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(54) Title: MATERIALS AND METHODS FOR REDUCING NUCLEIC ACID DEGRADATION IN BACTERIA

(57) Abstract: The present disclosure is directed to materials and methods for reducing heterologous DNA damage in bacteria (i.e., induce resistance to host restriction enzymes) by modifying the heterologous DNA to include one or more deazapurine bases.

MATERIALS AND METHODS FOR REDUCING NUCLEIC ACID DEGRADATION IN BACTERIA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority to U.S. Provisional Application No. 62/816,815, filed March 11, 2019, the disclosure of which is incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under GM070641 awarded by The National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present disclosure is directed to materials and methods for reducing heterologous DNA damage in bacteria by modifying the heterologous DNA to include one or more deazapurine bases.

BACKGROUND

[0004] DNA that is recognized as foreign to a given cell may be targeted for degradation within the cell, either by its lack of a host-like methylation pattern or by the presence of unusual base modifications relative to the host DNA (Bair and Black, 2007, J Mol Biol 366: 768-778). The subsequent degradation by restriction endonucleases reportedly constitutes effective barriers to the introduction of DNA into bacteria (Briggs et al. Appl. Environ. Microbiol. 1994, 60, 2006-2010; Accetto et al. FEMS Microbiol. Lett. 2005, 247, 177-183; Bair and Black, J. Mol. Biol. 2007, 366, 768-778; Corvaglia et al. Proc. Natl. Acad. Sci. U.S.A. 2010, 107, 1 1954-1 1958; Monk et al., 2012, mBio 3(2): e00277-1 1.doi: 10.1 128/mBio.00277-1 1).

[0005] These endonuclease-based systems are grouped into four main types, type I to type IV, by a number of criteria (Roberts et al. Nucleic Acids Res. 2003, 31, 1805-1812). Systems of type I to type III encompass paired methyltransferase and endonuclease activities, degrading foreign DNA that lacks the proper methylation pattern, whereas the type IV enzymes are endonucleases that only cleave DNA substrates that have been modified (Tock and Dryden, Curr. Opin. Microbiol. 2005, 8, 466-472).

[0006] Bacterial transformants provide a key platform for a variety of industrially relevant processes, such as metabolic engineering and biochemical production. However, the

introduction and expression of foreign DNA into some bacterial hosts can be an inefficient process. There is a need in the art for new strategies for maximizing the functionality of heterologous DNA in bacteria.

[0007] Bacteriophages (phages) are viruses that specifically infect and lyse bacteria. Phage therapy, a method of using whole phage viruses for the treatment of bacterial infectious diseases, was introduced in the 1920s by Felix d'Herelle. Initially, phage therapy was vigorously investigated and numerous studies were undertaken to assess the potential of phage therapy for the treatment of bacterial infection in humans and animals.

[0008] With the development of antibiotics in the 1940s, however, interest in phage-based therapeutics declined in the Western world. One of the most important factors that contributed to this decline was the lack of standardized testing protocols and methods of production. The failure to develop industry wide standards for the testing of phage therapies interfered with the documentation of study results, leading to a perceived lack of efficacy as well as problems of credibility regarding the value of phage therapy.

[0009] With the rise of antibiotic resistant strains of many bacteria, however, interest in phage-based therapeutics has returned. Even though novel classes of antibiotics may be developed, the prospect that bacteria will eventually develop resistance to the new drugs has intensified the search for non-chemotherapeutic means for controlling, preventing, and treating bacterial infections.

SUMMARY

[0010] In one aspect, described herein is a bacterial cell comprising a heterologous nucleic acid sequence comprising one or more deazapurine bases. In some embodiments, the one or more deazapurine bases are deazaguanine bases (e.g., 7-deazaguanine bases). Exemplary 7-deazaguanine bases include, but are not limited to, 7-amido-7-deazaguanine (ADG), 7-formamidino-7-deazaguanosine (G^+), 7-cyano-7-deazaguanine ($PreQ_0$) and 7- aminomethyl-7-deazaguanine ($PreQ_1$).

[0011] In another aspect, described herein is a method of protecting a heterologous nucleic acid sequence from cleavage by restriction enzymes in a host bacterium, the method comprising modifying the heterologous nucleic acid sequence to incorporate one or more deazaguanine bases; and introducing the modified heterologous nucleic acid sequence into the host bacterium, thereby protecting the heterologous nucleic acid sequence from cleavage by restriction enzymes in the host bacterium. In some embodiments, the modifying step

occurs *in vitro*. In this regard, in some embodiments, the modifying step comprises mixing the heterologous nucleic acid sequence with at least one enzyme that is involved in introducing deazaguanine bases in DNA for a time sufficient to promote modification of the heterologous nucleic acid sequence.

[0012] In some embodiments, the modifying step comprises introducing the heterologous nucleic acid into a bacterial cell that has been modified to encode at least one enzyme that is involved in introducing deazaguanine bases in DNA.

[0013] Exemplary enzymes that are involved in introducing deazaguanine bases in DNA include, but are not limited to, DpdA and Gat-QueC encoded by *Enterobacteria* phage 9g.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Figure 1: Queuosine and Archeosine synthesis pathways. $PreQ_0$ is synthesized from GTP in both bacteria and archaea through FolE, QueD, QueE and QueC as shown. In most bacteria, four more enzymatic steps lead to the insertion of Q in tRNAs at position 34 (dashed square on lower left). In archaea, $PreQ_0$ is transferred to position 15 of tRNA before being modified to G^+ (dashed rectangle on lower right). Bases identified in this study that are found in phage DNA include $PreQ_1$, $PreQ_0$, ADG and G^+. Molecule abbreviations: guanosine tri-phosphate (GTP), dihydroneopterin triphosphate (H_2NTP), 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4), 5-carboxy-deazaguanine (CDG), 7-amido-7-deazaguanine (ADG), 7-cyano-7-deazaguanine ($PreQ_0$), 7-aminomethyl-7-deazaguanine ($PreQ_1$), queuosine (Q) and archaeaosine (G^+).

[0015] Figures 2A-2C. Figure 2A is a Northern blot of an acrylamide electromobility gel shift assay showing the tRNA-Q complementation of *E. coli* mutants by *Enterobacteria* phage 9g orthologs. The WT strain modifies the $tRNA_{Asp}$ with Q and is shifted in its migration (Q line), but the *E. coli* mutant strains (Δ*folE*, Δ*queD*, Δ*queE*, Δ*queC* and Δ*tgt*) are not modified and migrate further (no Q line). In each mutant, the *Enterobacteria* phage 9g orthologs has been expressed *in trans*. The complementation of Δ*tgt* by *E. coli tgt* is shown as positive control of complementation. Figure 2B is an agarose gel of *Eco*RI digestion of plasmid extracted from different strains of *E. coli* (WT, Δ*queC*, Δ*queD*, Δ*tgt*) expressing variant of pBAD33 and pBAD24 (empty plasmid, 0, encoding *Enterobacteria* phage 9g *dpdA*, A, or encoding *Enterobacteria* phage 9g *gat-queC*, C). *Eco*RI cut pBAD24 once (4542 bp fragment) and pBAD33 twice (2479 bp and 2873 bp fragments). The resulting sizes for the digestion of pBAD24 are 5971 bp and 5509 bp when *qat-queC* or *dpdA* is inserted,

respectively. For pBAD33, the 2873 bp fragment stays unchanged but the 2479 bp fragment shifts to 3911 when *gat-queC* is inserted and 3449 bp when it is *dpdA*. The presence (+) or absence (-) of the modifications identified ($dPreQ_0$ and dG^+) by mass spectrometry are indicated under the gel. Figure 2C is an agarose gel of uncut (0) or EcoRI cut (D) pGH39/pGH66 couple of plasmids extracted from a WT strain of E. coli repressed in 0.4 % glucose (Glu) or induced in 0.4 % arabinose (Ara).

[0016] Figure 3. Genomic context of the dpdA and dG+/PreQ0 biosynthesis pathway genes of *Enterobacteria* phage 9g, *Streptococcus* phage Dp-1, *Vibrio* phage nt-1, *Mycobacterium* phage Rosebush, *Escherichia* phage CAjan, *Salmonell*a phage 7-11, *Mycobacterium* phage Orion and Halovirus HVTV-1. The genes are colored by functions: white is DpdA, shades of grey are the biosynthetic pathway of $PreQ_0$, and the genes coding for aminotransferases that synthetize G^+ from $PreQ_0$. In black are all other proteins. (*) Note that *Streptococcus* phage Dp-1 is grouped in the dG+ biosynthesis pathway in the bioinformatics analysis but it does not produce this modification.

[0017] Figures 4A-4C are gels showing the restriction pattern with different restriction enzymes on the DNA of *Enterobacteria* phage 9g (Figure 4A), *Mycobacterium* phage Rosebush (Figure 4B) and *Enterobacteria* phage CAjan (Figure 4C), as well as the representation of the expected restriction pattern.

[0018] Figure 5 provides a proposed synthesis pathway of the 2'-deoxy-7-deazaguanine modification. Percentages of modification identified for each phage are shown in boxes next to the modification of interest. Molecule abbreviations: guanosine tri-phosphate (GTP), 7-cyano-7-deazaguanine ($PreQ_0$), 2'-deoxy-7-cyano-7-deazaguanosine ($dPreQ_0$), guanine (G), 2'-deoxyguaonosine (dG), 2'-deoxy-7-aminomethyl-7-deazaguanosine ($dPreQ_1$), 2'deoxy-7-amido-7-deazaguanosine (dADG) and 2'-deoxyarchaeaosine (dG^+).

[0019] Figures 6A-6C are schematics showing means of introducing the modifications described herein. (A) The modified mobile genetic elements (MGE) will resist the degradation system from the bacteria of interest compared to the unmodified MGE, and then further be replicated and modified by the natural modification system of the bacteria. (B) *In vivo* modification strategy: an unmodified MGE is introduced in the strain expressing *Enterobacteria* phage 9g *dpdA* and *gat-queC*. The resulting modified MGE is then extracted. (C) As an *in vitro* modification strategy, an unmodified MGE DNA is mixed with the purified

Enterobacteria phage 9g DpdA and Gat-QueC protein and $PreQ_0$. The resulting modified MGE is then purified.

DETAILED DESCRIPTION

[0020] The present disclosure is based, at least in part, on the discovery that a deoxyribonucleic acid (DNA) sequence comprising one or more 7-deazaguanine modifications dramatically decreases the susceptibility of the DNA to endonucleases in bacterial host restriction-modification systems (RM) compared to the same nucleic acid sequence without the 7-deazaguanine modifications. Restriction-modification systems are one of the major defense systems for bacteria to prevent the invasion by foreign nucleic acids[5], such as phages, plasmids or integrons. Modifying nucleic acids (e.g., DNA) to incorporate the 7-deazaguanine modifications disclosed herein results in increased functionality or productivity of bacterial transformants because the modified DNA is less susceptible to host bacterial endonucleases.

[0021] Wild type bacteria encode for multiple defense systems against mobile genetic elements (MGEs). Many of these MGEs are used as tools for genetic engineering applications or as weapons against pathogens. Hence, the availability of a method that would protect these MGEs from bacterial defenses, particularly restriction enzymes, would greatly enhance their effectiveness. As demonstrated herein, nucleic acids (e.g., DNA) modified by $dPreQ_0$, $dPreQ_1$ or dG^+ are protected from cleavage by a wide variety of restriction enzymes.

[0022] In one aspect, described herein is a bacterial cell (or bacterium) comprising a heterologous nucleic acid sequence comprising one or more deazaguanine bases. In some embodiments, the deazaguanine bases are 7-deazaguanine bases. Exemplary 7-deazaguanine bases include, but are not limited to, 7-amido-7-deazaguanine (ADG), 7-cyano-7-deazaguanine ($PreQ_0$), 7-formamidino-7-deazaguanosine (G^+) and 7- aminomethyl-7-deazaguanine ($PreQ_1$).

[0023] In some embodiments, modifying the heterologous nucleic acid with one or more deazaguanine bases results in resistance to degradation by one or more restriction enzymes. In some embodiments, the one or more restriction enzymes is EcoRI (*E. coli*), EcoRII (*E. coli*), BamHI (*B. amyloiquefaciens*), HindIII (*H. influenzae*), NotI (*N. otitidis*), HinFI (*H. influenzae*), Sau3AI (*S. aureus*), PvuII (*P. vulgaris*), SmaI (*S. marcescens*), HaeIII (*H. aegyptius*), HgaI (*H. gallinarum*), AliI (*A. luteus*), EcoRV (*E. coli*), EcoP15I (*E. coli*), KpnI (*K. pneumonia*), PstI (*P. stuartii*), SacI (*S. achromogenes*), SalI (*S. albus),* ScaI (*S.*

caespitosus), SpeI (*S. natans*), SphI (*S. phaeochromogenes*), StuI (*S. tubercidicus*) and/or XbaI (*X. badrii*). Optionally, the heterologous nucleic acid comprising one or more deazaguanine bases is resistant to degradation by one or more of EcoRI, EcoRII, EcoRV and EcoP15I when transformed in *E. coli*.

[0024] The term "heterologous nucleic acid" is a nucleic acid that is not normally present in a particular wild type host cell. The bacterium has been "genetically modified" or "transformed" or "transfected" by heterologous nucleic acid when such nucleic acid(s) has been introduced inside the cell. Nucleic acids include DNA and RNA; can be single- or double-stranded; can be linear, branched or circular; and can be of any length. The heterologous nucleic acid described herein can be any DNA of interest. The DNA may be of genomic, cDNA, semisynthetic, synthetic origin, or any combinations thereof. The heterologous nucleic acid may encode any polypeptide having biological activity of interest or may be a DNA involved in the expression of the polypeptide having biological activity, e.g., a promoter. The heterologous nucleic acid encoding a polypeptide of interest may be obtained from any prokaryotic, eukaryotic, or other source. For purposes of the present disclosure, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted.

[0025] In some embodiments, the heterologous nucleic acid is a mobile genetic element. The term "mobile genetic element" or "MGE" as used herein refers to genetic elements that are not bound to a bacterial host and have the ability to move from one bacterial host to another. In some embodiments, the movement of DNA is within genomes (intracellular mobility). In some embodiments, the movement of DNA is between cells (intercellular mobility). Examples of MGEs include, but are not limited to, transposons, plasmids, bacteriophage nucleic acids, and pathogenicity islands. The MGE can be naturally occurring or engineered. The MGE can be cell-type specific, tissue specific, organism specific, or species specific (e.g., bacteria specific or human specific). The MGE can also be non-specific with respect to cell-type, tissue, organism and/or species.

[0026] A nucleic acid may be modified to incorporate one or more deazapurine bases in a cell-free environment or may be similarly modified in a bacterial cell. In some embodiments, the nucleic acid is modified in a bacterial cell. For example, in some embodiments, a nucleic acid (e.g., MGE) is introduced into a bacterial cell (e.g.,*E. coli, B. cereus,* or *B. subtilis*) that has been modified to encode a transglycosidase (e.g., *dpdA* gene) and an amidotransferase

(e.g.,*gat-queC* gene) from *Enterobacteria* phage 9g and express their respective proteins, DpdA and Gat-QueC. The bacterial cell in its native state expresses additional enzymes (e.g., FolE, QueD, QueE and QueC) that are involved in the four first steps of $PreQ_0$ synthesis. The expression of these native enzymes with a transglycosidase (and an amidotransferase) results in guanine(s) in the nucleic acid (e.g., MGE) being replaced with 7-cyano-7-deazaguanine ($PreQ_0$) and 7-formamidino-7-deazaguanosine (G^+)) . The modified nucleic acid (comprising one or more deazapurine bases) can be collected by lysing the bacterial cell, and then subsequently introduced into a strain of interest.

[0027] In some embodiments, the nucleic acid is modified in a cell free environment. In this regard, isolated and purified transglycosidase (e.g., DpdA) and amidotransferases (e.g., Gat-QueC) are mixed with the nucleic acid (e.g., MGE) and the $PreQ_0$ base (commercially available) for a time and temperature sufficient to promote modification of the nucleic acid by 7-formamidino-7-deazaguanosine (G^+). The modified nucleic acid (comprising one or more deazapurine bases) can then be purified and introduced into a strain of interest. The use of DpdA alone will provide a nucleic acid modified with $dPreQ_0$.

[0028] In some embodiments, a dGPT in a nucleic acid is modified into include a 7-substituted dazapurine dGTP, which DNA polymerases can use as a dNTP substrate to be integrated into newly created DNA (e.g., by PCR) (Cahove et al., ACS Chem. Biol. 11:3165-3171, 2016, the disclosure of which is incorporated herein by reference in its entirety).

[0029] In some embodiments, the heterologous nucleic acid is incorporated into a plasmid or other suitable expression vector (e.g., a bacteriophage-based vector). As used herein, the term "plasmid" or "vector" refers to an extrachromosomal nucleic acid, e.g., DNA, construct that is not integrated into a bacterial cell's chromosome. Plasmids are usually circular and capable of autonomous replication. Plasmids may be low-copy, medium-copy, or high-copy, as is well known in the art. Plasmids may optionally comprise a selectable marker, such as an antibiotic resistance gene, which helps select for bacterial cells containing the plasmid and which ensures that the plasmid is retained in the bacterial cell. A plasmid disclosed herein may comprise a nucleic acid sequence encoding a modified heterologous nucleic sequence e.g., a nucleotide sequence comprising one or more 7-deazaguanine bases.

[0030] The vector may contain one or more (e.g., two, several) selectable markers that permit easy selection of transformed bacterium (or bacterial cell). A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals,

prototrophy to auxotrophs, and the like. Examples of selectable markers include, but are not limited to, the dal genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3.

[0031] General methods, reagents and tools for transforming (e.g., bacteria) can be found, for example, in Sambrook et al (2001) Molecular Cloning: A Laboratory, Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York. Methods, reagents and tools for transforming yeast are described in "Guide to Yeast Genetics and Molecular Biology," C. Guthrie and G. Fink, Eds., Methods in Enzymology 350 (Academic Press, San Diego, 2002).

[0032] In some embodiments, introduction of the modified heterologous nucleic acid sequence (or vector comprising the modified heterologous nucleic acid sequence) of the present disclosure into a host cell is accomplished by calcium phosphate transfection, DEAE-dextran mediated transfection, electroporation, or other common techniques (See Davis et al., 1986, Basic Methods in Molecular Biology, which is incorporated herein by reference). In one embodiment, a preferred method used to transform *E. coli* strains is electroporation and reference is made to Dower et al., 1988) NAR 16: 6127-6145. Indeed, any suitable method for transforming host cells can be used. It is not intended that the present disclosure be limited to any particular method for introducing the modified heterologous nucleic acids into host cells.

[0033] In some embodiments, the bacterial cell (or bacterium) is modified via CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology to express the modified heterologous nucleic acid. A CRISPR genomic locus can be found in the genomes of many bacteria and archaea. The CRISPR locus encodes products that function as a type of immune system to help defend the cell against foreign invaders, such as virus and phage. There are three stages of CRISPR locus function: integration of new sequences into the locus, biogenesis of CRISPR RNA (crRNA), and silencing of foreign invader nucleic acid. Five types of CRISPR systems (e.g., Type I, Type II, Type III, Type U, and Type V) have been identified.

[0034] A CRISPR locus includes a number of short repeating sequences referred to as "repeats." The repeats can form hairpin structures and/or comprise unstructured single-stranded sequences. The repeats usually occur in clusters and frequently diverge between

species. The repeats are regularly interspaced with unique intervening sequences referred to as "spacers," resulting in a repeat-spacer-repeat locus architecture. The spacers are identical to or have high homology with known foreign invader sequences. A spacer-repeat unit encodes a crisprRNA (crRNA), which is processed into a mature form of the spacer-repeat unit. A crRNA comprises a "seed" or spacer sequence that is involved in targeting a target nucleic acid (in the naturally occurring form in prokaryotes, the spacer sequence targets the foreign invader nucleic acid). A spacer sequence is located at the 5' or 3' end of the crRNA.

[0035] A CRISPR locus also comprises polynucleotide sequences encoding CRISPR Associated (Cas) genes. Cas genes encode endonucleases involved in the biogenesis and the interference stages of crRNA function in prokaryotes. Some Cas genes comprise homologous secondary and/or tertiary structures.

[0036] crRNA biogenesis in a Type II CRISPR system in nature requires a trans-activating CRISPR RNA (tracrRNA). The tracrRNA is modified by endogenous RNaseIII, and then hybridizes to a crRNA repeat in the pre-crRNA array. Endogenous RNaseIII is recruited to cleave the pre-crRNA. Cleaved crRNAs are subjected to exoribonuclease trimming to produce the mature crRNA form (e.g., 5' trimming). The tracrRNA remains hybridized to the crRNA, and the tracrRNA and the crRNA associate with a site-directed polypeptide (e.g., Cas9). The crRNA of the crRNA-tracrRNA-Cas9 complex guides the complex to a target nucleic acid to which the crRNA can hybridize. Hybridization of the crRNA to the target nucleic acid activates Cas9 for targeted nucleic acid cleavage. The target nucleic acid in a Type II CRISPR system is referred to as a protospacer adjacent motif (PAM). In nature, the PAM facilitates binding of a site-directed polypeptide (e.g., Cas9) to the target nucleic acid. Type II systems (also referred to as Nmeni or CASS4) are further subdivided into Type II-A (CASS4) and II-B (CASS4a). Jinek et al., Science, 337(6096):816-821 (2012) showed that the CRISPR/Cas9 system is useful for RNA-programmable genome editing, and International Patent Application Publication Number WO2013/176772 (incorporated herein by reference) provides numerous examples and applications of the CRISPR/Cas endonuclease system for site-specific gene editing.

[0037] Exemplary CRISPR/Cas polypeptides include the Cas9 polypeptides in Fig. 1 of Fonfara et al., Nucleic Acids Research, 42: 2577-2590 (2014) (incorporated herein by reference). The CRISPR/Cas gene naming system has undergone extensive rewriting since the Cas genes were discovered. Fig. 5 of Fonfara, *supra*, provides PAM sequences for the Cas9 polypeptides from various species.

[0038] Cas9 polypeptides can introduce double-strand breaks or single-strand breaks in nucleic acids, e.g., genomic DNA. The double-strand break can stimulate a cell's endogenous DNA-repair pathways (e.g., homology-dependent repair (HDR) or non-homologous end joining (NHEJ) or alternative non-homologous end joining (A-NHEJ) or microhomology-mediated end joining (MMEJ)). NHEJ can repair cleaved target nucleic acid without the need for a homologous template. This can sometimes result in small deletions or insertions (indels) in the target nucleic acid at the site of cleavage, and can lead to disruption or alteration of gene expression. HDR can occur when a homologous repair template, or exogenous nucleic acid, is available.

[0039] Thus, in some embodiments, homologous recombination is used to insert heterologous nucleic acid into the genome of the host bacterium. The modifications of the target DNA due to NHEJ and/or HDR can lead to, for example, mutations, deletions, alterations, integrations, gene correction, gene replacement, gene tagging, transgene insertion, nucleotide deletion, gene disruption, translocations and/or gene mutation. The processes of deleting genomic DNA and integrating non-native nucleic acid into genomic DNA are examples of genome editing.

[0040] In some aspects, the Cas9 nuclease is introduced to the bacterium as a protein (i.e., a protein-based system). Typically, the bacteria is treated chemically, electrically, or mechanically to allow Cas9 nuclease entry into the cell. Alternatively, the Cas9 nuclease is introduced to the bacterium as a nucleic acid (e.g., DNA or mRNA) under conditions which allow production of the nuclease. Guide RNA also is introduced into the bacterium.

[0041] A genome-targeting RNA is referred to as a “guide RNA” or “gRNA” herein. A guide RNA comprises at least a spacer sequence that hybridizes to a target nucleic acid sequence of interest, and a CRISPR repeat sequence. In Type II systems, the gRNA also comprises a tracrRNA sequence. In the Type II guide RNA, the CRISPR repeat sequence and tracrRNA sequence hybridize to each other to form a duplex. The duplex binds a site-directed polypeptide, such that the guide RNA and site-direct polypeptide form a complex. The guide RNA provides target specificity to the complex by virtue of its association with the Cas9 nuclease. The guide RNA thus directs the activity of the Cas9 nuclease. In some embodiments, the guide RNA is a single molecule guide RNA (sgRNA).

[0042] A single-molecule guide RNA in a Type II system comprises, in the 5' to 3' direction, an optional spacer extension sequence, a spacer sequence, a minimum CRISPR

repeat sequence, a single-molecule guide linker, a minimum tracrRNA sequence, a 3' tracrRNA sequence and an optional tracrRNA extension sequence. The optional tracrRNA extension may comprise elements that contribute additional functionality (*e.g.*, stability) to the guide RNA. The single-molecule guide linker links the minimum CRISPR repeat and the minimum tracrRNA sequence to form a hairpin structure. The optional tracrRNA extension comprises one or more hairpins.

[0043] A nucleic acid encoding the Cas9 nuclease and/or guide RNA is typically delivered in an expression vector. The exogenous nucleic acid can be delivered in the same vector as the Cas9 nucleic acid, or in a second vector. Any of the expression vectors described herein may be used to deliver Cas9 nuclease-encoding nucleic acid into the bacterium. In many aspects, the expression vector is a plasmid. In some embodiments, an expression vector comprises one or more transcription and/or translation control elements. Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc., may be used.

[0044] The Cas9 nuclease-encoding nucleic acid is operably linked to a promoter that drives protein expression. Exemplary prokaryotic promoters include, but are not limited to, wMel WSP Promote , wDc WSP Promoter and T7. For expressing small RNAs, including guide RNAs used in connection with Cas or Cpf1 endonuclease, promoters such as RNA polymerase III promoters, including for example U6 and H1, can be advantageous. Suitable promoters, as well as parameters for enhancing the use of such promoters, are known in art, and additional information and approaches are regularly being described; see, *e.g.*, Ma, H. *et al.*, *Molecular Therapy - Nucleic Acids 3*, e161 (2014) doi:10.1038/mtna.2014.12.

[0045] In various aspects, the heterologous nucleic acid is of bacteriophage origin. Indeed, in some embodiments, the materials and methods described herein are used to efficiently generate stocks of phage for laboratory or therapeutic use. Phages are an attractive therapeutic option for treating bacterial infections, as phages are more specific than antibiotics, are generally harmless to animals and humans, and have been shown to be effective in combatting antibiotic-resistant bacterial infections. Antibiotic-resistant bacterial infections are an increasing concern in clinical and non-clinical settings. Current first-line treatments rely upon the administration of small-molecule antibiotics to induce bacterial cell death. These broad-spectrum treatments disrupt the patient's normal microflora, allowing resistant bacteria and fungal pathogens to take advantage of vacated niches.

[0046] In this regard, described herein is method of producing a bacteriophage composition (e.g., a stock of bacteriophage) comprising (a) modifying a nucleic acid of bacteriophage origin to incorporate one or more deazaguanine bases as described herein; (b) introducing the modified nucleic acid into a host bacteria cell; (c) incubating the host bacteria cell until phage-mediated bacterial lysis occurs; and (d) isolating bacteriophage lysate. Optionally, the bacteriophage lysate is purified to produce a pharmaceutical composition of bacteriophage. The bacteriophage may be further modified to produce one or more anti-bacterial toxins.

[0047] Any suitable means for culturing bacterial cells is contemplated. Conditions for the culture and production of bacterial cells are readily available and well-known in the art. Cell culture media in general are set forth in Atlas and Parks (eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, Fla. which is incorporated herein by reference. Additional information for cell culture is found in available commercial literature such as the Life Science Research Cell Culture Catalogue (1998) from Sigma-Aldrich, Inc (St Louis, Mo.) ("Sigma-LSRCCC") and, for example, The Plant Culture Catalogue and supplement (1997) also from Sigma-Aldrich, Inc (St Louis, Mo.) ("Sigma-PCCS"), all of which are incorporated herein by reference. Also reference is made to the Manual of Industrial Microbiology and Biotechnology. A. Demain and J. Davies Eds. ASM Press. 1999.

[0048] In some embodiments, the cell culture medium is a liquid medium. In some embodiments, the cell culture medium is a semi-solid medium (e.g., cultured in semi-solid agar on a plate of solid agar).

[0049] In some embodiments, the bacteria (or bacterial cells) are grown under batch or continuous fermentations conditions. Classical batch fermentation is a closed system, wherein the compositions of the medium is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. A variation of the batch system is a fed-batch fermentation. In this variation, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is likely to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Batch and fed-batch fermentations are common and well known in the art. Continuous fermentation is a system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium (e.g., containing the desired end-products) is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in the

growth phase where production of end products is enhanced. Continuous fermentation systems strive to maintain steady state growth conditions. Methods for modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

[0050] In some embodiments, the bacteriophage are isolated or purified from the lysate. For example, the culture medium can be filtered through a very small pore size filter to retain the bacteria and permit the smaller bacteriophage to pass through. Typically, a filter having a pore size in the range of from about 0.01 to about 1 μm can be used (or from about 0.1 to about 0.5 μm, or from about 0.2 to about 0.4 μm). Alternatively or in addition, the culture medium is purified from bacterial debris and endotoxins by dialysis using the largest pore membrane that retains bacteriophages, where the membrane preferably has a molecular cut-off of approximately 10^4 to about 10^7 daltons (or from about 10^5 to about 10^6 daltons). Many other suitable methods can be performed as disclosed for example in US 2001/0026795; US 2002/0001590; U.S. Pat. Nos. 6,121,036; 6,399,097; 6,406,692; 6,423,299; and WO 02/07742, the disclosures of which are incorporated herein by reference in their entireties.

[0051] Bacteria (or bacterial cells) for use according to the disclosure include, but are not limited to, *Bacillus, Bacteroides, Bifidobacterium, Brevibacteria, Caulobacter, Clostridium, Enterococcus, Escherichia coli, Lactobacillus, Lactococcus, Listeria, Mycobacterium, Saccharomyces, Salmonella, Staphylococcus, Streptococcus, Vibrio, Bacillus coagulans, Bacillus subtilis, Bacteroides fragilis, Bacteroides subtilis, Bacteroides thetaiotaomicron, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve UCC2003, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Clostridium acetobutylicum, Clostridium butyricum, Clostridium butyricum M-55, Clostridium cochlearum, Clostridium felsineum, Clostridium histolyticum, Clostridium multifermentans, Clostridium novyi-NT, Clostridium paraputrificum, Clostridium pasteureanum, Clostridium pectinovorum, Clostridium perfringens, Clostridium roseum, Clostridium sporogenes, Clostridium tertium, Clostridium tetani, Clostridium tyrobutyricum, Corynebacterium parvum, Escherichia coli MG1655, Escherichia coli Nissle 1917, Listeria monocytogenes, Mycobacterium bovis, Salmonella choleraesuis, Salmonella typhimurium, and Vibrio cholera.* In certain embodiments, the bacteria are selected from the group consisting of *Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum,*

Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, Oxalobacter formigenes and *Saccharomyces boulardii.* In some embodiments, the bacterium is *E. coli*, *B. cereus* or *L. acidophilus*.

[0052] In some embodiments, the bacterium is a species of the genus *Escherichia* (e.g., *E. coli*). In various embodiments, the *E. coli* bacterial strain used in the processes described herein are derived from strain W3110, strain MG1655, strain B766 (*E. coli* W) or strain BW25113.

[0053] Other examples of useful *E. coli* strains include, but are not limited to, *E. coli* strains found in the *E. coli* Stock Center from Yale University (at website cgsc.biology.yale. edu/index.php); the Keio Collection, available from the National BioResource Project at NBRP *E. coli*, Microbial Genetics Laboratory, National Institute of Genetics 1111 Yata, Mishima, Shizuoka, 411-8540 Japan (www at shigen.nig.ac.jp/ecoli/strain/top/top.jsp); or strains deposited at the American Type Culture Collection (ATCC).

[0054] The bacteriophage described herein are optionally used to treat a bacterial infection in a subject in need thereof. In this regard, a suitable method comprises administering a bacteriophage comprising a heterologous nucleic acid comprising one or more deazapurine bases to the subject. In some embodiments, the bacterial infection is an *Actinobacteria, Aquificae, Armatimonadetes, Bacteroidetes, Caldiserica, Chlamydiae, Chloroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Dictyoglomi, Elusimicrobia, Fibrobacteres, Firmicutes* (e.g., *Bacillus, Listeria, Staphylococcus*), *Fusobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria* (e.g., *Acidobacillus, Aeromonas, Burkholderia, Neisseria, Shewanella, Citrobacter, Enterobacter, Erwinia, Escherichia, Klebsiella, Kluyvera, Morganella, Salmonella, Shigella, Yersinia, Coxiella, Rickettsia, Legionella, Avibacterium, Haemophilus, Pasteurella, Acinetobacter, Moraxella, Pseudomonas, Vibrio, Xanthomonas*), *Spirochaetes, Synergistets, Tenericutes* (e.g., *Mycoplasma, Spiroplasma, Ureaplasma*), *Thermodesulfobacteria* or a *Thermotoga* infection. Optionally, the bacteriophage targets *Salmonella* spp., *Listeria monocytogenes*, MRSA, *E. coli*, *Mycobacterium tuberculosis*, *Campylobacter* spp., and/or *Pseudomonas syringae*. Alternatively, the bacteriophage is employed to destroy bacteria *ex vivo* (e.g., for surface sterilization).

[0055] In some embodiments, the heterologous nucleic acid (e.g., heterologous nucleic acid present in bacteriophage) is provided in a pharmaceutical composition, wherein the

delivery vehicle is a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known, and one skilled in the pharmaceutical art can easily select carriers suitable for particular routes of administration (Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985). Merely to illustrate, in the context of bacteriophage, the delivery vehicle optionally further stabilizes and/or enhances the efficacy of bacteriophage in inhibiting bacterial infection. In some embodiments, the delivery vehicle is a liquid vehicle suitable for administration by infusion or injection. In some embodiments, the delivery vehicle comprises a buffer. Exemplary buffers include, but are not limited to, phosphate buffered saline (PBS), lysogeny broth (LB), phage buffer (100 mM NaCl, 100 mM Tris-HCl, 0.01% (w/v) Gelatin), and Tryptic Soy broth (TSB). In some embodiments, the delivery vehicle is a solid vehicle suitable for administration, e.g., by inhalation or for application by spraying. In some embodiments, the delivery vehicle is a semi-solid or semi-liquid vehicle, such as a gel, cream, paraffin wax, or ointment, suitable for topical application.

[0056] All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification, are incorporated herein by reference, in their entireties.

[0057] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

EXAMPLES

[0058] **Materials/Methods**

[0059] Media composition: Lysogeny broth[1] (LB): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, powder order from fisher (BP1426).

[0060] Brain heart infusion[2] (BHI): Merck cat. 110493

[0061] BHI+[3]: BHI supplemented with 8 μM $MnCl_2$, 0.25 mM, $CaCl_2$, 0.2 mM $MgSO_4$, 50Mm Tris-HCl pH 7.5, 50 ng/μl choline chloride, 0.4% glycine and 100 μl/ml catalase.

[0062] Middlebrook 7H9 broth: 4.7 g Middlebrook 7H9 (Difco), 5 mL 40% glycerol, 900 mL ddH2O.

[0063] Middlebrook 7H10 agar: 19.0 g Middlebrook 7H10 (Difco), 12.5 mL 40% glycerol, 4.95 mL 40% dextrose, 5 drops anti-bubble, 990 mL ddH2O.

[0064] Middlebrook Top Agar: 4.7g Middlebrook 7H9 (Difco), 7.0 g BactoAgar, ddH2O up to 1000 mL, 4 drops of anti-bubble.

[0065] Salt water (SW) stock (30%): 240 g/L NaCl, 30 g/L $MgCl_2$, 35 g/L $MgSO_4$, 7 g/L KCl, 5 mM Tris-HCl pH 7.5.

[0066] Modified growth medium (Rodrigez-Valera 1983) (MGM): for liquid broth 23 % SW is used, 20 % for agar medium and 18 % for soft-agar medium. 5 g/L peptone and 1 g/L yeast extract are also added.

[0067] Difco nutrient broth: 3 g/L beef extract, 5 g/L peptone.

[0068] To these media, 15 g/L of agar are uses for solid medium and 7 g/L for top-agar medium.

[0069] *Construction of the E. coli Q⁻ mutants*: The *E. coli* BW25113 *folE::kan*, *queD::kan*, *queE::kan*, *queC::kan* and *tgt::kan* mutants were collected from the Keio collection[4]. Each mutation was transduced using phage P1[5] in *E. coli* MG1655. The transductions were verified by PCR (couple of primers used: GO119/GO120 and GO121/GO122 for *folE* mutation, GO123/GO124 and GO125/GO126 for *queD* mutation, GO127/GO128 and GO129/GO130 for *queE* mutation, GO111/GO112 and GO113/GO114 for *queC* mutation, GO107/GO108 and GO109/GO110 for *tgt* mutation). The kanamycin cassette was removed from all these strains but Δ*tgt* using pCP20 as described by Datsenko and Wanner[6]. The resulting strains are listed in Table 1.

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
NC_024146	Enterobacteria phage 9g	YP_009032326		YP_009032327	YP_009032328	YP_009032331		
NC_029021	Enterobacteria phage JenK1	YP_009219311		YP_009219312	YP_009219313	YP_009219316		
NC_029028	Enterobacteria phage JenP1	YP_009220002		YP_009220004	YP_009220005	YP_009220008		
NC_028997	Enterobacteria phage JenP2	YP_009216970		YP_009216971	YP_009216972	YP_009216975		
MG948468	Pantoea phage vB_PagS_Vid5	AVJ51789		AVJ51790	AVJ51791	AVJ51795		AVJ51796
KX898399	Pseudomonas phage JG012	ARB11100		ARB11102	ARB11103	ARB11105		
KX898400	Pseudomonas phage JG054	ARB11177		ARB11178	ARB11179	ARB11181		
NC_031058	Pseudomonas phage NP1	YP_009285845		YP_009285847	YP_009285848	YP_009285850		
JQ067084	Pseudomonas phage PaMx25	ALH23795		ALH23793	ALH23791	ALH23789		
KY926791	Salmonella phage SE1 (in:Nonagvirus)	ARM70139		ARM70137	ARM70136	ARM70133		
NC_020860	Cellulophaga phage phiSM	YP_007675729		YP_007675734	YP_007675726	YP_007675725	YP_007675727	
KY606587	Dinoroseobacter phage vB_DshS-R5C	ARB06140		ARB06137	ARB06133	ARB06149	ARB06136	
NC_015274	Streptococcus phage Dp-1	YP_004306895		YP_004306893	YP_004306891	YP_004306892	YP_004306890	
KC821610	Cellulophaga phage phi3ST:2	AGO47783		AGO47788	AGO47780	AGO47779	AGO47781	
NC_006268	Sulfolobus virus STSV1	YP_077211						
JQ287645	Sulfolobus virus STSV2	YP_007348259						
NC_019515	Bacillus phage BCD7	YP_007005872		YP_007005873	YP_007005879	YP_007005878	YP_007005876	
NC_009447	Burkholderia phage BcepGomr	YP_001210251		YP_001210253	YP_001210254	YP_001210257	YP_001210255	
NC_028776	Escherichia phage CAjan	YP_009196829		YP_009196831	YP_009196832	YP_009196839	YP_009196836	
KX534337	Escherichia phage Greed (partial genome)	ANY29829		ANY29831	ANY29832	ANY29839	ANY29835	
NC_027378	Escherichia phage Seurat	YP_009151977		YP_009151979	YP_009151980	YP_009151987	YP_009151983	
NC_028831	Escherichia phage slur01	YP_009201618		YP_009201620	YP_009201621	YP_009201628	YP_009201624	
LT907986	Escherichia phage vB_Eco_SLUR25	SOE45440			SOE45194	SOE45212	SOE45202	
JN699004	Mycobacterium phage Ares	AER48624		AER48628	AER48626	AER48627	AER48625	
MH051249	Mycobacterium phage Boyle	AVR76497		AVR76501	AVR76499	AVR76500	AVR76498	
KT880194	Mycobacterium phage Glass	AMB17316		AMB17320	AMB17318	AMB17319	AMB17317	

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
KR997932	Mycobacterium phage Godines	AKU45201		AKU45205	AKU45203	AKU45204	AKU45202	
JN698991	Mycobacterium phage Hedgerow	AER47234		AER47238	AER47236	AER47237	AER47235	
MG812490	Mycobacterium phage Holeinone	AUX82170		AUX82174	AUX82172	AUX82173	AUX82171	
MG812491	Mycobacterium phage ItsyBitsy1	AUX82212		AUX82216	AUX82214	AUX82215	AUX82213	
MH001452	Mycobacterium phage Kheth	AVO21849		AVO21853	AVO21851	AVO21852	AVO21850	
KX443696	Mycobacterium phage Laurie	ANZ52296		ANZ52300	ANZ52298	ANZ52299	ANZ52297	
KM101117	Mycobacterium phage LizLemon	AIK68776		AIK68780	AIK68778	AIK68779	AIK68777	
MG757162	Mycobacterium phage Opia	AVE00290		AVE00294	AVE00292	AVE00293	AVE00291	
DQ398048	Mycobacterium phage Qyrzula	YP_655682		YP_655686	YP_655684	YP_655685	YP_655683	
AY129334	Mycobacterium phage Rosebush	NP_817763		NP_817767	NP_817765	NP_817766	NP_817764	
KT365402	Mycobacterium phage Tres	ALF01287		ALF01291	ALF01289	ALF01290	ALF01288	
KF024722	Mycobacterium virus Ta17a	AGS81414		AGS81418	AGS81416	AGS81417	AGS81415	
NC_027296	Rhizobium phage RHEph06	YP_009145910		YP_009145913	YP_009145914	YP_009145916	YP_009145915	
MG962366	Rhodococcus phage Finch	AVO25132		AVO25134	AVO25133	AVO25170	AVO25169	
KY629621	Streptococcus virus MS1	AQY55385		ARQ32421	AQY55389	AQY55388	AQY55390	ARQ32422
MG592491	Vibrio phage 1.117.O._10N.261.45.E9	AUR88733		AUR88734	AUR88735	AUR88737	AUR88736	
KX198614	Vibrio phage vB_VhaS-tm	ANO57493		ANO57492	ANO57491	ANO57488	ANO57489	
NC_026610	Vibrio phage VpKK5	YP_009126591		YP_009126590	YP_009126589	YP_009126587	YP_009126588	
NC_031253	Mycobacterium phage Bipper	YP_009303151		YP_009303155	YP_009303153	YP_009303154		
MF417872	Uncultured phage clone 7AX_2 (uncomplete genome)	AGH13911		AGH13914	AGH13912	AGH13913		
KX228400	Clostridium phage CDKM15	ANT45221						ANT45222

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
JQ086369	Enterobacteria phage HK106	YP_007151699						
NC_027339	Enterobacteria phage SfI	YP_009147480						
JQ807243	environmental Halophage eHP-23 (partial genome)	AFH22355						
JQ807227	environmental Halophage eHP-6 (partial genome)	AFH21669						AFH21670
MG962362	Mycobacterium phage AltPhacts	AVO24577						
KX808132	Mycobacterium phage Amelie	APC43635						
MF140398	Mycobacterium phage Amohnition	ASR86319						
MF377440	Mycobacterium phage Bella96	ASR77972						
NC_028691	Mycobacterium phage Apizium	YP_009191212						
MH051264	Mycobacterium phage Cobra	AVR55838						
KX683293	Mycobacterium phage Daffy	AOZ64256						
MF140406	Mycobacterium phage DarthP	ASW31785						
KX576645	Mycobacterium phage Derpp	AOQ28581						
MF919503	Mycobacterium phage Dingo	ATN88731						
MF185719	Mycobacterium phage Edugator	ASR85778						
NC_028936	Mycobacterium phage Enkosi	YP_009211120						
KX576643	Mycobacterium phage FriarPreacher	AOQ28377						
MF185720	Mycobacterium phage Guillsminger	ASR85873						
KY087993	Mycobacterium phage Hammy	APD18205						

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
JF937095	Mycobacterium phage Harvey	AEK08772						
KT364588	Mycobacterium phage Hetaeria	ALA45631						
KJ538723	Mycobacterium phage KingVeVeVe	AHY84289						
NC_023724	Mycobacterium phage Larva	YP_009016449						
MG962371	Mycobacterium phage LaterM	AVO25553						
KX641264	Mycobacterium phage LindNT	AOT25056						
MF668276	Mycobacterium phage Lulumae	ASZ73467						
NC_026598	Mycobacterium phage Milly	YP_009125516						
NC_028759	Mycobacterium phage Mufasa	YP_009195289						
NC_028978	Mycobacterium phage Murucutumbu	YP_009215543						
NC_021310	Mycobacterium phage Newman	YP_008052097						
NC_023711	Mycobacterium phage Oline	YP_009014281						
JF704109	Mycobacterium phage Oosterbaan	AEK07224						
DQ398046	Mycobacterium phage Orion	YP_655116						
NC_028803	Mycobacterium phage OSmaximus	YP_009198878						
KR029086	Mycobacterium phage PDRPv	AKF12401						
KR029087	Mycobacterium phage PDRPxv	AKF12506						
MF185722	Mycobacterium phage Peanam	ASR85925						
NC_028681	Mycobacterium phage Pops	YP_009189977						
KX657794	Mycobacterium phage SamuelLPlaqson	AOZ61371						
KC661274	Mycobacterium phage	AGK87399						

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
	SDcharge11							
KY945355	Mycobacterium phage Shandong1	ARQ95482						
MF919530	Mycobacterium phage Sheila	ATN91769						
NC_025438	Mycobacterium phage Soto	YP_009100829						
NC_023563	Mycobacterium phage Suffolk	YP_009005667						
NC_028658	Mycobacterium phage Swish	YP_009187530						
NC_023498	Mycobacterium phage Validus	YP_009002693						
NC_023727	Mycobacterium phage Vista	YP_009016809						
NC_024147	Mycobacterium phage ZoeJ	YP_009032436						
JX042579	Mycobacterium virus MacnCheese	AFN37736						
NC_021558	Paenibacillus phage PG1	YP_008129913						
MG432137	Pectobacterium phage PEAT2	ATV25085						
NC_015938	Salmonella phage 7-11	YP_004782407						
MG873442	Salmonella phage SE131	AVJ48251						
NC_029003	Salmonella phage SEN1	YP_009217891						
NC_019545	Salmonella phage SPN3UB	YP_007011024						
LT714109	Salmonella virus BTP1	SIU02687						
NC_029046	Sinorhizobium phage phiLM21	YP_009221496						YP_009221497
KX925554	Streptomyces phage BRock	APC46298						
NC_025375	Stygiolobus rod-shaped virus	YP_009094239						
NC_004087	Sulfolobus islandicus rod-shaped virus 1	NP_666594						
NC_034625	Sulfolobus islandicus rod-shaped virus 10	YP_009362827						
NC_034624	Sulfolobus islandicus rod-shaped virus 11	YP_009362775						

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
NC_004086	Sulfolobus islandicus rod-shaped virus 2	NP_666547						
NC_034628	Sulfolobus islandicus rod-shaped virus 4	YP_009362962						
NC_034621	Sulfolobus islandicus rod-shaped virus 5	YP_009362657						
NC_034619	Sulfolobus islandicus rod-shaped virus 7	YP_009362545						
NC_034623	Sulfolobus islandicus rod-shaped virus 8	YP_009362725						
NC_034620	Sulfolobus islandicus rod-shaped virus 9	YP_009362602						
AJ748296	Sulfolobus islandicus rudivirus 1 variant XX	CAG38826						
NC_030884	Sulfolobus islandicus rudivirus 3	YP_009272960						
KT997866	uncultured Mediterranean phage uvDeep-CGR2-KM23-C246	ANS03705						
MG592472	Vibrio phage 1.100.O._10N.261.45.C3	AUR87363						
MG592540	Vibrio phage 1.173.O._10N.261.55.A11	AUR92499						
MG592572	Vibrio phage 1.201.B._10N.286.55.F1	AUR95132						
MG640035	Vibrio phage Athena1	AUG84879						
KU873925	Pseudomonas phage pf16		AND75003	AND75004	AND75007	AND75009	AND75001	
MG592456	Vibrio phage 1.081.O._10N.286.52.C2 (partial genome)		AUR85871	AUR85870	AUR85868	AUR86020	AUR85990	
NC_005083	Vibrio phage KVP40		NP_899370	NP_899369	NP_899368	NP_899531	NP_899504	
NC_028829	Vibrio phage ValKK3		YP_009201241	YP_009201242	YP_009201243	YP_009201467	YP_009201239	
NC_023568	Vibrio phage VH7D		YP_009006086	YP_009006085	YP_009006084	YP_009006244	YP_009006088	
KT919973	Vibrio phage phi-ST2		ALP47368	ALP47437	ALP47397	ALP47407	ALP47426	
JN849462	Vibriophage phi-pp2		AFN37353	AFN37351	AFN37350	AFN37515	AFN37486	
NC_021529	Vibrio phage nt-1		YP_008125322	YP_008125321	YP_008125319	YP_008125479	YP_008125323	

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
KR560069	Stenotrophomonas phage IME-SM1		AKO61693	AKO61689	AKO61579	AKO61694	AKO61692	
KY979132	Acidovorax phage ACP17		ASD50403	ASD50401	ASD50399	ASD50406		
NC_021330	Halovirus HCTV-1			YP_008059626	YP_008059634	YP_008059630	YP_008059635	YP_008059627
NC_021327	Halovirus HCTV-5			YP_008059110	YP_008059116	YP_008059113	YP_008059117	
NC_020158	Halovirus HVTV-1			YP_007378975	YP_007378981	YP_007378978	YP_007378982	
NC_019507	Campylobacter phage CP21			YP_007005301	YP_007005116	YP_007005215	YP_007005322	
NC_027997	Campylobacter phage CP220			YP_009169258	YP_009169203	YP_009169151	YP_009169248	
NC_027996	Campylobacter phage CPt10			YP_009169065	YP_009169010	YP_009168954	YP_009169054	
HM246724	Campylobacter phage IBB35			AEI88255	AEF56764	AEI88211	AEI88267	
LT598654	Phage NCTB			SBV38459	SBV38375	SBV38478	SBV38455	
KY487993	Uncultured virus clone CG99			ASF00408	ASF00598		ASF00594	
NC_023592671	Vibrio phage 2.275.O._10N.286.54.E11			AUS03055	AUS03064	AUS03059	AUS03053	AUS03052
NC_021803	Cellulophaga phage phi13:1			AGO49043	AGO49041	AGO49042		
KC821604	Cellulophaga phage phiST			AGO47177	AGO47175	AGO47176		
KT588073	Acinetobacter phage Ab105-3phi			ALJ98956				
NC_021858	Pandoravirus dulcis			YP_008318610				
NC_026440	Pandoravirus inopinatum			YP_009120778				
NC_022098	Pandoravirus salinus			YP_008436542				
NC_031245	Bacillus phage SP-15						YP_009302501	
LC373201	Enterobacter phage phiEM4						BBD52218	
NC_027340	Erwinia phage phiEa2809						YP_009147529	
NC_025446	Escherichia phage ECML-4						YP_009101458	
MG383452	Escherichia phage FEC14						ATW66911	
NC_019452	Escherichia phage PhaxI						YP_007002664	
JN593240	Escherichia virus CBA120						YP_004957727	
NC_022343	Klebsiella phage 0507-KN2-1						YP_008532008	
MG428990	Klebsiella phage Menlow						AUG87902	

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
NC_023744	Mycobacterium phage DS6A						YP_009018690	
NC_022054	Mycobacterium phage Muddy						YP_008408902	
NC_021063	Mycobacterium phage vB_MapS_FF47						YP_007869941	
MF063068	Pseudomonas phage Noxifer						ARV77198	
NC_028899	Ralstonia phage RSF1						YP_009207957	
AP014693	Ralstonia phage RSL2						YP_009212990	
JX006077	Saccharomonospora phage PIS 136						AFM10404	
NC_029042	Salmonella phage 38						YP_009220980	
NC_031045	Salmonella phage GG32						YP_009283840	
NC_019530	Salmonella phage PhiSH19						YP_007007999	
NC_016073	Salmonella phage SFP10						YP_004895194	
JX881828	Salmonella phage STML-13-1 (partial genome)						AFU64331	
NC_031128	Salmonella phage vB_SalM_PM10						YP_009293427	
NC_023856	Salmonella phage vB_SalM_SJ2						YP_009021339	
KX171211	Salmonella phage vB_SenM-2						ANT44593	
NC_015296	Salmonella phage Vi01						YP_004327394	
MF285619	Serratia phage 2050H1						ASZ78955	
NC_020083	Serratia phage phiMAM1						YP_007349154	
KX147096	Serratia phage vB_Sru_IME250						ANM47156	
MG592536	Vibrio phage 1.169.O._10N.261.52.B1 (partial genome)						AUR92104	
MG592554	Vibrio phage 1.188.A._10N.286.51.A6 (partial genome)						AUR93611	
MG592609	Vibrio phage						AUR97812	

Accession #	Name	DpdA	DpdA2	FolE	QueD	QueE	QueC	YhhQ
	1.244.A._10N.261.54.C3							
KY499642	Vibrio phage pVa-21						AQT27978	
JQ807233	environmental Halophage eHP-12							AFH21913

[0070] *Cloning E. coli tgt*: The *tgt* gene was amplified by PCR from *E. coli* MG1655 using tgt_pBAD24_KpnI_F and tgt_pBAD24_SphI_R primers. The resulting PCR product and pBAD24 were digested by *Kpn*I and *Sph*I. (NEB) following the recommendation of the manufacturer. The genes were then inserted by ligation using the T4 DNA ligase from NEB, following the manufacturer recommendations. The resulting plasmid was verified by sequencing (data not shown).

[0071] Cloning of 9g genes: *dpdA*, *folE*, *queD*, *queE* and *gat-queC* genes from *Enterobacteria* phage 9g (accession number: NC_024146) were amplified by PCR using the couple of primers GO80/GO81, GO92/GO93, GO94/GO95, GO100/GO101 and GO96/GO97, respectively. pBAD24 plasmid and the PCR products were digested by *Sal*I-HF and *Sbf*I-HF (NEB), following the recommendation of the manufacturer. The genes were then inserted by ligation using the T4 DNA ligase from NEB, following the manufacturer recommendations. *dpdA* and *gat-queC* were also cloned in pBAD33 using the same methods. The resulting plasmids were verified by sequencing (data not shown). Each resulting plasmid was transformed in different mutants of *E. coli* MG1655 as listed in Table 1 for the experiment showed in Figure 2A. Different couple of plasmids were co transformed in *E. coli* MG1655, *E. coli* MG1655 Δ*queC*, *E. coli* MG1655 Δ*queD* or *E. coli* MG1655 Δ*tgt* as listed in Table 1 for the experiment showed in Figure 2B.

[0072] *Plasmid DNA preparation for Mass spectrometry*: Overnight cultures were diluted 1/100-fold into 500 mL of LB supplemented with 0.4% arabinose, 100 µg/mL ampicillin and 20 µg/mL of chloramphenicol. Cells were grown overnight and pelleted. The Qiagen maxi-prep kit was used to extract the plasmid following the recommendations of the manufacturer.

[0073] *Rosebush and Orion DNA purification*: *Mycobacteriophages* and Rosebush and Orion were grown as described previously[13]. In brief, 30 mL of a dense *M. smegmatis* culture was mixed with approximately 106 phage particle, 270 mL of top-agar were added and the mixture was plated on 30 large (150 x 10mm) solid media plates. After incubation for 36-48 h at 37°C, 10 mls of phage buffer added, incubated for 4 hrs at room temperature, and the phage lysate collected. Following clarification by centrifugation, phage particles were precipitated with the addition of NaCl to a final concentration of 1M and polyethylene glycol 8000 to a final concentration of 10%. The precipitated particles were collected by centrifugation for 10 minutes at 5,500 x g at 4°C, and resuspended in 10 mls of phage buffer. The lysate was clarified by centrifuged at 5,500 x g for 10 minutes at 4°C, 8.5 g of CsCl was added, and placed in a heat-sealed tube. Samples were centrifuged at 38,000 RPM (98,000 x

g) for 16 hours, and the visible phage band removed with a syringe through the side of the tube.

[0074] Prior to DNA extraction, CsCl was removed by dialysis against phage buffer overnight at 4°C. For DNA extraction, 0.5 mls of phage lysate (~ 1012 particles) were incubated with 12.5 mM $MgCl_2$, 0.8 µU/mL DNAse I and 100 µg/mL RNAse at room temperature for 30 minutes. To this, 20 mM EDTA, 50 µg/mL of Proteinase K and 0.5 % of SDS were added, vortexed vigorously and incubated at 55°C for 60 minutes. An equal volume of phenol:chlorophorm:isoamyl-alcohol (25:24:1) was added and the mixture was inverted several time before being centrifuged for 5 minutes at room temperature at 13,000 rpm (16,000 x g). This step was repeated several times on the aqueous phase obtained until the white interphase was gone. The DNA was ethanol precipitated from the sample, pelleted, washed with 500 µL of 70% ethanol, dried, and the DNA pellet resuspended in 50 µL ddH2O. DNA concentrations were measured using NanoDrop (ThermoScientific).

[0075] *HVTV-1 DNA purification*: To 30 mL of a stationary phase *Haloarcula Valismoris* grown in MGM 23 %, enough phages were added to obtain confluent lysis on plates. 270 mL of MGM 18 % top-agar were added and the mixture was completely plated on MGM 20 % agar. The phages were grown for 4-5 days at 37°C then a top layer of HVTV-1 virus buffer[14] (1.2 M NaCl, 44 mM $MgCl_2$, 47 mM $MgSO_4$, 1.5 mM $CaCl_2$, 28 mM KCl, 24 mM Tris-HCl pH 7.2) was poured on top of each plate. Phages were allowed to diffuse to the liquid phase for 4 h at 4°C before being harvested. Debris were pelleted, and phages were precipitated over night at 4°C by adding 10 % polyethylene glycol (PEG 8000) to the supernatant. The phage suspension was centrifuged for 10 minutes at 4,500 x g at 4°C. The phage pellet was resuspended in 10 mL of HVTV-1 virus buffer and dialyzed in the same buffer over night at 4°C to eliminate the last traces of PEG. 12.5 mM $MgCl_2$, 0.8 µU/mL DNAse I and 100 µg/mL RNAse were added and the mixture were incubated at room temperature for ~ 30 minutes. 20 mM EDTA, 50 µg/mL of Proteinase K and 0.5 % of SDS were added to the mixture, which was then vortexed vigorously and incubate at 55°C for 60 minutes. A equal volume of phenol:chlorophorm:isoamyl-alcohol (25:24:1) was then added and the mixture was inverted several time before being centrifuged for 5 minutes at room temperature at 4,500 x g. This step was repeated several times on the aqueous phase obtained until the white interphase was gone. An equal volume of chloroform was added to the aqueous phase, vortexed and centrifuged again to eliminate the last traces of phenol. The DNA was then ethanol precipitated from the sample and pelleted. The pellet was washed with 500 µL of

70% ethanol. The dried DNA pellet was then resuspended in ~ 50 µL dH_2O. Concentrations were measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo scientific, Waltham, MA).

[0076] *9g DNA purification*: To 30 mL of a stationary phase *E. coli* MG1655 grown in LB, enough phages were added to obtain confluent lysis on plates. 270 mL of LB top-agar were added and the mixture was completely plated on LB agar. The phages were grown overnight at 37°C then a top layer of TM buffer (10 mM $MgSO_4$, 10 mM Tris-HCl pH 7.5) was poured on top of each plate. Phages were allowed to diffuse to the liquid phase for 4 h at 4°C before being harvested. Debris were pelleted, and phages were precipitated over night at 4°C by adding 1 M of NaCl and 10 % polyethylene glycol (PEG 8000) to the supernatant. The phage suspension was centrifuged for 10 minutes at 4,500 x g at 4°C. The phage pellet was resuspended in 10 mL of TM buffer and dialyzed in the same buffer over night at 4°C to eliminate the last traces of PEG. 12.5 mM $MgCl_2$, 0.8 µU/mL DNAse I and 100 µg/mL RNAse were added and the mixture were incubated at room temperature for ~ 30 minutes. 20 mM EDTA, 50 µg/mL of Proteinase K and 0.5 % of SDS were added to the mixture, which was then vortexed vigorously and incubate at 55°C for 60 minutes. A equal volume of phenol:chlorophorm:isoamyl-alcohol (25:24:1) was then added and the mixture was inverted several time before being centrifuged for 5 minutes at room temperature at 4,500 x g. This step was repeated several times on the aqueous phase obtained until the white interphase was gone. An equal volume of chloroform was added to the aqueous phase, vortexed and centrifuged again to eliminate the last traces of phenol. The DNA was then ethanol precipitated from the sample and pelleted. The pellet was washed with 500 µL of 70% ethanol. The dried DNA pellet was then resuspended in ~ 50 µL dH_2O. Concentrations were measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo scientific, Waltham, MA).

[0077] *Synthesis of 2-Amino-7-(2-deoxy-β-D-erythro-pentofuranosyl)-4,7-dihydro-4-oxo-1H-pyrrolo[2,3- d]pyrimidine-5-carboxamide (dADG):*

[0078] To a solution of compound i[16] (130 mg, 0.33 mmol, Figure 7) in 1:1 MeOH-dioxane (12 mL) was added Et_3N (0.2 mL, 1.5 mmol) and purged with CO gas for 10 min followed by addition of $Pd(PhCN)_2Cl_2$ (12.7 mg, 0.03 mmol). The reaction mixture was stirred at 60 °C for 24 h, cooled to ambient temperature and evaporated. To the resulting crude ester was added aqueous ammonia (15 mL) in a sealed tube, which was heated at 100

°C for 1 h. The reaction mixture was cooled to ambient temperature and evaporated to dryness. The crude reaction mixture was washed with hot methanol to afford dADG (60 mg, 58 %) as off-white solid. HRMS (ESI): m/z calculated for $C_{12}H_{16}N_5O_5$ $[M+H]^+$ 310.1151, observed 310.1152.

[0079] *Synthesis of 2-amino-7-(2-deoxy-β-D-erythro-pentofuranosyl)-4,7-dihydro-4-oxo-3H-pyrrolo[2,3- d]pyrimidine-5-carbonitrile (dPreQ$_0$)*[17]*:*

[0080] To a suspension of i[16] (600 mg, 1.53 mmol) in pyridine (10 mL) was added CuCN (1.37 g, 15.3 mmol) with stirring under reflux for 20 h. The reaction mixture was cooled to ambient temperature and solvent evaporated. The resulting solid was washed thoroughly with 20 % MeOH in dichloromethane, with the washings combined, evaporated and purified by column chromatography (100-200 mesh silica gel) eluting with 10 % to 20 % MeOH in dichloromethane to afford dPreq$_0$ (220 mg, 49 %) as off-white solid. HRMS (ESI): m/z calculated for $C_{12}H_{14}N_5O_4$ $[M+H]^+$ 292.1046, observed 292.1043.

[0081] *Synthesis of 2-Amino-7-(2-deoxy-β-D-erythro-pentofuranosyl)-4,7-dihydro-4-oxo-3H-pyrrolo[2,3- d]pyrimidine-5-carboximidamide (dG$^+$):*

[0082] Dry HCl gas was bubbled through a suspension of dPreQ$_0$ (100 mg, 0.34 mmol) in anhydrous MeOH (20 mL) at 0 °C for 2 h. Following stirring at ambient temperature for 16 h, the reaction mixture was evaporated and treated with 7N NH_3 in MeOH at 0 °C, with stirring for 16 h. The crude reaction mixture was evaporated under vacuum and purified by MPLC using C18 column eluting with acetonitrile and H_2O. The fractions containing product was lyophilized to afford dG$^+$ (20 mg, 18 %) as an off-white solid[18]. HRMS (ESI): m/z calculated for $C_{12}H_{17}N_6O_4$ $[M+H]^+$ 309.1311, observed 309.1306.

[0083] *Q detection in tRNA:* Overnight cultures were diluted 1/100-fold into 5 mL of LB supplemented with 0.4 % arabinose and 100 µg/mL ampicillin and grown for 2 h at 37 °C. Cells were harvested by centrifugation at 16,000 x g for 2 min at 4 °C. Cell pellets were immediately resuspended in 1 mL of Trizol (Life technologies, Carlsbad, CA). Small RNAs were extracted using PureLink™ miRNA Isolation kit from Invitrogen (Carlsbad, CA) according to manufacturer protocol. The purified RNAs were eluted in 50 µL of RNase free water and tRNA concentrations were measured by NanoDrop® ND-1000 Spectrophotometer (Thermo scientific, Waltham, MA). Then, 200 µg were used in 3-(Acrylamido)-phenylboronic acid (APB) assay described in detail previously[32] using the (5'-biotin-

CCCTCGGTGACAGGCAGG-3') probe that detects $tRNA_{Asp}$(GUC) at final concentration of 0.3 μM.

[0084] *Restriction assay for deazapurine presence in plasmid DNA*: *E. coli* strains containing different variation of pBAD24 and pBAD33 (with or without *dpdA* or *gat-queC* from *Enterobacteria* phage 9g) were grown overnight in LB supplemented with 0.2 % of glucose at 37 °C. Each strain was diluted 100-fold in LB supplemented with 0.4 % of arabinose and grown 6 h at 37°C. Plasmids were extracted using the Qiagen QIAprep Spin Miniprep Kit and 500 ng of plasmid were digested by *Eco*RI-HF (New England Biolabs, Ipswich MA) for 1 h at 37 °C in 20 mL CutSmart buffer. The enzyme was inactivated by 20 min incubation at 80 °C. The samples were run on a 0.5 % agarose gel, Tris-EDTA acetate (TAE) 1X. The gel was then stained 30 min in 0.5 μg/mL ethidium bromide, then washed 3 times for 15 min in water, and visualized with the Azur Biosystem c200 gel doc (Thermofisher, Waltham, MA, USA).

[0085] *Search for phage encoding queuosine and archaeosine biosynthesis proteins*: The Viruses nr database from NCBI was queried by three iterations of PSI-BLAST[37], default set up as previously suggested[50], using the proteins referenced in Table 2, known to be involved in Queuosine (Q) or Archaeosine (G^+) biosynthesis, as well as DpdA from *Enterobacteria* phage 9g, predicted to be involved in the modification of phage DNA, and another DpdA2 from *Vibrio* phage nt-1, part of a new family identified in this study.

[0086] *Table 2.*

accession #	protein name	Species
WP_001139613	FolE	*Escherichia coli*
WP_000987944	QueD	*Escherichia coli*
WP_001199973	QueE	*Escherichia coli*
WP_000817220	QueC	*Escherichia coli*
WP_000100421	QueF	*Escherichia coli*
WP_001266503	QueA	*Escherichia coli*
WP_001294219	QueG	*Escherichia coli*
WP_013679609	Gat-QueC	*Thermoproteus uzoniensis*
BAA80469	QueF-L	*Aeropyrum pernix K1*
WP_066380731	ArcS	*Halalkalicoccus paucihalophilus*
WP_011068173	QueH	*Bifidobacterium longum* NCC2705
WP_005315061	DUF3820 (QueD-like)	*Aeromonas salmonicida*
YP_009032326	DpdA	*Enterobacteria* phage 9g

YP_008125322	DpdA2	*Vibrio* phage nt-1

[0087] The $PreQ_0$ specific transporter YhhQ[27] was also added. For each virus identified with at least one of these genes, a reverse analysis was done (phage genome again the protein list) to ensure that no protein was missed during the first analysis. Each identified ortholog was verified by HHpred[38] for its annotation.

[0088] *Identification of the host and their gene content*: The Virus-Host DB[44] was used to gather the host of each phage identified in this study. For phages not referenced in this database, a manual investigation coupling RefSeq[42] and the literature was performed (data now shown) Each host identified was queried in the Globi database[43] (data not shown) The same analysis was done for the double strand DNA (dsDNA) phages, as only these phages were return in our analysis (data not shown). A list of genomes was created on PubSeed[45] from the hosts identified to create a new spreadsheet.

[0089] *Mass spectrometry analysis*: DNA analysis was performed as previously but with several modifications[16]. Purified DNA (20 µg) was hydrolyzed in 10 mM Tris-HCl (pH 7.9) with 1 mM MgCl2 with Benzonase (20U), DNase I (4U), calf intestine phosphatase (17U) and phosphodiesterase (0.2U) for 16 h at ambient temperature. Following passage through a 10 kDa filter to remove proteins, the filtrate was lyophilized and resuspended to a final concentration of 0.2 µg/µL (based on initial DNA quantity).

[0090] Quantification of the modified 2'-deoxynucleosides (dADG, dQ, $dPreQ_0$, $dPreQ_1$ and dG^+) and the four canonical 2'-deoxyribonucleosides (dA, dT, dG, and dC) was achieved by liquid chromatography-coupled triple quadrupole mass spectrometry (LC-MS/MS) and in-line diode array detector (LC-DAD), respectively. Aliquots of hydrolyzed DNA were injected onto a Phenomenex Luna Omega Polar C18 column (2.1 x 100 mm, 1.6 µm particle size) equilibrated with 98 % solvent A (0.1 % v/v formic acid in water) and 2 % solvent B (0.1 % v/v formic acid in acetonitrile) at a flow rate of 0.25 mL/min and eluted with the following solvent gradient: 12 % B for 10 min, 1 min ramp to 100 % B for 10 min, 1 min ramp to 2 % B for 10 min. The HPLC column was coupled to an Agilent 1290 Infinity DAD and an Agilent 6490 triple quadruple mass spectrometer (Agilent, Santa Clara, CA). The column was kept at 40 °C and the auto-sampler was cooled at 4 °C. The UV wavelength of the DAD was set at 260 nm and the electrospray ionization of the mass spectrometer was performed in positive ion mode with the following source parameters: drying gas temperature 200 °C with a flow of 14 L/min, nebulizer gas pressure 30 psi, sheath gas temperature 400 °C with a flow

of 11 L/min, capillary voltage 3,000 V and nozzle voltage 800 V. Compounds were quantified in multiple reaction monitoring (MRM) mode with the following m/z transitions: 310.1 → 194.1, 310.1 → 177.1, 310.1 → 293.1 for dADG, 394.1 → 163.1, 394.1 → 146.1, 394.1 → 121.1 for dQ, 292.1 → 176.1, 176.1 → 159.1, 176.1 → 52.1 for $dPreQ_0$, 296.1 → 163.1, 296.1 → 121.1, 296.1 → 279.1 for $dPreQ_1$, and 309.1 → 193.1, 309.1 → 176.1, 309.1 → 159.1 for dG^+. External calibration curves were used for the quantification of the modified canonical 2'-deoxynucleosides. The calibration curves were constructed from replicate measurements of eight concentrations of each standard. A linear regression with r2 > 0.995 was obtained in all relevant ranges. The limit of detection (LOD), defined by a signal-to-noise ratio (S/N) ≥ 3, ranged from 0.1 to 1 fmol for the modified 2'-deoxynucleosides. Data acquisition and processing were performed using MassHunter software (Agilent, Santa Clara, CA).

[0091] *Restriction assay of phage DNA:* 250 ng of phage DNA were digested by different enzymes (New England Biolabs) described in Figure 4 or 1 h at 37 °C in 20 mL CutSmart or 3.1 buffer solution, according to the manufacturer instructions. The enzymes were inactivated by a 20 min incubation at 80 °C. The samples were run on a 0.7 % agarose gel, Tris-EDTA acetate (TAE) 1X. The gel was then stained 30 min in 0.5 µg/mL ethidium bromide, then wash 3 times for 15 min in water, and visualized with the Azur Biosystem c200 gel doc.

Example 1 - Phage 9g encodes functional $PreQ_0$ synthesis genes

[0092] First, it was determined whether the phage 9g genes predicted to encode $PreQ_0$ synthesis enzymes could complement the Q deficiency phenotype of *E. coli* derivatives lacking the corresponding orthologs. As shown in Figure 2A, the expression *in trans* of *folE*, *queD* and *queE* from *Enterobacteria* phage 9g in *E. coli* MG1655 Δ*folE*, Δ*queD* and Δ*queE* strains respectively, successfully reestablished the production of queuosine (Q), demonstrating the isofunctionality of the tested pairs. However, this complementation was not observed when the viral *gat-queC* and *dpdA* genes were expressed in *E. coli* Δ*queC* and Δ*tgt*, respectively. The result was expected for *dpdA* as it was predicted to encode an enzyme that recognizes DNA and not tRNA[14,36]. However, it was unexpected for *gGat-QueC*, as it was shown previously that expression of an archaeal *gat-queC* homolog in *E. coli* could lead to G^+ in tRNA and hence formation of a $PreQ_0$ intermediate[20].

Example 2 - Phage 9g Gat-QueC and DpdA are needed for G^+ insertion in *E. coli* DNA genes

[0093] It was predicted that dual expression of the viral *gat-queC* and *dpdA* genes *in trans* would lead to the insertion of 7-deazaguanine derivatives, as dG^+, in *E. coli* DNA. Because the presence of dG^+ confers resistance to EcoRI digestion[34], restriction profiles were used as a first indication for the presence of modifications in plasmid DNA. The two phage genes were both cloned in pBAD24 and pBAD33. EcoRI cuts pBAD24 once and pBAD33 twice, as shown in the digestion profiles of plasmids extracted from an *E. coli* derivative co-transformed with the two empty plasmids (Figure 2B, lane 1). Because no EcoRI sites are present in the phage 9g *gat-queC* and *dpdA* genes, the restriction profiles of plasmids extracted from *E. coli* derivatives co-transformed with one empty plasmid and one plasmid containing one of the two genes are just shifted by the insert sizes with no additional bands (Figure 2B, lanes 2, 3, 5 and 6). However, an additional band corresponding to the uncut plasmid was observed for plasmid preparations from strains expressing both *gat-queC* and *dpdA* genes (Figure 2B, lanes 4 and 7). This band only appeared when the genes are induced (Figure 2C).

[0094] Analysis of dG^+, dADG, $dPreQ_0$ and $dPreQ_1$ profiles by liquid chromatography-coupled triple quadrupole mass spectrometry (LC-MS/MS) (Figure 2B, only $dPreQ_0$ and dG+ are presented as no dADG or dQ were found) revealed that plasmid DNA extracted from strains expressing only *dpdA* contained $dPreQ_0$, plasmid DNA extracted from strains expressing *dpdA* and *gat-queC* contained dG^+ (Figure 2B, lane 4 and 7), and $dPreQ_0$ when *gat-queC* was expressed at lower levels than *dpdA* (Figure 2B, lane 4). Taken together, these results showed that dG^+ but not $PreQ_0$ could confer resistance to EcoRI and that the phage 9g pathway that inserts dG^+ in its viral DNA can be transferred to *E. coli* genomic DNA.

[0095] Interestingly, whereas we had failed to complement the Q^- phenotype of the *E. coli queC* strain when expressing the phage 9g Gat-QueC gene, the EcoRI resistance phenotype caused by 7-deazapurine insertion in strains expressing both 9g *dpdA* and *gat-queC* was still observed in a *ΔqueC* background (Figure 2B, lanes 8 and 9) but not in a *ΔqueD* background (Figure 2B, lanes 10, 11). Furthermore, only dG^+ modification was observed in DNA of the *ΔqueC* strains by LC-MS/MS. This suggests that the Gat-QueC protein can produce $PreQ_0$ but that it is channeled to the putative DNA modifying enzyme DpdA and not to the tRNA modifying pathway enzyme QueF.

[0096] Finally, whether the *E. coli* TGT was required for DpdA activity in *E. coli* was tested as the active forms of TGT enzymes are known to be dimers[36]. This does not seem to

be the case as the restriction resistance phenotype was still observed in the Δ*tgt* background (Figure 2B, lanes 12 and 13).

Example 3 - A wide variety of phages harbor the dG$^+$ biosynthesis pathway

[0097] A new sub-family of DpdA encoded by the *Vibrio* phage nt-1was identified by investigating genes flanking $PreQ_0$ biosynthesis genes cluster. Indeed, phage nt-1 DpdA (YP_008125322) is not detected with PSI-BLAST when using the *E. coli* phage 9g DpdA as input sequence and it does not possess the conserved histidine found at position 196 but similarities with members of the TGT family could be detected using HHpred. This protein was renamed DpdA2.

[0098] An *in silico* search for phages that could harbor 7-deazaguanine derivatives in their genomic DNA revealed that a total of 182 viruses deposited in GenBank were found to encode a DpdA homolog and/or at least a G$^+$ synthesis gene (Table 1). Most of these viruses (163/182) were bacteriophages, while 16 archaeal viruses as well as the 3 eukaryotic viruses were found. The latter only encode for FolE, which is most likely to be linked to the folate pathway[39]. Analyses of the presence/absence patterns of the predicted Q/G$^+$ biosynthesis genes led to classification of these viruses in various groups and in some cases, predict the nature of the 7-deazaguanine base modification. It is important to note that no homologs to the proteins specifically involved in Q biosynthesis such as QueA, QueG, or QueH (see Figure 1) were found in viruses.

[0099] The first group contains 25 phages and is represented by *Enterobacteria* phage 9g (KJ419279), *Streptococcus* phage Dp-1 (NC_015274) and *Vibrio* phage nt-1 (NC_021529) in Figure 3. Those phages encode homologs of 9g DpdA or nt-1 DpdA2as well as homologs of FolE, QueD, QueE and QueC. In addition, they encode homologs of one of the three amidotransferases involved in the last steps of G$^+$ synthesis: ArcS, QueF-L (or QueF) or a Gat-QueC fusion, which replace the canonical QueC in this last case. These phages likely modify their DNA with dG$^+$, as phage 9g[14] does. It should be noted that the discrimination between the QueF-L homologs, predicted to produce the G$^+$ base from $PreQ_0$, and QueF homologs, predicted to produce $PreQ_1$ from $PreQ_0$, is difficult to establish based on the sequence similarity only. Therefore, the genome of phages encoding for these proteins might harbor dG$^+$ or $dPreQ_1$ (or both).

[00100] The second group includes 40 phages and is represented by *E. coli* phage CAjan (NC_028776) and *Mycobacterium* phage Rosebush (AY129334) in Figure 3. These phages

encode a homolog of one of the two types of DpdA, and of the $PreQ_0$ synthesis enzymes (FolE, QueD, QueE and QueC), but they are missing an amidotransferase. As such, it is predicted that these phages modify their DNA with $PreQ_0$ or ADG, like the bacteria that contain the *dpd* cluster[14]. *Mycobacterium* phage Bipper (KU728633) that misses only a gene encoding QueC was added to this group even if it could be modified by the QueC substrate (CDG, see Figure 1). The uncultured phage clone 7AX_2 (MF417872) was also added to this group as it also lacks a gene encoding QueC, although this may be due to the incomplete genomic sequence of this phage. Whether this phage also encodes an amidotransferase could not be excluded.

[00101] The third group contains 76 phages including *Salmonella* phage 7-11 (NC_015938) and *Mycobacterium* phage Orion (DQ398046) shown in Figure 3. These phages encode DpdA but no G^+ or $PreQ_0$ biosynthesis protein homologs. At this stage, their genome modification status, if any, was difficult to predict. Phages in this group could rely on $PreQ_0$ synthesized by the host or on uptake of exogenous 7-deazaguanine precursors. The large size of this group compared to the others might be caused by the relatively large number of Mycobacteriophages in the virus database due to the massive phage isolation and sequencing effort of PhagesDB and the SEA-PHAGES project.

[00102] The last group is composed of 48 phages encoding proteins of the $PreQ_0/G^+$ pathway but no DpdA. These phages could boost the production of the Q precursor to increase the level of Q in the host tRNA and increase translation efficiency[40]. However, it is possible that 7-deazaguanines are inserted in their DNA in a DpdA independent pathway as there is a recent report that the genomes of *Capylobacter* phages from this group are highly modified by dADG (data not shown).

[00103] Phages containing FolE and QueC singletons were discarded from further analysis because FolE is shared between folate and $PreQ_0$ synthesis[16] while QueC is also part of a superfamily of ATPase (COG) making their precise role to identify.

[00104] All the phages identified above are members of the *Caudovirales* order and are distributed into various families: *Siphoviridae* (95), *Myoviridae* (23), *Ackermannviridae* (20) and *Podoviridae* (3). For the Archaeal virus, 12 *Ligamenvirales* and 2 *Bicaudaviridae* were identified (data not shown).

Example 4 - The host may participate in the phage DNA modification

[00105] To study the interaction between phages containing 7-deazaguanine related genes and their bacterial hosts, metadata on the hosts and their habitat was gathered using RefSeq[42] and the Globi database[43], and the distribution of Q, G^+ and dADG synthesis genes in these organisms was analyzed (data not shown). Interestingly, 106 of the collected phages (~ 60%) infect a strain that is the model for a known bacterial pathogen, where only ~ 9% of the dsDNA viruses from the Virus-Host database[44] infect a strain related to pathogen (data not shown). No clear environment was found for the archaeal hosts.

[00106] All phage hosts predicted to modify their DNA with G^+ possess the pathway to produce Q in tRNA. Curiously the hosts of the phages coding for a QueF-L and a 9g DpdA homolog do not encode for the $PreQ_0$ biosynthetic pathway (QueDEC, see Figure 1), but encode for the specific $PreQ_0$ transporter YhhQ and the rest of the Q pathway (QueFAG and TGT, Figure 1). Conversely, all the hosts of the DpdA2 encoding phages encode the full Q pathway. As shown in Figure 1, 7-cyano-7-deazaguanine (PreQ0) is synthesized from GTP by four enzymes (FolE, QueD, QueE, QueC) and is the key intermediate in both the Q and G^+ pathways. The last step of $PreQ_0$ synthesis is catalyzed by 7-cyano-7-deazaguanine synthase (QueC) in a complex reaction that goes through the 7-amido-7-deazaguanine (ADG) intermediate. tRNA-guanine-transglycosylases (TGT in bacteria, arcTGT in archaea) are the signature enzymes in the Q and G+ tRNA modification pathways as they exchange the targeted guanines with the 7-deazaguanine precursors. In archaea, $PreQ_0$ is directly incorporated into tRNA by arcTGT before being further modified by different types of amidotransferases (ArcS, Gat-QueC or QueF-L). In bacteria, PreQ0 is reduced to 7-aminomethyl-7-deazaguanine ($PreQ_1$) by QueF before TGT incorporates it in tRNA, where it is further modified to Q in two steps (Figure 1).

[00107] There is no clear pattern for the bacterial hosts of phages encoding both DpdA and the whole $PreQ_0$ pathway. Most of them encode the full Q pathway enzymes except for *Streptococcus pneumoniae*, which lacks $PreQ_0$ pathway genes, *Rhodococcus erythropolis*, which encodes only TGT, and the *Mycobacteria*, that possess none of these genes.

[00108] The hosts of the phages encoding only DpdA also encode for the full set of Q synthesis enzymes except the *Clostridium* species, which lack the $PreQ_0$ pathway genes, and the *Mycobacterium* genus, that possess none of these genes. *Sulfolobi* were not referenced in PubSeed[45], but using BLASTp with default parameters with the genes listed in Table 2 above as queries, all G^+ pathway genes were identified. Hence, the 7-deazaguanine intermediates

produced by these hosts, *Clostridium* and *Mycobacterium* excluded, might be used by phages that lack the biosynthesis proteins to produce a 7-deazaguanine precursor.

[00109] Finally, the hosts of the phages that do not encode a DpdA but encode the $PreQ_0$ pathway proteins all encode the full Q synthesis pathway.

[00110] A few bacterial hosts, such as 46 different strains of *E. coli*, *Haloarcula valismortis* and *Vibrio harveyi* 1DA3, also harbor homologs of the bacterial DpdA. In these cases, infecting phages could be modified by the host modification machinery.

Example 5 - Different set of genes for different 7-deazaguanine modifications

[00111] To test predictions on the nature of phage DNA modification, a set of phages from each group was selected, and their genomic DNA were extracted for mass spectrometry analysis (Table 3).

[00112] Table 3.

Phage/Virus			Prediction based on gene content	Modification per 10^6 nuclotides				
Accession #	Name	GC content		$dpreQ_0$	dADG	dG^+	$dpreQ_1$	dQ
NC_028776	*Escherichia* phage CAjan	44.7%	$dPreQ_0$	70628	None	None	None	None
None	*Escherichia* phage CAjan ΔdpdA		None	None	None	None	None	None
NC_020158	*Halovirus* HVTV-1	58.3%	None/dG^+	None	152	22	420654	None
NC_008197	*Mycobacterium* phage Orion	66.5%	None	None	None	None	None	None
NC_004684	*Mycobacterium* phage Rosebush	69.0%	$dPreQ_0$	96530	9	None	None	None
NC_015938	*Salmonella* phage 7-11	44.1%	None/$PreQ_0$	None	50	None	None	None
NC_015274	*Streptococcus* phage Dp-1	40.3%	$dPreQ_1$/dG^+	None	None	None	9605	None
NC_021529	*Vibrio* phage nt-1	41.3%	dG^+	232	72	44	None	None

[00113] Interestingly, no 2'-deoxyqueuosine (dQ) was found in any of the tested samples, correlating with the fact that no phage or virus encodes the specific protein for Q synthesis (QueAGH).

[00114] First, phages encoding both a DpdA and one of the amidotransferase homologs were analyzed. *Streptococcus* phage Dp-1 DNA, encoding for a QueF-L, contained a large amount of $dPreQ_1$ (3,389 modifications per 10^6 nucleotides, ~ 1.7 % of the Gs) but no dG^+,

which would mean that the QueF-L of this phage would actually be functionally closer to the bacterial QueF than the archaeal QueF-L, as predicted by the SSN clustering. *Vibrio* phage nt-1, encoding an ArcS, was shown to harbor not only dG^+ (44 modifications per 10^6 nucleotides, ~ 0.02 % of the Gs) but also $dPreQ_0$ and dADG (232 modifications per 10^6 nucleotides, ~ 0.11 % of the Gs, and 72 modifications per 10^6 nucleotides, ~ 0.03 % of the Gs, respectively). This result might indicate that nt-1 DpdA is more promiscuous and could insert all intermediates of the pathway.

[00115] Next, phages of the second group that encode both a DpdA and the four proteins of the $PreQ_0$ biosynthesis pathway but no amidotransferase homolog were investigated. *Mycobacterium* phage Rosebush was found to harbor $dPreQ_0$ in its DNA (96,530 modifications per 10^6 nucleotides, ~ 28 % of the Gs) as does *Escherichia* phage CAjan (70,628 modifications per 10^6 nucleotides, ~ 32 % of the Gs). However, *Mycobacterium* phage Rosebush *was* found to also harbor a very small amount of dADG (9 modifications per 10^6 nucleotides, ~ 0.003 % of the Gs). These proportions are negligible for Rosebush and could be the result of the natural oxidation of the $PreQ_0$ base.

[00116] The genomic DNA of *Salmonella* phage 7-11 and *Mycobacterium* phage Orion from the third group of phage, which only encode a DpdA were also analyzed by LC-MS/MS. *Mycobacterium* phage Orion lacked any 7-deazaguanine modifications in its DNA. This result was expected as none of the phage nor the host encode for the $PreQ_0$ biosynthesis pathway (*Mycobacterium smegmatis*, Table 3). However, *Salmonella* phage 7-11 was unexpectedly modified by dADG (50 modifications per 10^6 nucleotides, ~ 0.02 % of the Gs), suggesting the presence of a protein responsible for the oxidation of $PreQ_0$ encoded by the phage.

[00117] Finally, *Halovirus* HVTV-1, which encodes the four proteins of the $PreQ_0$ biosynthesis pathway and an ArcS homolog but no DpdA, contained mainly $dPreQ_1$ (88,607 modifications per 10^6 nucleotides, ~ 30% of the Gs) but also relatively small amounts of dADG and dG^+ (152 modifications per 10^6 nucleotides, ~ 0.05 % of the Gs, and 22 modifications per 10^6 nucleotides, ~ 0.008 % of the Gs, respectively). As its host, *Haloarcula valismortis*, harbors a DpdA homolog, it is possible that the host DpdA inserts $PreQ_0$ in *Halovirus* HVTV-1 DNA before it is further modified to $dPreQ_1$ or dG^+ by the viral ArcS, that would have evolved to perform a nitrile reduction as well, or to dADG by another unidentified protein.

Example 6 - Exemplary modifications protect the phage genome from the restriction

[00118] The different modifications present in the phages analyzed above may lead to distinct resistance patterns to host defense mechanism such as RM systems. To test this hypothesis, phage DNA preparations were digested with a set of restriction enzymes that had been shown to be totally or partially inactivated in the presence of the dG^+ modification[34]. As a control, and as shown in Figure 4A, no digestion was observed with *Bam*HI, *Eco*RI, *Eco*RV, and *Swa*I while it was partially restricted with *Bst*XI, *Hae*III, *Mlu*I, *Nde*I, *Pci*I.

[00119] *Mycobacteria* phage Rosebush DNA that carries $PreQ_0$ showed a slightly different pattern of resistance. The restriction profiles for *Bam*HI, *Bst*XI and *Eco*RV were identical to those of *Enterobacteria* phage 9g. However, Rosebush DNA was fully sensitive to *Hae*III, *Mlu*I and *Pci*I and resisted to *Nde*I degradation (Figure 4B). *Eco*RI and *Swa*I could not be tested as the corresponding sites are absent in the *Mycobacterium* phage Rosebush genome.

[00120] *Discussion:*

[00121] As described herein, the presence of 7-deazaguanine modifications was directly linked with a restriction resistance phenotype.

[00122] In addition, all 7-deazaguanine modified DNA preparation tested were protected to various degrees from digestion by restriction enzymes. Transplanting the dG^+ modification in *E. coli* reproduced the resistance to cleavage by *Eco*RI (Figure 2).

[00123] Four 7-deazaguanine modifications in DNA were detected: dADG in bacteria, and dG^+, $dPreQ_1$ and $dPreQ_0$, all represented in phages. dADG was observed in phage genomes for the first time. The genes involved in the synthesis of these different modifications also were identified. FolE, QueD and QueE from *Enterobacteria* phage 9g were proven to functionally replace their *E. coli* orthologs (Figure 2A).

[00124] Most 7-deazaguanine containing phage genomes also harbor a gene coding for a DpdA homolog. As with its bacterial homolog[32], the phage DpdA introduces $PreQ_0$ in DNA (Figure 2B), most probably through a base exchange mechanisms similar to its TGT homolog[36]. DpdA2 proteins appear to share this function, as *Vibrio* phage nt-1 genome contains $dPreQ_0$. However, not all phages/viruses containing 7-deazaguanines encodes DpdA proteins, as seen with *Halovirus* HVTV-1 (Table 3 above). It is possible that in the HVTV-1 case, the host DpdA is responsible for the presence of modifications in its genome (EMA11768 in AOLQ01000002). Still, a DpdA is not always present in the host, and there could be cases where the phages encode a machinery to create modified dGTP for the DNA

polymerase to use, as proposed for *Campylobacter* phages (data not shown). Finally, one cannot rule out that some phages may harbor new families of 2'-deoxyribosyltransferase to be discovered.

[00125] The combination of comparative genomic analyses and experimental validations described herein has allowed to elucidate pathways for the insertion of $dPreQ_0$, $dPreQ_1$ and dG^+ in phage genomes (Figure 5). The presence of the minimal set of FolE, QueD, QueE QueC and DpdA proteins leads to the insertion of $dPreQ_0$, as seen in *Mycobacterium phage Rosebush* (Table 3 above). The replacement of QueC by Gat-QueC leads to the introduction of dG^+ (Figure 2B). However it is not known if Gat-QueC converts $PreQ_0$ into G^+ before or after it is inserted in DNA. The function of ArcS homologs in phages/viruses is less clear. Indeed, *Vibrio* phage nt-1 encodes an ArcS homolog and its DNA contains mostly. $dPreQ_0$ but also dG^+ and dADG (Figure 5). ArcS was the first G^+ synthase identified in archaea[19]. It is possible that some phage ArcS protein evolved to perform not only an amidotransferase reaction, like the archaeal ArcS[19], but either an nitrile reduction, like the bacterial QueF[22], or an amidohydrolase reaction, like the bacterial DpdC[32].

[00126] HHpred analysis predicted that a homolog of the archaeal QueF-L, that synthesizes G^+-tRNA from the $PreQ_0$-tRNA[49], was encoded by *Streptococcus* phage Dp-1. However, we found that this phage was modified by $dPreQ_1$. It is unclear if the reduction occurs on free $PreQ_0$, similarly to the bacterial QueF proteins[22], and then the free base $PreQ_1$ is inserted by DpdA, or if the phage QueF is able to modify the DNA-bounded $dPreQ_0$, as does the archaeal QueF-L with tRNA[49]. However, Halovirus HVTV-1 contains mainly $dPreQ_1$, but also small amounts of dADG and dG^+. It is possible that the QueF-L is on the verge of evolving from an amidohydrolase to an amidotransferase reaction, but one cannot rule out that the host ArcS could catalyze the reaction, although the specific PUA domain specific for tRNA bidding makes it highly unlikely.

[00127] Interestingly, 7-deazaguanine modifications seem to dramatically decrease the susceptibility of the phage genomes to the host restriction-modification systems (RM). These systems are one of the major defense systems for bacteria to prevent the invasion by foreign DNA[5]. Phages evolved to escape these RM systems by different methods including modification of their genomic DNA[11–14]. As demonstrated by the data provided herein, the presence of the dG^+ modification was directly linked with the restriction resistance phenotype. In addition, all 7-deazaguanine modified DNA preparations tested were protected to various degrees from digestion by restriction enzymes. It was also observed that

introducing the dG^+ modification in *E. coli* reproduced the resistance to cleavage by *Eco*RI (Figure 2).

Example 7 – *In Vivo* Modification System

[00128] The following Example describes an *in vivo* method for introducing 7-deazaguanine modifications into a heterologous nucleic acid.

[00129] Specific laboratory strains of the gram-negative bacteria *Escherichia coli* and the gram positive *Bacillus subtilis* will be engineered to encode the *dpdA* and *gat-queC* from *Enterobacteria* phage 9g and produce the respective proteins, DpdA and Gat-QueC, when voluntarily induced by the experimenter (Figure 6B). The MGE of interest can be then inserted in this strain, by transformation or conjugation for plasmids and integrons, or regular infection for phages, to be modified by dG^+, as seen in Figure 2. The MGE can then be collected by lysing the cells and will be ready to used to be introduced in the strain of interest. A system encoding only *dpdA* will also be created to obtain the $dPreQ_0$, and the necessary genes to produce dPreQ1 will be investigated to create a system inducing this modification.

[00130] The advantage of this system is that it requires only a few materials but the strain of interest has to have a compatible MGE with the modifying strain. The number of modifying strains used to produce the modification will be expanded as this technology grows to be more available to diverse species of bacteria.

Example 8 – *In Vitro* Modification System

[00131] The following Example describes an *in vitro* method for introducing 7-deazaguanine modifications into a heterologous nucleic acid.

[00132] The *Enterobacteria* phage 9g dpdA and gat-queC genes will be cloned in an expression plasmid, such as pET28. DpdA and Gat-QueC protein will be expressed in a specific strain of *E. coli*, such as BL21, and further purified to be used *in vitro* (Figure 6C). The MGE DNA will be mixed with the two purified enzymes and with the PreQ0 base and incubated to promote the modification of the MGE DNA by dG+, as seen *in vivo* in Figure 2. The MGE can be purified and introduced into the strain of interest. The use of DpdA alone will provide a MGE modified with dPreQ0, and the protein necessary for dPreQ1 will be purified to obtain this modification.

[00133] The advantage of this method is that all that is needed is the proteins and PreQ0 to modify a nucleic acid of interest, and thus it can be easily set up in form of a kit. However, this technique is not applicable to phage, unless the phage packaging system is available *in vitro*.

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[00189] 56. Kot, W., Vogensen, F. K., Sørensen, S. J. & Hansen, L. H. DPS - A rapid method for genome sequencing of DNA-containing bacteriophages directly from a single plaque. J. Virol. Methods 196, 152–156 (2014).

What is claimed is:

1. A bacterial cell comprising a heterologous nucleic acid sequence comprising one or more deazapurine bases.

2. The bacterial cell of claim 1, wherein the one or more deazapurine bases are deazaguanine bases.

3. The bacterial cell of claim 1, wherein the deazaguanine bases are 7-deazaguanine bases

4. The bacterial cell of claim 3, wherein the one or more 7-deazaguanine bases are 7-amido-7-deazaguanine (ADG), 7-formamidino-7-deazaguanosine (G^+), 7-cyano-7-deazaguanine (PreQ0) and/or 7- aminomethyl-7-deazaguanine (PreQ1).

5. The bacterial cell of claim 4, wherein the deazaguanine bases are 7-formamidino-7-deazaguanosine (G^+) or 7-cyano-7-deazaguanine ($PreQ_0$).

6. The bacterial cell of claim 1, wherein the bacterial cell is an *E. coli* bacterial cell or a *B. cereus* bacterial cell.

7 The bacterial cell of any one of claims 1-6, wherein the heterologous nucleic acid sequence is incorporated into the bacterial genome.

8. A method of protecting a heterologous nucleic acid sequence from cleavage by restriction enzymes in a host bacterium, the method comprising:

modifying the heterologous nucleic acid sequence to incorporate one or more deazaguanine bases; and

introducing the modified heterologous nucleic acid sequence into the host bacterium, thereby protecting the heterologous nucleic acid sequence from cleavage by restriction enzymes in the host bacterium.

9. The method of claim 8, wherein the modifying step comprises mixing the heterologous nucleic acid sequence with a transglycosidase, an amidotransferase and 7-cyano-7-deazaguanine ($PreQ_0$) for a time sufficient to promote modification of the heterologous nucleic acid sequence.

10. The method of claim 9, wherein the amidotransferase is Gat-QueC.

11. The method of claim 9, wherein the transglycosidase is DpdA.

12. The method of claim 8, wherein the modifying step comprises introducing the heterologous nucleic acid into a bacterial cell that has been modified to encode a transglycosidase and an amidotransferase.

13. The method of any one of claims 8-12, wherein the deazaguanine bases are 7-deazaguanine bases.

14. The method of claim 13 wherein the one or more 7-deazaguanine bases are 7-amido-7-deazaguanine (ADG), 7-formamidino-7-deazaguanosine (G^+), 7-cyano-7-deazaguanine ($PreQ_0$) and/or 7- aminomethyl-7-deazaguanine ($PreQ_1$).

15. A method of producing a bacteriophage composition, the method comprising (a) modifying a nucleic acid of bacteriophage origin to incorporate one or more deazaguanine bases; (b) introducing the modified nucleic acid into a host bacteria cell; (c) incubating the host bacteria cell until phage-mediated bacterial lysis occurs; and (d) isolating bacteriophage lysate.

FIG. 1

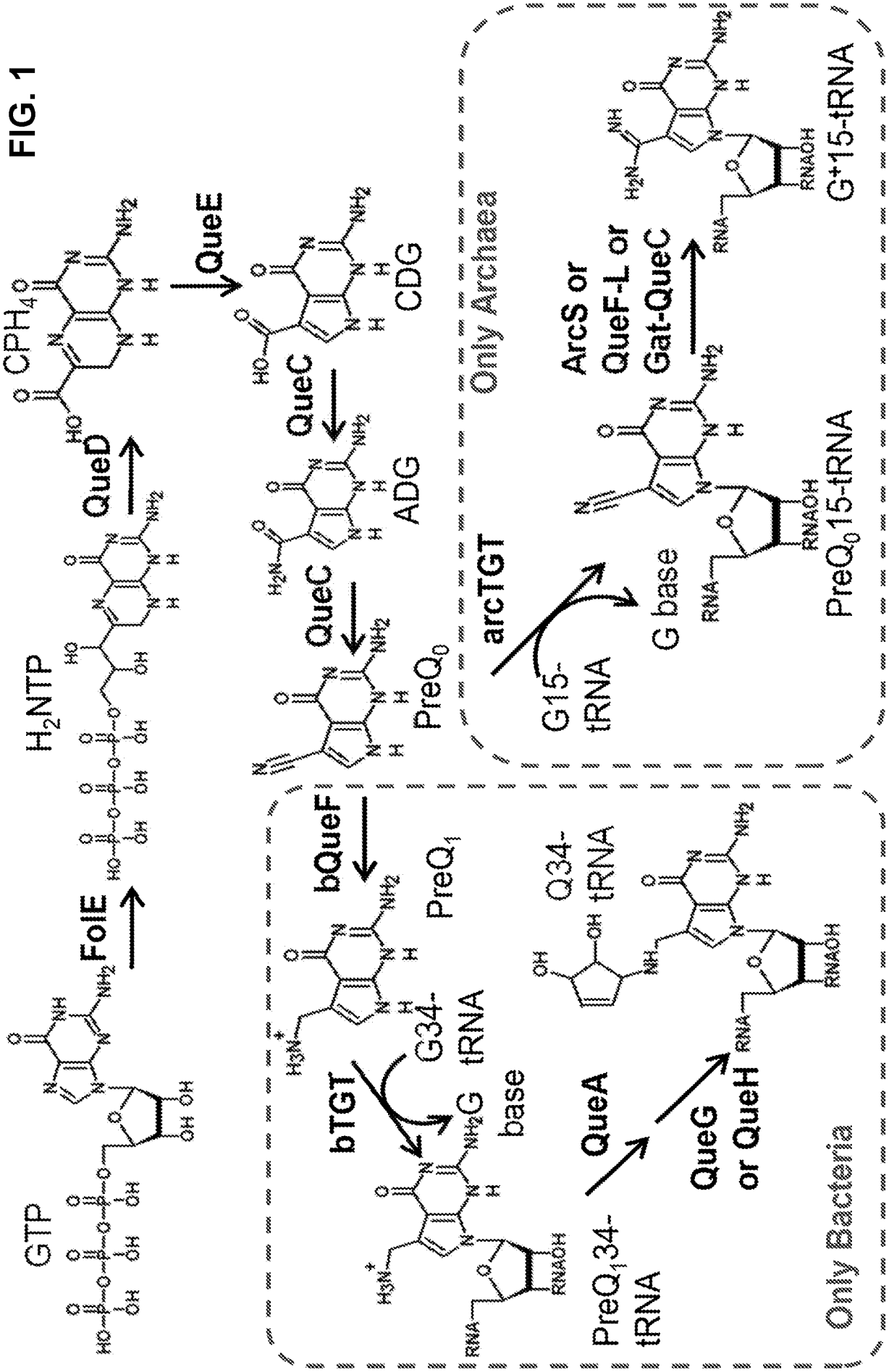

GTP
FolE
H2NTP
QueD
CPH4
QueE
CDG
QueC
ADG
QueC
PreQ0
arcTGT
G15-tRNA
G base
ArcS or QueF-L or Gat-QueC
PreQ0 15-tRNA
G+15-tRNA
Only Archaea
bQueF
PreQ1
bTGT
G34-tRNA base
Q34-tRNA
QueA
QueG or QueH
PreQ1 34-tRNA
Only Bacteria

FIG. 2

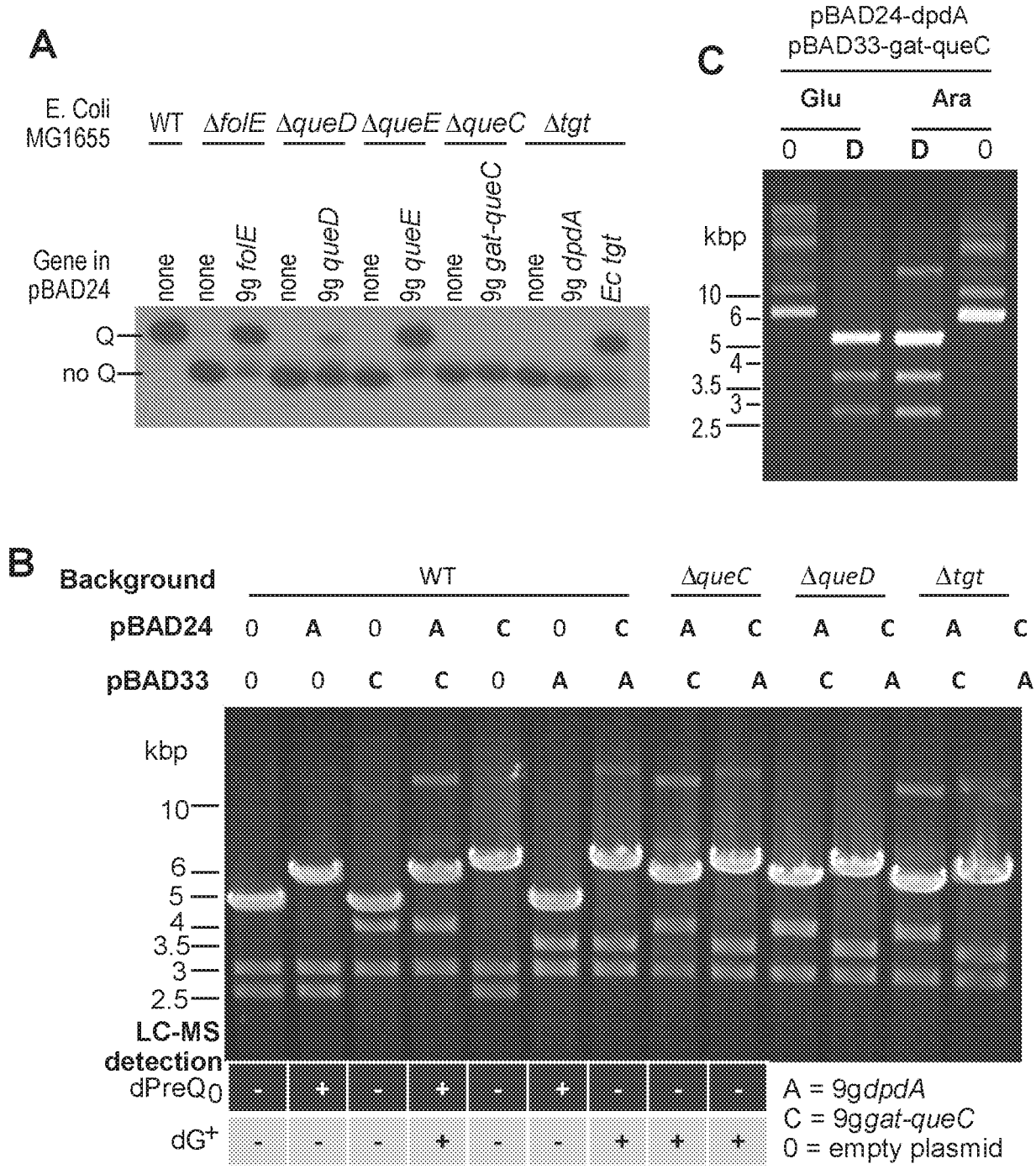

A
E. Coli MG1655
WT
ΔfolE
ΔqueD
ΔqueE
ΔqueC
Δtgt
Gene in pBAD24
none
9g folE
9g queD
9g queE
9g gat-queC
9g dpdA
Ec tgt
Q
no Q
C
pBAD24-dpdA
pBAD33-gat-queC
Glu
Ara
0 D D 0
kbp
10
6
5
4
3.5
3
2.5
B
Background
WT
ΔqueC
ΔqueD
Δtgt
pBAD24
pBAD33
LC-MS detection
dPreQ0
dG+
A = 9gdpdA
C = 9ggat-queC
0 = empty plasmid

FIG. 3

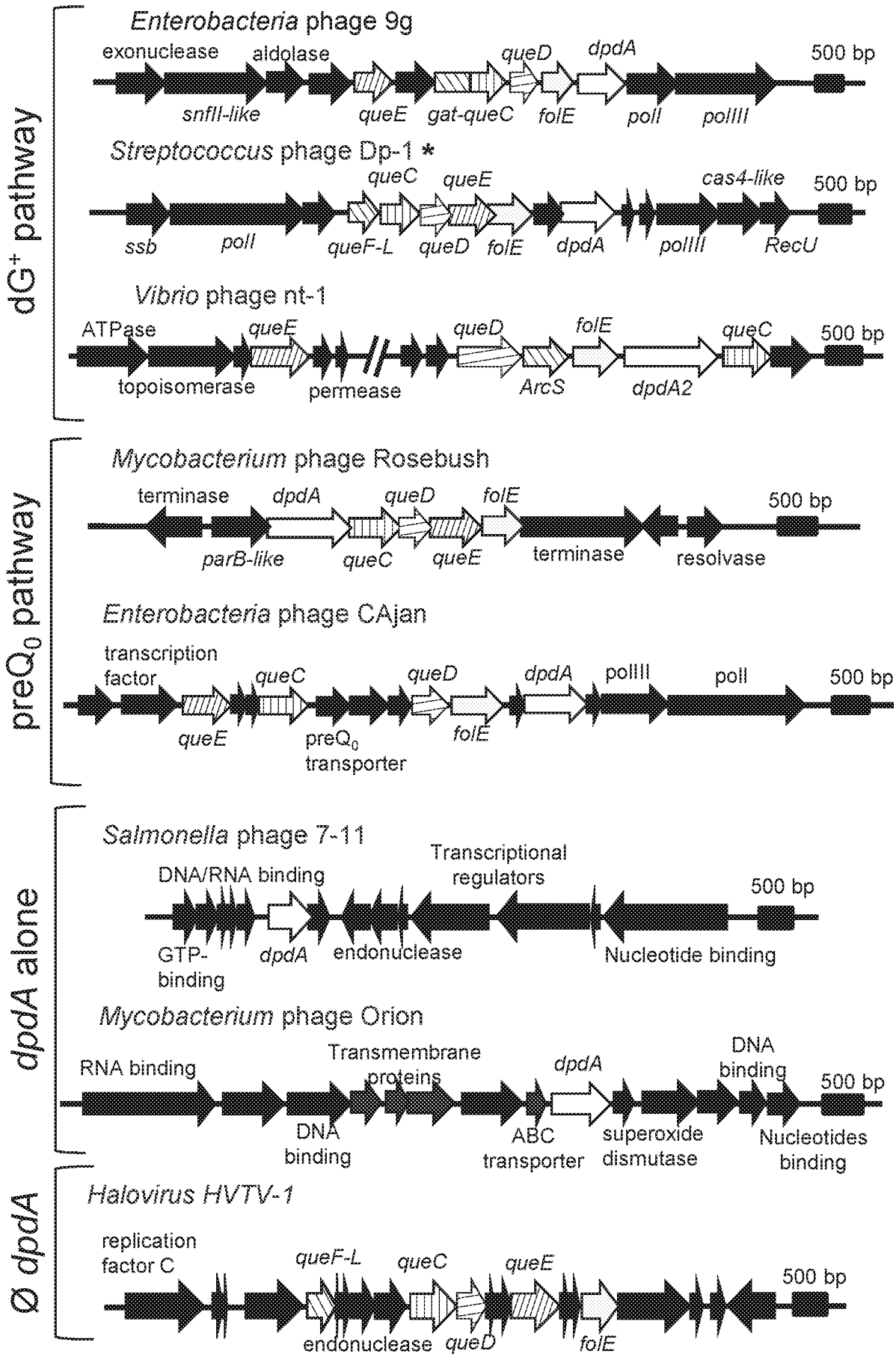
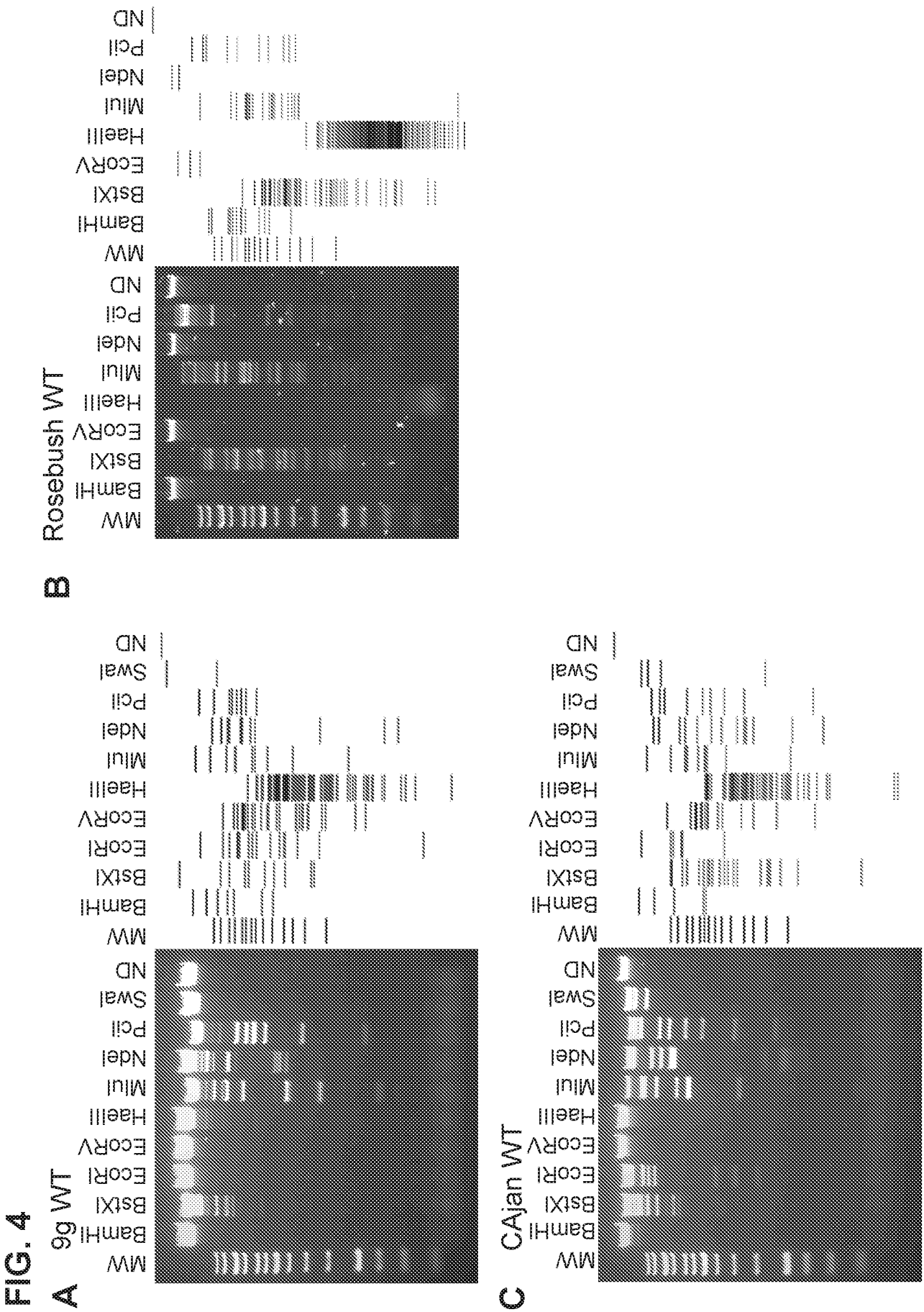

dG+ pathway
Enterobacteria phage 9g
exonuclease
aldolase
queD
dpdA
500 bp
snfII-like
queE
gat-queC
folE
polI
polIII
Streptococcus phage Dp-1 *
queC
queE
cas4-like
500 bp
ssb
polI
queF-L
queD
folE
dpdA
polIII
RecU
Vibrio phage nt-1
ATPase
queE
queD
folE
queC
500 bp
topoisomerase
permease
ArcS
dpdA2
preQ0 pathway
Mycobacterium phage Rosebush
terminase
dpdA
queD
folE
500 bp
parB-like
queC
queE
terminase
resolvase
Enterobacteria phage CAjan
transcription factor
queC
queD
dpdA
polIII
polI
500 bp
queE
preQ0 transporter
folE
dpdA alone
Salmonella phage 7-11
DNA/RNA binding
Transcriptional regulators
500 bp
GTP-binding
dpdA
endonuclease
Nucleotide binding
Mycobacterium phage Orion
RNA binding
Transmembrane proteins
dpdA
DNA binding
500 bp
DNA binding
ABC transporter
superoxide dismutase
Nucleotides binding
Ø dpdA
Halovirus HVTV-1
replication factor C
queF-L
queC
queE
500 bp
endonuclease
queD
folE

FIG. 4


A
9g WT
MW
BamHI
BstXI
EcoRI
EcoRV
HaeIII
MluI
NdeI
PciI
SwaI
ND
B
Rosebush WT
MW
BamHI
BstXI
EcoRV
HaeIII
MluI
NdeI
PciI
ND
C
CAjan WT
MW
BamHI
BstXI
EcoRI
EcoRV
HaeIII
MluI
NdeI
PciI
SwaI
ND

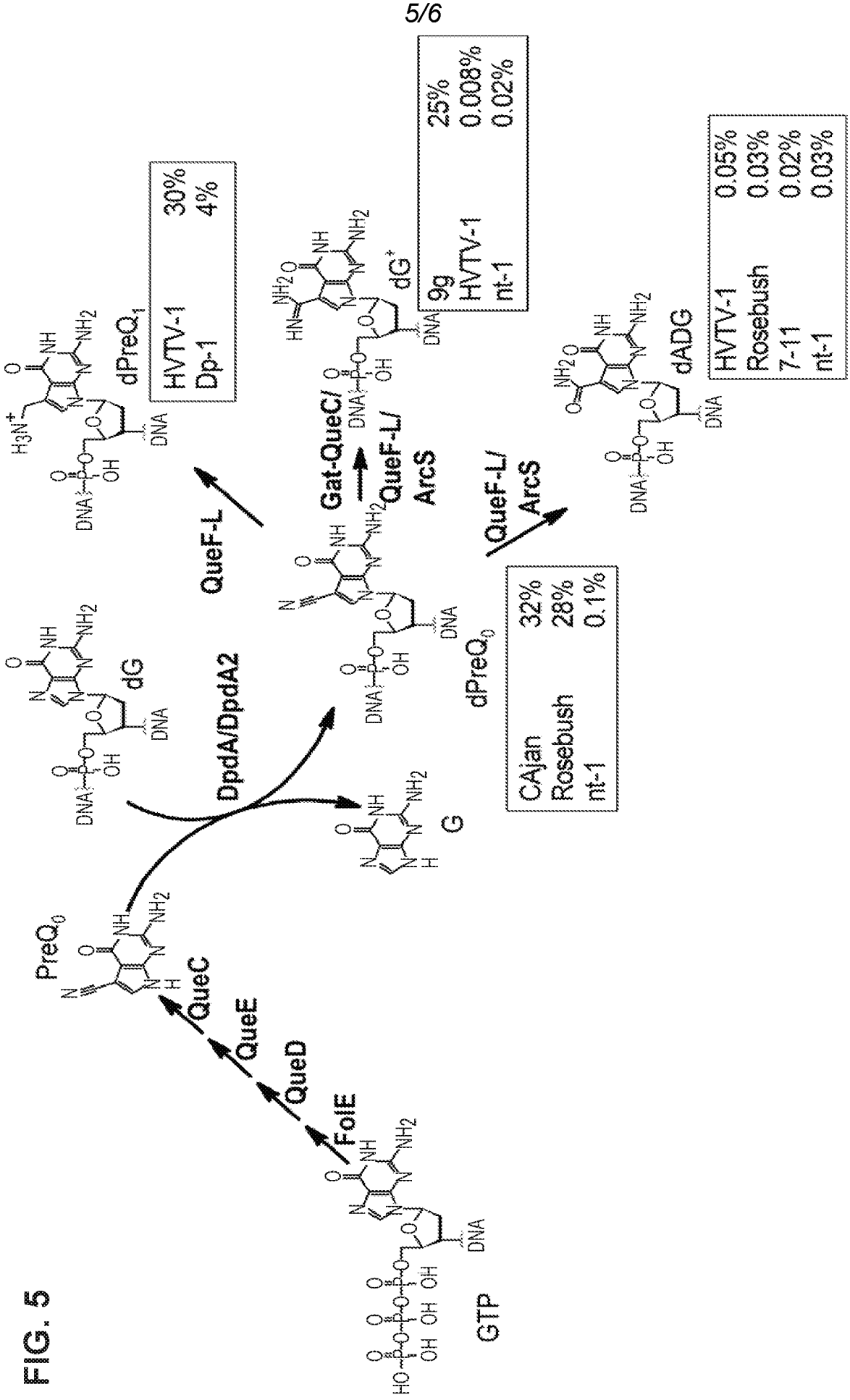
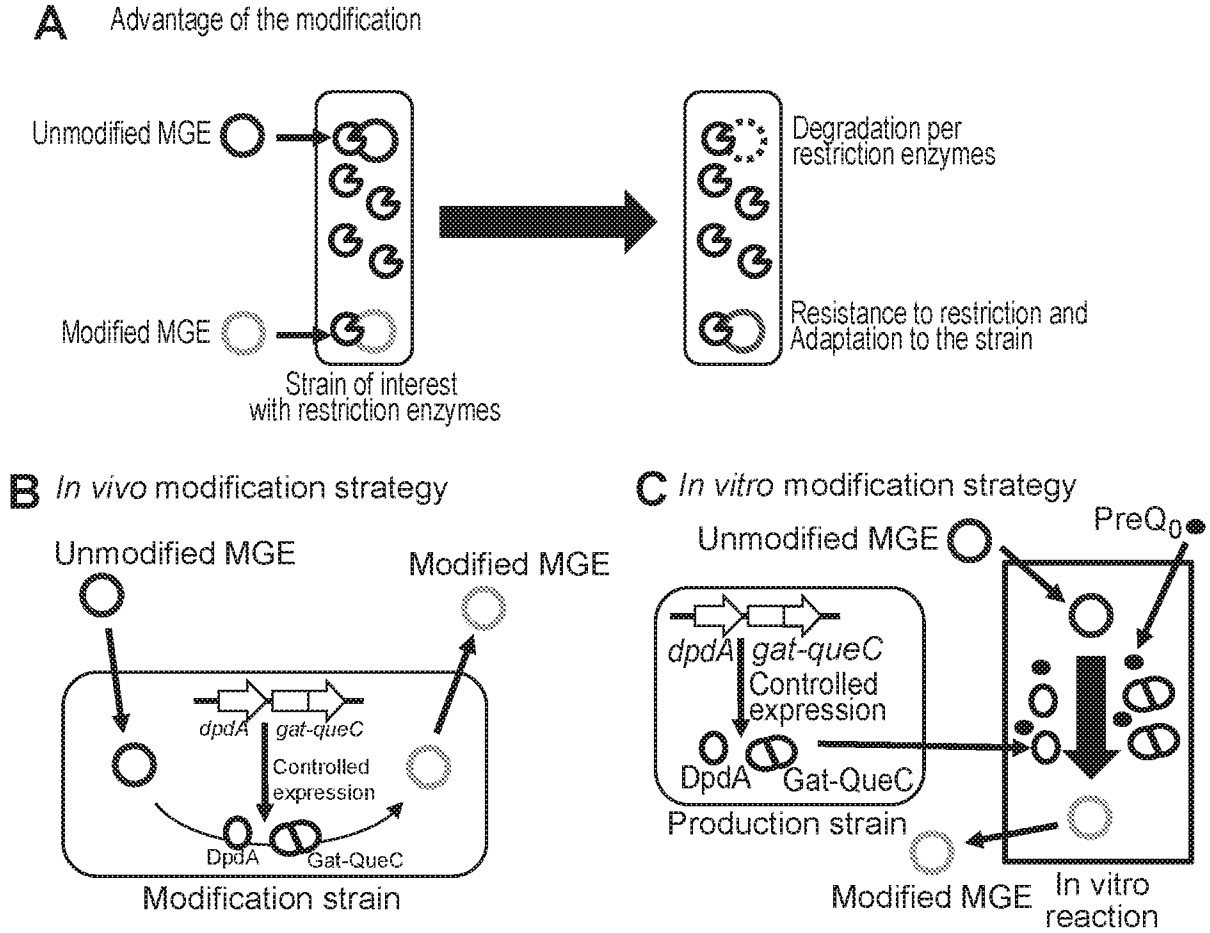

GTP
FolE
QueD
QueE
QueC
PreQ0
DpdA/DpdA2
dG
G
QueF-L
dPreQ1
HVTV-1 30%
Dp-1 4%
QueF-L/
ArcS
Gat-QueC/
dPreQ0
CAjan 32%
Rosebush 28%
nt-1 0.1%
dG+
9g 25%
HVTV-1 0.008%
nt-1 0.02%
QueF-L/
ArcS
dADG
HVTV-1 0.05%
Rosebush 0.03%
7-11 0.02%
nt-1 0.03%

FIG. 5

FIG. 6


A Advantage of the modification
Unmodified MGE
Modified MGE
Strain of interest with restriction enzymes
Degradation per restriction enzymes
Resistance to restriction and Adaptation to the strain
B In vivo modification strategy
Unmodified MGE
Modified MGE
dpdA
gat-queC
Controlled expression
DpdA
Gat-QueC
Modification strain
C In vitro modification strategy
Unmodified MGE
PreQ0
dpdA
gat-queC
Controlled expression
DpdA
Gat-QueC
Production strain
Modified MGE
In vitro reaction