biofuels in bacteria.

Thus, according to another aspect of the present invention, there is provided a method of generating an organic material in bacteria, the method comprising downregulating a gene which compromises the generation of the organic material in the bacteria.

It is expected that during the life of a patent maturing from this application many relevant CRISPR arrays and systems will be identified and the scope of the term CRISPR array is intended to include all such new technologies apriori

As used herein the term "about" refers to ± 10 %

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single
embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non-limiting fashion.

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non-limiting fashion.


**EXAMPLE 1**

*Down-regulation of two genes in E. coli bacteria*

As a proof of concept, a CRISPR system of E. coli may be used to silence two genes in E. coli which constitutively express GFP. The two genes are GFP and MaliF. Silencing of GFP is expected to result in loss of fluorescent emission, whilst silencing of MaliF is expected to result in loss of ability to grow on maltose as the sole carbon source. A typical system that may be used is one described in Bronus et al (Science 321, 960 (2008)), incorporated herein by reference. In the present case, the spacers will target the cellular genes MaliF and GFP. In each construct two spacers will be designed targeting the sense strand of each gene (C spacers) and 2 spacers will be targeting the antisense strand of each gene (T spacers).

In brief, the CRISPR/cas gene cluster from the E.coli strain K12W31 10 will be amplified using the following primers:

5'GGCGCGCCATGGGAAACAAAGAATT (SEQ ID NO: 13);
3'TTAATTAAGGTACCCTCCGTCTTGATGGGT (SEQ ID NO: 14).

The amplified DNA will be used to transform the naive GFP expressing E. coli strain BL21(DE3). In addition, CRISPR constructs will be transformed into the above described constructs.

The CRISPR constructs for silencing GFP expression and MaliF expression in E. coli are illustrated in Figures 1-4, 7 and 9. The GFP and MaliF sequences are illustrated in Figures 5-6, 8 and 10.
EXAMPLE 2

Selective silencing of endogenous genes in bacteria

As noted above, it has been proposed that the CRISPR system is analogous to the eukaryotic RNAi system and that the spacers function as prokaryotic siRNAs by base-pairing with foreign mRNAs and promoting their degradation. Manipulated CRISPR systems could selective gene knockdown without manipulation of the original microbial genome. Instead of knocking out the gene of interest, which is usually labour intensive, the same effect could be achieved by transforming a CRISPR-bearing plasmid into the organism of choice, with one of the spacers being changed to match the studied gene (Fig. 20). Moreover, the array nature of CRISPRs could allow the simultaneous knockdown of multiple endogenous genes. Similar RNAi-based applications have revolutionized eukaryotic genetic studies; we envisage that CRISPRs would have a similar impact in the field of microbial genetics.

Referring now to Fig. 19, fragments from a chromosome-encoded gene (green) are engineered into a CRISPR array as spacers. If, as suggested, the CRISPR system indeed functions by the silencing of RNA 16, this should lead to silencing of the endogenous gene.

The proposed analogy between the CRISPR system and eukaryotic RNAi raises another possible important role for CRISPRs. In eukaryotes, RNAi functions both in silencing foreign elements through siRNAs, as well as endogenous gene regulation through genome-encoded micro-RNAs. Analogously, it is possible that CRISPR systems regulate endogenous functions in different bacteria. Indeed, 7-35% of the spacers found in CRISPR arrays have homologues in the chromosomal DNA, which may indicate that CRISPR is being used to regulate the expression of chromosomally derived genes (Bolotin, A., Quinquis, B., Sorokin, A. & Ehrlich, S. D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151, 2551-2561 (2005); Horvath, P. et al. Diversity, activity and evolution of CRISPR loci in Streptococcus thermophilus. J. Bacteriol. 7 Dec 2007 (doi: 10.1128/JB.01415-07)). For example, the devTRS operon in Myxococcus xanthus, which encodes genes that are essential for spore differentiation inside fruiting bodies, is co-transcribed within a CRISPR operon, with DevS being a bona fide Cas5 protein (Haft, D. H., Selengut, J., Mongodin, E. F. & Nelson, K. E. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput. Biol. 1, e60 (2005); Viswanathan, P., Murphy, K., Julien, B., Garza, A. G. & Kroos, L. Regulation of dev, an operon that includes genes
essential for *Myxococcus xanthus* development and CRISPR-associated genes and repeats. *J. Bacterid.* 189, 3738-3750 (2007)). Use of the plasmid to silence or downregulate gene could be modeled after this possible example of a CRISPR system that regulates an endogenous mechanism.

**EXAMPLE 3**

**CRISPR Mechanism of Action**

maintaining its record in the form of CRISPR spacers. According to the proposed mechanism, the spacers are then used to identify and deactivate subsequent invasions by an RNAi-like mechanism. It was therefore speculated that the CRISPR/CAS system is a prokaryotic analog of an immune system [Mojica et al., J Mol Evol 2005, 60(2):174-182].

Despite the intriguing speculations based on bioinformatics findings, this hypothesis has not yet been verified experimentally. To date, the expression of CRISPR and subsequent cleavage of the transcript into repeat-spacer units are the only experimental results obtained [Tang TH, Bachellerie JP, Rozhdestvensky T, Bortolin ML, Huber H, Drungowski M, Elge T, Brosius J, Huttenhofer A: Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. Proc Natl Acad Sci USA 2002, 99(1):7536-7541]. Indirect evidence was also gained when a CRISPR-containing strain with a relevant spacer was found to be resistant against viral infection, while another strain lacking the CRISPR protection succumbed to the infection. However, other differences between the strains could contribute to the observed differences in reaction to phage.

1. Phage infection prevention by CRISPR/CAS system. The goals of the proposed work are to (1) verify the antiviral function of CRISPR/CAS system and (2) elucidate the mechanism of action. We, first, would like to verify whether the CRISPR/CAS system has an antiviral activity, then we intend to identify the molecule (DNA or RNA) on which antiviral action is performed. A description of the proposed work identifying specificity and components of the system follows.

The first step of the proposed work is to verify the antiviral effect of the system. According to the proposed mechanism, the fragments of viral DNA are stored as CRISPR spacers and used to recognize attacking viruses. A strain susceptible to a viral infection is expected to gain resistance by obtaining appropriate CRISPR spacers. Therefore, one can expect that an artificial insertion of a CRISPR cassette containing a conserved fragment of an essential viral gene should provide resistance against this particular phage. We, therefore, plan to identify a bacterial strain with a functional CRISPR susceptible to a phage, and then transform it with a CRISPR, containing an appropriate spacer, and challenge the strain with a phage and look for acquired immunity against for this phage. If the transformation induces resistance against phage, the anti-viral action of the CRISPR will be shown.

2. Identification of the level of CRISPR action. It was previously suggested the CRISPR/CAS system operates by an RNAi-like mechanism. However, even if it indeed provides immunity against phages the level on which this system acts on is still unknown, it
might operate on RNA as well as on DNA level. An RNA transcript of a CRISPR spacer can potentially identify invasive DNA and initiate a digestion that would result in the destruction of the intruder. We intend to determine whether the deactivation of the invader is performed on the DNA or RNA level. With this goal, a construct assembled from a plasmid containing a spacer from the relevant CRISPR species and a green florescent protein (GFP) under the control of an inducable promoter (Fig. 20) will be introduced into the CRISPR containing strains. A flow chart for such an experimental study is shown in Figure 2.

It is expected that if the immunity acts on the DNA level and does not require transcription of the spacer matching sequence, the transformation will result in nonviable cells. On the other hand, viable cells upon transformation indicate the action on RNA level. If the transformation is successful, the transformants will then be induced to express the spacer matching sequence, and the expression of GFP and the viability of the cells on the selective media will be observed. While GFP expression indicates the failure of the CRISPR system to prevent active transcripts, the lack of GFP expression will indicate the activity of the system. Viable cells with no GFP expression in the selective media confirm the RNAi-like mechanism. However, the absence of GFP expression due to the cell death shows that the presence of spacer matching sequence in the transcript caused degradation of the plasmid DNA. In short, any results obtained from the proposed work will provide insights into the mechanism of action of CRISPR/CAS system.

**MATERIALS & METHODS and PRELIMINARY DATA.** In order to carry out the work proposed above, we have obtained four bacterial strains each belonging to a different species, namely *Acinetobacter* sp. ADPI, *P. aeruginosa* UCBPP-PA 14, *S. typhimurium* LT2, and *E. coli* K12 MG1655. Candidate CRISPRs from each species were determined on IMG database (Joint Genome Institute, Walnut Creek, CA) and sequences of these CRISPRs were confirmed by sequencing. Upon verification of the sequences, several spacers from each strain were selected for further characterization.

A broad-host range vector, pZB, containing an inducible promoter (tet promoter, Pztl) and chloramphenicol resistance gene (CmR) was our vector of choice for the generation of the constructs will be used in this study. By transforming pZB into the four strains, we have confirmed that this plasmid is indeed broad-host range and can be transformed into our choice of microorganisms without any difficulties.

1. **Phage immunity study:** We will obtain viruses that lyse the strains in question. For each strain mentioned above, we will generate two alternative constructs, both containing
the same CRISPR but with an addition of a single oligonucleotide from a conserved area in a vital gene of a compatible phage as a CRISPR spacer. Both plasmids will have a single insertion of the same spacer, only differing in orientation of the spacer. The resulting plasmids will then be transformed into corresponding bacterial cells, and the transformants will be induced and their susceptibility to phage infection will be assessed. A plasmid containing both the CRISPR region and the oligo from the phage will be used as a control. The oligo in the control should NOT be embedded into the CRISPR as a spacer.

To distinguish DNA from RNA activity, we introduced a variety of plasmids into four different bacterial hosts. All plasmids contained an inducible promoter, selection marker (antibiotic resistance gene), reporter molecule (GFP) and one CRISPR spacer sequence (the presumed targets of CRISPR activity). Depending on the mode of action, we anticipated a number of different possible outcomes; i) no growth indicating that the plasmid had been degraded (DNA level action) and the cells would not grow on antibiotic-containing growth medium, ii) growth without GFP expression indicating that the messenger RNA containing the CRISPR spacer and GFP sequence in the open reading frame had been degraded, indicating that CRISPR acts on the RNA level.

We selected 4 microorganisms to work with, E. coli K12 MG1655, Acinetobacter sp. ADPI, Salmonella typhimurium LT2, and Pseudomonas aeruginosa UCBPP-PA14 all of which has at least one CRISPR array. Primers were designed for various spacers in these microorganisms which were amplified. pZB was the selected vector, which has a chloramphenical resistance cassette and gfp under the control of a PBAD promoter and rfp under the control of Pitzl (tet) promoter. (Lee et al., 2005. J Bac, 187(8): 2793-2800). Several constructs were made each containing a spacer from one of the four bacteria either in the sense or antisense strands. All these constructs was introduced back to each host and the response was observed after the induction. Expression inhibition was expected only when the spacer from the host was expressed in the right direction, and gfp expression in the rest with the spacer from other hosts. Some contradictory results were observed but testing of other constructs made must still be completed.

2. Mechanism of action of CRISPR/CAS system: Spacers of choices are fused into 5’ terminus of gfp by PCR. Forward primer used in PCR reaction contains a restriction site (PstI), an E.coli RBS, a spacer, and the part matching the 5’end of gfp in that order. Reverse primer on the other hand contains the part complementary to the 3’end of gfp along with Nhel restriction site. The amplicons were cloned into pZB and subsequently transformed into
strains corresponding to spacers. Then these recombinant strains will be induced with tetracycline and the presence / absence of GFP expression will be observed. Recombinant strains containing empty plasmid (pZB) or uninduced cells will be used as negative controls. The absence of transformants on selective plates might indicate the action of CRISPR/CAS system on DNA level while the GFP expression upon the induction shows that no immunity provided by that particular CRISPR.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.
WHAT IS CLAIMED IS:

1. An isolated polynucleotide, comprising a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of said CRISPR is sufficiently complementary to a portion of at least one prokaryotic gene so as to down-regulate expression of said prokaryotic gene.

2. The isolated polynucleotide of claim 1, wherein said at least one spacer comprises at least two spacers, each being sufficiently complementary to a portion of different prokaryotic genes so as to down-regulate expression of said different prokaryotic genes.

3. The isolated polynucleotide of claim 1, wherein said at least one spacer comprises 26-72 base pairs.

4. The isolated polynucleotide of claim 1, wherein said prokaryotic gene is a bacterial gene.

5. The isolated polynucleotide of claim 4, wherein said bacterial gene is associated with down-regulation of biofuel production.

6. The isolated polynucleotide of claim 5, wherein said bacterial gene is selected from the group consisting of acetate kinase, phosphate acetyltransferase and L-lactate dehydrogenase.

7. The isolated polynucleotide of claim 5, wherein said bacterial gene is a genetic repressor CcpN.

8. The isolated polynucleotide of claim 4, wherein said bacterial gene is an antibiotic resistance gene.
9. The isolated polynucleotide of claim 8, wherein said antibiotic resistance gene is a methicillin resistance gene or a vancomycin resistance gene.

10. The isolated polynucleotide of claim 4, wherein said bacterial gene is a bacterial virulence gene.

11. The isolated polynucleotide of claim 4, wherein said bacterial gene is a ribosomal RNA gene, a ribosomal protein gene or a tRNA synthetase gene.

12. The isolated polynucleotide of claim 4, wherein said bacterial gene is selected from the group consisting of dnaB, fabl, folA, gyrB, murA, pytH, metG, and tufA(B).

13. The isolated polynucleotide of claim 1, wherein said prokaryotic gene is an archaeal gene.

14. The isolated polynucleotide of claim 1, further comprising a nucleic acid sequence encoding a CRISPR leader sequence.

15. The isolated polynucleotide of claim 1, further comprising a nucleic acid sequence encoding at least one CRISPR associated (CAS) polypeptide.

16. The isolated polynucleotide of claim 1, being non-naturally occurring.

17. A nucleic acid construct comprising the isolated polynucleotide of claim 1.

18. The nucleic acid construct of claim 17, comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 6 and 11.

19. The nucleic acid construct of claim 17, comprising a cis regulatory element.

20. The nucleic acid construct of claim 19, wherein said cis regulatory element is a promoter.
21. The nucleic acid construct of claim 20, wherein said promoter is an inducible promoter.

22. A method of down-regulating expression of a gene of a prokaryotic cell, the method comprising introducing into the cell a CRISPR array polynucleotide, wherein a spacer of said CRISPR array is sufficiently complementary with a portion of the gene to down-regulate expression of the gene, and wherein the gene is not introduced into the cell by a bacteriophage, thereby down-regulating expression of gene of a prokaryotic cell.

23. The method of claim 22, wherein the gene is integrated into a chromosome of the cell.

24. The method of claim 22, wherein the gene is endogenous to the prokaryotic cell.

25. The method of claim 22, wherein the gene is epichromosomal.

26. The method of claim 22, further comprising introducing into the cell a naïve CRISPR array system.

27. A method of treating a bacterial infection in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an isolated polynucleotide, comprising a clustered, regularly interspaced short palindromic repeat (CRISPR) array nucleic acid sequence wherein at least one spacer of said CRISPR is sufficiently complementary to a portion of at least one bacterial gene so as to down-regulate expression of said bacterial gene, said bacterial gene being a vital bacterial gene or a bacterial virulence gene, thereby treating the bacterial infection.

28. A method of treating an antibiotic-resistant bacterial infection in a subject in need thereof, the method comprising administering to the subject the isolated polynucleotide of claim 8, thereby treating the antibiotic-resistant bacterial infection.
29. The method of claim 28, wherein the bacterial infection is induced by methicillin resistant Staphylococcus aureus or vancomycin resistant Staphylococcus aureus.

30. A method of annotating a prokaryotic gene, the method comprising:
   (a) introducing the isolated polynucleotide of claim 1 into a prokaryote under conditions that allow downregulation of the prokaryotic gene; and
   (b) assaying a phenotype of the prokaryote, wherein a change in phenotype following the introducing is indicative of the prokaryotic gene being associated with said phenotype.
FIG. 5
atgagtaagggagaagccacctttcaacctggagttgcccaatctttgttgaatggtaggtatgtaattg
atgacactctgcaaactcatcctgctatcaatctttctttctatcacaatgaggtcctttctttctttc
tagttgctccagagccacacactatctctcaataggtcagacaccaacacatgtgttgaatgtaggtatgtaat
atgagtagtctaatatatgtagggtaggtaggccatacactggagaataatattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
FIG. 8

GFP from BL21(DE3)-Nobu
544 bp
FIG. 9

MG1655 CRISPR / malF spacers
FIG. 13

CRISPR system: 2729021-2749374

CRISPR array

2729021
2732021
2735021
2738021
2741021
2744021
2747021

CAS genes
FIG. 14

CRISPR system: 3860684-3880305

CRISPR array

CAS genes
FIG. 16

Plasmid that carries an engineered CRISPR system.

Inducible promoter

Bacterial genome

Bacterial genome
FIG. 17

Plasmid that carries an engineered CRISPR array

Inducible promoter

Bacterial genome

Bacterial genome
FIG. 19

Spacer designed from an endogenous gene

Transformation of engineered CRISPR into studied bacteria

Endogenous gene normally expressed → Bacterial DNA

mRNA degraded

FIG. 20

CRISPR spacer
RBS
Inducible promoter
Antibiotic resistance gene

GFP