(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 224 353 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

(15) Correction information:
Corrected version no 1 (W1 B1)
Corrections, see
Sequence listing
Remarks
Sequence listing replaced or added

(48) Corrigendum issued on:
09.08.2023 Bulletin 2023/32

(45) Date of publication and mention of the grant of the patent:
07.06.2023 Bulletin 2023/23

(21) Application number: **15863631.6**

(22) Date of filing: **26.11.2015**

(51) International Patent Classification (IPC):
C12N 7/01 *(2006.01)* ***C12N 15/113*** *(2010.01)*
C12N 15/74 *(2006.01)* ***A61K 35/76*** *(2015.01)*
A61P 31/04 *(2006.01)*

(52) Cooperative Patent Classification (CPC):
C12N 15/902; A61K 35/76; A61P 31/04; C12N 15/111; C12N 15/63; C12N 2310/20; C12N 2320/30; Y02A 50/30

(86) International application number:
PCT/IL2015/051154

(87) International publication number:
WO 2016/084088 (02.06.2016 Gazette 2016/22)

(54) **TARGETED ELIMINATION OF BACTERIAL GENES**

GEZIELTE ENTFERNUNG VON BAKTERIELLEN GENEN

ÉLIMINATION CIBLÉE DE GÈNES BACTÉRIENS

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **26.11.2014 US 201462084703 P**

(43) Date of publication of application:
04.10.2017 Bulletin 2017/40

(73) Proprietor: **Technology Innovation Momentum Fund**
(Israel) Limited Partnership
6139201 Tel Aviv (IL)

(72) Inventors:
- **QIMRON, Ehud**
  6967124 Tel Aviv (IL)
- **YOSEF, Ido**
  7975500 Moshav Netiv Ha'Asara (IL)
- **MANOR, Miriam**
  9675764 Jerusalem (IL)

(74) Representative: **J A Kemp LLP**
80 Turnmill Street
London EC1M 5QU (GB)

(56) References cited:
WO-A1-2014/124226 **WO-A2-2008/108989**
US-A1- 2013 315 869

- **DAVID BIKARD ET AL: "Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials", NATURE BIOTECHNOLOGY, GALE GROUP INC, vol. 32, no. 11, 1 November 2014 (2014-11-01), pages 1146-1150, XP002742966, ISSN: 1087-0156, DOI: 10.1038/NBT.3043 [retrieved on 2014-10-05]**
- **ROBERT J CITORIK ET AL: "Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases", NATURE BIOTECHNOLOGY, vol. 32, no. 11, 21 September 2014 (2014-09-21), pages 1141-1145, XP55446719, ISSN: 1087-0156, DOI: 10.1038/nbt.3011**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Processed by Luminess, 75001 PARIS (FR)

(Cont. next page)

- **IDO YOSEF ET AL: "Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 112, no. 23, 9 June 2015 (2015-06-09) , pages 7267-7272, XP002742965, ISSN: 0027-8424, DOI: 10.1073/PNAS.1500107112 [retrieved on 2015-05-18]**
- **KRZYSZTOF CHYLINSKI ET AL: "The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems", RNA BIOLOGY, vol. 10, no. 5, 1 May 2013 (2013-05-01), pages 726-737, XP055116068, ISSN: 1547-6286, DOI: 10.4161/rna.24321**
- **KIMBERLEY D. SEED ET AL: "A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity", NATURE, vol. 494, no. 7438, 27 February 2013 (2013-02-27), pages 489-491, XP055281884, London ISSN: 0028-0836, DOI: 10.1038/nature11927**
- **Marie-?ve Dupuis ET AL: "CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance", Nature Communications, 2 July 2013 (2013-07-02), XP055162707, DOI: 10.1038/ncomms3087 Retrieved from the Internet: URL:http://www.nature.com/ncomms/2013/130702/ncomms3087/pdf/ncomms3087.pdf [retrieved on 2015-01-16]**
- **YOSEF, I. ET AL.: 'Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 112, no. 23, 09 June 2015, pages 7267 - 7272, XP002742965**
- **BIKARD, D. ET AL.: 'Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials.' NATURE BIOTECHNOLOGY vol. 32, no. 11, 05 October 2014, pages 1146 - 1150, XP002742966**
- **CITORIK, R. J. ET AL.: 'Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases.' NATURE BIOTECHNOLOGY vol. 32, no. 11, 21 September 2014, pages 1141 - 1145, XP055446719**

Remarks:

•The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

•The file contains technical information submitted after the application was filed and not included in this specification

Description

FIELD OF THE INVENTION

[0001] The invention relates to methods for a specific targeted elimination of bacterial genes. More specifically, the invention provides kits, systems, compositions and methods using engineered RNA guided nucleases (RGNs) systems for targeting and eliminating bacterial pathogenic genes.

BACKGROUND REFERENCES

[0002]

1. Lu TK & Collins JJ (2009) Proc Natl Acad Sci U S A 106(12):4629-4634.
2. Merril CR, et al. (2003) Nat Rev Drug Discov 2(6):489-497.
3. Qimron U, et al. (2006) Proc Natl Acad Sci USA 103(50):19039-19044.4
4. Lang LH (2006) Gastroenterology 131(5):1370.
5. Abuladze T, et al. (2008). Appl Environ Microbiol 74(20):6230-6238.
6. Hagens and Blassi (2003) Lett Appl Microbiol.;37(4):318-23.
7. Qimron U, et al. (2014) US Patent No. 8, 865,158.
8. Gomaa AA et al. (2014) MBio 5(1):e00928-00913.
9. Garneau JE et al. (2010) Nature 468(7320):67-71.
10. Citorik RJ et al. (2014) Nat. Biotechnol. 32(11): 1141-45
11. Bikard D et al. (2014) Nat Biotechnol. 32(11): 1146-50
12. Barrangou et al. (2007) Science 315(5819): 1709-12.
13. Bolotin et al. (2005) Microbiology 151(Pt 8): 2551-61.
14. Marraffini et al. (2008) Science 322: 1843-1845.
15. Yosef I et al. (2014) Bacteriophage 4(1):e28491.
16. Barrangou R, et al. (2007) Science 315(5819):1709-1712).
17. Hale CR, et al. (2009) Cell 139(5):945-956.
18. Pendleton JN, et al. (2013) Expert Rev Anti Infect Ther 11(3):297-308.
19. Boucher HW, et al. (2009) Clin Infect Dis 48(1):1-12.
20. Marraffini LA et al. (2010) Nat Rev Genet 11(3):181-190.
21. Hale CR et al. (2009) Cell 139(5):945-956.
22. Datsenko KA et al. (2000) Proc Natl Acad Sci U S A 97(12):6640-6645.
23. Yosef I et al. (2012) Nucleic Acids Res 40(12):5569-5576.
24. Sharan SK et al. (2009) Nat Protoc 4(2):206-223.
25. Yu D et al. (2000) Proc Natl Acad Sci U S A 97(11):5978-5983.
26. Yosef I et al. (2011) Proc Natl Acad Sci U S A 108(50):20136-20141.
27. Kiro R et al. (2013) Proc Natl Acad Sci U S A 110(48):19549-19554.
28. Bush K et al. (2011) Annu Rev Microbiol 65:455-478.
29. Brouns SJ et al. (2008) Science 321(5891):960-964.
30. Baba T et al. (2006) Mol Syst Biol 2:1-11.
31. Edgar R and Qimron U (2010) J Bacteriol 192(23):6291-6294.
32. WO 2014/124226 A1
33. Yousef I et al (2015) Proc Natl Acad Sci U S S 112(23): 7267-7272

[0003] Acknowledgement of the above references herein is not to be inferred as meaning that these are in any way relevant to the patentability of the presently disclosed subject matter.

BACKGROUND OF THE INVENTION

[0004] Bacteria have evolved to overcome a wide range of antibiotics, and resistance mechanisms against most of the conventional antibiotics have been identified in some bacteria. Accelerated development of newer antibiotics is being overrun by the pace of bacterial resistance. In the USA, for example, over 70 % of hospital-acquired infections involve bacteria resistant to at least one antibiotic, and in Japan over 50 % of the clinical isolates of *Staphylococcus aureus* are multidrug-resistant.

[0005] Antibiotic resistance of pathogen is a growing concern to human health, leading to renewed interest in phage therapy. This therapy uses phages, the natural bacterial enemies, to kill pathogens. However, the therapy is currently

not feasible mainly due to delivery barriers into the tissues as well as bacterial resistance to the phages. Major concerns over the use of phage therapy include neutralization of phages by the spleen/liver and by the immune system, their narrow host range, bacterial resistance to the phage, and lack of sufficient pharmacokinetic and efficacy studies in humans and animals.

[0006] Several studies used phages as a genetic tool to increase bacterial susceptibility to antibiotics. One study used phage M13, of the Gram-negative *Escherichia coli,* to genetically target several gene networks, thus rendering the bacteria more sensitive to antibiotics (1). It demonstrated that disrupting the SOS response by M13-mediated gene-targeting renders the bacteria several-fold more sensitive to a variety of antibiotics. It also demonstrated that phage-mediated gene transfer combined with antibiotics increases the survival of mice infected with pathogenic *E. coli.* Overall, the study showed that transferring genes by phage M13 weakens the bacteria, and render them more susceptible to killing by antibiotics. The end result is very similar to conventional phage-therapy practices, in which phages are used to directly kill the pathogen.

[0007] Different approaches make use of phages as "disinfectants" of pathogens present on edible foods, plants, and farm animals. In addition to increasing the shelf life of these products, the treatment is intended to prevent occasional outbreaks of disease. However, implementation of this strategy must overcome several barriers. One characteristic of most phage infections is their narrow host range. Most phages infect only one species of bacteria and some are limited to certain strains within a species (2). This feature can be advantageous, on the one hand, as it allows targeting specific pathogens without disrupting other bacterial populations (2). On the other hand, this narrow host range may constitute a significant shortcoming, as uninfected pathogens would remain untreated. One way to expand the host range of phages is to select for phage mutants that infect new hosts. In many cases, the selected mutants that adapted to new hosts also maintain their infectivity to the original host, and thus the range is extended (3). An additional way to partially overcome this issue is to use a mixture of phages to target an extended range of the same bacterial species. Successful examples of such approach is the use of mixtures of phages against *Listeria monocytogenes, E. coli, and Salmonella enterica* in the respective products ListShield, EcoShield, and SalmoFresh, all approved by the US Food and Drug Administration (FDA) (4). These phage mixtures were shown to effectively eradicate targeted pathogens on food and surfaces (5). Moreover, all of these products were given the "ready-to-eat" approval from the US FDA, demonstrating the safety of spreading phages on consumed products and on surfaces (4).

[0008] Other phage cocktails have been approved as food additives in Europe, and many are currently being developed by phage biotech companies. These applications demonstrate that phages can be dispersed in the environment and efficiently target pathogens in their surroundings.

[0009] Pathogen resistance to antibiotics is a rapidly growing problem, leading to an urgent need for novel antimicrobial agents. Unfortunately, development of new antibiotics faces numerous obstacles, and a method that will re-sensitize pathogens to approved antibiotics therefore holds key advantages.

[0010] Lu and Collins (1) teach genetically modified bacteriophage which serve to weaken bacteria such that they are more susceptible to antibiotics. Hagens and Blassi (6) teach genetically modified filamentous phage as bactericidal agents. The inventors have previously described (7) a genetically modified bacteriophage encoding a dominant sensitive resistance gene, for example, 30S ribosomal subunit protein S 12, gyrase, RNA Polymerase β Subunit and thymidylate synthase and additionally, a tellurite resistance gene, and uses thereof in reducing bacterial antibiotic resistance.

[0011] The clustered regularly interspaced short palindromic repeats (CRISPR) and their associated Cas proteins (CRISPR-Cas) have revolutionized molecular biology by providing an efficient tool to precisely delete and edit the genome of human, primate, rodent, fish, fly, worm, plant, yeast, bacterial cells, and bacteriophages. The CRISPR-Cas system has also recently been used to phenotypically correct a genetic disease in live animals and its utility is being explored for various therapeutic approaches in mammals. Nevertheless, only limited studies have shown the use of the CRISPR-Cas system to target antibiotic-resistance genes or specific population of virulent bacterial strains (8, 10, 11).

[0012] CRISPR is a genetic system comprised of a cluster of short repeats interspersed by similarly sized non repetitive sequences (called spacers). Additional components of the system include CRISPR-associated (*cas*) genes and a leader sequence. Transcribed spacers guide Cas proteins to homologous sequences within the foreign nucleic acid, called protospacers, which are subsequently cleaved. 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.

[0013] 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.

[0014] It has been previously demonstrated 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 (12). Indeed, spacers found in naturally occurring CRISPR arrays are frequently derived from phages and other extrachromosomal elements (13).

[0015] Marraffini et al. 2008 (14) teach manipulation of CRISPR arrays for impeding the spread of antibiotic resistance genes and virulence factors in bacterial pathogens. Garneau et al., (9) teach that CRISPR arrays cleave plasmid DNA encoding antibiotic resistance genes.

[0016] Sequence-specific antimicrobials have been described previously (32). Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria have also been described previously (33). Two recent elegant studies demonstrated that phage-transferable CRISPR-Cas systems are capable of specifically killing pathogens or re-sensitizing them to antibiotics (10, 11). These, and another study (8), also showed that the transferred CRISPR-Cas system is capable of enriching specific bacterial populations. Furthermore, they demonstrated that the system might be used against pathogens to effectively treat infected animals. Consequently, it was suggested that the system could be used as a potent antimicrobial agent. Nevertheless, while the results of these studies highlight the potential of a transferable CRISPR-Cas system, the concept of using the system as a direct antimicrobial is similar to conventional phage therapy, which suffers from various difficulties (15). One may argue that it would be more efficient to directly kill a pathogen by a lytic phage if it were possible to deliver a specific DNA into this pathogen by a phage. Moreover, using the proposed system in an infected patient to resensitize the pathogens to antibiotics while antibiotics counter-select for these sensitized pathogens would most likely fail due to escape mutants that are selected by the antibiotics.

[0017] Thus, there is a pressing need to develop efficient antimicrobial approach to specifically target bacterial resistant genes and moreover, to eliminate horizontal transfer of antibiotic resistance.

SUMMARY OF THE INVENTION

[0018] A first aspect of the invention relates to a kit comprising:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and
(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one clustered, regularly interspaced short palindromic repeat (CRISPR) array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component.

[0019] A second aspect of the invention relates to an *in vitro* method of interfering with a genetic element comprising at least one pathogenic gene between bacteria, the method comprises the steps of: contacting at least one of a surface, a substance or an article containing bacteria harboring said pathogenic gene with:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage, or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and
(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component, or
(iii) at least one kit comprising (i) and (ii), thereby inactivating said pathogenic gene and interfering with horizontal transfer thereof, preferably, said kit is as defined in the first aspect.

[0020] A third aspect of the invention relates to least one first component (i), that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and at least one second component (ii), comprising at least one recombinant vector comprising a nucleic acid

sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component; or

(i) at least one kit comprising (i) and (ii), preferably, said kit is as defined in the first aspect;
for use in a method of interfering with a horizontal transfer of a genetic element comprising at least one pathogenic gene between bacteria, the method comprising the steps of: contacting at least one of a surface, a substance or an article containing bacteria harboring said pathogenic gene with (i) and (ii) or (iii). A fourth aspect of the invention relates to at least one first component (i), that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and at least one second component (ii) comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component; or
(iii) at least one kit comprising (i) and (ii), thereby targeting and inactivating said pathogenic gene and preventing said pathologic condition, preferably, said kit is as defined in the first aspect; for use in a method of preventing a pathologic condition in a mammalian subject caused by a bacterial infection of bacteria containing a pathogenic gene, the method comprising contacting at least one of surface, a substance or an article, in the vicinity of said subject with (i) and (ii), or (iii).

[0021] Also disclosed is a method of preventing a pathologic condition in a mammalian subject caused by a bacterial infection of bacteria containing a pathogenic gene, using at least one of the selective and the sensitizing componentsor any kits comprising the same, specifically, any of the kits or systems provided herein.

[0022] The disclosure also relates to a genetically modified, temperate bacteriophage comprising an engineered CRISPR. The disclosure also relates to any genetically modified lytic bacteriophage comprising the protospacers, specifically, any of the lytic phages described.

[0023] 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. 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

[0024] 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.

[0025] In the drawings:

Figure 1. Schematics of the lysogenizing phages
The CRISPR associated genes of type I-E: *cas3, cse1, cse2, cas7, cas5,* and *cas6e* (dark bars) were inserted in place of nucleotides at position 19014-27480 of the λ chromosome (NCBI Reference Sequence: NC_001416.1, SEQ ID NO. 36) yielding the control lysogenizing phage λ_{cas} (bottom). The $\lambda_{cas\text{-}CRISPR}$ phage (top) encodes in addition to the *cas* genes, a CRISPR array with spacers targeting the genes *ndm-1* (N_1, N_2, N_3, as denoted by SEQ ID NO. 37, 38, 39 respectively) and *ctx-M-15* (C_1, C_2, C_3, as denoted by SEQ ID NO. 40, 41, 42, respectively). P_{T7}, T7 promoter.

Figure 2A-2D. Competitive Fitness of a lysogen compared to bacteria harboring resistance plasmid/s
Fig. 2A shows cultures of bacteria encoding the λcas-CRISPR prophage and pVec plasmids, mixed at a 1:1 ratio.
Fig. 2B shows cultures of bacteria encoding the λcas-CRISPR prophage and pCTX plasmids, mixed at a 1:1 ratio.
Fig. 2C shows cultures of bacteria encoding the λcas-CRISPR prophage and pNDM plasmids, mixed at a 1:1 ratio.
Fig. 2D shows cultures of bacteria encoding the λcas-CRISPR prophage and pNDM*+pCTX plasmids, mixed at a 1:1 ratio.
Bacteria from the panels **A, B, C,** and **D,** were cultured together in LB at 32 oC for 14 h. The cells were then diluted

1/800 in LB and grown for an additional 14 h at 32°C. Samples from the mixed cultures were taken at the indicated time points and plated on either kanamycin or streptomycin or streptomycin+gentamicin agar plates to differentiate between lysogens (kanamycin^r) and plasmid-harboring bacteria (streptomycin^r for panels A, B, C or streptomycin^r+gentamicin^r for panel D). The CFU ratio of each strain was then determined by calculating the number of each type of resistant CFU out of the total resistant CFU.

Figure 3. Lysogenization effect on transformation of antibiotic resistance plasmids *E. coli* K-12 were lysogenized with λ_{cas} (light grey bars) or $\lambda_{cas\text{-}CRISPR}$ (dark grey bars). These lysogens were transformed with a control (pVEC), *ndm-1* (pNDM), or *ctx-M-15* (pCTX) encoding plasmids and plated on agar plates supplemented with streptomycin. Bars represent average and standard deviation of the number of colony forming units (CFU) per ml counted after plating serial dilutions of the cultures in three independent experiments.

Figure 4. Sensitization of antibiotic resistant bacteria by lysogenization

E. coli K-12 harboring a control (pVEC), *ndm-1* (pNDM), *ctx-M-15* (pCTX), or *ndm-1* + *ctx-M-15* (pNDM*/pCTX) encoding plasmids were treated with λ_{cas} (light grey bars) or $\lambda_{cas\text{-}CRISPR}$ (dark grey bars) and plated on LB plates supplemented with 5μg/ml tetracycline and 0.2% arabinose. Colonies (24 of each strain) were then inoculated on plates supplemented with 5 μ g/ml tetracycline and 0.2% arabinose and having or lacking streptomycin or gentamicin. Bars represent percentage and standard deviation from three independent experiments of streptomycin- or gentamicin-sensitive bacteria scored as CFU unable to grow on plates with streptomycin or gentamicin out of the total number of CFU able to grow on plates lacking these antibiotics.

Figure 5. Lysogenization effect on protection against lytic phages

E. coli K-12 were lysogenized with λ_{cas} (light grey bars) or $\lambda_{cas\text{-}CRISPR}$ (dark grey bars). These lysogens were infected with a control T7-gp8 lacking targeted protospacers, or with T7 phages encoding two protospacers of *ndm-1* (T7-N_1N_2, as denoted by SEQ ID NO. 55) or two protospacers of *ctx-M-15* (T7-C_2C_1, as denoted by SEQ ID NO. 56) or one spacer of each (T7-N_1C_1, as denoted by SEQ ID NO. 57 and T7-C_2N_2, as denoted by SEQ ID NO. 58). Bars represent average and standard deviation of the number of plaque forming units (PFU) per ml counted after plating serial dilutions of the phages in three independent experiments.

Figure 6A-6C. Enrichment of antibiotic-sensitized bacteria by lytic phages

Fig. 6A. shows schematic presentation of the procedure to enrich for antibiotic-sensitive bacteria. A bacterial culture is mixed with lysogenizing phages, resulting in both lysogens and non-lysogens in the culture. Lysogens are both antibiotic-sensitized and phage resistant, as the CRISPR-Cas system degrades the antibiotic-resistance-conferring plasmid and the lytic-phage chromosome. The treated culture is inoculated on agar containing lytic phages that selectively kill the non-lysogens and enrich for antibiotic-sensitive bacteria.

Fig. 6B. Enrichment of phage-resistant *E. coli. E. coli* K-12 harboring a control (pVEC), *ndm-1* (pNDM), *ctx-M-15* (pCTX) or *ndm-1* + *ctx-M-15* (pNDM*/pCTX) encoding plasmids were treated with λ_{cas} (light grey bars) or $\lambda_{cas\text{-}CRISPR}$ (dark grey bars) and plated on T7-N1C1 (as denoted by SEQ ID NO. 57)-coated plates as shown in the scheme presented in panel A. Bars represent average and standard deviation of the number of surviving CFU per ml counted in three independent experiments.

Fig. 6C. Enrichment of antibiotic-sensitive *E. coli.* Surviving colonies (20-48 CFU) from each culture described in panel B were inoculated on plates having or lacking streptomycin or gentamycin. Bars represent percentage and standard deviation of from three independent experiments of streptomycin-or gentamycin-sensitive bacteria scored as CFU unable to grow on plates with streptomycin or gentamycin out of the total number of CFU unable to grow on plates lacking these antibiotics.

DETAILED DESCRIPTION

[0026] The present inventors use the CRISPR/Cas system both to confer selective advantage and as a genetic tool to destroy specific DNAs which confer antibiotic resistance or any pathogenicity to bacteria. The CRISPR/Cas system has been recently shown to function as an adaptive immune system in bacteria (16). The system's physiological role is to protect from phage attack and from undesired plasmid replication by targeting foreign DNA or RNA (16-17). CRISPR/Cas can be rationally designed to specifically target any DNA molecule, based on short homologous DNA sequences in a unique DNA array called CRISPR (see below, and Figure 1). Rational design of the CRISPR array enables targeting any DNA molecule that encodes resistance determinants. In addition, the system, originally evolved as a defense mechanism against phages, can be designed to protect against lytic phages of choice. This allows the present inventors to genetically link a trait that is beneficial to the bacteria (i.e., genes conferring phage resistance) with DNA that reverses drug resistance and eliminates resistance determinants. This genetic linkage enables selecting for the sensitized bacterial population by using lytic phages as selection agents. The lytic phages may be engineered to contain sequences displaying an identity to at least one spacer in the engineered CRISPR array system. Such artificial phages that are used for selection, ultimately linking antibiotic sensitivity and phage resistance. Bacteria harboring defense against the lytic phages along with the sensitizing construct will survive, whereas other bacteria will be killed

by the lytic phages, specifically, the engendered lytic phages. The integrated construct is designed to actively eradicate existing resistance genes and also eliminate horizontal transfer of these genes between pathogens. The CRISPR/Cas system proposed herein combining two elements, the sensitizing component being the CRISPR array and the selective element, being the lytic phage, has all of the components of a genetic tool to reverse drug resistance.

[0027] More specifically, the present invention provides a specific and effective technology to counteract the emerging threat of antibiotic resistant bacteria, which overcomes the above shortcomings. Instead of directly targeting the pathogens, a sophisticated approach is provided herein, an approach that sensitizes the pathogens on surfaces or in the human natural flora, enriches for specific sensitive populations, and thus enables the consequent use of traditional antibiotics in infected patients. In this technology, the CRISPR-Cas system is used to destroy specific DNAs that confer antibiotic resistance and to concomitantly confer a selective advantage to antibiotic-sensitive bacteria. The selective advantage enables efficient replacement of populations of antibiotic sensitive bacteria by selecting against untreated bacteria. The approach differs from conventional phage therapy in that it does not aim to directly kill treated bacteria, but rather to sensitize them to antibiotics and to kill the untreated bacteria. Therefore, there is no counter selection against the treatment. By using a selective advantage, the efficiency of delivery is maximized, as bacteria escaping the treatment are killed by the selection agent. By this strategy the inventors propose to sensitize the pathogens on surfaces or in the human skin flora while concomitantly enriching for these sensitized populations. Patients infected by these antibiotic-sensitive bacteria would thus be treatable by traditional antibiotics.

[0028] Thus, a first aspect of the invention relates to a kit comprising:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and

(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one clustered, regularly interspaced short palindromic repeat (CRISPR) array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component.

[0029] "Selective component" as used herein, refers to an element or component of the kit of the invention that enables, facilitates, leads to and acts on selecting, choosing, electing or enriching a specific population of bacterial cells, specifically, a population of cells that carry the cas-CRISPR system, more specifically, a population of bacterial cells that carry the sensitizing component. The selective component provides selective advantage to the desired population, for example by imposing conditions that enable and allow only the survival of the selected desired population (such as, any population or cells that carry the sensitizing component).

[0030] "Sensitizing component" as used herein refers to an element of the kit of the invention that enables an increased sensitivity or susceptibility and/or a reduced resistance of an organism that carry said element or component, to a certain substance, for example, to an antibiotic substance. The sensitizing component may specifically target, inactivate and/or destroy pathogenic bacterial-genes, for example, genes encoding antibiotic resistance or genes encoding a toxic compound, enables sensitization of the cells and reversion thereof to less resistant and more susceptible cells. In certain embodiments, "targeting" should be understood as to make an element or object or group of elements or objects a target, to elect or choose it or them to be acted upon, where the elected or chosen object/s or element/s is/are to be attacked, taken, degraded, inactivated or destroyed.

[0031] Moreover, at least one spacer of the CRISPR array may be sufficiently complementary to a nucleic acid sequence (or a proto-spacer) comprised within the selective component of the kit of the invention, so as to target and inactivate the selective component, where "inactivate" means delay, decrease, inhibit, eliminate, attenuate or stop the activity of the selective component. It should be noted that such inactivation renders a bacterium comprising said sensitizing element insensitive and resistant to the selective component of the kit of the invention. It should be appreciated that sufficient complementarity as used herein reflects any complementarty of between about 10% to 100%, more specifically, complementarity of about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% and 100%.

[0032] In certain embodiments, "Complementarity" refers to a relationship between two structures each following the lock-and-key principle. In nature complementarity is the base principle of DNA replication and transcription as it is a property shared between two DNA or RNA sequences, such that when they are aligned antiparallel to each other, the nucleotide bases at each position in the sequences will be complementary (e.g., A and T or U, C and G).

[0033] The present invention, in some embodiments thereof, relates to KITS and methods of down regulating (e.g. eliminating) bacterial genes using CRISPR constructs. More specifically, the invention provides kits and methods for

enriching bacterial populations with antibiotic sensitive bacteria that carry the sensitizing component of the kit of the invention.

[0034] 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.

[0035] The term "bacteria" (in singular a "bacterium") in this context refers to any type of a single celled microbe. Herein the terms "bacterium" and "microbe" are interchangeable. This term encompasses herein bacteria belonging to general classes according to their basic shapes, namely spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes), as well as bacteria that exist as single cells, in pairs, chains or clusters.

[0036] It should be noted that the term "bacteria" as used herein refers to any of the prokaryotic microorganisms that exist as a single cell or in a cluster or aggregate of single cells. In more specific embodiments, the term "bacteria" specifically refers to Gram positive, Gram negative or Acid fast organisms. The Gram-positive bacteria can be recognized as retaining the crystal violet stain used in the Gram staining method of bacterial differentiation, and therefore appear to be purple-colored under a microscope. The Gram-negative bacteria do not retain the crystal violet, making positive identification possible. In other words, the term "bacteria" applies herein to bacteria with a thicker peptidoglycan layer in the cell wall outside the cell membrane (Gram-positive), and to bacteria with a thin peptidoglycan layer of their cell wall that is sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane (Gram-negative). This term further applies to some bacteria, such as Deinococcus, which stain Gram-positive due to the presence of a thick peptidoglycan layer, but also possess an outer cell membrane, and thus suggested as intermediates in the transition between monoderm (Gram-positive) and diderm (Gram-negative) bacteria._Acid fast organisms like Mycobacterium contain large amounts of lipid substances within their cell walls called mycolic acids that resist staining by conventional methods such as a Gram stain.

[0037] As indicated above, the kit of the invention may comprise at least two components, a selective component that enables the enrichment and selection of a bacterial population that carry the sensitizing component that is therefore a population that is sensitive to antibiotics, or a population having reduced or eliminated resistance.

[0038] It should be appreciated that the selective component may be any genetic element or vector that carry or comprise at least one protospaces displaying at least minimal identity (specifically, of about 70% or more) to at least one protospacer comprised within a pathogenic bacterial gene and/or is recognized by at least one spacer comprised within the sensitizing component. Such selective component may be for example a plasmid that further encodes a toxic element or protein that may harm, kill or eliminate bacterial cells. More specifically, it can also be a DNA-injected entity encoding genes that kill bacteria by inactivating its essential elements or otherwise disrupting essential components for growth of the bacterium. E.g. a DNA encoding such genes, and injected by specialized protein machineries derived of phage products.

[0039] In yet some specific embodiments, the selective component used by the kit of the invention may comprise at least one lytic bacteriophage. In more specific embodiments, such bacteriophage may comprise a nucleic acid sequence comprising at least one proto-spacer that serves as a target for the spacers of the sensitizing component.

[0040] Under the term bacteriophage is meant a virus that infects and replicates within prokaryotes, such as bacteria. It should be note that the term "bacteriophage" is synonymous with the term "phage". Phages are composed of proteins that encapsulate a DNA or RNA genome, which may encode only a few or hundreds of genes thereby producing virions with relatively simple or elaborate structures. Thus, bacteriophages are among the most common and diverse entities in the biosphere. Phages are classified according to the International Committee on Taxonomy of Viruses (ICTV) considering morphology and the type of nucleic acid (DNA or RNA, single- or double-stranded, linear or circular). About 19 phage families have been recognized so far that infect bacteria and/or archaea (a prokaryotic domain previously classified as archaebacteria). Many bacteriophages are specific to a particular genus or species or strain of cell.

[0041] As noted above, in certain specific and non-limiting embodiments, the bacteriophage used as the selective component of the kit of the invention may be a lytic bacteriophage. A lytic bacteriophage is one that follows the lytic pathway through completion of the lytic cycle, rather than entering the lysogenic pathway. A lytic bacteriophage undergoes viral replication leading to lysis of the cell membrane, destruction of the cell, and release of progeny bacteriophage particles capable of infecting other cells.

[0042] In certain embodiments, the lytic bacteriophage of the selective component of the kit of the invention may be genetically modified bacteriophage comprising at least one proto-spacer having an identity of at least 70% to at least one nucleic acid sequence comprised within the bacterial pathogenic gene. In more specific embodiments, such bacteriophage may comprise at least one proto-spacer having an identity of about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% to at least one nucleic acid sequence comprised within the bacterial pathogenic gene.

[0043] In further embodiments, the sensitizing component may comprise at least one recombinant vector comprising a recombinant nucleic acid sequence encoding at least one *cas* protein. It should be noted that the vector may further comprise nucleic acid sequence of at least one of said CRISPR array/s. Such vector may be in certain embodiments,

any plasmid, construct, phagemid or an engendered bacteriophage comprising the CRISPR system described herein.

[0044] As used herein, the term "recombinant DNA", "recombinant nucleic acid sequence" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding one of the CRISPR system.

[0045] Thus, in some embodiments, the sensitizing element may be any vector that comprises the *cas* proteins and at least one of said CRISPR array/s. *"Vectors"* or *"Vehicles",* as used herein, encompass vectors such as plasmids, phagemides, viruses, bacteriophage, integratable DNA fragments, and other vehicles, which enable the integration of DNA fragments into the genome of the host, or enable expression of genetic elements that are not integrated. Vectors are typically self-replicating DNA or RNA constructs containing the desired nucleic acid sequences, and operably linked genetic control elements that are recognized in a suitable host cell and effect the translation of the desired spacers. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system. Such system typically includes a transcriptional promoter, transcription enhancers to elevate the level of RNA expression. Vectors usually contain an origin of replication that allows the vector to replicate independently of the host cell. The expression vectors used may comprise elements necessary for integration of the desired the CRISPR system into the bacterial chromosome.

[0046] Accordingly, the term control and regulatory elements includes promoters, terminators and other expression control elements. Such regulatory elements are described in Goeddel; [Goeddel., et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990)]. For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding any desired protein using the method.

[0047] A vector may additionally include appropriate restriction sites, antibiotic resistance or other markers for selection of vector-containing cells. Plasmids are the most commonly used form of vector but other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels et al., Cloning Vectors: a Laboratory Manual (1985 and supplements), Elsevier, N.Y.; and Rodriquez, et al. (eds.) Vectors: a Survey of Molecular Cloning Vectors and their Uses, Buttersworth Boston, Mass , (1988).

[0048] The sensitizing element may be a phagemid comprising the CRISPR system. "Phagemids" as used herein are plasmids modified to carry a phage packaging site and may also encode phage proteins. Phagemids may comprise, in general at least a phage packaging site and an origin of replication (ori). Phagemids of the present disclosure may further encode phage packaging sites and/or proteins involved in phage packaging.

[0049] The sensitizing element may be a genetically modified bacteriophage. More specifically, such genetically modified bacteriophage may comprise at least one CRISPR spacer that targets at least one nucleic acid sequence comprised within said lytic bacteriophage and at least one CRISPR spacer that targets a nucleic acid sequence comprised within said at least one pathogenic gene. In such way the sensitizing component may target and/or inactivate both, the lytic phage/s that serve as the selective component and the pathogenic gene of interest.

[0050] More specifically, the present inventors contemplate use of lytic (as the selective component) or temperate (as the sensitizing component), specifically, temperature-sensitive temperate bacteriophage. A "temperate phage", as used herein, relates to a phage where at a particular temperature (e.g. at 36 °C or below, for example, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 °C) the phage favors lysogeny, whereas higher temperatures induce lytic production of the phage. As noted herein above, lytic phage is a phage that uses the lytic cycle. The lytic cycle results in the destruction of the infected cell and its membrane. A key difference between the lytic and lysogenic phage cycles is that in the lytic phage, the viral DNA exists as a separate molecule within the bacterial cell, and replicates separately from the host bacterial DNA. The location of viral DNA in the lysogenic phage cycle is within the host cell, therefore in both cases the virus/phage replicates using the host DNA machinery, but in the lytic phage cycle, the phage is a free floating separate molecule to the host cell.

[0051] The phages used in the kits, either as the selective or the sensitizing components, may be bacteriophages of a type that selectively infect a pathogenic type of bacteria, or a type of bacteria that can have pathogenic and nonpathogenic members in a mixed bacteria population, or can infect different types of bacteria in a mixed bacterial population. Such mixed bacterial populations are found in Hospital surfaces. Importantly, a few resistant pathogens such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumanni, Pseudomonas aeruginosa, and Enterobacter species* (also referred to herein as ESKAPE organisms or bacteria) are responsible for the lion's share of nosocomial infections in both the developed and developing countries. Those pathogens cause the majority of nosocomial infections and effectively escape the effects of antibiotics (18, 19). Thus, an efficient and effective treatment against these species, or even only a few of them, could significantly reduce fatalities and financial burden caused by resistant pathogen infections.

[0052] Thus, the phages used by the kit may be bacteriophage/s specific for a particular bacterial genus, species or strains. The phage may be specific for a bacterial strain that may be a member of at least one of: *Escherichia coli, Streptococcus spp., Staphylococcus spp., Clostridium spp., Bacillus spp., Salmonella spp.*, *Helicobacter spp., Neisseria spp.* (specifically, *N. gonorrhoeae* and *N. meningitidis*), or *Pseudomonas aeruginosa.*

[0053] A non-limiting example of this type of phage may be the λgt11 phage. Other λ phages having their *cI* gene

changed to the *cI857* allele are also contemplated since they will exhibit similar growth pattern. Preferably, the phage is selected such that it allows stable insertion of at least 1 kb of foreign DNA and more preferably at least 5 kb of foreign DNA. The phage may comprise deletion mutants with minimal genes and may be capable of efficient lysogenization.

[0054] Identification of phages capable of infecting additional bacteria is within the scope of one skilled in the art. The phages used for infecting the bacteria may be capable of integrating into a Gram-positive, Gram-negative bacteria or Acid fast organism and the like.

[0055] As noted above, in some embodiments, the lytic phage that serves as the selective element in the systems or kits of the invention may be a genetically modified phage. Such phage may be genetically engineered to comprise at least one nucleic acid sequence that is a proto-spacer. In more specific embodiments, such proto spacer may display an identity of at least 70% to at least one proto-spacer, or in other words, nucleic acid sequence comprised within the pathogenic gene of a bacterium, or any fragment, part or portion thereof.

[0056] The vector comprised within the sensitizing component may be a genetically modified, temperate bacteriophage comprising at least one CRISPR spacer that targets a nucleic acid sequence comprised within the lytic bacteriophage (that serve as the selective component) and at least one CRISPR spacer that targets a nucleic acid sequence comprised within said bacterial pathogenic gene.

[0057] Ideally, at least one spacer of the CRISPR array should be sufficiently complementary to the nucleic acid sequence that is a proto-spacer comprised within the lytic genetically modified bacteriophage (the selective component) and also within the target pathogenic gene of a bacterium. In such a way, the CRISPR array of the sensitizing component targets and inactivates both, the lytic phage and the target pathogenic gene.

[0058] The target pathogenic gene of a bacterium or any RNA transcribed therefrom targeted by the CRISPR system, may be a bacterial endogenous gene. It should be noted that "endogenous gene" as used herein, refers to DNA originated from the specific organism, in the current case, bacteria, and therefore may be a part of its chromosomal DNA.

[0059] The target pathogenic gene of a bacterium may be epichromosomal. Such non-endogenous gene may be acquired by horizontal transfer. An "epichromosomal gene" as used herein, relates to a unit of genetic material, specifically, DNA in bacteria, for example a plasmid, that can either replicate independently as an extrachromosomal DNA, or may be integrated into the host chromosome.

[0060] In some specific embodiments, at least one target pathogenic gene of a bacterium may be a gene encoding a virulence factor or toxin, thereby rendering said bacteria virulent.

[0061] The term *"virulent"* as used herein means bacteria that can cause a bacterial disease or infection. Virulent bacteria are those that cause a bacterial disease or infection in a human subject, or any other organism including but not limited to mammal, rodent, bird, fish, reptile, insect or a plant, who does not have a compromised immune system. Typically, virulent bacteria will produce certain proteins which are referred to as *"virulence factors."* Virulent bacteria are distinguishable from those bacteria that normally colonize one or more of a healthy host's tissue and for which they are thus undesirable to kill under ordinary therapeutic circumstances because the latter generally do not express virulence factors, or express lower amounts of virulence factors relative to virulent bacteria. As discussed above, the present disclosure includes CRISPR systems which comprise sequences encoding targeting RNA directed to bacterial DNA sequences which encode virulence factors. Such *virulence factors* include but are not necessarily limited to bacteria proteins that are involved in pathogenic adhesion, colonization, invasion, biofilm formation or immune response inhibitors, or toxins. Examples of virulence genes include, but are not limited to genes encoding toxins (e.g. Shiga toxin and cholera toxin), hemolysins, fimbrial and afimbrial adhesins, proteases, lipases, endonucleases, endotoxins and exotoxins cytotoxic factors, microcins and colicins and also those identified in the art. The sequences of bacterial genes from a wide array of bacteria types that encode these and other virulence factors are known in the art. Virulence factors can be encoded on the bacterial chromosome, or on a plasmid in the bacteria, or both. The virulence factor may be encoded by a bacterial superantigen gene, such as a superantigen enterotoxin gene, one non-limiting example of which is the *S. aureus* Sek gene. Additional virulence factors for *S. areus* include but are not limited to cytolitic toxins, such as a-hemolysin, β-hemolysin, γ-hemolysin, leukocidin, Panton-Valentine leukocidin (PVL); exotoxins, such as toxic shock syndrome toxin- 1 (TSST-1); enterotoxins, such as SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI, and exfoliative toxins, such as ETA and ETB. Homologues of all of these toxins expressed by other types of bacteria are contemplated herein as virulence gene targets as well.

[0062] More specifically, the term *"toxin"* as used herein means a substance generated by bacteria, which can be classified as either exotoxin or endotoxin. Exotoxins are generated and actively secreted; endotoxins remain part of the bacteria. Usually, an endotoxin is part of the bacterial outer membrane, and it is not released until the bacterium is killed by the immune system.

[0063] The bacterial virulence gene may be selected from the group consisting of actA (example is given in genebank accession no: NC_003210.1), Tern (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 (aadA1) (example is given in genebank

accession no: DQ489717), ant(2")-Ia (aadB)b (example is given in genebank accession no: DQ176450), 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 (aphA1) (example is given in genebank accession no: NC_007682), aph(3')-IIa (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: FJ411076), 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), catI (example is given in genebank accession no: NC_005773), catII NC_010119, catIII (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), dhfrXIII (example is given in genebank accession no: NC_000962), dhfrXV (example is given in genebank accession no: Z83311), suII (example is given in genebank accession no: NC_000913), suIII (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_011742), vatC (example is given in genebank accession no: AF015628), 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: AF117259), vgb (example is given in genebank accession no: AF117258), and vgbB (example is given in genebank accession no: AF015628).

[0064] As noted above, the kit of the invention may specifically target any pathogenic bacterial gene, for example, any gene/s that provides resistance or in other words, inhibits, reduces, suppress or attenuates the susceptibility of the bacteria to any antimicrobial agent. The term "antimicrobial agent" as used herein refers to any entity with antimicrobial activity (either bactericidal or bacteriostatic), i.e. the ability to inhibit the growth and/or kill bacterium, for example Gram positive- and Gram negative bacteria. An antimicrobial agent may be any agent which results in inhibition of growth or reduction of viability of a bacteria by at least about 10%, 20%, 30% or at least about 40%, or at least about 50% or at least about 60% or at least about 70% or more than 70%, for example, 75%, 80%, 85%, 90%, 95%, 100% or any integer between 30% and 70% or more, as compared to in the absence of the antimicrobial agent. Stated another way, an antimicrobial agent is any agent which reduces a population of microbial cells, such as bacteria by at least about 30% or at least about 40%, or at least about 50% or at least about 60% or at least about 70% or more than 70%, or any integer between 30% and 70% as compared to in the absence of the antimicrobial agent. An antimicrobial agent may be an agent which specifically targets a bacteria cell. An antimicrobial agent may modify (i.e. inhibit or activate or increase) a pathway which is specifically expressed in bacterial cells. An antimicrobial agent can include any chemical, peptide (i.e. an antimicrobial peptide), peptidomimetic, entity or moiety, or analogues of hybrids thereof, including without limitation synthetic and naturally occurring non-proteinaceous entities. An antimicrobial agent may be a small molecule having a chemical moiety. For example, chemical moieties include unsubstituted or substituted alkyl, aromatic or heterocyclyl moieties including macrolides, leptomycins and related natural products or analogues thereof. Antimicrobial agents can be any entity known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[0065] As noted above, the sensitizing element of the kits, systems and methods may target any gene that provides antibiotic resistance. As used herein, the term *"resistance"* is not meant to imply that the bacterial cell population is 100% resistant to a specific antibiotic compound, but includes bacteria that are tolerant of the antibiotics or any derivative thereof. More specifically, the term "bacterial resistance gene/s" refers to gene/s conferring about 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10% protection from an antibiotic compound, thereby reversing susceptibility and sensitivity thereof to said antibiotic compound.

[0066] Thus, in some embodiments, the bacterial pathogenic gene may be any gene that provides resistance to any of the anti-bacterial compounds described herein above.

[0067] Still further, in other embodiments, the at least one target pathogenic gene of a bacterium, may be a gene encoding an antibiotic resistance factor.

[0068] The phrase *"antibiotic resistance genes"* as used herein refers to genes that confer resistance to antibiotics, for example by coding for enzymes which destroy said antibiotic compound, by coding for surface proteins which prevent the entrance of an antibiotic compound to the microorganism, actively exports it, or by being a mutated form of the antibiotic's target thereby preventing its antibiotic function.

[0069] Antibiotic resistance genes carried by a variety of bacteria are known in the art and the sequences of antibiotic resistance genes in any particular bacteria can be determined if desired. The present disclosure includes CRISPR systems which comprise spacers encoding targeting RNA that is directed to bacterial DNA sequences which comprise antibiotic resistance genes. The resistance gene may confer resistance to a narrow-spectrum beta-lactam antibiotic of the penicillin class of antibiotics. The resistance gene may confer resistance to methicillin (e.g., methicillin or oxacillin), or flucloxacillin, or dicloxacillin, or some or all of these antibiotics. Thus, the CRISPR system may suitable for selectively targeting antibiotic resistant genes in what has colloquially become known as methicillin-resistant S. aureus (MRSA) which in practice refers to strains of *S. aureus* that are insensitive or have reduced sensitivity to most or all penicillins.

The CRISPR system may be suitable for targeting vancomycin resistance in vancomycin resistant *S. aureus* (VRSA). Vancomycin resistant S. aureus may also be resistant to at least one of linezolid (ZYVOX™), daptomycin (CUBICIN ™), and quinupristin/dalfopristin (SYERCID™).

[0070] Additional antibiotic resistant genes include but are not limited to fosfomycin resistance gene fosB, tetracycline resistance gene tetM, kanamycin nucleotidyltransferase aadD, bifunctional aminoglycoside modifying enzyme genes aacA-aphD, chloramphenicol acetyltransferase cat, mupirocin-resistance gene ileS2, vancomycin resistance genes vanX, vanR, vanH, vraE, vraD, methicillin resistance factor femA, fmtA, mecl, streptomycin adenylyltransferase spcl, spc2, antl, ant2, pectinomycin adenyltransferase spd, ant9, aadA2, and any other resistance gene.

[0071] The pathogenic gene may be a gene encoding any gene conferring resistance to any β-lactam antibiotic compound. Such gene may encode at least one β-lactamase. As used herein, the term "β-lactamase" denotes a protein capable of catalyzing cleavage of a β-lactamase substrate such as a β-lactam containing molecule (such as a β-lactam antibiotic) or derivative thereof.

[0072] β-lactamases are organized into four molecular classes (A, B, C and D) based on their amino acid sequences. Class A enzymes have a molecular weight of about 29 kDa and preferentially hydrolyze penicillins. Examples of class A enzymes include RTEM and the β-lactamase of *Staphylococcus aureus.* Class B enzymes include metalloenzymes that have a broader substrate profile than the other classes of β-lactamases. Class C enzymes have molecular weights of approximately 39 kDa and include the chromosomal cephalosporinases of gram-negative bacteria, which are responsible for the resistance of gram-negative bacteria to a variety of both traditional and newly designed antibiotics. In addition, class C enzymes also include the lactamase of P99 *Enterobacter cloacae,* which is responsible for making this Enterobacter species one of the most widely spread bacterial agents in United States hospitals. The class D enzymes are serine hydrolases, which exhibit a unique substrate profile.

[0073] As noted above, the kits of the invention and systems may be directed against any gene that may confer resistance to any β lactam antibiotics. The term "β-lactam" or "β lactam antibiotics" as used herein refers to any antibiotic agent which contains a β-lactam ring in its molecular structure.

[0074] β-lactam antibiotics are a broad group of antibiotics that include different classes such as natural and semi-synthetic penicillins, clavulanic acid, carbapenems, penicillin derivatives (penams), cephalosporins (cephems), cephamycins and monobactams, that is, any antibiotic agent that contains a β-lactam ring in its molecular structure. They are the most widely-used group of antibiotics. While not true antibiotics, the β-lactamase inhibitors are often included in this group.

[0075] β-lactam antibiotics are analogues of D-alanyl-D-alanine the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis.

[0076] Under normal circumstances peptidoglycan precursors signal a reorganisation of the bacterial cell wall and, as a consequence, trigger the activation of autolytic cell wall hydrolases. Inhibition of cross-linkage by β-lactams causes a build-up of peptidoglycan precursors, which triggers the digestion of existing peptidoglycan by autolytic hydrolases without the production of new peptidoglycan. As a result, the bactericidal action of β-lactam antibiotics is further enhanced.

[0077] Generally, β-lactams are classified and grouped according to their core ring structures, where each group may be divided to different categories. The term "penam" is used to describe the core skeleton of a member of a penicillin antibiotic. i.e. a β-lactam containing a thiazolidine rings. Penicillins contain a β-lactam ring fused to a 5-membered ring, where one of the atoms in the ring is sulfur and the ring is fully saturated. Penicillins may include narrow spectrum penicillins, such as benzathine penicillin, benzylpenicillin (penicillin G), phenoxymethylpenicillin (penicillin V), procaine penicillin and oxacillin. Narrow spectrum penicillinase-resistant penicillins include methicillin, dicloxacillin and flucloxacillin. The narrow spectrum β-lactamase-resistant penicillins may include temocillin. The moderate spectrum penicillins include for example, amoxicillin and ampicillin. The broad spectrum penicillins include the co-amoxiclav (amoxicillin+clavulanic acid). Finally, the penicillin group also includes the extended spectrum penicillins, for example, azlocillin, carbenicillin, ticarcillin, mezlocillin and piperacillin.

[0078] Other members of this class include pivampicillin, hetacillin, bacampicillin, metampicillin, talampicillin, epicillin, carbenicillin, carindacillin, ticarcillin, azlocillin, piperacillin, mezlocillin, mecillinam, pivmecillinam, sulbenicillin, clometocillin, procaine benzylpenicillin, azidocillin, penamecillin, propicillin, pheneticillin, cloxacillin and nafcillin.

[0079] β-lactams containing pyrrolidine rings are named carbapenams. A carbapenam is a β-lactam compound that is a saturated carbapenem. They exist primarily as biosynthetic intermediates on the way to the carbapenem antibiotics.

[0080] Carbapenems have a structure that renders them highly resistant to β-lactamases and therefore are considered as the broadest spectrum of β-lactam antibiotics. The carbapenems are structurally very similar to the penicillins, but the sulfur atom in position 1 of the structure has been replaced with a carbon atom, and hence the name of the group, the carbapenems. Carbapenem antibiotics were originally developed from thienamycin, a naturally-derived product of *Streptomyces cattleya.* The carbapenems group includes: biapenem, doripenem, ertapenem, imipenem, meropenem, panipenem and PZ-601.

[0081] β-lactams containing 2, 3-dihydrothiazole rings are named penems. Penems are similar in structure to carbap-

enems. However, where penems have a sulfur, carbapenems have another carbon. There are no naturally occurring penems; all of them are synthetically made. An example for penems is faropenem.

[0082] β-lactams containing 3, 6-dihydro-2H-1, 3-thiazine rings are named cephems. Cephems are a sub-group of β-lactam antibiotics and include cephalosporins and cephamycins. The cephalosporins are broad-spectrum, semisynthetic antibiotics, which share a nucleus of 7-aminocephalosporanic acid. First generation cephalosporins, also considered as the moderate spectrum includes cephalexin, cephalothin and cefazolin.

[0083] Second generation cephalosporins that are considered as having moderate spectrum with anti-*Haemophilus* activity may include cefaclor, cefuroxime and cefamandole. Second generation cephamycins that exhibit moderate spectrum with anti-anaerobic activity include cefotetan and cefoxitin. Third generation cephalosporins considered as having broad spectrum of activity includes cefotaxime and cefpodoxime.

[0084] Finally, the fourth generation cephalosporins considered as broad spectrum with enhanced activity against Gram positive bacteria and β-lactamase stability include the cefepime and cefpirome. The cephalosporin class may further include: cefadroxil, cefixime, cefprozil, cephalexin, cephalothin, cefuroxime, cefamandole, cefepime and cefpirome.

[0085] Cephamycins are very similar to cephalosporins and are sometimes classified as cephalosporins. Like cephalosporins, cephamycins are based upon the cephem nucleus. Cephamycins were originally produced by *Streptomyces,* but synthetic ones have been produced as well. Cephamycins possess a methoxy group at the 7-alpha position and include: cefoxitin, cefotetan, cefmetazole and flomoxef.

[0086] β-lactams containing 1, 2, 3, 4-tetrahydropyridine rings are named carbacephems. Carbacephems are synthetically made antibiotics, based on the structure of cephalosporin, a cephem. Carbacephems are similar to cephems but with a carbon substituted for the sulfur. An example of carbacephems is loracarbef.

[0087] Monobactams are b-lactam compounds wherein the β-lactam ring is alone and not fused to another ring (in contrast to most other β-lactams, which have two rings). They work only against Gram-negative bacteria. Other examples of monobactams are tigemonam, nocardicin A and tabtoxin.

[0088] β-lactams containing 3, 6-dihydro-2H-1, 3-oxazine rings are named oxacephems or clavams. Oxacephems are molecules similar to cephems, but with oxygen substituting for the sulfur. Thus, they are also known as oxapenams. An example for oxapenams is clavulanic acid. They are synthetically made compounds and have not been discovered in nature. Other examples of oxacephems include moxalactam and flomoxef.

[0089] Another group of β-lactam antibiotics is the β-lactamase inhibitors, for example, clavulanic acid. Although they exhibit negligible antimicrobial activity, they contain the β-lactam ring. Their sole purpose is to prevent the inactivation of β-lactam antibiotics by binding the β-lactamases, and, as such, they are co-administered with β-lactam antibiotics. β-lactamase inhibitors in clinical use include clavulanic acid and its potassium salt (usually combined with amoxicillin or ticarcillin), sulbactam and tazobactam.

[0090] It should be therefore understood that the kit of the invention, by targeting and destroying antibiotic resistance genes, lead to sensitization of bacterial populations to any of the antibiotic compounds indicated herein above. It should be thus appreciated that such sensitization increase the sensitivity of the bacteria to said compound thereby enhancing its effectivity that may lead to reduction in the amounts required. A combined treatment with the kit of the invention and any of the antibiotic compounds disclosed herein is also contemplated. The kits of the invention may further comprise at least one antibiotic compound. Ssuch compound may be any of the antibiotic compounds disclosed.

[0091] The antibiotic resistance factor or gene, that is the target pathogenic gene for the kit may be any one of an extended-spectrum beta-lactamase resistance factor (ESBL factor), CTX-M-15, beta lactamase, New Delhi metallo-β-lactamase (NDM)-1,2,5,6 and tetracycline A (tetA).

[0092] New Delhi metallo-β-lactamase (NDM-1) is an enzyme that renders bacteria resistant to all currently used β-lactam antibiotics. The NDM-1 resistance spectrum includes the antibiotics of the carbapenem family, which are a mainstay for the treatment of antibiotic-resistant bacterial infections. The gene for NDM-1 is one member of a large gene family that encodes β-lactamase enzymes called carbapenemases. Bacteria that produce carbapenemases are notoriously difficult to treat. Importantly, the gene for NDM-1 can spread from one strain of bacteria to another by horizontal gene transfer, and can therefore spread easily. The NDM-1 protein may be the *Klebsiella pneumoniae* metallo-beta-lactamase gene blaNDM-1, of protein_id CAZ39946.1. Said NDM-1 protein may comprise the amino acid sequence encoded by the nucleic acid sequence as denoted by SEQ ID NO. 75. The NDM-1 protein may comprise the amino acid sequence as denoted by SEQ ID NO. 74.

[0093] Still further, CTX-M-15, as used herein is a member of the CTX-M family (Cefotaximases (CTX-M-ases)) of extended-spectrum β-lactamases (ESBLs) that were initially described in E. coli, *Klebsiella pneumoniae,* and *Salmonella* spp. but rapidly emerged in other *Enterobacteriaceae*, as well as in non *Enterobacteriaceae* species including *Pseudomonas aeruginosa.* This family includes the CTX-M-3, CTX-M-9, CTX-M-14, and CTX-M-15 enzymes. The CTX-M-15 used as a target for the kits of the invention may be the *Escherichia coli* beta-lactamase CTX-M-15, of protein_id AAL02127.1. Said CTX-M-15 protein may comprise the amino acid sequence encoded by the nucleic acid sequence as denoted by SEQ ID NO. 77. The CTX-M-15 protein may comprise the amino acid sequence as denoted by SEQ ID

NO. 76.

[0094] At least one spacer of the CRISPR array in the sensitizing element of the kit of the invention or system of may comprise a nucleic acid sequence that targets at least one of: at least one proto-spacer of CTX-M-15, at least one proto-spacer of NDM-1 and at least one nucleic acid sequence comprised within a lytic phage genome.

[0095] It must be appreciated that any sequence, sub-sequence or fragment comprising about 10 to about 50 nucleotides, specifically, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 56, 47, 48, 49 or 50 nucleotides, or more specifically, about 35 nucleotides of any of the resistance conferring genes described herein before, may be use as a protospacer, and therefore as a target to the sensitizing component and specifically to the particular spacers comprised within. Any sequence, sub-sequence or fragment comprising about 10 to about 50 nucleotides of at least one of the NDM-1 and the CTX-M-15 genes, and specifically of those having or comprising the nucleic acid sequences as denoted by any one of SEQ ID NO. 74 and 77, respectively, may be used as an appropriate and effective protospacer. Such protospacers may comprise at least one protospacer adjacent motif (PAM) sequence. Such PAM sequences may be any one of AAA, AAC, AAG, AAT, CAG, GAA, GAC, GAG, TAA, TAC, TAG, AGA ,AGC, AGG, GGG, TGG, ATA, ATC, ATG, ATT, CTG, GTG, TTG. Such PAM may be AWG, wherein "W" may represent any one of "A" or "T". Still further, the protospacers may comprise at last one of said PAM sequences at the 5' end of the protospacer sequence.

[0096] The CRISPR system, specifically, the sensitizing component thereof, may comprise at least one of: at least spacer that targets at least one proto-spacer of CTX-M-15. Such protospacer/s may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51 (also referred to herein as C1, C2 and C3, respectively), at least one spacer that targets at least one proto-spacer of NDM-1, specifically, such protospacer may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48 (also referred to herein as N1, N2 and N3, respectively).

[0097] Still further, the genetically modified lytic phage that is the selective component of the kit of the invention may comprise at least one proto-spacer of at least one of: (a) at least one proto-spacer of CTX-M-15. Such protospacer may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51 (also referred to herein as C1, C2 and C3, respectively). The genetically modified lytic phage may comprise in addition or instead, (b) at least one proto-spacer of NDM-1. In more specific embodiments, such proto spacer may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48 (also referred to herein as N1, N2 and N3, respectively). It should be noted that in certain embodiments, the protospacers of NDM-1 comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48 may be targeted by spacers as denoted by SEQ ID NO. 37, 38 and 39, respectively. Still further, the proto-spacer of CTX-M-15, comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51 may be targeted by spacers as denoted by SEQ ID NO. 40, 41 and 42, respectively.

[0098] The selective component may comprise at least one lytic phage. Such lytic phage may be at least one of T7-likevirus and T4-like virus. The lytic phage used by the kit of the invention may be a T7-like-virus, specifically, at least one Enterobacteria phage T7. Bacteriophage T7 are DNA viruses having a lytic life cycle. These phages belong to the order *Caudovirales,* family*Podoviridae* and the genus *T7-like viruses.*

[0099] It should be appreciated that any suitable lytic phage may be used by the kits, systems and methods. One non-limiting example may be the phages that are members of the *Myoviridae* family. Members of *Myoviridae* are non-enveloped phages having a characteristic structure of head-and-tail separated by a neck. *Myoviridae* genome is a linear dsDNA of about 33.6-170 Kb in length, which encodes up to 200-300 proteins that are transcribed in operons. Most of the *Myoviridae* are lytic phages, lacking the genes required to become lysogenic (become integrated into the host bacterium's genome or forming a circular replicon in the bacterium's cytoplasm) but a number of lysogenic species are known. *Myoviridae* have been divided into four subfamilies, of which the most relevant to the present context is the *Tevenvirinae* subfamily (also *Teequatrovirinae,* Taxonomy ID: 1198136) to which, among others, belong the T4likevirus phages.

[0100] Therefore, lytic phage may be at least one host-DNA degrading bacteriophage, for example any member of the *Tevenvirinae* phages.

[0101] Members of *Tevenvirinae* have similar morphology characterized by moderately elongated heads of about 110 nm in length, 114 nm long tails with a collar, base plates with short spikes and six long kinked tail fibers. This subfamily is divided into two genera on the basis of head morphology (i.e. T4likevirus and Schizot4likevirus) and within the genera - on the basis of protein homology the species have been divided into a number of groups. The complete *Tevenvirinae* lineage includes phages from the genus *T4likevirus* and *Schizot4likevirus.*

[0102] Specifically, the present disclosure pertains to the *T4likevirus* bacteriophages including *Acinetobacter phage 133, Aeromonas phage 25, Aeromonas phage 31, Aeromonas phage 44RR2.8t, Aeromonas phage 65, Aeromonas phage Aeh1, Enterobacteria phage SV14, Enterobacteria phage T4 sensu lato, Vibrio phage nt-1 sensu lato, Unclassified T4-like viruses* species (according to ICTV). More specifically, the present disclosure pertains to bacteriophages from the *Enterobacteria phage T4 sensu lato* species, including *Enterobacteria phage C16, Enterobacteria phage FSalpha, Enterobacteria phage MV 72, Enterobacteria phage MV SS, Enterobacteria phage MV12, Enterobacteria phage MV13, Enterobacteria phage MV14, Enterobacteria phage MV9, Enterobacteria phage PST, Enterobacteria phage T2, Entero-*

bacteria phage T4, Enterobacteria phage T6 subspecies; and further to bacteriophages from the Unclassified *T4-like viruses species,* including *Acinetobacter phage Ac42, Acinetobacter phage Acj61, Acinetobacter phage Acj9, Acinetobacter phage ZZ1, Aeromonas phage Aes002, Aeromonas phage Aes007, Aeromonas phage Aes012, Aeromonas phage Aes120, Aeromonas phage Aes123, Aeromonas phage Aes144, Aeromonas phage Aes151, Aeromonas phage Aes508, Aeromonas phage Aes509, Aeromonas phage Aes512, Aeromonas phage Aes516, Aeromonas phage Aes517, Aeromonas phage CC2, Aeromonas phage phiAS4, Aeromonas phage phiAS5, Aeromonas phage PX29, Burkholderia phage 42, Citrobacter phage Miller, Cronobacter phage vB_CsaM_GAP161, Cyanophage 2B096, Cyanophage 2Bnp, Cyanophage 2Gdp, Cyanophage 4B092, Cyanophage 4B09p, Cyanophage 5Bd2, Cyanophage 5Bnp, Cyanophage 6Bnp, Cyanophage 7E02p, Cyanophage 7G09p, Cyanophage 7Gmp, Cyanophage 8B026, Cyanophage 8B092, Cyanophage 8G092, Cyanophage P-TIM3, Cyanophage S-TIM4, Enterobacteria phage 1, Enterobacteria phage Ac3, Enterobacteria phage AR1, Enterobacteria phage Baker, Enterobacteria phage Bp7, Enterobacteria phage CC31, Enterobacteria phage CEV1, Enterobacteria phage DD VI, Enterobacteria phage ELY-1, Enterobacteria phage GEC-3S, Enterobacteria phage HX01, Enterobacteria phage IME08, Enterobacteria phage ime09, Enterobacteria phage JS, Enterobacteria phage JS10, Enterobacteria phage JS98-C3, Enterobacteria phage JSE, Enterobacteria phage K3, Enterobacteria phage KC69, Enterobacteria phage LZ1, Enterobacteria phage LZ10, Enterobacteria phage LZ2, Enterobacteria phage LZ3, Enterobacteria phage LZ4, Enterobacteria phage LZ5, Enterobacteria phage LZ6, Enterobacteria phage LZ7, Enterobacteria phage LZ8, Enterobacteria phage LZ9, Enterobacteria phage M1, Enterobacteria phage Mi, Enterobacteria phage MV BS, Enterobacteria phage nvv1, Enterobacteria phage Ox2, Enterobacteria phage Phil, Enterobacteria phage Pol, Enterobacteria phage RB1, Enterobacteria phage RB10, Enterobacteria phage RB14, Enterobacteria phage RB15, Enterobacteria phage RB16, Enterobacteria phage RB18, Enterobacteria phage RB2, Enterobacteria phage RB21, Enterobacteria phage RB23, Enterobacteria phage RB25, Enterobacteria phage RB26, Enterobacteria phage RB27, Enterobacteria phage RB3, Enterobacteria phage RB30, Enterobacteria phage RB32, Enterobacteria phage RB33, Enterobacteria phage RB42, Enterobacteria phage RB43, Enterobacteria phage RB49, Enterobacteria phage RB5, Enterobacteria phage RB51, Enterobacteria phage RB6, Enterobacteria phage RB61, Enterobacteria phage RB62, Enterobacteria phage RB68, Enterobacteria phage RB69, Enterobacteria phage RB70, Enterobacteria phage RB8, Enterobacteria phage RB9, Enterobacteria phage SC1, Enterobacteria phage SCI, Enterobacteria phage SV76, Enterobacteria phage Tula, Enterobacteria phage Tulb, Enterobacteria phage U4, Enterobacteria phage U5, Enterobacteria phage vB_EcoM-VR7, Enterobacteria phage vB_EcoM_ACG-C40, Escherichia phage e11/2, Escherichia phage IME08, Escherichia phage Lw1, Escherichia phage LZ, Escherichia phage LZ1, Escherichia phage LZ9, Escherichia phage vB_EcoM_JS09, Escherichia phage vB_EcoM_PhAPEC2, Escherichia phage wV7, Klebsiella phage KP15, Klebsiella phage KP27, Phage LZ, Phage LZ11, Prochlorococcus phage P-SSM2, Prochlorococcus phage P-SSM4, Salmonella phage S16, Serratia phage PS2, Shigella phage Shfl2, Shigella phage SP18, Sinorhizobium phage phiM12, Stenotrophomonas phage IME13, Stenotrophomonas phage Smp14, Synechococcus phage metaG-MbCM1, Synechococcus phage S-MbCM100, Synechococcus phage S-MbCM6, Synechococcus phage S-MbCM7, Synechococcus phage S-PM2, Synechococcus phage S-RSM4, Synechococcus phage syn9* and *Yersinia phage PST* subspecies (according to ICTV).

[0103] It should be appreciated that the selective component may comprise any lytic phage that infects E. coli. Such lytic phages may be any phages that target any pathogenic bacteria.

[0104] As noted above, the selective element may be a DNA sequence encoding bacterial killers as described herein before and at least one protospacer. The selective component may comprise at least one lytic bacteriophage, specifically, a genetically modified lytic phage. Such lytic phage may be at least one T7 bacteriophage. The lytic phage used as the selective component in the kit of the invention may be a T7 genetically modified phage comprising one proto-spacer of NDM-1, N1 as denoted by SEQ ID NO. 46, and a proto-spacer of CTX-M-15, C1, as denoted by SEQ ID NO. 49, said phage is referred to herein as T7-N1C1. The recombinant or genetically modified lytic phage T7-N1C1 may comprise the nucleic acid sequence as denoted by SEQ ID NO. 57. In yet another embodiment, the selective element may be a genetically modified lytic phage, specifically, a T7 bacteriophage comprising two proto-spacers of NDM-1, N1 and N2, as denoted by SEQ ID NO. 46 and 47, respectively, said phage is referred to herein as T7-N1N2. The recombinant or genetically modified lytic phage T7-N1N2 may comprise the nucleic acid sequence as denoted by SEQ ID NO. 55. Still further, the selective element may be a genetically modified lytic phage, specifically, a T7 bacteriophage comprising two proto-spacers of CTX-M-15, for example, C2 and C1, as denoted by SEQ ID NO. 50 and 49, respectively, said phage is referred to herein as T7 C2C1. The recombinant or genetically modified lytic phage T7- C2C1 may comprise the nucleic acid sequence as denoted by SEQ ID NO. 56. The selective element may be a genetically modified lytic phage, specifically, a T7 bacteriophage comprising one proto-spacer of CTX-M-15, C2, as denoted by SEQ ID NO. 50, and a proto-spacer of NDM-1, N2, as denoted by SEQ ID NO. 47, said phage is referred to herein as T7-C2N2. IThe recombinant or genetically modified lytic phage T7- C2N2 may comprise the nucleic acid sequence as denoted by SEQ ID NO. 58.

[0105] The selective component of the kit or a system may be a non-engineered phage. Accordingly, the CRISPR array of the sensitizing component should be designed to target such phages. As such, at least one CRISPR spacer must be sufficiently complementary to a nucleic acid sequence comprised within an essential gene of said lytic phage.

[0106] Thus, at least one CRISPR spacer may target a nucleic acid sequence comprised within an essential gene of said lytic phage. Such lytic phage may be at least one of T7-like-virus and T4like virus. More specific disclosures relate to at least one of Enterobacteria phage T7 and Enterobacteria phage T4. In more specific disclosures, the lytic phage used as the selective component of the kit or a system may be at least one of T4 and T7.

[0107] In such specific cases, the CRISPR array may comprise spacers that target and recognize nucleic acid sequences of these phages.

[0108] In some specific and non-limiting embodiments, such spacers may comprise spacers that target proto-spacers in T7 bacteriophage. More specific embodiments relate to spacers comprising any one of SEQ ID NO. 43, 44 and 45. In yet more specific embodiments, such spacers target proto-spacers that comprise the nucleic acid sequence of any one of SEQ ID NO. 52, 53 and 54, respectively. In more specific embodiments, these proto-spaces are comprised within the lytic phage.

[0109] The disclosure also relates to any one of the genetically modified or engineered lytic bacteriophages as described herein before.

[0110] The bacteriophage used for the sensitizing component of the kit or system may be a lambda phage. Such phage may be a lambda temperate phage.

[0111] An example for a temperate phage may be a lambda phage having the nucleic amino acid sequence as denoted by SEQ ID NO. 36 (NCBI Reference Sequence: NC_001416.1).

[0112] By way of example, the bacteriophages used for the kits of the invention and systems include, but are not limited to, those bacteriophage, either lytic or temperate bacteriphages, capable of infecting any nosocomial bacteria.

[0113] By way of another example, the bacteriophage include, but are not limited to, those bacteriophage (lytic or temperate) capable of infecting a bacterium including but not limited to any one of the *proteobacteria, Firmicutes* and *Bacterioidetes* phyla.

[0114] By way of further example, the bacteriophage include but are not limited to, those bacteriophage capable of infecting bacteria belonging to the following genera: *Escherichia coli, Pseudomonas, Streptococcus, Staphylococcus, Clostidium, Enterococcus, Klebsiella Acinetobacter* and *Enterobacter.*

[0115] Other lytic phages infecting other organisms, and particularly the ESKAPE organisms listed above could also be used as a selective agent in a kit targeting these organisms. The selective agent can also be a DNA encoding a bacterial-killing agent that is injected through a phage capsid or another method. Other temperate phages infecting other organisms, and particularly the ESKAPE organisms listed above could also be used as the sensitizing component in a kit targeting these organisms. More specifically, it should be appreciated that any bacteriophage, either a lytic phage or a temperate bacteriophage, may be applicable for the purpose of the invention, specifically, as the selective and the sensitizing components. Of particular interest are bacteriophages that specifically target any of the "ESKAPE" pathogens. As used herein, these pathogens include but are not limited to *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter.* To name but few, these bacteriophages, either lytic or temperate, may include but are not limited to bacteriophages specific for *Staphylococcus aureus,* specifically, at least one of vB_Sau. My D1, vB_Sau My 1140, vB_SauM 142, Sb-1, vB_SauM 232, vB_SauS 175, vB_SauM 50, vB_Sau 51/18 , vB_Sau.M. 1, vB_Sau.M. 2, vB_Sau.S. 3, vB_Sau.M. 4, vB_Sau.S. 5, vB_Sau.S. 6, vB_Sau.M.7, vB_Sau.S.8, vB_Sau.S.9, vB_Sau.M.10, vB_Sau.M.11. Lytic or temperate bacteriophages specific for *Klebsiella pneumoniae,* may be also applicable for the present invention. These phages may include vB_Klp 1, vB_Klp 2, vB_Klp. M.1, vB_Klp. M.2, vB_Klp. P.3, vB_Klp. M.4, vB_Klp. M.5, vB_Klp. M.6, vB_Klp. 7, vB_Klp. M.8, vB_Klp. M.9, vB_Klp. M.10, vB_Klp. P.11, vB_Klp. P.12, vB_Klp. 13, vB_Klp. P.14, vB_Klp. 15, vB_Klp. M.16. Bacteriophages specific for *Pseudomonas aeruginosa,* may be applicable for the selective and/or sensitizing components or any kits or methods using these components. Non-limiting examples for such bacteriophages include but are not limited to vB_Psa.Shis 1 , vB_PsaM PAT5, vB_PsaP PAT14, vB_PsaM PAT13, vB_PsaM ST-1, vB_Psa cT 27, vB_Psa CT 44 K, vB_Psa CT 44 M, vB_Psa 16, vB_Psa Ps-1, vB_Psa 8-40, vB_Psa 35 K, vB_Psa 44, vB_Psa 1, vB_Psa 9, vB_Psa 6-131 M, vB_Psa cT 37, vB_Psa cT 45 S, vB_Psa CT 45 M, vB_Psa CT 16 MU, vB_Psa CT 41, vB_Psa CT 44 MU, vB_Psa cT 43, vB_Psa CT 11 K, vB_Psa 1638, vB_Psa Ps-2, vB_Psa 35 CT, vB_Psa 35 M, vB_Psa S.Ch.L, vB_Psa R1, vB_Psa SAN, vB_Psa L24, vB_Psa F8, vB_Psa BT - 4, vB_Psa BT-2(8), vB_Psa BT-1(10), vB_Psa BT-4-16, vB_Psa BT-5, vB_Psa F-2, vB_Psa B-CF, vB_Psa Ph7/32, vB_Psa Ph7/63, vB_Psa Ph5/32, vB_Psa Ph8/16, vB_Psa Ph11/1, vB_Psa, vB_Psa 3, vB_Psa 4, vB_Psa 5, vB_Psa 6, vB_Psa 7, vB_Psa.P. 15, vB_Psa.17, vB_Psa.M. 18, vB_Psa. 28, vB_Psa.M .2, vB_Psa.M 3, vB_Psa.23, vB_Psa.P. 8, vB_Psa.M. PST7, vB_Psa.M .C5, vB_Psa.M .D1038. Bacteriophages specific for *Acinetobacter baumanii*, may be applicable for the present invention. Such lytuic or temperate phages may include any one of vB_Aba B37, vB_Aba G865, vB_Aba G866, vB_Aba U7, vB_Aba U8, vB_Acb 1, vB_Acb 2. Bacteriophages specific for *Enterobacter* may be used for the kits and methods, specifically, any one of vB_Eb 1, vB_Eb 2, vB_Eb 3, vB_Eb 4 bacteriophages. *Enterococcus faecalis* specific bacteriophages may be used. Several non-limiting examples include any one of, vB_Ec 1, vB_Ec 2, vB_Enf.S.4, vB_Enf.S.5 bacteriophages.

[0116] Bacteriophages that specifically infect *Bacillus anthracis,* for example, vB_BaK1, vB_BaK2, vB_BaK6,

vB_BaK7, vB_BaK9, vB_BaK10, vB_BaK11, vB_BaK12, vB_BaGa4, vB_BaGa5, vB_BaGa6, may be also applicable for the present invention. Still further, bacteriophages specific for *Brucella abortus* for example, Tb, vB_BraP IV, vB_BraP V, vB_BraP VI, vB_BraP VII, vB_BraP VIII, vB_BraP IX, vB_BraP X, vB_BraP XII, vB_BraP 12(b), vB_BraP BA, vB_BraP 544, vB_BraP 141 Я , vB_BraP 141m, vB_BraP 19 Я , vB_BraP 19m, vB_BraP 9, bacteriophages specific for *Brucella canis,* specifically, vB_BrcP 1066, bacteriophages specific for *Clostridium perfigenes A.B.C.D.E,* for example, vB_CpPI, vB_CpII, vB_CpIII, vB_CpIV, bacteriophages specific for Desulfovibrio vulgaris, specifically, vB_DvRCH1/M1, vB_DvH/P15, vB_DvH/M15, those specific for *Enterococcus faecalis,* specifically, vB_Ec 1, vB_Ec 2, vB_Enf.S.4, vB_Enf.S.5, bacteriophages specific for *Escherichia coli,* specifically, vB_Eschc.pod 9, vB_Eschc.Pod 4, vB_Eschc.Shis 7, vB_Eschc.Shis 14, vB_Eschc.Shis 5, vB_Eschc.My 2, PhI-1, PhI-2, PhI3, PhI4, PhI5, T2, T4, T5, DDII, DDVI, DDVII, vB_Eschc.Shis 7/20, vB_Eschc.Shis 1161, vB_Eschc.Shis 8963, vB_Eschc 4, vB_Eschc 11/24, vB_Eschc.Shis 18, vB_Shis 3/14, vB_Sau A, vB_Shis G, vB_Eschc.Shis W, vB_Shis GE25, vB_Eschc.Shis 8962, vB_Eschc 90/25, vB_Eschc 5/25, vB_Eschc 12/25, vB_Eschc H, T3, T6, T7, vB_Eschc 4, vB_Eschc 121, vB_Eschc BaK2, vB_Eschc L7-2, vB_Eschc L7-3, vB_Eschc L7-7, vB_Eschc L7-8, vB_Eschc L7-9, vB_Eschc L7-10, vB_Eschc Φ8, vB_Eschc.Shis 20, vB_Eschc.Shis 25, vB_Eschc.Shis 27, vB_Eschc.Shis MY, vB_Eschc 11, vB_Eschc 12, vB_Eschc 13, vB_Eschc 17, vB_Eschc 18, vB_Eschc 19, vB_Eschc 20, vB_Eschc 21, vB_Eschc 22, vB_Eschc 23, vB_Eschc 24, vB_Eschc 25, vB_Eschc 26, vB_Eschc 27, vB_Eschc 28, vB_Eschc 29, vB_Eschc 30, vB_Eschc 31, vB_Eschc 32, vB_Eschc 34, vB_Eschc 35, vB_Eschc 37, vB_Eschc 38, vB_Eschc 39, vB_Eschc 44, vB_Eschc 45, vB_Eschc 46, vB_E.coli.M. 1, vB_E.coli.M. 2, vB_E.coli. P.3, vB_E.coli. P.4, vB_E.coli. P.5, vB_E.coli. P.6, vB_E.coli. P.7, vB_E.coli. P.8, phages specific for *Salmonella paratyphi,* specifically, vB_ SPB Diag 1, vB_ SPB Diag 2, vB_ SPB Diag 3, vB_ SPB Diag 3b, vB_ SPB Diag Jersey, vB_ SPB Diag Beecles, vB_ SPB Diag Taunton, vB_ SPB DiagB.A.O.R, vB_ SPB Diag Dundee, vB_ SPBDiagWorksop, vB_ SPB Diag E, vB_ SPB Diag D, vB_ SPB Diag F, vB_ SPB Diag H, specific for *Salmonella typhi abdominalis* vB_ Sta Diag A, vB_ Sta Diag B1, vB_ Sta Diag B2, vB_ Sta Diag C1, vB_ Sta Diag C2, vB_ Sta Diag C3, vB_ Sta Diag C4, vB_ Sta Diag C5, vB_ Sta Diag C6, vB_ Sta Diag C7, vB_ Sta Diag D1, vB_ Sta Diag D2, vB_ Sta Diag D4, vB_ Sta Diag D5, vB_ Sta Diag D6, vB_ Sta Diag D7, vB_ Sta Diag D8, vB_ Sta Diag E1, vB_ Sta Diag E2, vB_ Sta Diag E5, vB_ Sta Diag E10, vB_ Sta Diag F1, vB_ Sta Diag F2, vB_ Sta Diag F5, vB_ Sta Diag G, vB_ Sta Diag H, vB_ Sta Diag J1, vB_ Sta Diag J2, vB_ Sta Diag K, vB_ Sta Diag L1, vB_ Sta Diag L2, vB_ Sta Diag M1, vB_ Sta Diag M2, vB_ Sta Diag N, vB_ Sta Diag O, vB_ Sta Diag T, vB_ Sta Diag Vi1, vB_ Sta Diag27, vB_ Sta Diag 28, vB_ Sta Diag 38, vB_ Sta Diag 39, vB_ Sta Diag 40, vB_ Sta Diag 42, vB_ Sta Diag 46, *Salmonella typhimurium,* specifically, vB_Stm.My 11, vB_Stm.My 28, vB_Stm.Shis 13, vB_Stm.My 760, vB_Stm.Shis 1, IRA, vB_Stm 16 , vB_Stm 17 , vB_Stm 18 , vB_Stm 19 , vB_Stm 20, vB_Stm 21 , vB_Stm 29, vB_Stm 512 , vB_Stm Diag I, vB_Stm Diag II, vB_Stm Diag III, vB_Stm Diag IV, vB_Stm Diag V, vB_Stm Diag VI, vB_Stm Diag VII, vB_Stm Diag VIII, vB_Stm Diag IX, vB_Stm Diag X, vB_Stm Diag XI, vB_Stm Diag XII, vB_Stm Diag XIII, vB_Stm Diag XIV, vB_Stm Diag XV, vB_Stm Diag XVI, vB_Stm Diag XVII, vB_Stm Diag XVIII, vB_Stm Diag XIX, vB_Stm Diag XX, vB_Stm Diag XXI, vB_Stm Diag 1, vB_Stm Diag 2, vB_Stm Diag 3, vB_Stm Diag 4, vB_Stm Diag 5, vB_Stm Diag 6, vB_Stm Diag 7, vB_Stm Diag 8, vB_Stm Diag 9, vB_Stm Diag 10, vB_Stm Diag 11, vB_Stm Diag 12, vB_Stm Diag 13, vB_Stm Diag 14, vB_Stm Diag 15, vB_Stm Diag 16, vB_Stm Diag 17, vB_Stm Diag 18, vB_Stm Diag 19, vB_Stm Diag 20, vB_Stm Diag 21, vB_Stm Diag 22, vB_Stm Diag 23, vB_Stm Diag 24, vB_Stm Diag 25, vB_Stm Diag 26, vB_Stm Diag 27, vB_Stm Diag 28, vB_Stm Diag 29, vB_Stm Diag 30, vB_Stm Diag 31, vB_Stm Diag 32, vB_Stm Diag 33, vB_Stm Diag 34, vB_Stm Diag 35, vB_Stm Diag 36, vB_Stm Diag 37, vB_Stm Diag 38, vB_Stm Diag 39, vB_Stm Diag 40, vB_Stm Diag 41, vB_Stm Diag 42, vB_Stm Diag 43, vB_Stm Diag 44, vB_Stm Diag 45, vB_Stm Diag 46, vB_Stm Diag 47, vB_Stm Diag 48, vB_Stm Diag 49, vB_Stm Diag 50, vB_Stm Diag 51, vB_Stm Diag 52, vB_Stm Diag 53, vB_Stm Diag 54, vB_Stm Diag 55, vB_Stm Diag 56, vB_Stm Diag 57, vB_Stm Diag 58, vB_Stm Diag 59, vB_Stm Diag 60, vB_Stm Diag 61, vB_Stm Diag 62, vB_Stm Diag 63, vB_Stm Diag 64, vB_Stm Diag 65, vB_Stm. P. 1, vB_Stm. P. 2, vB_Stm. P. 3, vB_Stm. P. 4, *Shigella sonnei,* specifically, vB_Shs.Pod 3, vB_Eschc.Shis 7/20, vB_Eschc.Shis 1161, vB_Eschc.Shis 8963, vB_Eschc.Shis 8962, vB_Shis GE25, vB_Eschc.Shis W, vB_Shis G, vB_Shis 3/14, vB_Eschc.Shis 18, vB_Shis 1188, vB_Shis 1188 Г, vB_Shis 1188 Y, vB_Shis 1188 X, vB_Shis 5514, vB_Shis L7-2, vB_Shis L7-4, vB_Shis L7-5, vB_Shis L7-11, vB_Shis K3, vB_Shis TuI A, vB_Shis Ox2, vB_Shis SCL, vB_Shis Bak C2, vB_Shis 4/1188, vB_Shis 8962, vB_Shis 8963, vB_Shis XIV, vB_Shis 116, vB_Shis 106/8, vB_Shis 20, vB_Shis 90/25, vB_Shis 87/25, vB_Shis 16/25, vB_Shs 7, vB_Shs 38, vB_Shs 92, vB_Shs 1391, vB_Shs. P. 1, vB_Shs. P. 2, vB_Shs. P. 3. It should be further appreciated that any bacteriophage, either lytic or temperate, specific for any pathogenic bacteria, and specifically to any of the pathogenic bacteria disclosed herein, may be applicable for the kits and methods of the invention or to any component thereof, specifically, the selective and the sensitizing components.

[0117] The CRISPR-Cas system has evolved in prokaryotes to protect against phage attack and undesired plasmid replication by targeting foreign DNA or RNA (16, 20, 21). The *Escherichia coli* CRISPR-Cas system, targets DNA molecules molecules based on short homologous DNA sequences, called spacers that exist between repeats within the bacterial genome. These spacers guide CRISPR-associated (Cas) proteins to matching (and/or complementary) sequences within the foreign DNA, called protospacers, which are subsequently cleaved. The spacers can be rationally

designed to target any DNA sequence, including those that encode resistance genes and lytic phages. This allows genetically linking a trait that is beneficial to the bacteria (i.e., elements conferring phage resistance) with a trait that reverses drug resistance (i.e. elements eliminating resistance genes). This genetic linkage enables selecting a sensitized bacterial population by using lytic phages as selection agents. Bacteria harboring both a defense against the lytic phages and a sensitizing construct survive on the treated surfaces, whereas those that lack these factors are killed by the lytic phages. The integrated construct/s are designed not only to actively eradicate existing resistance genes but also to eliminate horizontal transfer of genes between bacteria.

[0118] The temperate bacteriophage may further comprise a nucleic acid sequence encoding a CRISPR leader sequence.

[0119] As indicated above, the sensitizing component of the kit of the invention may comprise at least one Cas gene and the CRISPR system. With respect to CRISPR systems, as will be recognized by those skilled in the art, the structure of a naturally occurring CRISPR locus includes a number of short repeating sequences generally referred to as "repeats". The repeats occur in clusters and up to 249 repeats have been identified in a single CRISPR locus and are usually regularly spaced by unique intervening sequences referred to as "spacers." Typically, CRISPR repeats vary from about 24 to 47 base pair (bp) in length and are partially palindromic. The CRISPR repeats may be included in the sensitizing component of the kit and may comprise repeats having about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or more bp. The repeats are generally arranged in clusters (up to about 20 or more per genome) of repeated units. The spacers are located between two repeats and typically each spacer has unique sequences that are from about 20 or less to 72 or more bp in length. Thus, the CRISPR spacers used in the sensitizing component may comprise between 20 to 72 nucleotides (nt.) each. More specifically, about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71 72 or more. Many spacers are identical to or have high homology with known phage sequences. In addition to repeats and spacers, a CRISPR locus also includes a leader sequence and often at least one associated Cas gene, specifically, a set of two to six or more associated Cas genes. The leader sequence typically is an AT-rich sequence of up to 550 bp directly adjoining the 5' end of the first repeat. New repeat-spacer units are believed to be almost always added to the CRISPR locus between the leader and the first repeat.

[0120] As indicated above, the engineered temperate phage used as the sensitizing element in the kit of the invention or a system may comprise CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) arrays together with the *cas* genes form the CRISPR system. As used herein, 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.* In subsequent years, similar CRISPR arrays were found in *Mycobacterium tuberculosis, Haloferax mediterranei, Methanocaldococcus jannaschii, Thermotoga maritima* and other bacteria and archaea. It should be understood that the use of any of the known CRISPR systems is contemplated, particularly and of the CRISPR systems disclosed herein.

[0121] 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 down regulating (e.g. eliminating) a complementary gene.

[0122] The CRISPR array polynucleotide may comprise at least 2 repeats with 1 spacer between them. The CRISPR array of the sensitizing component may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, specifically, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 or more spacers. It should be further understood that the spacers of the sensitizing component may be either identical or different spacers. These spacers may target either an identical or different target bacterial pathogenic gene. Such spacer may target at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more pathogenic bacterial gene/s.

[0123] The CRISPR array polynucleotide may comprise 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.

[0124] 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.

[0125] It has been revealed that CRISPR systems are found in approximately 40 % and 90 % of sequenced bacterial and archaeal genomes, respectively, and the present inventor contemplates the use of CRISPR arrays from all such CRISPR systems.

[0126] The CRISPR array polynucleotide may comprise a nucleic acid sequence which, apart from the spacer, (or

spacers) which is replaced so as to down-regulate (e.g. eliminate) the gene of interest, is 100 % homologous to the naturally occurring (wild-type) sequence.

[0127] The CRISPR array polynucleotide may comprise 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. The CRISPR array polynucleotide may comprise a nucleic acid sequence which, apart from the spacer, (or spacers) which is replaced so as to down-regulate or eliminate a gene of interest and specifically, RNA encoded thereby, is 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% homologous to the naturally occurring (wild-type) sequence.

[0128] 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. CRISPR spacers may be located in between two identical CRISPR repeats. CRISPR spacers may be identified by sequence analysis at the DNA stretches located in between two CRISPR repeats.

[0129] A CRISPR spacer may be naturally present in between two identical, short direct repeats that are palindromic. It should be noted that the spacers may be located or present between two identical or not identical repeats.

[0130] The phrase "portion of a gene" or "a nucleic acid sequence comprise within a gene" relates to a portion from the coding or non-coding region of the gene.

[0131] The phrase "sufficiently complementary" as used herein, refers to the sequence of the spacer being adequately complementary such that it is capable of down regulating expression of the gene.

[0132] A sequence which is sufficiently complementary to a portion of the gene and specifically, RNA encoded by said gene may be 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 gene. The sequence may be 100 % complementary to the gene.

[0133] The targeting RNA encoded by the CRISPR system may be a CRISPR RNA (crRNA). The sequence of the targeting RNA encoded by the CRISPR spacers is not particularly limited, other than by the requirement for it to be directed to (i.e., having a segment that is the same as or complementarity to) a target sequence in a pathogenic gene of a bacteria that is also referred to herein as a "proto-spacer". Such proto-spacers comprise nucleic acid sequence having sufficient complementarity to a targeting RNA encoded by the CRISPR spacers comprised within the sensitizing system.

[0134] A crRNA may comprise or consist of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nt of the spacer (targeting) sequence followed by 19-36 nt of repeat sequence. The targeting spacer may comprise or consist of a segment that targets any one of the genes for which representative spacer sequences are indicated herein.

[0135] The spacers of the CRISPR system may encode a targeting RNA. A "targeting RNA" is an RNA that, when transcribed from the portion of the CRISPR system encoding it, comprises at least a segment of RNA sequence that is identical to (with the exception of replacing T for U in the case of RNA) or complementary to (and thus "targets") a DNA sequence in the bacterial chromosome, or a sequence on a plasmid within the targeted bacteria. The CRISPR systems of the present disclosure can encode more than one targeting RNA, and the targeting RNAs can be directed to one or more sequences in the bacterial chromosome, or plasmid, or combinations thereof. The sequence of the targeting RNA thus dictates what is targeted by the CRISPR system carried by the sensitizing element, specifically, the genetically modified phage.

[0136] The modified CRISPR array may also comprise a nucleic acid sequence encoding one or more Cas proteins (i.e. *cas* genes).

[0137] 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.

[0138] CRISPR arrays are typically found in the vicinity of four genes named *cas1* to *cas4.* The most common arrangement of these genes is *cas3-cas4-cas l-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 *cas1* gene (NCBI COGs database code: COG1518) is especially noteworthy, as it serves as a universal marker of the CRISPR system (linked to all CRISPR systems except for that of *Pyrococcus abyssii*). *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.

[0139] In addition, there is another cluster of three genes associated with CRISPR structures in many bacterial species, referred to herein as *cas IB, cas5* and *cas6.* The cas gene may be selected from *casl, cas2, cas3, cas4, cas IB, cas5* and/or *cas6,* fragments, variants, homologues and/or derivatives thereof. A combination of two or more cas genes be useful, including any suitable combinations.

[0140] The cas genes may comprise DNA. Alternatively, the cas may comprise RNA. The nucleic acid may be of genomic origin, or of synthetic or recombinant origin. The cas genes may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof.

[0141] The cas gene may be 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.

[0142] 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).

[0143] Still further, three major types of CRISPR-Cas system are delineated: Type I, Type II and Type III.

[0144] **Type I CRISPR-Cas systems** contain the *cas3* gene, which encodes a large protein with separate helicase and DNase activities, in addition to genes encoding proteins that probably form Cascade-like complexes with different compositions. These complexes contain numerous proteins that have been included in the repeat-associated mysterious proteins (RAMPs), which form a large superfamily of Cas proteins, and contain at least one RNA recognition motif (RRM; also known as a ferredoxin-fold domain) and a characteristic glycine-rich loop. RAMP superfamily encompasses the large Cas5 and Cas6 families on the basis of extensive sequence and structure comparisons. Furthermore, the Cas7 (COG1857) proteins represent another distinct, large family within the RAMP superfamily.

[0145] The type I CRISPR-Cas systems seem to target DNA where the target cleavage is catalysed by the HD nuclease domains of Cas3. As the RecB nuclease domain of Cas4 is fused to Cas1 in several type I CRISPR-Cas systems, Cas4 could potentially play a part in spacer acquisition instead. It should be noted that any type I CRISPR-Cas systems may be applicable in the present invention, specifically, any one of type I-A, B, C, D, E, and F.

[0146] **The type II CRISPR-Cas systems** include the 'HNH'-type system (Streptococcus-like; also known as the Nmeni subtype, for *Neisseria meningitidis serogroup A str.* Z2491, or CASS4), in which Cas9, a single, very large protein, seems to be sufficient for generating crRNA and cleaving the target DNA, in addition to the ubiquitous Cas1 and Cas2. Cas9 contains at least two nuclease domains, a RuvC-like nuclease domain near the amino terminus and the HNH (or McrA-like) nuclease domain in the middle of the protein, but the function of these domains remains to be elucidated. However, as the HNH nuclease domain is abundant in restriction enzymes and possesses endonuclease activity, it is likely to be responsible for target cleavage.

[0147] Type II systems cleave the pre-crRNA through an unusual mechanism that involves duplex formation between a tracrRNA and part of the repeat in the pre-crRNA; the first cleavage in the pre-crRNA processing pathway subsequently occurs in this repeat region. This cleavage is catalysed by the housekeeping, double-stranded RNA-specific RNase III in the presence of Cas9. Still further, it should be noted that type II system comprise at least one of *cas9, cas1, cas2 csn2, and cas4* genes. It should be appreciated that any type II CRISPR-Cas systems may be applicable in the present invention, specifically, any one of type II-A or B.

[0148] **The type III CRISPR-Cas systems** contain polymerase and RAMP modules in which at least some of the RAMPs seem to be involved in the processing of the spacer-repeat transcripts, analogous to the Cascade complex. Type III systems can be further divided into sub-types III-A (also known as Mtube or CASS6) and III-B (also known as the polymerase-RAMP module). Subtype III-A systems can target plasmids, as has been demonstrated *in vivo* for *S. epidermidis,* and it seems plausible that the HD domain of the polymerase-like protein encoded in this subtype (COG1353) might be involved in the cleavage of target DNA. There is strong evidence that, at least in vitro, the type III-B CRISPR-Cas systems can target RNA, as shown for a subtype III-B system from *furiosus.* It should be appreciated that any cas gene that belongs to the type III CRISPR system may be used for the purpose of the invention, for example, any one of *cas6, cas10, csm2, csm3, csm4, csm5, csm6, cmr1, cmr3, cmr4, cmr5, cmr6, cas1* and *cas2.* Still further, any one of typeIII-A or typeIII-B systems may be used for the kits, components and method. Of particular interest, specifically in cases where endogenous pathogenic genes are targeted by the kits and methods of the invention, the typeIII-B system may be used.

[0149] The three types of CRISPR systems show a distinctly non-uniform distribution among the major lineages of the Archaea and the Bacteria. In particular, the type II systems have been found exclusively in the Bacteria so far, whereas type III systems are more common in the Archaea.

[0150] Typically, a repeat cluster is preceded by a 'leader' sequence, an AT-rich region several hundred base pairs long with intraspecies but not interspecies conservation. CRISPR-associated (*"cas"*) genes, a set of conserved protein-coding genes that are associated with these loci, are usually present on one side of the array. Analysis of spacer sequences in several CRISPR loci revealed that spacers match sequences from foreign, mobile genetic elements, such as bacteriophages and plasmids. Approximately 40% of sequenced bacterial genomes, and about 90% of those from archaea (prokaryotes), contain at least one CRISPR locus.

[0151] CRISPR-Cas immune systems must discriminate between self and non-self to avoid an autoimmune response. In ***"type I and II CRISPR-Cas systems",*** foreign DNA which contain the protospacer adjacent motif (PAM) sequences are targeted for degradation, whereas potential targets in CRISPR loci of the host do not contain PAMs and are thereby avoided by RNA-guided interference complexes.

[0152] The sensitizing components of the kits of the invention may comprise at least one *cas* gene. Such cas gene/s may be at least one cas gene of at least one of type I, type II and type III CRISPR systems.

[0153] Such at least one *cas* gene may be at least one cas gene of type I-E CRISPR system. The ***"type-IE CRISPR"***

system refers to native to K-type *Escherichia coli.* It has been shown to inhibit phage infection, cure plasmids, prevent conjugal element transfer and kill cells. This CRISPR machinery can be used to degrade specific intracellular DNA in an inducible and targeted manner, leaving the remainder DNA intact.

[0154] The at least one type I-E *cas* gene comprised within the temperate phage may be at least one of *cse1, cse2, cas7, cas5 cas6e* and *cas3* genes. In addition to at least one of *cse1, cse2, cas7, cas5 cas6e* and *cas3* genes, the sensitizing component may further comprise at least one of *cas1* and *cas2* genes.

[0155] The cas genes of the sensitizing component includes *cse1,* gene. Such *cse1* gene encodes the Cse1 protein of *Escherichia coli* str. K-12 substr. MG1655, as denoted by protein_id AAC75802.1. The *cse1* gene may comprise the nucleic acid sequence as denoted by SEQ ID NO. 60. The *cse1* gene may encode the Cse1 protein that comprises the amino acid sequence as denoted by SEQ ID NO. 67. The sensitizing component may include the *cse2* gene. Such Cse2 protein may be the *Escherichia coli* str. K-12 substr. MG1655, as denoted by protein_id AAC75801.1. The Cse2 protein may be encoded by the nucleic acid sequence as denoted by SEQ ID NO. 61. The cse2 protein may comprise the amino acid sequence as denoted by SEQ ID NO. 68. Still further, the sensitizing component may comprise cas7. Said cas7 protein may be the Escherichia coli str. K-12 substr. MG1655 Cas7 protein of id AAC75800.1. The Cas7 protein may be encoded by the nucleic acid sequence as denoted by SEQ ID NO. 62, or related to the Cas7 protein comprising the amino acid sequence as denoted by SEQ ID NO. 69. Still further, the sensitizing component may comprise the cas5. More specifically, the Escherichia coli str. K-12 substr. MG1655 Cas5 protein_of idAAC75799.2. The Cas5 protein may encoded by the nucleic acid sequence as denoted by SEQ ID NO. 63. The Cas5 protein may comprise the amino acid sequence as denoted by SEQ ID NO. 70.

[0156] The sensitizing component may comprise cas6e. The Cas6e protein may be the Escherichia coli str. K-12 substr. MG1655 Cas6e protein of _id AAC75798.1. The Cas6e protein may be encoded by a nucleic acid sequence as denoted by SEQ ID NO. 64.Tthe Cas6e protein may comprise the amino acid sequence as denoted by SEQ ID NO. 71. The temperate phage may further comprise the cas3 gene. The cas3 gene may encode the Escherichia coli str. K-12 substr. MG1655 Cas3 protein of id AAC75803.1. The Cas3 protein may be encoded by the nucleic acid sequence as denoted by SEQ ID NO. 65. The Cas3 protein may comprise the amino acid sequence as denoted by SEQ ID NO. 72.

[0157] It should be noted that the kits of the invention and particularly, the sensitizing components thereof in accordance with the present invention applies to a plurality of CRISPR-cas proteins orthologs or homologues having a sequence homology or identity to the *cas* proteins used as described herein before, of at least 50%, at least 60% and specifically 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher.

[0158] There are a number of well known approaches for identification and isolation of candidate orthologs or functional homologues from different species, most of which use sequence similarity on a nucleotide or protein levels. Isolated candidates are then subjected to sequencing and sequence comparisons using customary software programs such as *Basic Local Alignment Search Tool (BLAST)* at *NCBI* (The National Center for Biotechnology Information), LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wis.), or other methods (e.g. Wu et al. (eds.) "Information Superhighway and Computer Databases of Nucleic Acids and Proteins" in Methods in Gene Biotechnology (CRC Press, Inc. 1997) and Bishop (ed.) "Guide to Human Genome Computing" 2nd Edition (Academic Press, Inc. 1998).

[0159] The CRISPR cascade genes used by the kits, systems and methods may be of the E.coli type I-E CRISPR system. Nevertheless, as also indicated above, it should be appreciated that any other CRISPR systems may be applicable.

[0160] Thus, the at least one *cas* gene used in the kits and systems may be at least one *cas* gene of type II CRISPR system (either typeII-A or typeII-B). At least one *cas* gene of type II CRISPR system used by the kits of the invention may be the *cas9* gene. It should be appreciated that such system may further comprise at least one of *cas1, cas2, csn2* and *cas4* genes.

[0161] Double-stranded DNA (dsDNA) cleavage by Cas9 is a hallmark of ***"type II CRISPR-Cas"*** immune systems. The CRISPR-associated protein Cas9 is an RNA-guided DNA endonuclease that uses RNA:DNA complementarity to identify target sites for sequence-specific doublestranded DNA (dsDNA) cleavage. The targeted DNA sequences are specified by the CRISPR array, which is a series of B30-40 bp spacers separated by short palindromic repeats. The array is transcribed as a pre-crRNA and is processed into shorter crRNAs that associate with the Cas protein complex to target complementary DNA sequences known as proto-spacers. These proto-spacer targets must also have an additional neighbouring sequence known as a proto-spacer adjacent motif (PAM) that is required for target recognition. After binding, a Cas protein complex serves as a DNA endonuclease to cut both strands at the target and subsequent DNA degradation occurs via exonuclease activity.

[0162] The Cas9 of Streptococcus pyogenes M1 GAS, specifically, the Cas9 of protein id: AAK33936.1 may be applicable in the kit of the invention. The Cas9 protein may be encoded by the nucleic acid sequence as denoted by SEQ ID NO. 66. The Cas9 protein may comprise the amino acid sequence as denoted by SEQ ID NO. 73. As noted above, it should be recognized that with few adaptations, the use of the strategy may be further broadened. For example, the system may be designed to specifically eliminate phage lysogenizations and transductions by targeting specific phages, thus reducing a significant source of virulence-genes transfer. Another alteration of this strategy may deal with resistance

genes encoded by chromosomal elements (as also referred to herein, endogeneous gene) rather than those transferred on mobile elements. In such cases, targeting the DNA would counter select against the transferred CRISPR-Cas as it will kill the host. However, elimination of the resistance element can still be achieved using CRISPR-Cas system that target RNA, for example, the type III-B system. While targeting the RNA will eliminate the resistance conferred by the encoded gene, it will not kill the pathogen, and would thus avoid counter selection against the delivering temperate phage. The flexibility and ease of genetically engineering spacers combined with the availability of various types of CRISPR-Cas systems may thus allow many useful variations of the strategy. In this respect, the fact that the inventors used the CRISPR-Cas subtype I-E, rather than the more frequently used subtype-IIA, demonstrates that desired outcomes may be obtained with different subtypes. For targeting epichromosomal or extrachromosomal pathogenic genes, the CRISPR-Cas type I, TYPE II and type III-A systems may be used, however, where the target pathogenic gene is an endogeneous, or chromosomal gene, the type III-B system may be applicable for the components, kits and methods.

[0163] The genetically modified lysogenic phage lambda of the sensitizing component may be a phage designated IYMMPh3. Such genetically modified phage may comprise the nucleic acid sequence as denoted by SEQ ID No. 59.

[0164] Provided are efficient kits, systems and methods targeting and destroying pathogenic genes of bacterial pathogens. More specifically, such bacteria or bacterial populations may be antibiotic resistant bacteria. Of particular interest are any bacteria involved in nosocomial infections. The term ***"Nosocomial Infections"*** refers to Hospital-acquired infections, namely, an infection whose development is favored by a hospital environment, such as surfaces and/or medical personnel, and is acquired by a patient during hospitalization. Nosocomial infections are infections that are potentially caused by organisms resistant to antibiotics. Nosocomial infections have an impact on morbidity and mortality, and pose a significant economic burden. In view of the rising levels of antibiotic resistance and the increasing severity of illness of hospital inpatients, this problem needs an urgent solution.

[0165] In the United States, the Centers for Disease Control and Prevention estimated roughly 1.7 million hospital-associated infections, from all types of microorganisms, however, Gram-negative infections are estimated to account for two-thirds of the annual patients' deaths. A Gram-negative bacterium *Clostridium difficile* is now recognized as the chief cause of nosocomial diarrhea in the US and Europe. Other common nosocomial organisms include methicillin-resistant *Staphylococcus aureus,* coagulase-negative *Staphylococci,* vancomycin-resistant *Enteroccocci,* resistant *Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter* and *Stenotrophomonas maltophilia.*

[0166] The nosocomial-infection pathogens could be subdivided into Gram-positive bacteria (*Staphylococcus aureus, Coagulase-negative staphylococci*), Gram-positive cocci (*Enterococcus faecalis and Enterococcus faecium*), Gram-negative rod-shaped organisms (*Klebsiella pneumonia, Klebsiella oxytoca, Escherichia coli, Proteus aeruginosa, Serratia spp.*), Gram-negative bacilli (*Enterobacter aerogenes, Enterobacter cloacae*), aerobic Gram-negative coccobacilli (*Acinetobacter baumanii, Stenotrophomonas maltophilia*) and Gram-negative aerobic bacillus (*Stenotrophomonas maltophilia,* previously known as *Pseudomonas maltophilia*). Among many others *Pseudomonas aeruginosa* is an extremely important nosocomial Gram-negative aerobic rod pathogen.

[0167] Some disclosures relate to the kit of the invention or a system for use in targeting and eliminating pathogenic genes in bacteria of any strain of at least one of *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Clostidium difficile, Enterococcus faecium, Klebsiella pneumonia, Acinetobacter baumanni and Enterobacter species* (specifically, ESKAPE bacteria).

[0168] The bacterium may be any one of *Pseudomonas aeruginosa, Streptococcus pyogenes, Clostidium difficile* and *Staphylococcus aureus.*

[0169] The bacteria as referred to herein may include *Yersinia enterocolitica, Yersinia pseudotuberculosis, Salmonella typhi, Pseudomonas aeruginosa, Vibrio cholerae, Shigella sonnei, Bordetella Pertussis, Plasmodium falciparum, Chlamydia trachomatis, Bacillus anthracis, Helicobacter pylori* and *Listeria monocytogens.*

[0170] The kit or a system may be particularly suitable for use in any *E. coli* strain, specifically, any one of O157:H7, enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and diffuse adherent (DAEC) E. coli.

[0171] It should be appreciated that any unit or component or element of the kits of the invention or systems may be comprised or presented in any composition, preparation or device.

[0172] Still further, it should be understood that any composition or preparation may comprise at least one of the kits of the invention, or at least one of its elements, components or units. The kits, or any elements or components thereof, for example, at least one selective component and at least one sensitizing component/s may be presented at any suitable ration. For example, between about 0.0001-10,000:0.0001-10,000. More specifically, 0.0001:10000 and 10000:0.0001. A cocktail of kits that are directed against different pathogenic genes, and/or different pathogenic bacteria may be used.

[0173] The disclosure includes CRISPR systems on the genetically modified phage which target virulent bacterial genes in bacteria within a bacterial population. The bacterial population can comprise one type of bacteria, but with virulent and non-virulent members, or the bacterial population can comprise a plurality of bacterial species, with only certain species having virulent and non-virulent members in the population. A mixed bacterial population may comprise at least two different strains or species of bacteria. The mixed bacterial population may comprise from between two

distinct types of bacteria, to up to a thousand distinct types of bacteria, or more. The kits of the invention and systems may specifically target only bacteria comprising pathogenic genes and lead to specific and targeted elimination of said pathogenic gene from any homogenous or heterogeneous bacterial population.

[0174] The proof of principle presented here is a step toward decreasing the threat of emerging drug-resistant pathogens, against which limited weapons have been developed. It demonstrates that with simple genetic engineering, bacteria can be sensitized to approved and useful antibiotics. The system may be a simple treatment for hospital surfaces and useful in hand sanitizers and possibly as a probiotic food additive to reverse the resistance of pathogens residing on hospital surfaces and in the normal flora of the medical personnel. In contrast to antibiotics and disinfectants that select for resistant pathogens, the proposed treatment enriches and selects for sensitive pathogens. Moreover, as shown by the Examples, the system enriches for pathogens that cannot transfer or receive resistance determinants horizontally, and may thus reduce the spread of antibiotic resistance. The enriched, sensitive population could prevent newly introduced resistant pathogens from becoming established by overtaking their ecological niche.

[0175] Since the CRISPR-Cas system can be programmed to eliminate any gene of interest, the system could be used to restrict transfer of any antibiotic-resistance gene. In fact, the short sequence of spacer required to eliminate an antibiotic-resistant gene enables the construction of dozens of such spacers in a single array, thus re-enabling the use of a vast number of antibiotics against which resistance has developed. It can also be programmed to simultaneously protect against several lytic phages that will be used for selection, thus reducing the occurrence of unsensitized mutants that escaped these lytic phages. Moreover, the system may be used to target lysogenic phages or any plasmid or DNA element carrying virulence genes, and by using a CRISPR-Cas system that targets RNA, it can even target virulence genes encoded by the pathogen itself.

[0176] The activity of the CRISPR-Cas system against plasmid DNA as well as against lytic phages is well established. Nevertheless, its utility in clinical settings as a tool to render pathogens sensitive to antibiotics and to reduce horizontal gene transfer of resistance determinants is novel. The proof of principle provided herein can be applied to different pathogen-phage systems as temperate phages can be found for most of the pathogens, and a compatible CRISPR-Cas system should work in many pathogens. Broad use of the proposed system, in contrast to antibiotics and phage therapy, will potentially change the nature of nosocomial infections by making the bacteria more susceptible rather than more resistant to antibiotics.

[0177] The present inventors contemplate use of the above described temperate bacteriophages to infect bacterial populations on surfaces, for example solid or liquid surface/s, or solid support, any substance, or any article, rendering antibiotic insensitive bacteria residing thereon to become sensitive to antibiotic.

[0178] Cocktails of different temperate bacteriophages that serve as the sensitizing components may be applied to surfaces, for example solid or liquid surfaces or solid support, any substance ar any article, each temperate bacteriophage having different host specificity, each carrying a CRISPR array which specifically targets antibiotic resistance genes by encoding homologous sequences to these genes in the spacers of the CRISPR array. To select and enrich for the bacteria carrying the arrays, the arrays may also carry spacers against lytic phages, thus protecting them from these agents. These lytic phages may be sprayed in the environment to exert selection pressure for the pathogens to take the sensitizing CRISPR array. It will be appreciated that the temperate bacteriophages are not bactericidal to their hosts since the bacteriophages are not modified to express agents that are toxic to bacteria.

[0179] The enriched, antibiotic-sensitive populations might then interfere with the establishment of newly introduced resistant pathogens by overtaking their ecological niche. The present approach differs from conventional phage therapy in the sense that it does not use phages to kill the pathogens directly. Consequently, there is no selection against the used phage, but rather selection for pathogens harboring the phage because it contains resistance to a lytic phage. Moreover, the approach avoids the use of phages inside the patient's body, thus overcoming toxicity issues and other drawbacks of phage therapy, such as phage neutralization by the spleen and the immune system. Extended use of this treatment will result in replacing also the natural bacterial flora of the hospital personnel (skin, respiratory and GI tracts), to carry less resistant pathogens.

[0180] It should thus be appreciated that the temperate bacteriophage used as the sensitizing component of the kit or the system may be formulated as a spray, a stick, a paint, a gel, a cream, wash, a wipe, a foam, a soap, an oil, a solution, a lotion, an ointment, a hand sanitizer or a paste.

[0181] The lytic bacteriophage used as the selective component of the kit or a system may be formulated as a spray, a stick, paint, a gel, a cream, a wash, a liquid, a wipe, foam, soap, oil, a solution, a lotion, an ointment or a paste.

[0182] It should be noted that the temperate bacteriophages of the sensitizing component of the kit of the invention and the lytic bacteriophages that serve as the selective component of the kit of the invention may be applied concurrently, or one following the other. Alternatively, the temperate and lytic bacteriophages may be applied on consecutive days. It should be further noted that each of these components may be comprised within any composition, formulation or vehicle that may optionally comprise at least one of pharmaceutically acceptable carrier/s, excipient/s, auxiliaries, and/or diluent/s. It should be further appreciated that in accordance with routine procedures as compositions adapted for external or internal application. Where necessary, the composition may also include a solubilizing agent or any compound facil-

itating application thereof.

[0183] The active agents can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0184] Still further, the kits of the invention and systems and any components thereof may be applied as a single daily dose or multiple daily doses, preferably, every 1 to 7 days. It is specifically contemplated that such application may be carried out once, twice, thrice, four times, five times or six times daily, or may be performed once daily, once every 2 days, once every 3 days, once every 4 days, once every 5 days, once every 6 days, once every week, two weeks, three weeks, four weeks or even a month. The application of the kits of the invention or of any component thereof may last up to a day, two days, three days, four days, five days, six days, a week, two weeks, three weeks, four weeks, a month, two months three months or even more. Specifically, application may last from one day to one month. Most specifically, application may last from one day to 7 days. Application of the kits of the invention and systems or any component thereof may be a routine procedure, specifically, daily procedure of treating surfaces, articles or any substance, for example, in a hospital environment.

[0185] Single or multiple applications of the kits of the invention and systems and any components thereof are applied depending on the amount and frequency as required. In any event, the kits of the invention and systems and any components thereof should provide a sufficient quantity to effectively prevent horizontal transfer of a pathogenic bacterial gene and most importantly, to prevent any pathologic disorder in a mammalian subject, caused by bacteria comprising said pathogenic gene. Preferably, the effective amount may be applied once but may be applied periodically until a result is achieved.

[0186] Hospital surfaces contain complex mixtures of bacterial populations: some of them are resistant pathogens belonging to different species. Spraying surfaces may be an effective method to target these pathogens. The spray may contain both the temperate CRISPRCas-encoding phages and the lytic phages. Delivery may also be carried out in the form of liquid added to soaps or other hand sanitizers in hospitals. These delivery methods avoid the use of phages inside the patient's tissues, thus overcoming toxicity issues and other drawbacks of phage therapy.

[0187] As noted above, this strategy may be applied for treating hospital surfaces and hand sanitizers soaps or other liquids for targeting the skin flora of medical personnel. In contrast to antibiotics and disinfectants that select for resistant pathogens, the proposed treatment enriches and selects for sensitive pathogens. Specifically, this strategy may be further broadened to Medical Departments where immune compromised patients are hospitalized in whom antibiotic resistance is a life threatening condition. In contrast to antibiotics and disinfectants that select for resistant pathogens, the proposed treatment enriches and selects for sensitive pathogens. Moreover, the system enriches for pathogens that cannot receive or transfer resistance determinants horizontally and may thus further reduce the spread of antibiotic resistance. The enriched sensitive population could prevent newly introduced resistant pathogens from becoming established by overtaking their ecological niche.

[0188] It should be further noted that contacting the bacterial cells with a specific CRISPR-Cas construct that targets resistance-conferring plasmids, can discriminate between antibiotic resistant and -sensitive pathogens within the same strain in complex bacterial populations.

[0189] A second aspect of the invention relates to an *in vitro* method of interfering with a genetic element comprising at least one pathogenic gene between bacteria, the method comprises the steps of: contacting at least one of a surface, a substance or an article containing bacteria harboring said pathogenic gene with:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage, or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and
(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component, or
(iii) at least one kit comprising (i) and (ii), thereby inactivating said pathogenic gene and interfering with horizontal transfer thereof, preferably, said kit is as defined in the first aspect.

[0190] Thus, provided is a method for preventing, reducing, attenuating, inhibiting and eliminating horizontal transfer of pathogenic genes in bacterial populations. "Horizontal gene transfer" (HGT), as used herein refers to the transfer of genes between organisms in a manner other than traditional reproduction. Also termed *lateral gene transfer* (LGT), it contrasts with *vertical transfer,* the transmission of genes from the parental generation to offspring via sexual or asexual

reproduction. As noted above, horizontal gene transfer is the primary reason for bacterial antibiotic resistance and transmission of virulence. This horizontal gene transfer often involves temperate bacteriophages and plasmids. Genes that are responsible for antibiotic resistance in one species of bacteria can be transferred to another species of bacteria through various mechanisms (e.g., via F-pilus). Horizontal transfer interfered, inhibited, eliminated or reduced by the kits and methods may be HGT affected by conjugative pili that allow for the transfer of DNA between bacteria, in the process of bacterial conjugation. More specifically, a pilus is typically 6 to 7 nm in diameter. During conjugation, a pilus emerging from the donor bacterium ensnares the recipient bacterium, draws it in close, and eventually triggers the formation of a mating bridge, which establishes direct contact and the formation of a controlled pore that allows transfer of DNA from the donor to the recipient. Occasionally, the DNA transferred consists of antibiotic resistance genes (often encoded on a plasmid). It should be further understood that the kits of the invention and method may interfere with any pathway or mechanism that leads to transfer of pathogenic genes, specifically horizontal transfer, between bacteria. It should be further noted that the method may be applicable for interfering with HGT mediated either by transduction, by natural competence or by trasposons.

[0191] It should be understood that interfering with horizontal transfer may encompass any elimination, inhibition, reduction, moderation, decrease, attenuation, restraining or retardation of any transfer of pathogenic gene/s between bacteria (for example, horizontal transfer) by any one of about 1% to 99.9%, specifically, about 1% to about 5%, about 5% to 10%, about 10% to 15%, about 15% to 20%, about 20% to 25%, about 25% to 30%, about 30% to 35%, about 35% to 40%, about 40% to 45%, about 45% to 50%, about 50% to 55%, about 55% to 60%, about 60% to 65%, about 65% to 70%, about 75% to 80%, about 80% to 85% about 85% to 90%, about 90% to 95%, about 95% to 99%, or about 99% to 99.9%.

[0192] Such interference may be of at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or about 100% of the horizontal transfer of a bacterial pathogenic gene as compared to the transfer occurred in the absence of the kits of the invention and methods.

[0193] Further disclosed is a method of preventing a pathologic condition in a mammalian subject caused by a bacterial infection of bacteria containing a pathogenic gene. The method comprises contacting at least one solid or liquid surface, substance, article or support in the vicinity of the subject with at least one of (i) at least one selective component; (ii) at least one sensitizing component; and (iii) any kit comprising at least one of (i) and (ii), , thereby targeting and inactivating the pathogenic gene that may be comprised in bacteria that exist in the environment of such subject. In such a way, the method leads to inactivation of these pathogenic genes in the bacteria, thereby preventing pathologic condition that may be caused by bacteria expressing at least one intact pathogenic gene. Any of the kits described by the invention may be used for any of the methods.

[0194] The methods may involve the steps of contacting a surface, specifically a solid or liquid surface, article, or any substance (specifically, in the vicinity of the subject) with the temperate bacteriophage, that is the sensitizing component, and subsequently contacting the solid surface with the lytic bacteriophage, that is the selective component of the kit of the invention or a system.

[0195] As used herein the term "contacting" refers to the positioning of the temperate bacteriophages (and optionally, the lytic bacteriophage) such that they are in direct or indirect contact with the bacterial cells. Thus, the present invention contemplates both applying the temperate bacteriophages (and optionally the lytic bacteriophages) to a desirable surface and/or directly to the bacterial cells.

[0196] Contacting surfaces with the kits of the invention, and specifically with the temperate bacteriophages (sensitizing component) and the lytic bacteriophages (selective component) can be effected using any method known in the art including spraying, spreading, wetting, immersing, dipping, painting, ultrasonic welding, welding, bonding or adhering.

[0197] The present invention envisages contacting a wide variety of surfaces with the bacteriophages including fabrics, fibers, foams, films, concretes, masonries, glass, metals, plastics, polymers, and like.

[0198] The bacteriophages may be contacted with surfaces present in a hospital, hospice, old age home, or other such care facility.

[0199] 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 in a food or beverage factory.

[0200] 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. Thus, the bacteriophages may also be used for disinfecting toilet bowls, catheters, NG tubes, inhalators and the like.

[0201] The kit of the invention may be applied in the vicinity of the treated subject. The expression "vicinity of the treated subject" relates to the perimeter surrounding said subject onto which the kit according to the invention may be

applied in order to prevent horizontal transfer of antibiotic resistance gene/s. Therefore, it is understood that the "vicinity of said subject" encompasses all objects present within a range of up to at least about 1 centimeter (cm), 2 cm, 3 cm, 4 cm, 5 cm, 6 cm, 7 cm, 8 m, 9 m, 10 cm, 20 cm, 30 cm, 40 cm, 50 cm, 60 cm, 70 cm, 80 cm, 90 cm, 1 meter (m), 2 m, 3 m, 4 m, 5 m, 6 m, 7 m, 8 m, 9 m, 10 m, 11 m, 12 m, 13 m, 14 m, 15 m, 16 m, 17, m 18 m, 19 m, 20 m, 30 m, 40 m or even 50 m of said subject. The term "vicinity of said subject" also relates to objects to which the kit of the invention is applied to prior to their placement in said range of the treated subject.

[0202] The kits of the invention or any components or any bacteriophages may be applied every 12 hours, daily, 6 times a week, 5 times a week, four times a week, three times a week, twice a week or even once a week to the solid surface.

[0203] The methods may use any of the kits as defined by the invention and systems, herein above. More specifically, the selective component of the kits used by the methods may be any DNA sequence comprising at least one protospacer recognized by at least one spacer of the sensitizing component, and a sequence encoding at least one toxic agent or any bacterial killer. The selective component may comprise at least one lytic bacteriophage. In some specific embodiments such lytic bacteriophage may be at least one genetically modified bacteriophages comprising at least one proto-spacer having an identity of at least 70% to at least one nucleic acid sequence comprised within said bacterial pathogenic gene. The sensitizing component of the kits used by the methods may comprise at least one recombinant vector comprising a nucleic acid sequence encoding at least one *cas* protein. Such vector may further comprise a nucleic acid sequence of at least one of said CRISPR array.

[0204] Still further, such vector may be at least one genetically modified bacteriophage comprising at least one CRISPR spacer that targets at least one nucleic acid sequence comprised within said lytic bacteriophage and at least one CRISPR spacer that targets a nucleic acid sequence comprised within said at least one pathogenic gene, thereby targeting and inactivating both, the lytic phage and said pathogenic gene.

[0205] The target bacterial pathogenic gene may be at least one bacterial endogenous gene or alternatively, an epichromosomal gene. The pathogenic gene may be an antibiotic resistance gene. Alternatively, the pathogenic gene may be a gene encoding at least one of a virulence factor and at least one toxin. The antibiotic resistance gene targeted by the kits of used by the methods may encode a resistance factor selected from the group consisting of CTX-M-15, New Delhi metallo-β-lactamase (NDM)-1, 2, 5, 6, an extended-spectrum beta-lactamase resistance factor (ESBL factor), beta lactamase, and tetracycline A (tetA). The at least one CRISPR spacer of the sensitizing component of the kits used by the methods may comprise a nucleic acid sequence that targets at least one of: at least one proto-spacer of CTX-M-15, at least one proto-spacer of NDM-1, 2, 5, 6, at least one proto-spacer of ESBL factor, at least one proto-spacer of beta lactamase, at least one proto-spacer of tetA and at least one at least one proto-spacer of a lytic bacteriophage.

[0206] More specifically, at least one of the proto-spacer of CTX-M-15, may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51 and at least one of said proto-spacer of NDM-1, may comprise a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48.

[0207] The genetically modified lytic bacteriophage used as the selective component of the kits used by the methods may comprise at least one of: (a) at least one proto-spacer of CTX-M-15, comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51; and (b) at least one proto-spacer of NDM-1 comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48.

[0208] The sensitizing component of the kits used by the methods may comprise at least one CRISPR spacer that targets a nucleic acid sequence comprised within an essential gene of the lytic bacteriophage. More specifically, such lytic bacteriophage may be at least one of T7like-virus and T4like-virus. Such T7like-virus may be at least one Enterobacteria phage T7.

[0209] The sensitizing component of the kits used by the methods may comprise a bacteriophages, specifically, a lambda temperate bacteriophage. Still further, the at least one *cas* gene of the sensitizing component of the kits used by the methods may be at least one cas gene of at least one of type I, type II and type III CRISPR systems.

[0210] The sensitizing component of the kits used by the methods may comprise at least one *cas* gene of type I-E CRISPR system. Such type I-E *cas* gene may be at least one of cse1, cse2, cas7, cas5e cas6 and cas3 genes. The at least one *cas* gene of the sensitizing component of the kits used by the methods may be at least one *cas* gene of type II CRISPR system. At least one *cas* gene of type II CRISPR system may be cas9 gene. It should be further noted that the kits of the invention and methods may target at least one bacterium of any strain of at least one of *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Clostidium difficile, Enterococcus faecium, Klebsiella pneumonia, Acinetobacter baumanni and Enterobacter species.* Such bacteria may be at least one *E. coli* strain selected from the group consisting of O157:H7, enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and diffuse adherent (DAEC) E. coli.

[0211] The at least one of the temperate bacteriophage and the lytic bacteriophage of the kits used by the methods may be formulated as a spray, a stick, a paint, a gel, a cream, wash, a wipe, a foam, a soap, a liquid, an oil, a solution, a lotion, an ointment or a paste. The disclosure also relates to a genetically modified, temperate bacteriophage. More specifically, the temperate bacteriophage may comprise at least one CRISPR array. At least one spacer of the CRISPR may be complementary to a nucleic acid sequence comprised within at least one pathogenic gene of a bacterium (that

is a portion of said gene), so as to target and inactivate the at least one pathogenic gene in the bacterium. It should be further noted that at least one spacer of said CRISPR array is sufficiently complementary to a nucleic acid sequence comprised within a lytic bacteriophage so as to target and inactivate the lytic phage. In such a way, a bacterium infected by the temperate phage is insensitive and resistant to the lytic phage.

[0212] The disclosure also relates to the genetically modified lytic phages described.

[0213] As used herein, the term 'polynucleotide' or a 'nucleic acid sequence' refers to a polymer of nucleic acids, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). As used herein, 'nucleic acid' (also or nucleic acid molecule or nucleotide) refers to any DNA or RNA polynucleotides, oligonucleotides, fragments generated by the polymerase chain reaction (PCR) and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action, either single- or doublestranded. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., alpha-enantiomeric forms of naturally-occurring nucleotides), or modified nucleotides or any combination thereof. Herein this term also encompasses a cDNA, i.e. complementary or copy DNA produced from an RNA template by the action of reverse transcriptase (RNA-dependent DNA polymerase).

[0214] In this connection an 'isolated polynucleotide' is a nucleic acid molecule that is separated from the genome of an organism. For example, a DNA molecule that encodes the *cas* gens used by the kit of the invention or any derivatives or homologs thereof, as well as the sequences comprised within the CRISPR spacers and repeats of the kit of the invention, that has been separated from the genomic DNA of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemicallysynthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

[0215] Also disclosed are recombinant DNA constructs comprising the polynucleotides, specifically, those encoding the cas-CRISPR system, or any variants, homologues or derivatives thereof. The constructs may further comprise additional elements such as promoters, regulatory and control elements, translation, expression and other signals, operably linked to the nucleic acid sequence. As used herein, the term "recombinant DNA" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding one of the proteins.

[0216] Still further, disclosed are therapeutic methods comprising the step of administering a therapeutically effective amont of the kit of the invention, optionally in combination with at least one antibiotic compound, specifically, any of the antibiotics disclosed herein before), to a subject suffering from an infectious disease. It should be further noted that the application of the kit of the invention or any component thereof, may form a complementary treatment regimen for subjects suffering from an infectious disease or condition.

[0217] The term "treatment" in accordance with disorders associated with infectious conditions may refer to one or more of the following: elimination, reducing or decreasing the intensity or frequency of disorders associated with said infectious condition. The treatment may be undertaken when disorders associated with said infection, incidence is beginning or may be a continuous administration, for example by administration every 1 to 14 days, to prevent or decrease occurrence of infectious condition in an individual prone to said condition.

[0218] The term "prophylaxis" refers to prevention or reduction the risk of occurrence of the biological or medical event, specifically, the occurrence or re occurrence of disorders associated with infectious disease, that is sought to be prevented in a tissue, a system, animal or human by a researcher, veterinarian, medical doctor or other clinician, and the term "prophylactically effective amount" is intended to mean that amount of a pharmaceutical composition that will achieve this goal. Thus, the methods may be particularly effective in the prophylaxis, i.e., prevention of conditions associated with infectious disease. Thus, subjects administered with said compositions are less likely to experience symptoms associated with said infectious condition that are also less likely to re-occur in a subject who has already experienced them in the past.

[0219] The term "amelioration" as referred to herein, relates to a decrease in the symptoms, and improvement in a subject's condition brought about by the compositions and methods, wherein said improvement may be manifested in the forms of inhibition of pathologic processes associated with bacterial infections, a significant reduction in their magnitude, or an improvement in a diseased subject physiological state.

[0220] The term "inhibit" and all variations of this term is intended to encompass the restriction or prohibition of the progress and exacerbation of pathologic symptoms or a pathologic process progress, said pathologic process symptoms or process are associated with.

[0221] The term "eliminate" relates to the substantial eradication or removal of the pathologic symptoms and possibly pathologic etiology, optionally, according to the methods described below.

[0222] The terms "delay", "delaying the onset", "retard" and all variations thereof are intended to encompass the slowing of the progress and/or exacerbation of a pathologic disorder or an infectious disease and their symptoms slowing their progress, further exacerbation or development, so as to appear later than in the absence of the treatment.

[0223] 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.

[0224] 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.

[0225] As used herein, "disease", "disorder", "condition" and the like, as they relate to a subject's health, are used interchangeably and have meanings ascribed to each and all of such terms.

[0226] The present disclosure relates to the treatment of subjects, or patients, in need thereof. By "patient" or "subject in need" it is meant any organism who may be infected by the above-mentioned pathogens, and to whom the preventive and prophylactic kit/s, system/s and methods herein described is desired, including humans, domestic and non-domestic mammals such as canine and feline subjects, bovine, simian, equine and murine subjects, rodents, domestic birds, aquaculture, fish and exotic aquarium fish. It should be appreciated that the treated subject may be also any reptile or zoo animal. More specifically, the kit/s of the invention and method/s are intended for preventing pathologic condition in mammals. By "mammalian subject" is meant any mammal for which the proposed therapy is desired, including human, equine, canine, and feline subjects, most specifically humans. It should be noted that specifically in cases of non-human subjects, the method may be performed using administration via injection, drinking water, feed, spraying, oral gavage and directly into the digestive tract of subjects in need thereof.

[0227] Still further, it should be noted that further provided are methods for sensitizing bacterial population or increasing the sensitivity of said population to at least one antibiotic compound, by applying the kits of the invention and any components thereof on said bacterial population.

[0228] Disclosed are methods for preventing or reducing resistance of bacteria or bacterial population/s to at least one antibiotic compound using the kits of the invention and any component thereof.

[0229] Further disclosed is a method for treating outbreak of pathogenic bacteria by applying the kits of the invention or any components thereof on surfaces comprising said bacteria.

[0230] 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 sub combination 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.

[0231] 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.

[0232] All scientific and technical terms used herein have meanings commonly used in the art unless otherwise specified. The definitions provided herein are to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

[0233] As used herein the term "about" refers to $\pm$ 10 % The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not 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 systems, kit, composition, method or structure.

[0234] The term "about" as used herein indicates values that may deviate up to 1%, more specifically 5%, more specifically 10%, more specifically 15%, and in some cases up to 20% higher or lower than the value referred to, the deviation range including integer values, and, if applicable, non-integer values as well, constituting a continuous range. As used herein the term "about" refers to $\pm$ 10 %.

[0235] The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to". This term encompasses the terms "consisting of" and "consisting essentially of". The phrase "consisting essentially of" means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the composition or method. Throughout this specification and the Examples and claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0236] It should be noted that various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers

within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range. Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals there between.

[0237] 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 sub combination 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.

[0238] 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.

[0239] Disclosed and described, it is to be understood that this invention is not limited to the particular examples, methods steps, and compositions disclosed herein as such methods steps and compositions may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

[0240] It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

[0241] The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that these techniques are exemplary of preferred embodiments for the practice of the invention.

EXAMPLES

[0242] 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.

[0243] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996). Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

Experimental procedures

Reagents, strains, and plasmids

[0244] Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and agar were from Acumedia. 2YT medium contained 1.6% (w/v) Bacto-tryptone (Acumedia), 1% (w/v) Bacto-yeast extract (Acumedia), and 0.5% (w/v) NaCl (Acumedia) in distilled water. Antibiotics, lysozyme, L-arabinose, and maltose were from Calbiochem. Sodium chloride and magnesium sulphate were from Merck. Restriction enzymes, ligation enzymes, and Phusion® High-Fidelity

DNA Polymerase were from New England Biolabs. The bacterial strains, plasmids, and phages used in this study are listed in **Table 1.**

Table 1. *Bacterial strains, plasmids and phages*

Bacterial strains	**Description**	**Source of reference**
NEB5α	F ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_k^-, m_k^+) *gal- phoA supE44* λ- *thi -1 gyrA96 relA1*	New England Biolabs
DY378	W3110λ*cI857* Δ(*cro-bioA*)	(25)
BW25113Δ yeeX	F-,Δ(*araD-araB*)*567*, Δ*yeeX*::*kan,* Δ*lacZ4787(::rrnB-3),* λ-, *rph-1*, Δ(*rhaD- rhaB) 568, hsdR514*	(30)
RE1001	K12-*araB::*T7*RNAP-tetA*	(26)
RK6471	K12-*araB::*T7*RNAP-tetA*, T7*cas3*::*kan,* T7 *cse1::cm*	The present invention
IYB5101	BW25113 araB::T7-RNAp-tetA, tet^r	(26)
IYB5666	IYB5101 Δ*(cas3-cas2)*:: *cm*	The present invention
IYB5670	BW25113 araB::T7-RNAp-tetA, tet^r . harbors λcas prophage.	The present invention
IYB5671	BW25113 araB::T7-RNAp-tetA, tet^r . harbors λcas-CRISPR prophage.	The present invention
BL21-AI	F- *ompT hsdSB*(rB-, mB-) *gal dcm arab*::*T7RNAP-tetA,* tet^r	Invitrogen (25)
IYB5297	F- *ompT hsdSB*(rB-, mB-) *gal dcm arab*::*T7RNAP-tetA,* tet^r. harbors λcI857-kan prophage.	The present invention
IYB5614	F- *ompT hsdSB*(rB-, mB-) *gal dcm arab*::*T7RNAP-tetA,* tet^r. harbors λcas prophage.	The present invention
IYB5656	F- *ompT hsdSB*(rB-, mB-) *gal dcm arab*::*T7RNAP-tetA,* tet^r. harbors λcas-CRISPR prophage.	The present invention
Phages		
λcI857-kan	cI857 Kan^{R-}	(31)
λcas-cm	cI857 Kan^R, cm^R. Contains *cas3* under T7 promoter and *casABCDE* under T7 promoter	The present invention
λ_{cas}	cI857 Kan^R. Contains *cas3* under T7 promoter and *casABCDE* under T7 promoter	The present invention
λcas-CRISPR	cI857 Kan^R, cm^R. Contains *cas3* under T7 promoter, *casABCDE* under T7 promoter and engineered CRISPR array under T7 promoter.	The present invention
$T7_{FRT\ trxA}$	T7 with trxA flanked by FRT sites	(27)
Plasmids		
pCas 1+2	pCDF-1b (Novagen) cloned with *cas1,2* under T7 promoter, str^r	(23)
pIYEC1	pUC57 cloned with anti NDM-1, CTX-M-15 and T7 phage spacers under T7 promoter, Amp^R	The present invention
pIYEC2	pUC57 cloned with anti NDM-1, CTX-M-15 and T7 phage spacers under T7 promoter, Cam^R, Amp^R	The present invention
pNDM	pCDF-1b (Novagen) based containing New Delhi Metallo-beta-lactamase-1 (NDM-1).	The present invention

(continued)

Plasmids		
pCTX	pCDF-1b (Novagen) based containing CTX-M-15 beta-lactamase.	The present invention
pVEC	pCDF-1b (Novagen) based plasmid.	The present invention
pNDM	Pbil2c BASED CONTAINING New Delhi Metallo-bata-lactamase-1 Gentamicin^r, Carbapenem^r	The present invention
pTRX1	pOEM t-vector (promega) cloned with gp8 proto-spacer (ref severinov).	The present invention
PTRX2	pGEM T-vector (promega) cloned with NDM-1 proto-spacer.	The present invention
PTRX3	pGEM T-vector (promega) cloned with CTX-M-15 proto-spacer.	The present invention
pTRX4	pOEM T-vector (promega) cloned with NDM-1 and CTX-M-15 protospacer.	The present invention
pTRX5	pOEM T-vector (promega) cloned with NDM-1 and CTX-M-15 protospacer.	The present invention
pKD3	pSC101 encoding chloramphenicol resistance marker flanked by FRT sites.	(22)

Plasmid construction

[0245] Plasmids were constructed using standard molecular biology techniques. DNA segments were amplified by PCR. Standard digestion of the PCR products and vector by restriction enzymes was carried out according to the manufacturer's instructions. pIYEC1 plasmid, synthesized by GenScript, encodes a CRISPR array transcribed by a T7 promoter encoding three spacers targeting the *ndm-1* gene (N_1, N_2, N_3, the spacers are denoted by SEQ ID NO. 37, 38, 39 and their corresponding proto-spacers as denoted by SEQ ID NO. 46, 47, 48), three spacers targeting the *ctx-M-15* gene (C_1, C_2, C_3, the spacers are denoted by SEQ ID NO. 40, 41, 42. Said spacers target proto-spacers comprising the nucleic acid sequence of any one of SEQ ID NO. 49, 50, 51)), and three spacers targeting the T4 phage genome (T1, T2, T3 the spacers are denoted by SEQ ID NO. 43, 44, 45. Said spacers target proto-spacers comprising the nucleic acid sequence of any one of SEQ ID NO. 52, 53, 54). pIYEC2 is similar to pIYEC1 except that it also encodes a chloramphenicol resistance marker. To construct pIYEC2, the chloramphenicol resistance marker from pKD3 (22) was amplified using oligonucleotides IY344F and IY344R. The amplified DNA and pIYEC1, both digested by *Hind*III, were ligated to yield pIYEC2. pNDM and pCTX plasmids were constructed by ligating PCR fragments encoding *ndm-1* or *ctx-M-15* to another PCR fragment containing an origin of replication and a str^r marker derived from plasmid pCas1+2 (23) using oligonucleotides IY246F and IY246R for pNDM and IY346F and IY346R for pCTX. Plasmid pVEC was constructed by ligating an irrelevant DNA fragment to the origin of replication and the str^r marker derived from plasmid pCas1+2. Plasmids pTRX1, pTRX2, pTRX3, pTRX4, and pTRX5 were constructed to insert protospacers into the T7 genome (Table 1). The plasmids encode the *trxA* gene, a positive selection marker for T7 grown on hosts lacking *trxA,* flanked by desired protospacers and followed by 50 bp upstream and downstream of a DNA sequence corresponding to the end of T7 gene 1.3 and the beginning of T7 gene 1.4, respectively. The plasmids were constructed by PCR amplification of T7 phage encoding a *trxA* gene flanked by Flippase recognition target sites using the primers indicated in Tables 2 and 3. The resulting PCR product was used as a template for PCR using primers IY260F and IY260R (SEQ ID NO. 24 and 25 Table 2). The final PCR fragment was ligated into pGEM-T vector (Promega). Constructed plasmids were validated as encoding the desired fragments by DNA sequencing.

Table 2. *Oligonucleotide primers*

Oligonucleotides	5'→3'	SEQ ID. NO:
IY344F	ACCGAAGCTTTGAATATCCTCCTTAGTTCC	1
IY344R	CGCCAAGCTTACGGGGGCAACCTCATGTCAAGTGTAGGCTGGAGCTGCTTC	2
IY246F	ATGGAATTGCCCAATATTAT	3
IY246R	TCAGCGCAGCTTGTCGGCCA	4
IY247F	GAACTAAATCAGGCACTTGAGCATCAAGATTGGTG	5
IY247R	CACCAATCTTGATGCTCAAGTGCCTGATTTAGTTC	6
IY346F	ATGGTTAAAAAATCACTGCGCCAGT	7
IY346R	TTACAAACCGTCGGTGACGA	8
IY142Fb	CACACGGTCACACTGCTTCC	9
MG110R	CGATGCCCTTGAGAGCCTTC	10
MG17F	ATAAGTCGGACACCATGGCA	11
IY80F	AATAGCCCGCTGATATCATCGATAATACTAAAAAAAACAGGGAGGCTATTAG TGTAGGCTGGAGCTGCTTC	12
IY80R	ACCTTAATGTAACATTTCCTTATTATTAAAGATCAGCTAATTCTTTGTTTTGA ATATCCTCCTTAGTTCC	13
IY333F	ATGCGTAATGTGTGTATTGCCGTTGCTGTCTTTGCCGCACTTGCGGTGACCC GGAATGAAATTAATACGACTC	14
IY333R	AACCTGTCGCACTCCAGAGAAGCACAAAGCCTCGCAATCCAGTGCAAAGCT CACAGTGGAGCCAAAGATA	15
IY347F	GGCCAGCTAAATCGATGGGATGTGGCTTGCTATCTTTGGCTCCACTGTGAGG GATGTGCTGCAAGGCGAT	16
IY347R	AACCTGTCGCACTCCAGAGAAGCACAAAGCCTCGCAATCCAGTGCAAAGCA CGGGGCAACCTCATGTCAA	17

(continued)

Oligonucleotides	5'→3'	SEQ ID. NO:
IY309F	ACCCTCAAGAGAAAATGTAAAAGCTGTCTTTCGCTGCTGAGGGTGACGATC CCGCGATCCGTCAGCCTGCAGTTC	18
IY309R	CCGAAGGTGAGCCAGTGTGAAAGCTGTCTTTCGCTGCTGAGGGTGACGATC CCGCTGTAGGCTGGAGCTGCTTCG	19
IY340Fa	ACCCTCAAGAGAAAATGTAAAAGCTGAGCACCGCATTAGCCGCTGCATTGA TGCTGATCCGTCAGCCTGCAGTTC	20
IY340Fb	ACCCTCAAGAGAAAATGTAAAAGCTGATTGCTCACGTTGGCGGCCCGGCTA GCGTGATCCGTCAGCCTGCAGTTC	21
IY340Ra	CCGAAGGTGAGCCAGTGTGAGTACGTCCGCCGTTTGCGCATACAGCGGCAC ACTTTGTAGGCTGGAGCTGCTTCG	22
IY340Rb	CCGAAGGTGAGCCAGTGTGAACCGCCAGCGCGACCGGCAGGTTGATCTCCT GCTTTGTAGGCTGGAGCTGCTTCG	23
IY260F	TGGCTCTTTGCGGCACCCATCGTTCGTAATGTTCCGTGGCACCGAGGACAAC CCTCAAGAGAAAATGTAA	24
IY260R	CCAACCTTCTTAAACATAAAGTGTCTCCTTATAAACGCAGAAAGGCCCACCC GAAGGTGAGCCAGTGTGA	25
RK41F	GGAATTACTTCGCTTCGCC	26
RK41R	CCTCCTTATCTCCCTATAGTGAGTCGTATTAATTTCATTCCGGGGATCCGTCG ACC	27
RK42F	AAACGCGTTTCTTTGGCTTAAAAAGGGAATGTGGGTTACACGAAGGGTAAT GTAGGCTGGAGCTGCTTCG	28
RK42R	GGATTTTCCCCAGTAATGGCATATATATTTAAAAGGTTCCATTAATAGCCCC TCCTTATCTCCCTATAGT	29

(continued)

Oligonucleotides	5'→3'	SEQ ID. NO:
RK43F	GCAGCATTACACGTCTTGAG	30
RK43R	CTCCTTATCTCCCTATAGTGAGTCGTATTAATTTCTGAATATCCTCCTTAGTTCC	31
RK44F	TTCGGGAATGATTGTTATCAATGACGATAATAAGACCAATAACGGTTTATGTGTAGGCTGGAGCTGCTTC	32
RK44R	CGCGGGCGTACAGGGATCCAGTTATCAATAAGCAAATTCATTTGTTCTCCCTCCTTATCTCCCTATAGTG	33
RK29R	GACTCTCGAGGCCACTGATCTCTACTGCAG	34
RK33R	GACTCTCGAGGCAACAGCAGCAACATCAAG	35

Homologous recombination-based genetic engineering

[0246] Homologous recombination using short-homology flanking ends was carried out as previously described (24). To insert the six *cas* genes required for CRISPR interference under T7 control, the inventors first cloned the T7 promoters upstream of the *cas3* and *cse1* genes in *Escherichia coli* K-12. An overnight culture of *E. coli* RE1001 (Table 1) harboring the pSIM6 plasmid was diluted 1:100 in 50 mL fresh LB supplemented with 100 μg/mL ampicillin at 32°C and aerated until the OD_{600} reached 0.4-0.6. The culture was then heat-induced for expression of the *red* recombination enzymes at 42°C for 15 min in a shaking water bath followed by incubation in ice water for 10 min. The culture was then centrifuged at 4600 × g for 10 min at 4°C. The supernatant was removed and the pellet was washed three times in ice-cold double-distilled water (ddH_2O). The pellet was resuspended in 200 μL of ice-cold ddH2O and kept on ice. The cultures were then electroporated with ~500 ng of PCR products encoding a T7 promoter fused to either kanamycin or chloramphenicol resistance markers flanked by 50 bp of sequences flanking the original promoters of *cas3* (fragment T7*cas3::kan*) and *cse1* (fragment *T7cse1::cm*) genes. *T7cas3::kan* fragment was constructed by PCR amplification of the kanamycin resistance gene encoding FRT sites from BW25113Δ*yeeX* (Table 1) by using primers RK41F and a primer encoding the T7 promoter in its 5' end, RK41R (Table 1). The PCR fragment was then amplified with RK42F and RK42R (Table 1), encoding 50-bp homology to the immediate 5' region of *cas3.* The *T7cse1::cm* fragment was constructed by PCR amplification of the chloramphenicol fragment encoding FRT sites from the pKD3 plasmid (**Table 1**) by using primers RK43F and a primer encoding the T7 promoter in its 5' end, RK43R (**Table 1**). The PCR fragment was then amplified with RK44F and RK44R (**Table 1**), encoding 50-bp homology of the immediate 5' region of *cse1.* Electroporation of these fragments was carried out using a 50-μL aliquot of electrocompetent bacteria in a 0.2-cm cuvette at 25 μF, 2.5 kV, and 200 Ω. After electroporation 1 mL of 2YT medium was added to the cuvette, followed by aeration at 32°C for 3 h. The cultures were then inoculated on LB agar plates supplemented with 25 μg/mL kanamycin and 17.5 μg/mL chloramphenicol and incubated overnight at 32°C. Recombinant colonies were streaked on 25 μg/mL kanamycin and 17.5 μg/mL chloramphenicol plates and incubated at 42°C to eliminate the temperature-sensitive pSIM6 plasmid. A single colony was validated as encoding the desired substitutions by DNA sequencing using RK33R and RK29R. The entire manipulated cassette encoding *cas3* and *cse1* under the T7 promoters was transduced to the RE1001 strain and selected using both antibiotic markers to yield the RK6471 strain.

[0247] The *cas* genes were deleted as described previously (25). Briefly, *E. coli* DY378 was electroporated with about 500 ng of PCR product generated by amplifying plasmid pKD3 using primers IY80F and IY80R (Tables 1 and 2). This amplified DNA encoded a chloramphenicol resistance marker flanked on one end by 50 bp of sequences of the *cas3* promoter and on another end by 50 bp of the CRISPR leader sequence. Desired recombinants were selected on LB agar plates supplemented with 17 μg/ml chloramphenicol. The deletion was then transferred to IYB5101 using P1 transduction as described (26), yielding IYB5666.

[0248] To construct a λ phage encoding the *cas* genes under T7 promoters, an overnight culture of IYB5297/pSIM6 was diluted 50-fold in 25 mL of LB medium with appropriate antibiotics and grown at 32°C to an OD_{600} of 0.5. The culture was then heat-induced for expression of recombination enzymes from both the λ prophage and the plasmid at 42°C for exactly 4 min in a shaking water bath. The induced samples were immediately cooled on ice slurry and then pelleted at 4600 × g at 4°C for 10 min. The pellet was washed twice in ice-cold ddH_2O, resuspended in 200 μL of ice-cold ddH_2O, and kept on ice until electroporation with ~1600 ng of a gel-purified PCR product obtained by amplifying the genomic DNA of RK6471 using primers IY333F and IY333R. A 25-μL aliquot of electrocompetent cells was used for each electroporation in a 0.2-cm cuvette at 25 μF, 2.5 kV, and 200 Ω. After electroporation, the bacteria were grown in 1 mL LB for 1 h in a 32°C shaking water bath and inoculated on selection plates containing 17 μg/mL chloramphenicol. The chloramphenicol resistance marker was removed using the Flippase recombination enzyme encoded by plasmid pCP20 (24) and chloramphenicol-sensitive colonies were used for phage induction at 42°C. The resulting phage, λ_{cas}, encoding the six *cas* genes transcribed from T7 promoters but lacking a CRISPR array, was used to lysogenize BL21-AI, yielding IYB5614. The engineered CRISPR array was inserted into IYB5614/pSIM6 as described above by using a PCR fragment obtained from amplifying pIYEC2 by primers IY347F and IY347R. The resulting strain, IYB5656, harbors $\lambda_{cas\text{-}CRISPR}$, which encodes the six *cas* genes transcribed from T7 promoters and the CRISPR array encoding spacers against *ndm-1, ctx-M-15,* and the T4 phage genome.

Table 3. Oligonucleotides and templates used for construction of bacteria, phages and plasmids

Constructed phage/ plasmid	Oligonucleotides for PCR	DNA template
IYB5300	IY80F, IY80R	pKD3
RK6471	RK41F, RK41R, RK42F, RK42R, RK43F, RK43R, RK44F, RK44R	pKD3 and Genomic DNA of BW25II3ΔyeeX

(continued)

Constructed phage/ plasmid	Oligonucleotides for PCR	DNA template
λcas-cm	IY333F, IY333R	Genomic DNA of RK6471
λcas-CRISPR	IY347F, IY347R	pIYEC2
T7-gp8	IY309F, IY309R	$T7_{FRTtrxA}$ (24)
T7-N_1N_2	IY340Fa, IY340Rb	$T7_{FRTtrxA}$
T7- C_2C_1	IY340Fb, IY340Ra	$T7_{FRTtrxA}$
T7- N_1C_1	IY340Fa, IY340Ra	$T7_{FRTtrxA}$
T7- C_2N_2	IY340Fb, IY340Rb	$T7_{FRTtrxA}$
pTRX1	IY309F, IY309R	$T7_{FRTtrxA}$
pTRX2	IY340Fa, IY340Rb	$T7_{FRTtrxA}$
pTRX3	IY340Fb, IY340Ra	$T7_{FRTtrxA}$
pTRX4	IY340Fa, IY340Ra	$T7_{FRTtrxA}$
pTRX5	IY340Fb, IY340Rb	$T7_{FRTtrxA}$

Homologous recombination of bacteriophage T7

[0249] T7 phages encoding desired protospacers were constructed as described (29) by using plasmids pTRX1, pTRX2, pTRX3, pTRX4, and pTRX5.

Transformation efficiency assays

[0250] Overnight cultures of *E. coli* IYB5670 and IYB5671 were diluted 1:50 and aerated at 32°C in 10 mL of LB medium supplemented with 25 μg/mL kanamycin and 10 μg/mL chloramphenicol. When the culture reached an OD_{600} of 0.2, 0.2% L-arabinose was added, and the cultures were incubated at 32°C until an OD_{600} of 0.5-0.6 was reached. Bacteria were then centrifuged at 4600 × g at 4°C, the supernatant was disposed, and the bacteria were resuspended in 1 mL of ice-cold ddH_2O and transferred to a 1.5-mL tube. The cells were spun down for 1 min at 13000 × g at 4 °C. After an additional washing step, the cells were suspended in 250 μL of ice-cold ddH_2O. Bacterial cells (50 μL) were then mixed in an ice-cold 0.2-mm electroporation cuvette (Bio-Rad) with 12 ng of pVEC, pNDM, or pCTX plasmids. The mixture was pulsed in a Bio-Rad micropulser at 200 S2, 25 μF, and 1.8 kV. Immediately after the pulse, 0.1 mL of 2YT broth containing 0.2% L-arabinose was added, and the cells were aerated for 1 h at 32°C. Various dilutions of the reaction were plated on LB-agar plates supplemented with 50 μg/mL streptomycin and 0.2% L-arabinose. Plates were incubated overnight at 32°C. Colonies emerging on the selection plates were counted, and the CFU number per mL was calculated accordingly.

Assays of lytic phage growth efficiency

[0251] Overnight cultures of *E. coli* IYB5670 and IYB5671 were diluted 1:50 and aerated at 32°C in 10 mL of LB medium supplemented with 25 μg/mL kanamycin. When the culture reached an OD_{600} of 0.2, 0.2% L-arabinose was added and the cultures were incubated at 32°C until an OD_{600} of 0.5-0.6 was reached. The bacteria were harvested by centrifugation and concentrated to an OD_{600} of ~3. One mL from the concentrated cultures IYB5670 and IYB5671 was mixed with 10 mL of soft agar supplemented with 0.2% L-arabinose and spread onto LB agar plates supplemented with 25 μg of kanamycin and 0.2% L-arabinose. After the agar solidified, the plates were incubated at 32°C for 40 min. Fifteen microlitres of phage dilutions was plated onto the soft agar, allowed to dry, and then incubated at 32°C for 15 h. Plaque-forming units were counted on several dilutions, and their number per mL was calculated accordingly.

Lysogenization

[0252] Overnight culture of IYB5666 harboring pNDM, pCTX or control plasmid (pVEC) were diluted 1:50 in LB medium supplemented with 50 μg/mL of streptomycin, 10 mM $MgSO_4$, and 0.2% (w/v) maltose. Culture was grown to an OD_{600}

of 0.5 and then centrifuged at 13000 g for 1 min. The supernatant was discarded and the pellet was resuspended in LB medium supplemented with 10 mM $MgSO_4$, and 0.2% (w/v) maltose. 10 μL of the treated culture was mixed with 10 μL of phage λ_{cas} or $\lambda_{cas\text{-}CRISPR}$ at a multiplicity of infection of ~10 in a 1.5-mL tube and incubated at room temperature for 30 min. 60 μL of LB medium supplemented with 0.2% L-arabinose was then added, and the cultures were aerated at 32°C for an additional 2.5 h. Cultures were then diluted 1:10 and 84 μL was spread onto LB plates containing 5 μg/mL of tetracycline and 0.2% L-arabinose and 3 mL of soft agar containing 5.5×10^5 T7-C_1N_1 phage. The plates were incubated 36 h at 32°C. To determine plasmid loss, 20-48 of the surviving colonies were resuspended in 0.1 mL of LB, and using a plate replicator the suspension was plated on LB agar plates supplemented with 5 μg/mL tetracycline and 0.2% L-arabinose either with or without 50 μg/mL streptomycin. Colonies sensitive to streptomycin were determined as those grown on medium lacking streptomycin but not on medium having streptomycin.

Example 1

CRISPR-Cas system delivery by a λ phage

[0253] CRISPR/Cas systems have evolved in bacteria to limit the transfer of nucleic acids, such as phages, plasmids, or other parasitic elements. These systems consist of an array of short repeats of about 30 bp flanked by similarly sized sequences, called spacers (Figure 1). The spacers serve as molecular "labels" of undesired nucleic acids. An invading DNA molecule will be eliminated from the cell if the system encodes a spacer that is identical in sequence to any part of this DNA molecule (26, 27). The elimination is carried out by specific proteins which "sense" the alignment, and target and destroy the invading molecule. Recently, a CRISPR/Cas system consisting of a single gene adjacent to a CRISPR array was reported to be active in *Escherichia coli* (27). The system was shown to target a plasmid which had a sequence identical to a spacer in the CRISPR array.

[0254] The system comprises two components or elements, the first component is a sensitizing element that is a temperate phage designed to induce CRISPR-mediated inactivation of pathogenic genes, for example, genes conferring resistance to antibiotics. As such, inactivation of antibiotics resistant genes renders the bacteria sensitive to such antibiotic agents. The second component of the is a lytic phage that is used for selection. Some embodiments encompass genetically modified lytic phage comprising proto-spacers identical to proto-spacers in pathogenic genes that are recognized by the spacers of the CRISPR-array in the temperate phage of the sensitizing component.

[0255] To sensitize bacteria carrying antibiotic resistance genes, a transferable CRISPR-Cas system that targets the *ndm-1* and *ctx-M-15* genes (as shown in Fig. 1) was first constructed. These genes encode extended spectrum β lactamases that confer resistance to carbapenems, β lactam antibiotics which are often the last line of effective antibiotics against resistant pathogens (28). Polymerase chain reaction (PCR) was used to amplify the CRISPR cascade genes (*cse1, cse2, cas7, cas5,* and *cas6e*) and *cas3* of the *E. coli* type I-E CRISPR system. The PCR product was introduced by homologous recombination into a λ prophage. These genes encode proteins that are sufficient to eliminate DNA molecules encoding targeted protospacers (29). A CRISPR array, encoding spacers that target conserved sequences of the resistance genes *ndm-1* and *ctx-M-15* was also introduced into the same lysogen, immediately downstream of the *cas* genes, as illustrated by Figure 1. The prophage was then induced, and its progeny were used to lysogenize naive *E. coli* bacteria. The engineered CRISPR-Cas system, designed to target and destroy plasmids encoding genes *ndm-1* and *ctx-M-15,* was thus made transferable to bacteria by lysogenization.

[0256] Lysogenized bacteria could outcompete bacteria harboring resistance plasmids, indicating that the genetic fitness cost of the transferred prophage is smaller than that of the tested plasmids (Fig. 2).

Example 2

Lysogenized bacteria block transformation

[0257] Naïve *E. coli* lysogenized with the λ phage encoding the CRISPR-Cas system ($\lambda_{cas\text{-}CRISPR}$) or with a similar phage lacking the CRISPR array (λ_{cas}) as a negative control were made competent and transformed with a control plasmid or plasmids encoding *ndm-1* or *ctx-M-15,* all conferring streptomycin resistance. Transformation efficiency was determined by counting colonies that acquired streptomycin resistance. Lysogens of the $\lambda_{cas\text{-}CRISPR}$ were transformed equally well with the control plasmid compared to lysogens of the λ_{cas}. In contrast, as shown in Figure 3, these lysogens were transformed less efficiently with the targeted plasmids by approximately three orders of magnitude. These results clearly indicate that the lysogenized CRISPR-Cas system can be transferred into bacteria and moreover, this system specifically prevents horizontal gene transfer of antibiotic-resistance elements by plasmid transformations.

[0258] To demonstrate that lysogenization can also cure established resistance plasmids, the inventors lysogenized resistant bacteria and determined plasmid loss. Plasmids were cured specifically from bacteria lysogenized with λcas-CRISPR but not with λcas (Fig. 4). Together, these results indicate that the CRISPR-Cas system can be transferred by

temperate phages into bacteria to specifically prevent horizontal gene transfer of antibiotic resistance elements.

Example 3

Protection from lytic bacteriophages

[0259] A desired feature of the sensitizing CRISPR-Cas system is the ability to concomitantly confer advantage to the pathogens harboring it. For example, resistance to lytic phages would enable selection and enrichment of sensitized pathogens exposed to them. Therefore, the inventors next genetically engineered lytic T7 phages encoding protospacers identical to the *ndm-1* and *ctx-M-15* spacers targeted by the transferred CRISPR-Cas system. These engineered phages would thus be targeted concomitantly with the resistance genes. These similar protospacers were intentionally cloned to ensure that the lysogens could not lose the sensitizing element without also losing phage resistance. In addition, targeting a synthetic protospacer of the phage rather than a naturally occurring sequence does not provide the lysogens with protection against the wild-type phage, and thus does not interfere with the natural ecological balance. Naive *E. coli* were lysogenized with $\lambda_{cas\text{-}CRISPR}$ or λ_{cas}, and the bacteria were then infected with the engineered T7 phages. As clearly shown in Figure 5, bacteria lysogenized with $\lambda_{cas\text{-}CRISPR}$ did not resist growth of a control T7-gp8 phage compared to bacteria lysogenized with the control λ_{cas} phage. In contrast, these lysogens resisted growth of the T7 phages encoding either two protospacers of *ndm-1* (T7-N_1-N_2, SEQ ID NO. 55) or two protospacers of *ctx-M-15* (T7-C_2C_1, SEQ ID NO. 56) or one of each (T7-N_1C_1, SEQ ID NO.57or T7-C_2N_2, SEQ ID NO.58) by at least four orders of magnitude (Fig. 5). These results indicate that the lysogenized CRISPR-Cas system can be transferred to bacteria and protect them from a modified T7 bacteriophage, thus linking pathogen sensitization to antibiotics with resistance to lytic phage. Moreover, the system confers resistance only to phages encoding artificial matching protospacers, demonstrating that the system does not interfere with natural ecological interactions.

Example 4

Lytic phage selection of sensitized bacteria

[0260] The transferred CRISPR-Cas system prevented plasmid transformation and concomitantly protected the lysogenized bacteria from lytic phages. This indicates that lysogenization can be used to sensitize antibiotic-resistant bacteria, and that the population of sensitized bacteria may be enriched by lytic phages. To simulate treatments that could be applied on hospital surfaces or skin flora, bacteria harboring control, *ctx-M-15,* or *ndm1* encoding plasmids were next propagated.

[0261] Lysogenizing phages encoding the CRISPR-Cas system ($\lambda_{cas\text{-}CRISPR}$) or control ($\lambda_{cas}$) phages were then added to the cultures. The cultures were then overlaid on agar plates containing the T7-N_1C_1 lytic phage, against which the lysogenized bacteria have CRISPR-Cas-mediated protection. Surviving colonies were counted after overnight incubation (Fig. 6A). In all cultures, more than 20-fold more colonies treated with the targeting $\lambda_{cas\text{-}CRISPR}$ phage were resistant to the engineered T7-N_1C_1 phage compared with those treated with the control λ_{cas} phage (Fig. 6*B*). Phage-resistant colonies treated with either $\lambda_{cas\text{-}CRISPR}$ or λ_{cas} were inoculated on plates having or lacking streptomycin to test for loss of the antibiotic resistance conferring plasmid. As expected, cultures harboring the nontargeted plasmid (pVEC) remained streptomycin resistant in both types of lysogenizations. However, all of the bacteria lysogenized with $\lambda_{cas\text{-}CRISPR}$ and harboring targeted plasmids (pNDM or pCTX) concomitantly became sensitive to streptomycin, whereas all of the bacteria treated with λ_{cas} maintained this resistance (Fig. 6*C*). Finally, to demonstrate that multiple resistances in the same bacterium can also be eliminated, the inventors repeated the above described procedure using bacteria harboring two different antibiotic resistance plasmids (pNDM*+pCTX). As expected, in this case also, bacterial cultures treated with the $\lambda_{cas\text{-}CRISPR}$ resisted the lytic phages, as they carry antiphage spacers (Fig. 6*B*). Bacteria surviving the lytic phage infection and treated with $\lambda_{cas\text{-}CRISPR}$ were cured from both resistance plasmids, whereas survivors treated with λ_{cas} maintained the resistance plasmids (Fig. 6*C*). Altogether, these experiments provide a proof of principle that an engineered temperate phage delivering the CRISPR-Cas system can be used along with an engineered lytic phage to facilitate the simultaneous loss of multiple resistance determinants, reduce their horizontal transfer, and enrich for bacterial populations that exhibit both features. Altogether, these experiments demonstrate the feasibility of using an engineered temperate phage delivering the CRISPR-Cas system, along with an engineered lytic phage to facilitate the loss of multiple resistance determinants, reduce their horizontal transfer, and enrich for bacterial populations that exhibit both features.

Example 5

In vivo testing

[0262] The present inventors next test the technology's ability to reduce infection of mice by drug-resistant pathogens. Mouse cages are used to simulate hospital rooms, and mice are used to simulate patients. ESBF-resistant pathogens are spread throughout all of the cages. The engineered phages' efficiency for enrichment of drug-sensitive pathogen populations in cages is assessed by spraying the sensitizing phages followed by the lytic phages for several days. Mice are then placed into these or untreated cages. Mice developing bacterial disease are treated with antibiotics. It is expected that the mice in the phage-treated cages will be cured by the antibiotics whereas mice in the control cages will succumb to the bacterial disease.

[0263] It should be noted that the CRISPR system can also target virulence factors such as shiga and cholera toxins, which are horizontally transferred by phages, thereby reducing the severity of pathogen infections. In addition, CRISPR/Cas systems targeting RNA molecules can be used to target genomic resistance determinants and virulence factors. RNA targeting systems can be used without counter selection since the bacterial genome remains intact whereas only the specific virulence genes are silenced. These possibilities are examined by the inventors.

[0264] 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.

Claims

1. A kit comprising:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and
(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one clustered, regularly interspaced short palindromic repeat (CRISPR) array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component.

2. The kit according to claim 1, wherein said first component comprises at least one lytic bacteriophage, preferably, said lytic bacteriophage is at least one genetically modified bacteriophage comprising at least one proto-spacer having an identity of at least 70% to at least one nucleic acid sequence comprised within said bacterial pathogenic gene, preferably, said genetically modified lytic bacteriophage comprises at least one of: (a) at least one proto-spacer of CTX-M-15, comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51; and (b) at least one proto-spacer of NDM-1 comprising a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48.

3. The kit according to claim 1, wherein said vector of the second component (ii), is at least one genetically modified bacteriophage comprising at least one CRISPR spacer that targets at least one nucleic acid sequence comprised within said lytic bacteriophage and at least one CRISPR spacer that targets a nucleic acid sequence comprised within said at least one pathogenic gene, thereby targeting and inactivating both, said lytic phage and said pathogenic gene, preferably, said bacteriophage is a lambda temperate bacteriophage.

4. The kit according to claim 1, wherein:

(a) said at least one bacterial pathogenic gene is at least one bacterial endogenous gene; or
(b) said at least one bacterial pathogenic gene is at least one epichromosomal gene.

5. The kit according to claim 4, wherein:

(a) at least one of said pathogenic gene is an antibiotic resistance gene; or

(b) at least one of said pathogenic gene is a gene encoding at least one of a virulence factor and at least one toxin.

6. The kit according to claim 5, wherein at least one of said pathogenic gene is an antibiotic resistance gene, and wherein said at least one antibiotic resistance gene encodes a resistance factor selected from the group consisting of CTX-M-15, New Delhi metallo-β-lactamase (NDM)-1, 2, 5, 6, an extended-spectrum beta-lactamase resistance factor (ESBL factor), beta lactamase, and tetracycline A (tetA).

7. The kit according to claim 6, wherein said at least one CRISPR spacer comprises a nucleic acid sequence that targets at least one of: at least one proto-spacer of CTX-M-15, at least one proto-spacer of NDM-1, 2, 5, 6, at least one proto-spacer of ESBL factor, at least one proto-spacer of beta lactamase, at least one proto-spacer of tetA; and at least one at least one proto-spacer of a lytic bacteriophage, preferably, at least one of said proto-spacer of CTX-M-15, comprises a nucleic acid sequence as denoted by any one of SEQ ID NO. 49, 50 and 51 and at least one of said proto-spacer of NDM-1, comprises a nucleic acid sequence as denoted by any one of SEQ ID NO. 46, 47 and 48.

8. The kit according to claim 3, wherein said at least one CRISPR spacer targets a nucleic acid sequence comprised within an essential gene of said lytic bacteriophage, preferably, said lytic bacteriophage is at least one of T7like-virus and T4like-virus, preferably, said T7like-virus is at least one Enterobacteria phage T7.

9. The kit according to claim 1, wherein said at least one *cas* gene is at least one cas gene of at least one of type I, type II and type III CRISPR systems.

10. The kit according to claim 9, wherein said at least one *cas* gene is at least one cas gene of type I-E CRISPR system, preferably, said at least one type I-E *cas* gene is at least one of cse1, cse2, cas7, cas5e cas6 and cas3 genes.

11. The kit according to claim 9, wherein said at least one *cas* gene is at least one *cas* gene of type II CRISPR system, preferably, said at least one *cas* gene of type II CRISPR system is cas9 gene.

12. The kit according to claim 1, wherein said bacterium is at least one bacterium of any strain of at least one of *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Clostidium difficile, Enterococcus faecium, Klebsiella pneumonia, Acinetobacter baumanni and Enterobacter* species, preferably, said bacteria are at least one *E.coli* strain selected from the group consisting of O157:H7, enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and diffuse adherent (DAEC) E. coli.

13. An *in vitro* method of interfering with a horizontal transfer of a genetic element comprising at least one pathogenic gene between bacteria, the method comprises the steps of: contacting at least one of a surface, a substance or an article containing bacteria harboring said pathogenic gene with:

(i) at least one first component that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage, or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and
(ii) at least one second component comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component, or
(iii) at least one kit comprising (i) and (ii), thereby inactivating said pathogenic gene and interfering with horizontal transfer thereof, preferably, said kit is as defined in any one of claims 2-12.

14. At least one first component (i), that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and at least one second component (ii), comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic

gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component; or

(iii) at least one kit comprising (i) and (ii), preferably, said kit is as defined in any one of claims 2-12;
for use in a method of interfering with a horizontal transfer of a genetic element comprising at least one pathogenic gene between bacteria, the method comprising the steps of: contacting at least one of a surface, a substance or an article containing bacteria harboring said pathogenic gene with (i) and (ii) or (iii).

15. At least one first component (i), that is a selective component comprising at least one genetic element or vector comprising a nucleic acid sequence comprising at least one proto-spacer, wherein said selective component comprises a lytic bacteriophage or a plasmid that further encode a toxic element or protein that kill bacterial cells, and wherein said proto-spacer serve as a target for at least one spacer of the second component of (ii); and at least one second component (ii) comprising at least one recombinant vector comprising a nucleic acid sequence comprising at least one *cas* gene and at least one CRISPR array, wherein at least one spacer of said CRISPR targets a proto-spacer comprised within at least one pathogenic gene of a bacterium so as to specifically inactivate said pathogenic gene in said bacterium and wherein at least one spacer of said CRISPR targets a proto-spacer comprised within said selective component of (i) so as to specifically inactivate said selective component; or
(iii) at least one kit comprising (i) and (ii), thereby targeting and inactivating said pathogenic gene and preventing said pathologic condition, preferably, said kit is as defined in any one of claims 2-12; for use in a method of preventing a pathologic condition in a mammalian subject caused by a bacterial infection of bacteria containing a pathogenic gene, the method comprising contacting at least one of surface, a substance or an article, in the vicinity of said subject with (i) and (ii), or (iii).

Patentansprüche

1. Kit, das Folgendes umfasst:

(i) mindestens eine erste Komponente, die eine selektive Komponente ist, die mindestens ein genetisches Element oder einen Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens einen Proto-Spacer umfasst, wobei die selektive Komponente einen lytischen Bakteriophagen oder ein Plasmid umfasst, das ferner für ein toxisches Element oder Protein codiert, das bakterielle Zellen abtötet, und wobei der Proto-Spacer als Target für mindestens einen Spacer der zweiten Komponente von (ii) dient; und
(ii) mindestens eine zweite Komponente, die mindestens einen rekombinanten Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens ein *cas-Gen* und mindestens ein geclustertes regulär beabstandetes kurzes palindromisches Wiederholungs- (Clustered, Regularly Interspaced Short Palindromic Repeat, CRISPR) Array umfasst, wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in mindestens einem pathogenen Gen eines Bakteriums enthalten ist, um das pathogene Gen in dem Bakterium spezifisch zu inaktivieren und wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in der selektiven Komponente von (i) enthalten ist, um so die selektive Komponente spezifisch zu inaktivieren.

2. Kit nach Anspruch 1, wobei die erste Komponente mindestens einen lytischen Bakteriophagen umfasst, wobei der lytische Bakteriophage vorzugsweise mindestens ein genetisch modifizierter Bakteriophage ist, der mindestens einen Proto-Spacer umfasst, der eine Identität von mindestens 70 % zu mindestens einer Nukleinsäuresequenz aufweist, die in dem bakteriellen pathogenen Gen enthalten ist, wobei vorzugsweise der gentechnisch modifizierte lytische Bakteriophage mindestens eines der Folgenden umfasst: (a) mindestens einen Proto-Spacer von CTX-M-15, der eine Nukleinsäuresequenz umfasst, wie sie in einer der SEQ ID NO. 49, 50 und 51 angegeben ist; und (b) mindestens einen Proto-Spacer von NDM-1, der eine Nukleinsäuresequenz umfasst, wie sie in einer der SEQ ID NO. 46, 47 und 48 angegeben ist.

3. Kit nach Anspruch 1, wobei der Vektor der zweiten Komponente (ii) mindestens ein genetisch modifizierter Bakteriophage ist, der mindestens einen CRISPR-Spacer umfasst, der auf mindestens eine Nukleinsäuresequenz abzielt, die in dem lytischen Bakteriophagen enthalten ist, und mindestens einen CRISPR-Spacer umfasst, der auf eine Nukleinsäuresequenz abzielt, die in dem mindestens einen pathogenen Gen enthalten ist, wodurch sowohl auf den lytischen Phagen als auch das pathogene Gen abgezielt wird und diese inaktiviert werden, wobei der Bakteriophage vorzugsweise ein gemäßigter Lambda-Bakteriophage ist.

4. Kit nach Anspruch 1, wobei:

(a) es sich bei dem mindestens einen bakteriellen pathogenen Gen um mindestens ein bakterielles endogenes Gen handelt; oder
(b) es sich bei dem mindestens einen bakteriellen pathogenen Gen um mindestens ein epichromosomales Gen handelt.

5. Kit nach Anspruch 4, wobei:

(a) mindestens eines der pathogenen Gene ein Antibiotikaresistenzgen ist; oder
(b) mindestens eines der pathogenen Gene ein Gen ist, das für mindestens einen Virulenzfaktor oder mindestens ein Toxin codiert.

6. Kit nach Anspruch 5, wobei mindestens eines der pathogenen Gene ein Antibiotikaresistenzgen ist, und wobei das mindestens eine Antibiotikaresistenzgen für einen Resistenzfaktor codiert, der ausgewählt ist aus der Gruppe bestehend aus CTX-M-15, Neu-Delhi-Metallo-β-Lactamase (NDM)-1, 2, 5, 6, einem Extended-Spectrum-Beta-Lactamase-Resistenzfaktor (ESBL-Faktor), Beta-Lactamase und Tetracyclin A (tetA).

7. Kit nach Anspruch 6, wobei der mindestens eine CRISPR-Spacer eine Nukleinsäuresequenz umfasst, die auf mindestens eines der Folgenden abzielt: mindestens einen Proto-Spacer von CTX-M-15, mindestens einen Proto-Spacer von NDM-1, 2, 5, 6, mindestens einen Proto-Spacer von ESBL-Faktor, mindestens einen Proto-Spacer von Beta-Laktamase, mindestens einen Proto-Spacer von tetA; und wobei mindestens ein Proto-Spacer eines lytischen Bakteriophagen, vorzugsweise mindestens einer der Proto-Spacer von CTX-M-15, eine Nukleinsäuresequenz umfasst, wie sie in einer der SEQ ID NO. 49, 50 und 51 angegeben ist, und wobei mindestens einer der Proto-Spacer von NDM-1 eine Nukleinsäuresequenz umfasst, wie sie in einer der SEQ ID NO. 46, 47 und 48 angegeben ist.

8. Kit nach Anspruch 3, wobei der mindestens eine CRISPR-Spacer auf eine Nukleinsäuresequenz abzielt, die in einem essentiellen Gen des lytischen Bakteriophagen enthalten ist, wobei der lytische Bakteriophage vorzugsweise mindestens ein T7-ähnliches Virus oder ein T4-ähnliches Virus ist, wobei das T7-ähnliche Virus vorzugsweise mindestens ein Enterobakterien-Phage T7 ist.

9. Kit nach Anspruch 1, wobei das mindestens eine *cas-Gen* mindestens ein cas-Gen von mindestens einem der CRISPR-Systeme vom Typ I, Typ II und Typ III ist.

10. Kit nach Anspruch 9, wobei das mindestens eine *cas-Gen* mindestens ein cas-Gen des Typ I-E CRISPR-Systems ist, wobei das mindestens eine Typ I-E *cas-Gen* vorzugsweise mindestens eines der Gene cse1, cse2, cas7, cas5e cas6 und cas3 ist.

11. Kit nach Anspruch 9, wobei das mindestens eine *cas-Gen* mindestens ein *cas-Gen* des Typ II CRISPR-Systems ist, wobei das mindestens eine *cas-Gen* des Typ II CRISPR-Systems vorzugsweise das cas9-Gen ist.

12. Kit nach Anspruch 1, wobei das Bakterium mindestens ein Bakterium eines beliebigen Stammes von mindestens einem der Folgenden ist: *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Clostidium difficile, Enterococcus faecium, Klebsiella pneumonia, Acinetobacter baumanni und Enterobacter* species, wobei die Bakterien vorzugsweise mindestens ein *E.coli-Stamm* sind, ausgewählt aus der Gruppe bestehend aus 0157:H7, enteroaggregativen (EAEC), enterohämorrhagischen (EHEC), enteroinvasiven (EIEC), enteropathogenen (EPEC), enterotoxigenen (ETEC) und diffus adhärenten (DAEC) E. coli.

13. *In* vitro-Verfahren zur Störung eines horizontalen Transfers eines genetischen Elements, das mindestens ein pathogenes Gen zwischen Bakterien umfasst, wobei das Verfahren folgende Schritte umfasst: Inkontaktbringen mindestens einer Oberfläche, einer Substanz oder eines Gegenstandes, der Bakterien enthält, die das pathogene Gen beherbergen, mit:

(i) mindestens einer ersten Komponente, die eine selektive Komponente ist, die mindestens ein genetisches Element oder einen Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens einen Proto-Spacer umfasst, wobei die selektive Komponente einen lytischen Bakteriophagen oder ein Plasmid umfasst, das ferner für ein toxisches Element oder Protein codiert, das bakterielle Zellen abtötet, und wobei der Proto-Spacer als Target für mindestens einen Spacer der zweiten Komponente von (ii) dient; und
(ii) mindestens einer zweiten Komponente, die mindestens einen rekombinanten Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens ein *cas-Gen* und mindestens ein geclustertes regulär beabstan-

detes kurzes palindromisches Wiederholungs-, CRISPR, Array umfasst, wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in mindestens einem pathogenen Gen eines Bakteriums enthalten ist, um das pathogene Gen in dem Bakterium spezifisch zu inaktivieren und wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in der selektiven Komponente von (i) enthalten ist, um so die selektive Komponente spezifisch zu inaktivieren, oder
(iii) mindestens ein Kit, das (i) und (ii) umfasst, wodurch das pathogene Gen inaktiviert und seine horizontale Übertragung gestört wird, wobei das Kit vorzugsweise wie in einem der Ansprüche 2 bis 12 definiert ist.

14. Mindestens eine erste Komponente (i), die eine selektive Komponente ist, die mindestens ein genetisches Element oder einen Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens einen Proto-Spacer umfasst, wobei die selektive Komponente einen lytischen Bakteriophagen oder ein Plasmid umfasst, das ferner für ein toxisches Element oder Protein codiert, das bakterielle Zellen abtötet, und wobei der Proto-Spacer als Target für mindestens einen Spacer der zweiten Komponente von (ii) dient; und mindestens einer zweiten Komponente (ii), die mindestens einen rekombinanten Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens ein *cas-Gen* und mindestens ein geclustertes regulär beabstandetes kurzes palindromisches Wiederholungs-, CRISPR, Array umfasst, wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in mindestens einem pathogenen Gen eines Bakteriums enthalten ist, um das pathogene Gen in dem Bakterium spezifisch zu inaktivieren und wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in der selektiven Komponente von (i) enthalten ist, um so die selektive Komponente spezifisch zu inaktivieren; oder

(iii) mindestens ein Kit, das (i) und (ii) umfasst, wobei das Kit vorzugsweise wie in einem der Ansprüche 2 bis 12 definiert ist;
zur Verwendung in einem Verfahren zur Störung eines horizontalen Transfers eines genetischen Elements, das mindestens ein pathogenes Gen zwischen Bakterien umfasst, wobei das Verfahren folgende Schritte umfasst: Inkontaktbringen mindestens einer Oberfläche, einer Substanz oder eines Gegenstands, der Bakterien enthält, die das pathogene Gen beherbergen, mit (i) und (ii) oder (iii).

15. Mindestens eine erste Komponente (i), die eine selektive Komponente ist, die mindestens ein genetisches Element oder einen Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens einen Proto-Spacer umfasst, wobei die selektive Komponente einen lytischen Bakteriophagen oder ein Plasmid umfasst, das ferner für ein toxisches Element oder Protein codiert, das bakterielle Zellen abtötet, und wobei der Proto-Spacer als Target für mindestens einen Spacer der zweiten Komponente von (ii) dient; und mindestens einer zweiten Komponente (ii), die mindestens einen rekombinanten Vektor umfasst, der eine Nukleinsäuresequenz umfasst, die mindestens ein *cas-Gen* und mindestens ein geclustertes regulär beabstandetes kurzes palindromisches Wiederholungs-, CRISPR, Array umfasst, wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in mindestens einem pathogenen Gen eines Bakteriums enthalten ist, um das pathogene Gen in dem Bakterium spezifisch zu inaktivieren und wobei mindestens ein Spacer des CRISPR auf einen Proto-Spacer abzielt, der in der selektiven Komponente von (i) enthalten ist, um so die selektive Komponente spezifisch zu inaktivieren; oder
(iii) mindestens ein Kit, das (i) und (ii) umfasst, wodurch auf das pathogene Gen abzielt und dieses inaktiviert wird und der pathologische Zustand verhindert wird, wobei das Kit vorzugsweise wie in einem der Ansprüche 2 bis 12 definiert ist; zur Verwendung in einem Verfahren zur Verhinderung eines pathologischen Zustands in einem Säuger, der durch eine bakterielle Infektion von Bakterien, die ein pathogenes Gen enthalten, verursacht wird, wobei das Verfahren das In-Kontakt-Bringen mindestens einer Oberfläche, einer Substanz oder eines Gegenstands in der Nähe des Subjekts mit (i) und (ii) oder (iii) umfasst.

Revendications

1. Kit comprenant :

(i) au moins un premier composant qui est un composant sélectif comprenant au moins un élément génétique ou vecteur comprenant une séquence d'acide nucléique comprenant au moins un proto-espaceur, dans lequel ledit composant sélectif comprend un bactériophage lytique ou un plasmide qui code en outre pour un élément ou une protéine toxique qui tue les cellules bactériennes, et dans lequel ledit proto-espaceur sert de cible pour au moins un espaceur du deuxième composant de (ii) ; et
(ii) au moins un deuxième composant comprenant au moins un vecteur recombinant comprenant une séquence d'acide nucléique comprenant au moins un gène *cas* et au moins un réseau de répétitions palindromiques courtes groupées régulièrement espacées, (CRISPR), dans lequel au moins un espaceur de ladite CRISPR

cible un proto-espaceur compris dans au moins un gène pathogène d'une bactérie de manière à inactiver spécifiquement ledit gène pathogène dans ladite bactérie et dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans ledit composant sélectif de (i) de manière à inactiver spécifiquement ledit composant sélectif.

2. Kit selon la revendication 1, dans lequel ledit premier composant comprend au moins un bactériophage lytique, de préférence, ledit bactériophage lytique est au moins un bactériophage génétiquement modifié comprenant au moins un proto-espaceur ayant une identité d'au moins 70 % avec au moins une séquence d'acide nucléique comprise dans ledit gène pathogène bactérien, de préférence, ledit bactériophage lytique génétiquement modifié comprend au moins l'un parmi : (a) au moins un proto-espaceur de CTX-M-15, comprenant une séquence d'acide nucléique telle qu'indiquée par l'une quelconque de SEQ ID NO. 49, 50 et 51 ; et (b) au moins un proto-espaceur de NDM-1 comprenant une séquence d'acide nucléique telle qu'indiquée par l'une quelconque de SEQ ID NO. 46, 47 et 48.

3. Kit selon la revendication 1, dans lequel ledit vecteur du deuxième composant (ii) est au moins un bactériophage génétiquement modifié comprenant au moins un espaceur CRISPR qui cible au moins une séquence d'acide nucléique comprise dans ledit bactériophage lytique et au moins un espaceur CRISPR. qui cible une séquence d'acide nucléique comprise dans ledit au moins un gène pathogène, ciblant et inactivant ainsi à la fois ledit phage lytique et ledit gène pathogène, de préférence, ledit bactériophage est un bactériophage tempéré lambda.

4. Kit selon la revendication 1, dans lequel :

(a) ledit au moins un gène pathogène bactérien est au moins un gène endogène bactérien ; ou
(b) ledit au moins un gène pathogène bactérien est au moins un gène épichromosomique.

5. Kit selon la revendication 4, dans lequel :

(a) au moins l'un desdits gènes pathogènes est un gène de résistance aux antibiotiques ; ou
(b) au moins l'un desdits gènes pathogènes est un gène codant pour au moins l'un parmi un facteur de virulence et au moins une toxine.

6. Kit selon la revendication 5, dans lequel au moins l'un desdits gènes pathogènes est un gène de résistance aux antibiotiques, et dans lequel ledit au moins un gène de résistance aux antibiotiques code pour un facteur de résistance choisi dans le groupe constitué par CTX-M-15, New Delhi metallo-β-lactamase (NDM)-1, 2, 5, 6, un facteur de résistance à la bêta-lactamase à spectre étendu (facteur BLSE), la bêta-lactamase et la tétracycline A (tetA).

7. Kit selon la revendication 6, dans lequel ledit au moins un espaceur CRISPR comprend une séquence d'acide nucléique qui cible au moins l'un parmi : au moins un proto-espaceur de CTX-M-15, au moins un proto-espaceur de NDM-1, 2, 5, 6, au moins un proto-espaceur du facteur BLSE, au moins un proto-espaceur de bêta lactamase, au moins un proto-espaceur de tetA ; et au moins un proto-espaceur d'un bactériophage lytique, de préférence, au moins un desdits proto-espaceurs de CTX-M-15, comprend une séquence d'acide nucléique telle qu'indiquée par l'une quelconque de SEQ ID NO. 49, 50 et 51 et au moins l'un desdits proto-espaceurs de NDM-1, comprend une séquence d'acide nucléique telle qu'indiquée par l'une quelconque des SEQ ID NO. 46, 47 et 48.

8. Kit selon la revendication 3, dans lequel ledit au moins un espaceur CRISPR cible une séquence d'acide nucléique comprise dans un gène essentiel dudit bactériophage lytique, de préférence, ledit bactériophage lytique est au moins l'un parmi le virus de type T7 et le virus de type T4, de préférence, ledit virus de type T7 est au moins une entérobactérie phage T7.

9. Kit selon la revendication 1, dans lequel ledit au moins un gène *cas* est au moins un gène *cas* d'au moins l'un des systèmes CRISPR de type I, de type II et de type III.

10. Kit selon la revendication 9, dans lequel ledit au moins un gène *cas* est au moins un gène *cas* du système CRISPR de type I-E, de préférence, ledit au moins un gène *cas* de type I-E est au moins l'un parmi les gènes cse1, cse2, cas7, cas5e cas6 et cas3.

11. Kit selon la revendication 9, dans lequel ledit au moins un gène *cas* est au moins un gène *cas* du système CRISPR de type II, de préférence, ledit au moins un gène *cas* du système CRISPR de type II est le gène cas9.

12. Kit selon la revendication 1, dans lequel ladite bactérie est au moins une bactérie de n'importe quelle souche d'au moins l'une des espèces *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Clostidium difficile, Enterococcus faecium, Klebsiella pneumonia, Acinetobacter baumanni* et *Enterobacter,* de préférence, lesdites bactéries sont au moins une souche *E. coli* choisie dans le groupe constitué par E. coli O157:H7, entéroagrégative (EAEC), entérohémorragique (EHEC), entéroinvasive (EIEC), entéropathogène (EPEC), entérotoxinogène (ETEC) et adhérente diffuse (DAEC).

13. Procédé *in vitro* d'interférence avec un transfert horizontal d'un élément génétique comprenant au moins un gène pathogène entre des bactéries, le procédé comprend les étapes consistant à : mettre en contact au moins une surface, une substance ou un article contenant des bactéries hébergeant ledit gène pathogène avec :

(i) au moins un premier composant qui est un composant sélectif comprenant au moins un élément génétique ou un vecteur comprenant une séquence d'acide nucléique comprenant au moins un proto-espaceur, dans lequel ledit composant sélectif comprend un bactériophage lytique, ou un plasmide qui code en outre pour un élément ou une protéine toxique qui tue les cellules bactériennes, et dans lequel ledit proto-espaceur sert de cible pour au moins un espaceur du deuxième composant de (ii) ; et
(ii) au moins un deuxième composant comprenant au moins un vecteur recombinant comprenant une séquence d'acide nucléique comprenant au moins un gène *cas* et au moins un réseau CRISPR, dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans au moins un gène pathogène d'une bactérie de manière à inactiver spécifiquement ledit gène pathogène dans ladite bactérie et dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans ledit composant sélectif de (i) afin d'inactiver spécifiquement ledit composant sélectif, ou
(iii) au moins un kit comprenant (i) et (ii), inactivant ainsi ledit gène pathogène et interférant avec son transfert horizontal, de préférence, ledit kit est tel que défini selon l'une quelconque des revendications 2 à 12.

14. Au moins un premier composant (i), qui est un composant sélectif comprenant au moins un élément génétique ou vecteur comprenant une séquence d'acide nucléique comprenant au moins un proto-espaceur, ledit composant sélectif comprenant un bactériophage lytique ou un plasmide qui code en outre pour un élément ou une protéine toxique qui tue les cellules bactériennes, et dans lequel ledit proto-espaceur sert de cible pour au moins un espaceur du deuxième composant de (ii) ; et au moins un deuxième composant (ii), comprenant au moins un vecteur recombinant comprenant une séquence d'acide nucléique comprenant au moins un gène *cas* et au moins un réseau CRISPR, dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans au moins un gène pathogène d'une bactérie de manière à inactiver spécifiquement ledit gène pathogène dans ladite bactérie et dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans ledit composant sélectif de (i) afin d'inactiver spécifiquement ledit composant sélectif ; ou
(iii) au moins un kit comprenant (i) et (ii), de préférence, ledit kit est tel que défini selon l'une quelconque des revendications 2 à 12 ;
destiné à être utilisé dans un procédé d'interférence avec un transfert horizontal d'un élément génétique comprenant au moins un gène pathogène entre des bactéries, le procédé comprenant les étapes consistant à : mettre en contact au moins une surface, une substance ou un article contenant des bactéries hébergeant ledit gène pathogène avec (i) et (ii) ou (iii).

15. Au moins un premier composant (i), qui est un composant sélectif comprenant au moins un élément génétique ou vecteur comprenant une séquence d'acide nucléique comprenant au moins un proto-espaceur, ledit composant sélectif comprenant un bactériophage lytique ou un plasmide qui code en outre pour un élément ou une protéine toxique qui tue les cellules bactériennes, et dans lequel ledit proto-espaceur sert de cible pour au moins un espaceur du deuxième composant de (ii) ; et au moins un deuxième composant (ii) comprenant au moins un vecteur recombinant comprenant une séquence d' acide nucléique comprenant au moins un gène *cas* et au moins un réseau CRISPR, dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans au moins un gène pathogène d'une bactérie de manière à inactiver spécifiquement ledit gène pathogène dans ladite bactérie et dans lequel au moins un espaceur de ladite CRISPR cible un proto-espaceur compris dans ledit composant sélectif de (i) afin d'inactiver spécifiquement ledit composant sélectif ; ou
(iii) au moins un kit comprenant (i) et (ii), ciblant et inactivant ainsi ledit gène pathogène et prévenant ledit état pathologique, de préférence, ledit kit est tel que défini selon l'une quelconque des revendications 2 à 12 ; destiné à être utilisé dans un procédé de prévention d'un état pathologique chez un sujet mammifère causé par une infection bactérienne de bactéries contenant un gène pathogène, le procédé comprenant la mise en contact d'au moins une surface, une substance ou un article, à proximité dudit sujet avec (i) et (ii), ou (iii).

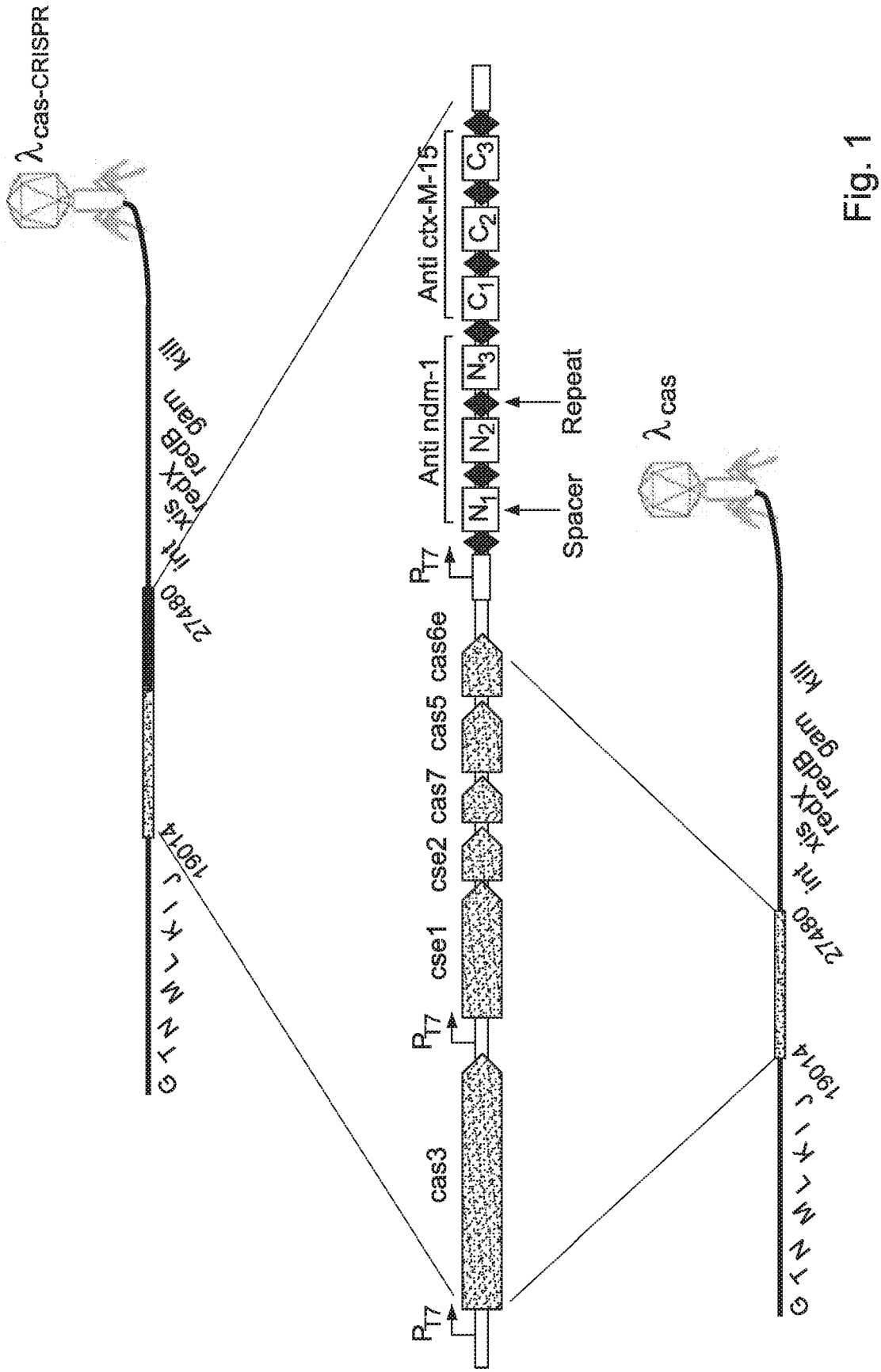
λ cas-CRISPR
kIII
gam
redB
redX
xis
int
27480
19014
G T N M L K I J
Anti ctx-M-15
Anti ndm-1
Repeat
Spacer
C_3
C_2
C_1
N_3
N_2
N_1
P_{T7}
cas6e
cas5
cas7
cse2
cse1
cas3
λ cas

Fig. 1

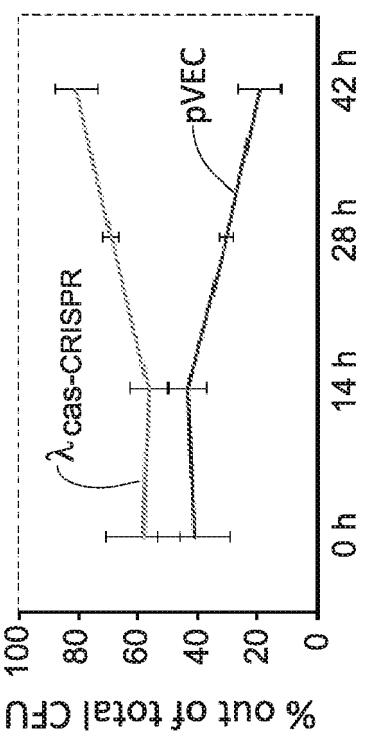

Fig. 2A

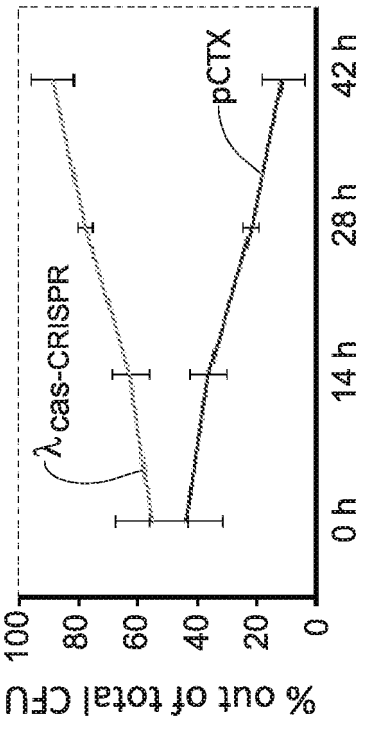

Fig. 2B

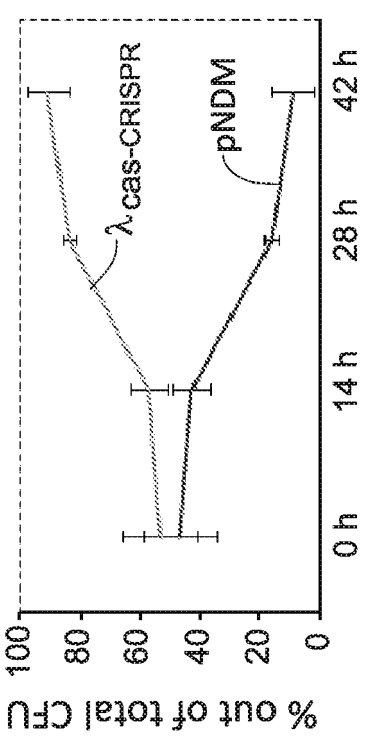

Fig. 2C

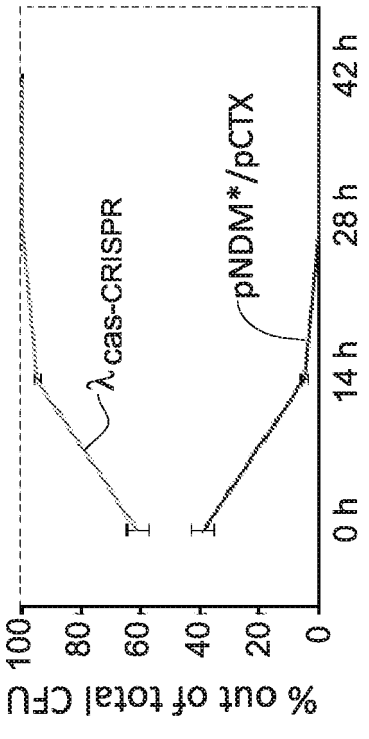

Fig. 2D

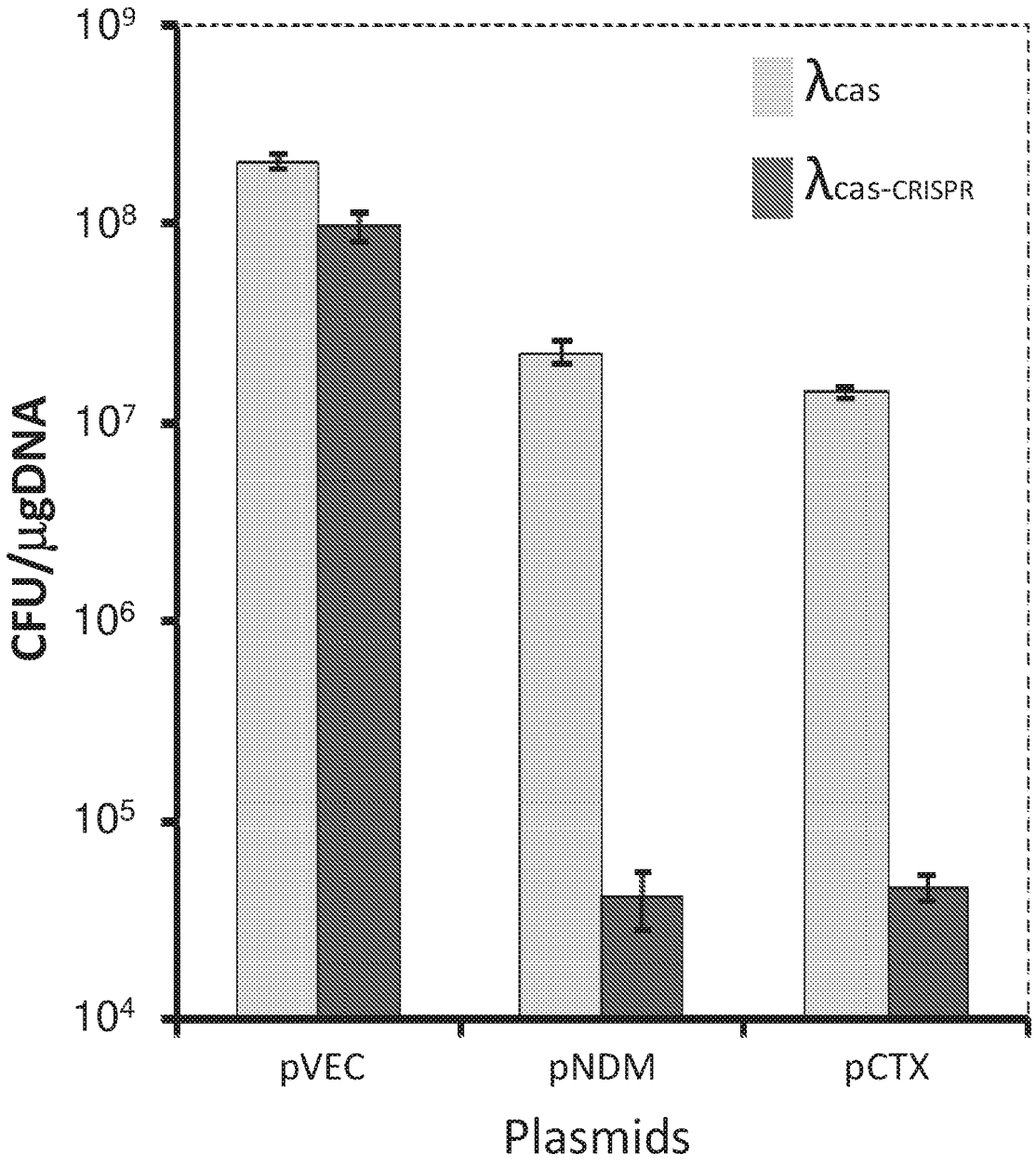
10^9
10^8
10^7
10^6
10^5
10^4
CFU/μgDNA
λcas
λcas-CRISPR
pVEC
pNDM
pCTX
Plasmids

Fig. 3

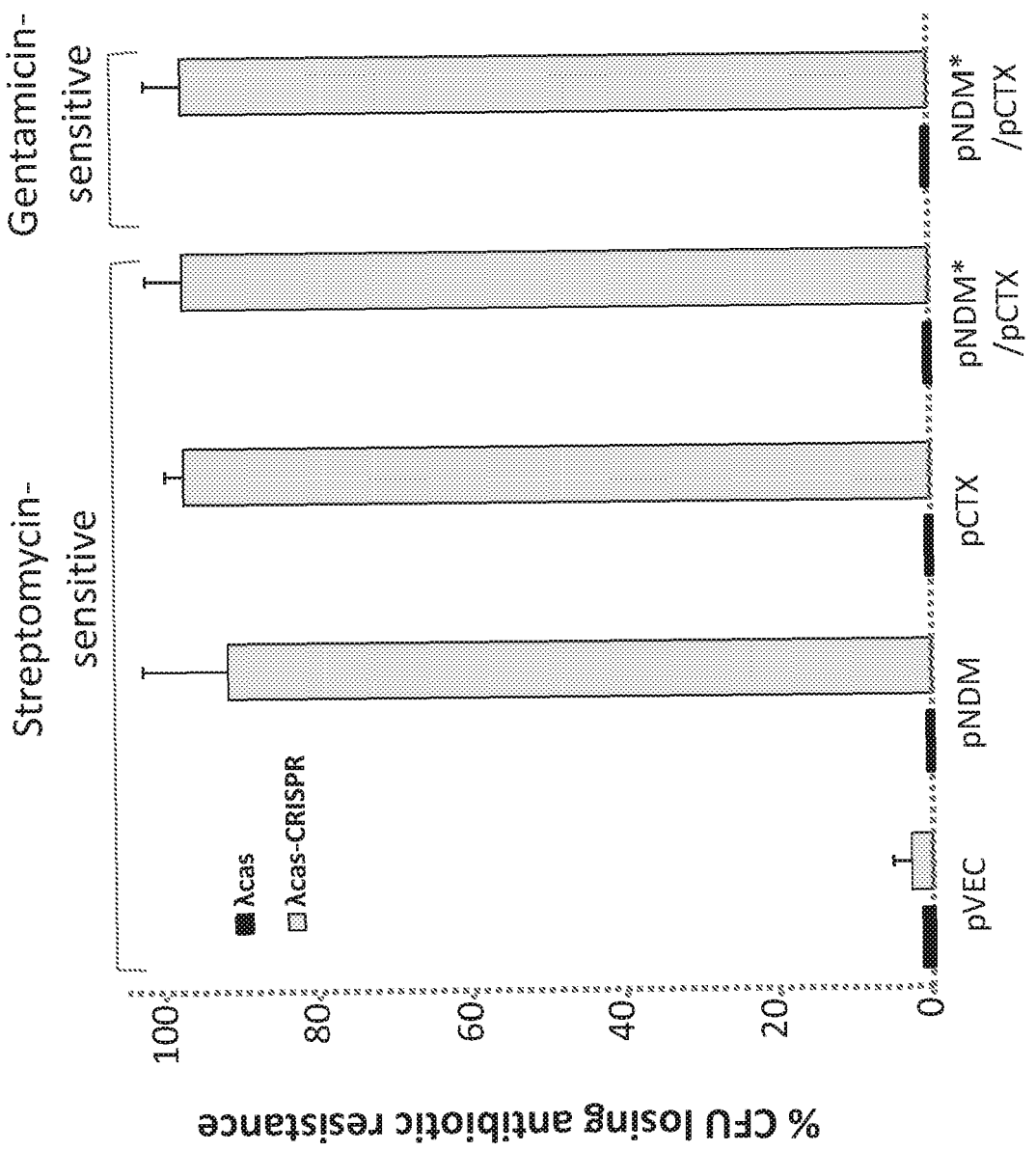
Streptomycin-sensitive
Gentamicin-sensitive
λcas
λcas-CRISPR
% CFU losing antibiotic resistance
100
80
60
40
20
0
pVEC
pNDM
pCTX
pNDM*/pCTX
pNDM*/pCTX
Plasmids

Fig. 4

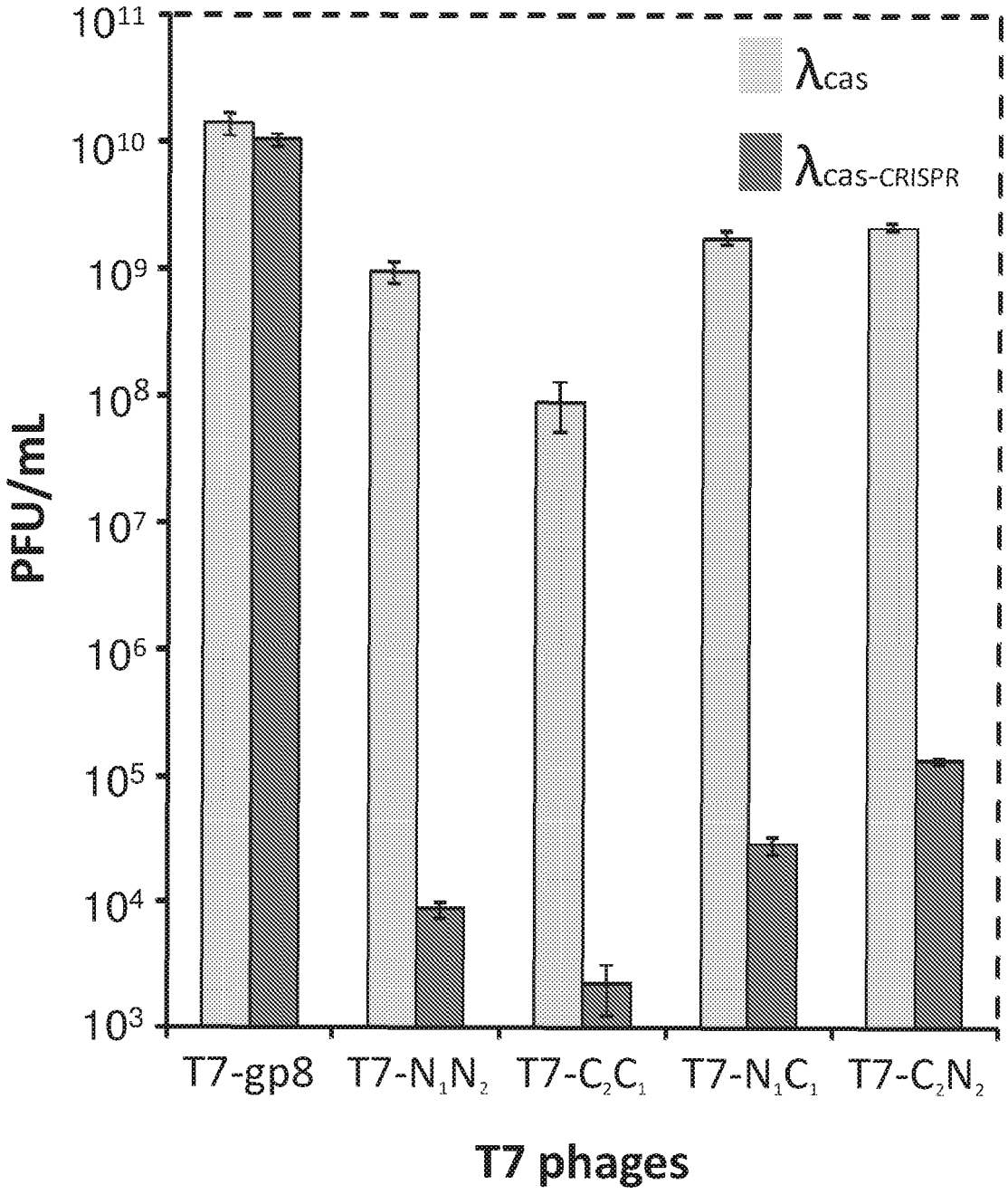
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
PFU/mL
λ_{cas}
$\lambda_{cas\text{-}CRISPR}$
T7-gp8
T7-N_1N_2
T7-C_2C_1
T7-N_1C_1
T7-C_2N_2
T7 phages

Fig. 5

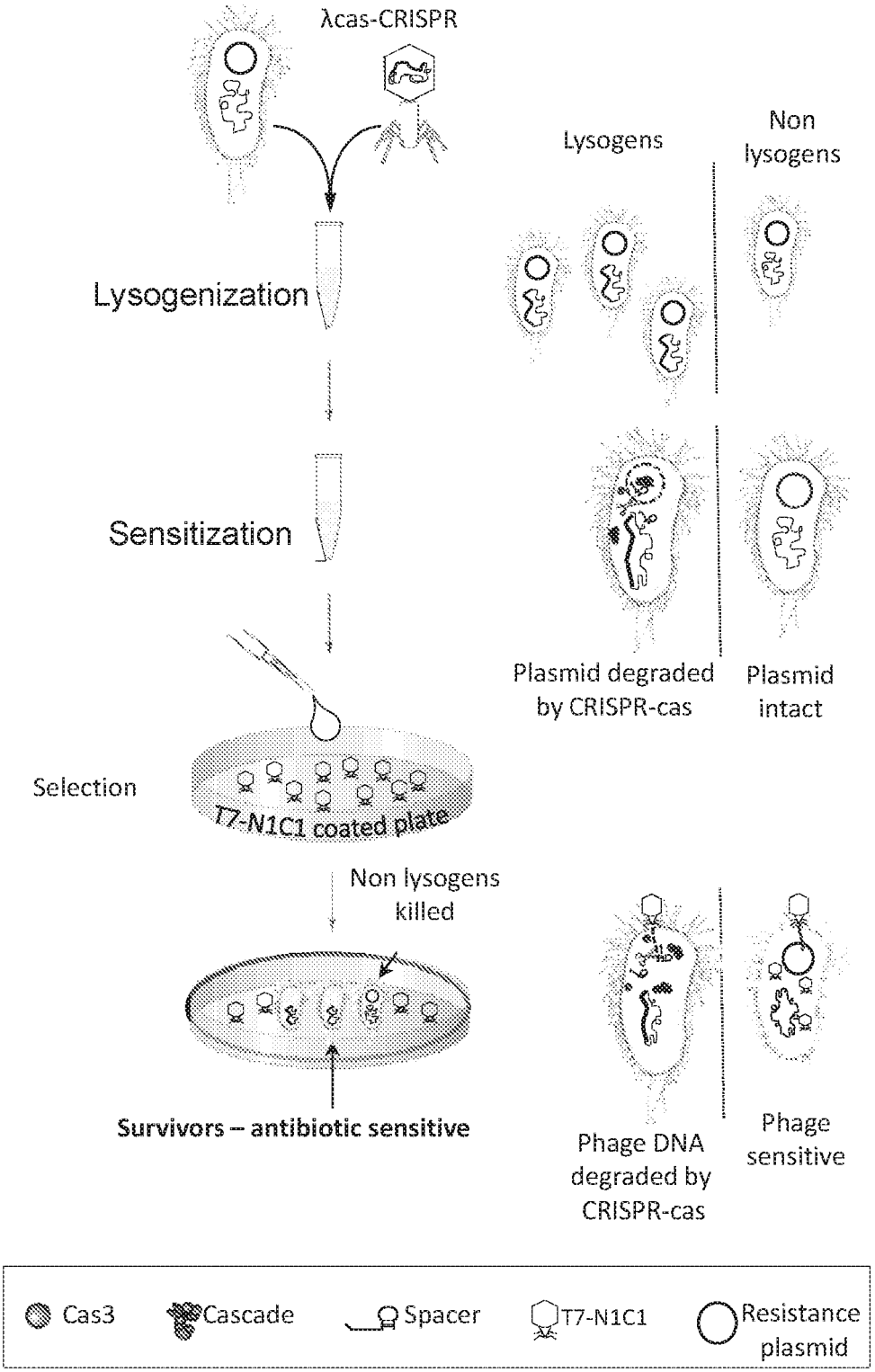
λcas-CRISPR
Lysogenization
Sensitization
Selection
T7-N1C1 coated plate
Non lysogens killed
Survivors – antibiotic sensitive
Lysogens
Non lysogens
Plasmid degraded by CRISPR-cas
Plasmid intact
Phage DNA degraded by CRISPR-cas
Phage sensitive
Cas3
Cascade
Spacer
T7-N1C1
Resistance plasmid

Fig. 6A

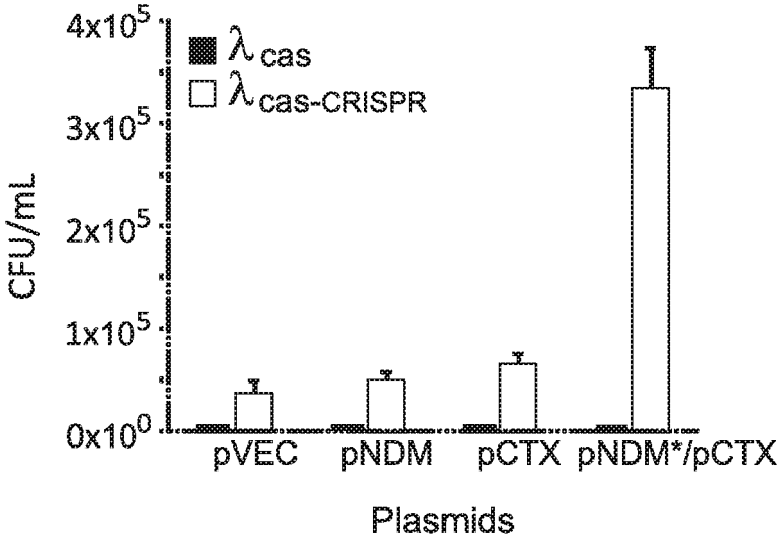
4x10^5
3x10^5
2x10^5
1x10^5
0x10^0
CFU/mL
λ cas
λ cas-CRISPR
pVEC
pNDM
pCTX
pNDM*/pCTX
Plasmids

Fig. 6B

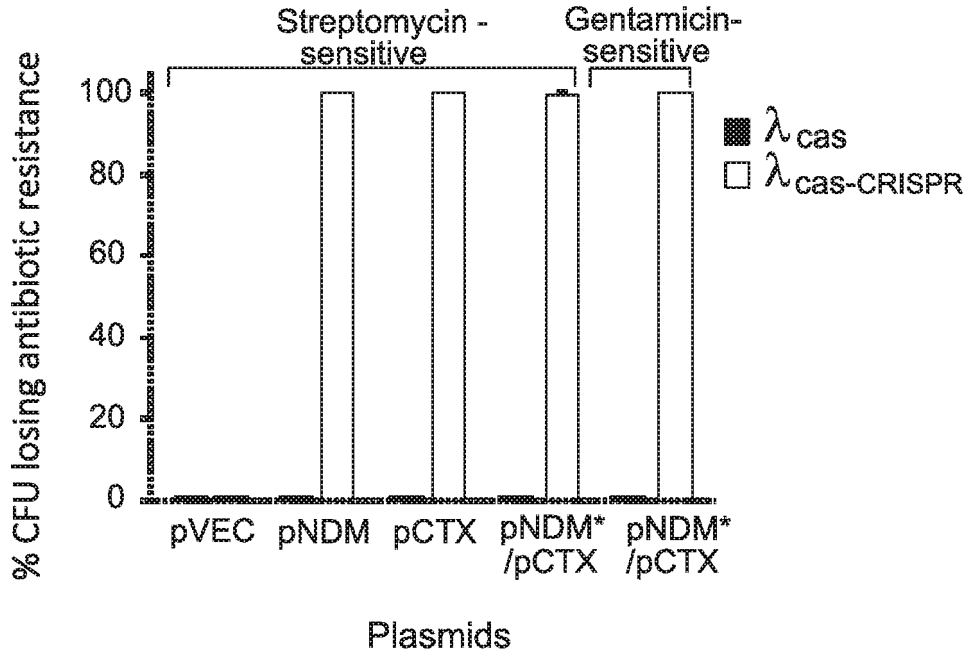
Streptomycin - sensitive
Gentamicin-sensitive
% CFU losing antibiotic resistance
100
80
60
40
20
0
λ cas
λ cas-CRISPR
pVEC
pNDM
pCTX
pNDM* /pCTX
pNDM* /pCTX
Plasmids

Fig. 6C

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 8865158 B, Qimron U **[0002]**
- WO 2014124226 A1 **[0002]**
- US 4666828 A **[0243]**
- US 4683202 A **[0243]**
- US 4801531 A **[0243]**
- US 5192659 A **[0243]**
- US 5272057 A **[0243]**
- US 3791932 A **[0243]**
- US 3839153 A **[0243]**
- US 3850752 A **[0243]**
- US 3850578 A **[0243]**
- US 3853987 A **[0243]**
- US 3867517 A **[0243]**
- US 3879262 A **[0243]**
- US 3901654 A **[0243]**
- US 3935074 A **[0243]**
- US 3984533 A **[0243]**
- US 3996345 A **[0243]**
- US 4034074 A **[0243]**
- US 4098876 A **[0243]**
- US 4879219 A **[0243]**
- US 5011771 A **[0243]**
- US 5281521 A **[0243]**

Non-patent literature cited in the description

- **LU TK ; COLLINS JJ.** *Proc Natl Acad Sci U S A,* 2009, vol. 106 (12), 4629-4634 **[0002]**
- **MERRIL CR et al.** *Nat Rev Drug Discov,* 2003, vol. 2 (6), 489-497 **[0002]**
- **QIMRON U et al.** *Proc Natl Acad Sci USA,* 2006, vol. 103 (50), 19039-19044.4 **[0002]**
- **LANG LH.** *Gastroenterology,* 2006, vol. 131 (5), 1370 **[0002]**
- **ABULADZE T et al.** *Appl Environ Microbiol,* 2008, vol. 74 (20), 6230-6238 **[0002]**
- **HAGENS ; BLASSI.** *Lett Appl Microbiol,* 2003, vol. 37 (4), 318-23 **[0002]**
- **GOMAA AA et al.** *MBio,* 2014, vol. 5 (1), e00928-00913 **[0002]**
- **GARNEAU JE et al.** *Nature,* 2010, vol. 468 (7320), 67-71 **[0002]**
- **CITORIK RJ et al.** *Nat. Biotechnol.,* 2014, vol. 32 (11), 1141-45 **[0002]**
- **BIKARD D et al.** *Nat Biotechnol.,* 2014, vol. 32 (11), 1146-50 **[0002]**
- **BARRANGOU et al.** *Science,* 2007, vol. 315 (5819), 1709-12 **[0002]**
- **BOLOTIN et al.** *Microbiology,* 2005, vol. 151, 2551-61 **[0002]**
- **MARRAFFINI et al.** *Science,* 2008, vol. 322, 1843-1845 **[0002]**
- **YOSEF I et al.** *Bacteriophage,* 2014, vol. 4 (1), e28491 **[0002]**
- **BARRANGOU R et al.** *Science,* 2007, vol. 315 (5819), 1709-1712 **[0002]**
- **HALE CR et al.** *Cell,* 2009, vol. 139 (5), 945-956 **[0002]**
- **PENDLETON JN et al.** *Expert Rev Anti Infect Ther,* 2013, vol. 11 (3), 297-308 **[0002]**
- **BOUCHER HW et al.** *Clin Infect Dis,* 2009, vol. 48 (1), 1-12 **[0002]**
- **MARRAFFINI LA et al.** *Nat Rev Genet,* 2010, vol. 11 (3), 181-190 **[0002]**
- **DATSENKO KA et al.** *Proc Natl Acad Sci U S A,* 2000, vol. 97 (12), 6640-6645 **[0002]**
- **YOSEF I et al.** *Nucleic Acids Res,* 2012, vol. 40 (12), 5569-5576 **[0002]**
- **SHARAN SK et al.** *Nat Protoc,* 2009, vol. 4 (2), 206-223 **[0002]**
- **YU D et al.** *Proc Natl Acad Sci U S A,* 2000, vol. 97 (11), 5978-5983 **[0002]**
- **YOSEF I et al.** *Proc Natl Acad Sci U S A,* 2011, vol. 108 (50), 20136-20141 **[0002]**
- **KIRO R et al.** *Proc Natl Acad Sci U S A,* 2013, vol. 110 (48), 19549-19554 **[0002]**
- **BUSH K et al.** *Annu Rev Microbiol,* 2011, vol. 65, 455-478 **[0002]**
- **BROUNS SJ et al.** *Science,* 2008, vol. 321 (5891), 960-964 **[0002]**
- **BABA T et al.** *Mol Syst Biol,* 2006, vol. 2, 1-11 **[0002]**
- **EDGAR R ; QIMRON U.** *J Bacteriol,* 2010, vol. 192 (23), 6291-6294 **[0002]**
- **YOUSEF I et al.** *Proc Natl Acad Sci U S S,* 2015, vol. 112 (23), 7267-7272 **[0002]**
- **GOEDDEL et al.** Gene Expression Technology: Methods in Enzymology. Academic Press, 1990, vol. 185 **[0046]**
- **POUWELS et al.** Cloning Vectors: a Laboratory Manual. Elsevier, 1985 **[0047]**

- Vectors: a Survey of Molecular Cloning Vectors and their Uses. Buttersworth Boston, Mass, 1988 **[0047]**
- Information Superhighway and Computer Databases of Nucleic Acids and Proteins. Methods in Gene Biotechnology. CRC Press, Inc, 1997 **[0158]**
- Guide to Human Genome Computing. Academic Press, Inc, 1998 **[0158]**
- **SAMBROOK et al.** *Molecular Cloning: A laboratory Manual,* 1989 **[0243]**
- Current Protocols in Molecular Biology. 1994, vol. I-III **[0243]**
- **AUSUBEL et al.** Current Protocols in Molecular Biology. John Wiley and Sons, 1989 **[0243]**
- **PERBAL.** A Practical Guide to Molecular Cloning. John Wiley & Sons, 1988 **[0243]**
- **WATSON et al.** Recombinant DNA. Scientific American Books **[0243]**
- Genome Analysis: A Laboratory Manual Series. Cold Spring Harbor Laboratory Press, 1998, vol. 1-4 **[0243]**
- Cell Biology: A Laboratory Handbook. 1994, vol. I-III **[0243]**
- **FRESHNEY.** Culture of Animal Cells - A Manual of Basic Technique. Wiley-Liss, 1994 **[0243]**
- Current Protocols in Immunology. 1994, vol. I-III **[0243]**
- Basic and Clinical Immunology. Appleton & Lange, 1994 **[0243]**
- Selected Methods in Cellular Immunology. W. H. Freeman and Co, 1980 **[0243]**
- Oligonucleotide Synthesis. 1984 **[0243]**
- Nucleic Acid Hybridization. 1985 **[0243]**
- Transcription and Translation. 1984 **[0243]**
- Animal Cell Culture. 1986 **[0243]**
- Immobilized Cells and Enzymes. IRL Press, 1986 **[0243]**
- **PERBAL, B.** A Practical Guide to Molecular Cloning. 1984 **[0243]**
- Methods in Enzymology. Academic Press, vol. 1-317 **[0243]**
- PCR Protocols: A Guide To Methods And Applications. Academic Press, 1990 **[0243]**
- **MARSHAK et al.** Strategies for Protein Purification and Characterization - A Laboratory Course Manual. CSHL Press, 1996 **[0243]**