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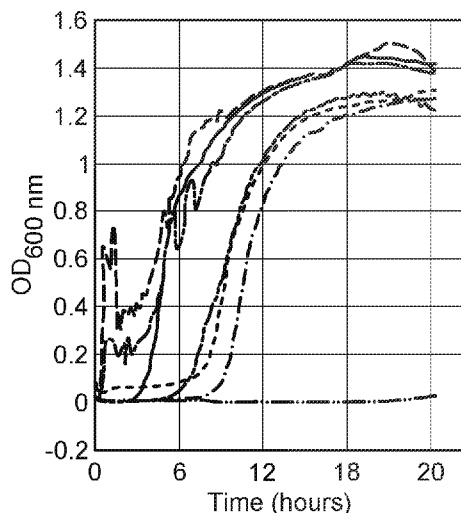


FIG. 1A

(57) Abstract: Described herein are compositions and methods of use related to oligonucleotides with modified nucleic acid backbones and conjugated to cell penetrating peptides that are complementary to 5'-GGTGGTGG-3' for the treatment of antibiotic-resistant bacterial infection.

SINGLE-STRANDED NUCLEIC ACID ANALOGS FOR USE AS MUTATION RESISTANT ANTIBACTERIAL TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 63/524,274, filed June 30, 2023, the contents of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on June 27, 2024, is named “002806-000102WOPT_SL.xml” and is 65,913 bytes in size.

TECHNICAL FIELD

[0003] The technology described herein relates to peptide-nucleic acid oligonucleotides and their use in treatment of antibiotic resistant bacteria.

BACKGROUND

[0004] Antibiotic resistance is approaching a crisis as the efficacy of existing antibiotics wanes and the discovery of new antibiotics stagnates. New antibiotics are urgently needed to counteract the increasing prevalence of infections caused by bacteria resistant to currently available antibiotics. Recent research has demonstrated bacterial inhibition using single-stranded nucleic acids and nucleic acid analogs that bind to target mRNA and inhibit the function of the essential gene encoded by that mRNA.

[0005] To be effective, the short oligonucleotides need to efficiently enter the cells and bind to the target sequence with high specificity and affinity. Furthermore, targeting genes that promote virulence or antibiotic resistance can also improve clinical outcomes. Previous work has emphasized targets near the start codon of a target gene because those regions are likely to offer single-stranded regions that are available to bind the single-stranded nucleic acid analog; however, a drawback to targeting regions near start codons is that they have diverse sequences, so multiple oligonucleotides would need to be used to target different essential genes.

SUMMARY

[0006] It was found that oligonucleotides substantially complementary to 5'-GGTGGTGG-3', comprising one or more modifications to the nucleic acid backbone and conjugated to a cell penetrating peptide, can inhibit the growth of multiple bacteria and/or render bacteria that are antibiotic resistant susceptible to antibiotic activity. Thus, in one aspect, described herein are compositions comprising an oligonucleotide, with a modified nucleic acid backbone, that is substantially complementary to 5'-GGTGGTGG-3' and is conjugated to a cell penetrating peptide. Further described herein are methods of inhibiting bacterial cell growth by contacting with such an oligonucleotide composition and/or treating bacterial infection or antibiotic resistant bacterial infection by administering such an oligonucleotide composition.

[0007] Accordingly, in one aspect, described herein is a composition comprising an oligonucleotide with a modified nucleic acid backbone wherein the oligonucleotide is conjugated to a cell penetrating peptide, and wherein the oligonucleotide is substantially complementary to 5'-GGTGGTGG-3'.

[0008] In some embodiments of any of the aspects, the modified nucleic acid backbone is a peptide-nucleic acid (PNA), a locked nucleic acid (LNA), or a bridged nucleic acid (BNA) backbone. In some embodiments, the modified nucleic acid backbone is a PNA backbone.

[0009] In some embodiments of any of the aspects, the cell penetrating peptide is selected from those listed in Table 1.

[0010] In some embodiments of any of the aspects, the cell penetrating peptide is selected from the group consisting of 5'-CCACCACC-3', 5'-CCACCAAC-3', 5'-CCACCAGC-3', 5'-CCACCATC-3', 5'-CCACCAGC-3', 5'-CCACCAGC-3', 5'-CCACCAGC-3', 5'-CCAGCGCC-3', 5'-CTAGTGGA-3', 5'-CTAGTGGA-3', 5'-CGCTGGCG-3', 5'-GCTGGTGG-3', 5'-CCACCAGCGC-3', 5'-ACCACCACCG-3', 5'-ACCACCACCC-3', 5'-ACCACC-3', 5'-ACCAGC-3'.

[0011] In some embodiments of any of the aspects, the oligonucleotide has a sequence of 6 to 10 nucleotides.

[0012] In some embodiments of any of the aspects, the oligonucleotide sequence is complementary to 5'-GGTGGTGG-3'.

[0013] In some embodiments of any of the aspects, the oligonucleotide sequence is complementary to 5'-GXTGGTGG-3', and wherein the X is A, T, or G.

[0014] In some embodiments of any of the aspects, the oligonucleotide sequence is substantially complementary to 5'-GGTGGTGG-3', and wherein one nucleotide differs from the complementary sequence.

[0015] In some embodiments of any of the aspects, the oligonucleotide sequence differs from the sequence complementarity with 5'-GGTGGTGG-3' by one nucleotide, and wherein that different nucleotide is A, T, G, or C.

[0016] In some embodiments of any of the aspects, the composition further comprises an antibiotic.

[0017] In some embodiments of any of the aspects, the antibiotic is selected from the group consisting of: nalidixic acid, ampicillin, and tetracycline.

[0018] In one aspect of any of the embodiments, described herein is a pharmaceutical composition comprising a composition as described herein. In some embodiments, the pharmaceutical composition comprises a composition as described herein and a pharmaceutically-acceptable carrier.

[0019] In one aspect of any of the embodiments, described herein is a method of inhibiting growth or replication of a bacterium, the method comprising contacting the bacterium with a composition as described herein.

[0020] In one aspect of any of the embodiments, described herein is a method of treating a bacterial infection, the method comprising administering a composition as described herein to a subject in need thereof.

[0021] In some embodiments of any of the aspects, the bacterium is an antibiotic resistant bacterium.

[0022] In some embodiments of any of the aspects, the bacterium is selected from enterobacteriaceae.

[0023] In some embodiments of any of the aspects, the bacterium is selected from the group consisting of: bacteria expressing extended-spectrum beta-lactamases (ESBLs), *Escherichia coli*, *E. coli* MG1655, *Citrobacter freundii*, and *Haemophilus influenzae*.

[0024] In some embodiments of any of the aspects, the method as described herein further comprises contacting the bacterium with an antibiotic.

[0025] In some embodiments of any of the aspects, the antibiotic is selected from quinolone antibiotics.

[0026] In some embodiments of any of the aspects, the quinolone antibiotic is selected from nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid, ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin, balofloxacin, grepafloxacin, levofloxacin, pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.

[0027] In some embodiments of any of the aspects, the antibiotic is selected from penicillins.

[0028] In some embodiments of any of the aspects, the penicillin is selected from amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.

[0029] In some embodiments of any of the aspects, the antibiotic is selected from tetracyclins.

[0030] In some embodiments of any of the aspects, the tetracyclin antibiotic is selected from tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.

Definitions

[0031] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology, and

molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of which are all incorporated by reference herein in their entireties.

[0032] As used herein, the term “modified nucleic acid backbone” refers to a nucleic acid molecule with one or more modifications to the sugar, the phosphate group, or the phosphodiester linkages that form the backbone of the nucleic acid molecule, relative to nucleic acids that generally occur in nature. As the term is used herein, a nucleic acid molecule with a modified nucleic acid backbone will retain the ability to base pair to a complementary sequence of a nucleic acid (including, but not limited to a complementary sequence of a naturally-occurring nucleic acid).

[0033] As used herein, “peptide nucleic acid” (PNA) refers to a nucleic acid mimetic (including, but not limited to an oligonucleotide mimetic), in which the sugar-backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an aminoethylglycine backbone. In a PNA, the nucleobases are retained and are bound directly or indirectly to atoms of the amide portion of the backbone.

[0034] As used herein, “locked nucleic acid” (LNA) refers to nucleic acid comprising a modified nucleotide, in which the ribose moiety of an LNA nucleotide is modified with an extra bridge (*e.g.*, a methylene bridge or an ethylene bridge) connecting the 2' hydroxyl to the 4' carbon of the same ribose sugar. Such a bridge can, for instance, “lock” the ribose in the 3'-endo North conformation.

[0035] As used herein, “bridged nucleic acid” (BNA) refers to a nucleic acid comprising a modified nucleotide, in which the nucleotide contains a 5-, 6-, or even a 7-membered bridged structure with a “fixed” C_{3'}-endo sugar pucker. The bridge is typically incorporated at the 2'-, 4'-position of the ribose to afford a 2', 4'-BNA nucleotide.

[0036] As used herein, the terms “protein” and “polypeptide” are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms “protein”, and “polypeptide” refer to a polymer of amino acids, including modified amino acids (*e.g.*, phosphorylated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. “Protein” and “polypeptide” are often used in reference to relatively large polypeptides, whereas the term “peptide” is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms “protein” and “polypeptide” are used interchangeably herein when referring to a gene product and fragments thereof.

[0037] As used herein, the term “cell penetrating peptide” (CPP), refers to relatively short peptides that facilitate cellular intake and uptake of molecules through endocytosis. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or have sequences that contain an alternating pattern of polar, charged amino acids and non-polar, hydrophobic amino acids. Non-limiting examples of CPPs are discussed herein below.

[0038] As used herein, the term “substantially complementary to” refers to the capacity for precise base pairing between two nucleotides. In some embodiments, an oligonucleotide that is substantially complementary to 5'-GGTGGTGG-3' is complementary over its full length to the 5'-GGTGGTGG-3' sequence. In some embodiments, the oligonucleotide may be at least 80% complementary to (optionally one of at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% complementary to) the consecutive nucleotides of 5'-GGTGGTGG-3'. It is understood in the art that a complementary nucleotide sequence need not be 100% complementary to that of its target to be specifically hybridizable. In some embodiments, the oligonucleotide may contain 1 or more base mismatches relative to 5'-

GGTGGTGG-3' sequence while retaining hybridization energy that permits hybridization as predicted by S-fold 2.2 under conditions specified as 37°C temperature, ionic conditions 1 M NaCl, no divalent ions; length specified as 8 or 10 nucleotides as appropriate for the specific PNA oligo in question, and prokaryotes as organism, with sequence of the target gene entered).

[0039] As used herein, the term “differs from the complementary sequence”, refers to an oligonucleotide sequence that encompasses at least a single non-complementary nucleotide mismatch relative to 5'-GGTGGTGG-3' sequence. In some embodiments a single nucleotide mismatch includes one additional nucleotide on either its 5' or 3' end. In some embodiments a single nucleotide mismatch includes one deleted nucleotide on either its 5' or 3' end. In some embodiments, a single nucleotide mismatch includes one non-complementary nucleotide at any position relative to the 5'-GGTGGTGG-3' sequence.

[0040] As used herein, the term “antibiotic resistant” refers to a bacterium that can tolerate (e.g., continue to grow and divide) the presence of a given antibiotic. A “resistant” bacterium can continue to grow and divide, whether at the same or a slower rate in the presence of an antibiotic as compared to the growth or division of the bacterium that is not in the presence of the given antibiotic.

[0041] The terms “decrease”, “reduced”, “reduction”, or “inhibit” are all used herein to mean a decrease by a statistically significant amount. In some embodiments, “reduce,” “reduction” or “decrease” or “inhibit” typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, “reduction” or “inhibition” does not encompass a complete inhibition or reduction as compared to a reference level. “Complete inhibition” is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for a subject without a disorder, e.g., a bacterial infection.

[0042] The terms “increased”, “increase”, “enhance”, or “activate” are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms “increased”, “increase”, “enhance”, or “activate” can mean an increase of at least 10% as

compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, an “increase” is a statistically significant increase in such level.

[0043] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomolgous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, “individual,” “patient” and “subject” are used interchangeably herein.

[0044] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of bacterial infections. A subject can be male or female.

[0045] A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment or one or more complications related to such a condition, and optionally, have already undergone treatment for the condition or the one or more complications related to the condition. Alternatively, a subject can also be one who has not been previously diagnosed as having the condition or one or more complications related to the condition. For example, a subject can be one who exhibits one or more risk factors for the condition or one or more complications related to the condition or a subject who does not exhibit risk factors.

[0046] As used herein, a “subject in need” of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition, or at increased risk of developing that condition.

[0047] As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorder, e.g. a bacterial infection. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with, e.g., a bacterial infection. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[0048] As used herein, the term "pharmaceutical composition" refers to an active agent in combination with a pharmaceutically acceptable carrier e.g. a carrier commonly used in the pharmaceutical industry. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0049] As used herein, the term "administering," refers to the placement of a compound as disclosed herein into a subject by a method or route which results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising the compounds disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject.

[0050] The term "effective amount" as used herein refers to the amount of a composition needed to alleviate at least one or more symptom of a disease or disorder, and relates to a sufficient amount of a pharmaceutical composition to provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of a composition that is sufficient to provide a particular effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay

the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slowing the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not generally practicable to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[0051] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[0052] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean $\pm 1\%$.

[0053] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[0054] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[0055] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0056] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin *exempli gratia* and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

BRIEF DESCRIPTION OF THE DRAWINGS

[0057] **Figs. 1A-1C** demonstrate the effect of PNA molecules as described herein on bacterial growth measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate. (Fig. 1A) Results for ESBL *E. coli* treated with 10 μ M of various peptide conjugate PNAs. The solid

line curve shows the growth for untreated cells. The other curves show results for cells treated with peptide conjugate PNAs. The long dash-short dash curve, long dash curve, long dash-double short dash curve, short dash curve, long dash-dot curve, and long dash-double dot curve correspond to the following PNAs: P-PNA_{LF}, P-PNA_{CCA}, P-PNA_{MM1A}, P-PNA_{MM1C}, P-PNA_{MM1T}, P-PPNA_{GGT}. (Fig. 1B) Same as A but for *E. coli* MG1655. (Fig. 1C) Same as B but with 40 μ M P-PNA_{LF} and 20 μ M P-PPNA_{GGT}.

[0058] Figs. 2A-2C demonstrate the effect of PNAs as described herein on cell morphology (Fig. 2A) Image of untreated cells. (Fig. 2B) Image of treated cells. (Fig. 2C) Same as B.

[0059] Figs. 3A-3B demonstrate the effect of PNA molecules as described herein measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate. (Fig. 3A) Results for *E. coli* MG1655. The solid curve shows growth for untreated cells. The short dash curve shows results for cells treated with 10 μ M P-PNA_{MM1C}. The dot curve shows results for cells treated with 10 μ M P-BNA_{MM1C} (dotted line). The dash-dot curve shows results for cells treated with 30 μ M P-BNA_{MM1C}. (Fig. 3B) Results for *E. coli* AS19 cells. The solid curve shows the growth for untreated cells. The long dash-short dash and spaced short dash curves show results for cells treated with 3 μ M P-PNA_{LF} and P-PNA_{MM1C}, respectively. The two tight short dash and dash-dot curves show results for P-BNA_{LF} and P-BNA_{MM1C}, respectively. Cells were treated with with 0.3, 1, and 3 μ M PNAs with results being similar at all concentrations. The dot curve shows results for cells treated with 0.3 μ M PNAs. The P-BNA_{LF} has no effect, but the P-BNA_{MM1C} completely inhibits growth even with 1 μ M PNA.

[0060] Fig. 4 demonstrates the optical density at 600 nm vs. time for PNA with ESBL *E. coli* cells. A. The solid curve shows the result for untreated cells (negative control). The dashed line shows the result for treating the cells with 3x MIC ampicillin.

[0061] Fig. 5 demonstrates the concentration dependence measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate. A. The solid line represents the growth for untreated *E. coli* MG1655 and the dot line, dash line, and long dash-double dot line are results for cells treated with 1, 3, and 10 μ M P-PNA_{GGT} respectively.

[0062] Figs. 6A-6B show the effect of PNA molecules complementary to sequences that are frequent in the genome measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate. (Fig. 6A) The solid line represents the growth for untreated *E. coli* MG1655 cells. Long dash-short dash curve and tight short dash curve are the results for 10 μ M P-PNA_{LF} and 10 μ M

P-PNA_{MG}, respectively. The spaced short dash and the long dash-double short dash line are the results for 10 μ M P-PNA_{MM1C} and 10 μ M P-PNA_{MM1T}, respectively. (Fig. 6B) Same as A except the tight short dash line is missing and the long dash line shows the result for 10 μ M P-PNA_{HF}.

[0063] **Figs. 7A-7C** show frequency of target sequence and ASO binding. (Fig. 7A) Distribution of target sequences in the *E.coli* MG1655 genome giving a graphical representation of the information shown in Table 1. The bars show the results for the target sequences with subscripts GGT, MM1T, MM1C, MM1A, MM2, LF, and HF, from left to right. (Fig. 7B) Histogram of the number of 8 bp repeats on either strand. The most probable number of repeats per strand is between 30 and 40 and is indicated by the black horizontal lines in A. 71 is the average number of times an 8 bp sequence is repeated on one strand. The most frequent repeat appears 778 times on one strand. Inset shows the histogram for x-axis values only up to 200. The arrows indicate the histogram bins corresponding to each of the oligonucleotide sequences on each strand. The letters above the arrow indicate the strand on which the target sequence appears. (Fig. 7C) Table of 8 bp repeats in the *E.coli* MG1655 genome. The first column is the number of repeats on each strand. Repeats targeted by oligonucleotides used in this work are shaded. The second and third columns are the sequence of the repeats on the Watson and Crick strands, respectively. The fourth column indicates the oligonucleotide name used in the brief descriptions of the drawings and in the examples. Coding is the same as A.

[0064] **Fig. 8A-8B** show *E.coli* MG1655 and RecA minus *E.coli* MG1655 with 30 μ M P-PNA_{MM1C} (long dash-double dot line) and untreated cells (solid line). The error bars show results for independent experiments of separate aliquots obtained from dilution of the same initial sample of cells to a final cell concentration of $\sim 10^4$ cells/mL.

[0065] **Figs. 9A-9E** show OD vs time measured at 600 nm for 20 μ M PNA interacting with RecBCD mutants. (Fig. 9A) Control for *E.coli* MG1655. The solid curve shows results for an untreated control. The long dash-short dash and long dash-double dot curves show results for P-PNA_{LF} and P-PNA_{MM1C}, respectively. Error bars show the rms deviations for two separately treated aliquots from the same initial cell sample. (Fig. 9B) Same as A but for V66 mutant that lacks RecF [37-40] (Fig. 9C) same as B but for V73 mutant (Fig. 9D) A second independent run of A. (Fig. 9E) Same as D but for V2831 Δ RecBCD mutant,

[0066] **Figs. 10A-10D** show microscopy of unstained and stained *E.coli* MG1655 cells. (Fig. 10A) Untreated cells and unstained. (Fig. 10B) Untreated cells incubated with NADA

during growth and with Hoescht dye after growth. (Fig. 10C) Same as A but after 3-h exposure to 10 μ M P-PNA_{GGT}. (Fig. 10D) Same as C but incubated with NADA during growth and finally stained with Hoescht.

[0067] **Figs. 11A-11B** demonstrate synergy between nalidixic acid and PNAs for *E.coli* MG1655 treated cells. (Fig. 11A) OD vs. time measured at 600 nm for 10 μ M P-PNAs. The solid curve, dash-dot curve, spaced short dash curve, long dash-short dash curve, and long dash-double short dash curves correspond to untreated controls, P-PNA_{LF}, P-PNA_{MM1C}, P-BNA_{MM1C}, P-LNA_{MM1C}, respectively. (Fig. 11B) Same as A but with 1/5x MIC nalidixic acid; cells treated with 1/5x MIC nalidixic acid are shown by the tight short dash curve. Only P-PNA_{MM1C} shows a strong synergistic interaction.

[0068] **Figs. 12A-12B** demonstrate synergy between tetracycline and PNAs for *E.coli* MG1655 treated cells. (Fig. 12A) OD vs. time measured at 600 nm for 10 μ M P-PNAs. The solid curve, long dash-short dash curve, spaced long dash curve, and short dash curves correspond to untreated controls, P-PNA_{LF}, P-PNA_{HF}, P-PNA_{MM1C}, respectively. (Fig. 12B) Same as A but with 1/5x MIC tetracycline; cells treated with 1/5x MIC tetracycline are shown by the tight long dash curve. The synergy and the sequence dependence of the synergy is smaller than with nalidixic acid.

[0069] **Figs. 13A-13B** demonstrate synergy with ampicillin. (Fig. 13A) OD vs. time measured at 600 nm for 10 μ M P-PNA treating *E.coli* MG1655 cells. The solid, long dash curve, and dash-double dot curves correspond to untreated controls, 1xMIC ampicillin, and P-PNA_{GGT}, respectively. The short dash curve shows the results with both 1x MIC ampicillin and 10 μ M P-PNA_{GGT}, respectively, and indicates substantial synergy. (Fig. 13B) Same as A but for the ESBL *E. coli*. There is no indication of synergy.

[0070] **Figs. 14A-14C** show effect of P-PNA on AS19 cells (Fig. 14A) OD vs time measured at 600 nm different PNAs concentrations. The solid curve shows results for untreated controls. Cells treated with 0.5 % SDS had results similar to the spaced short dash curve. The dotted, small dash, and long dash-short dash, represent results for 3, 10, and 30 μ M PNA, respectively. The long dash-double dash, long dash-double small dash, and small dash, represent results for 3, 10, and 30 μ M of PNA of BNA, respectively (Fig. 14B) OD vs. time measured at 600 nm different PNAs concentrations. The solid curve shows results for untreated controls. The dotted line represents results for 0.3 μ M P-BNA_{MM1C}. The dash-dot curves represent results for 3 μ M P-BNA_{MM1C}. The long dash-small dash, tight small dash, and spaced

small dash curves correspond to P-PNA_{LF}, P-PNA_{MM1C}, and P-PNA_{HF}, respectively. The long dash-double small dash corresponds to P-PNA_{MF} (Fig. 14C) OD vs. time measured at 600 nm for different PNAs concentrations. The solid curve shows results for untreated controls. The lines represent results for 3 μ M PNAs. The long dash-small dash, long dash-double dot, long dash-double small dash, tight small dash, and spaced short dash curves represent results for P-PNA_{LF}, P-PNA_{MM1C}, P-PNA_{MM1C+2}, P-PNA_{MG}, and P-PNA_{HF}, respectively.

[0071] **Fig. 15** relate to probing formation of triple helix products with FRET. (Top Left) Control for rhodamine labelled PNA added to fluorescein labeled ssDNA containing the complementary sequence. FRET signal shows a decrease when the sequence binds and FRET takes place. (Top Right) Control for buffer addition to dsDNA labeled with fluorescein (same sequence as in A) and annealed to its fully complementary sequence. (Bottom) Experiment probing triplex formation. The decrease in fluorescence observed after adding rhodamine-PNA to the fluorescein labeled dsDNA is similar to the control shown in B, suggesting no triplex formation.

[0072] **Fig. 16** demonstrates P-PNA induced inhibition for *Citrobacter freundii*. OD vs time measured at 600 nm for different PNAs concentrations. The solid curve shows results for the untreated control. The lines represent results for 10 μ M PNAs. The long dash-small dash, long dash-double dot, tight small dash, and spaced small dash represent results for P-PNA_{LF}, P-PNA_{MM1C}, P-PNA_{MG}, and P-PNA_{HF}, respectively.

[0073] **Figs. 17A-17B** demonstrate *Citrobacter freundii* morphology changes in response to treatment with P-PNA_{MM1C}. (Fig. 17A) Untreated cells. (Fig. 17B) Treated cells

[0074] **Fig. 18** shows calculated binding energies for P-PNA_{GGT} to divisome genes and genes that maintain cell shape in several Gram-negative bacteria.

[0075] **Fig. 19** shows P-PNA induced inhibition for several Gram-negative bacteria. OD vs time measured at 600 nm for different PNAs. The solid curve shows results for the untreated control. The spaced dash is a replicate for untreated control. The lines represent results for 10 μ M PNAs. The dash, long dash-double dot, long dash-dot, and long dash-double small dash curves represent results for P-PNA_{MM1C}, P-PNA_{GGT}, P-PNA_{MM1T}, and P-PNA_{MM1A} respectively.

[0076] **Fig. 20** shows the effect of 10 nucleotide PNA molecules on *E. coli* MG1655 cells measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate.

[0077] **Fig. 21** shows the effect of 10 nucleotide PNA molecules on AS19 cells measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate. Figure discloses SEQ ID NOS 15 and 16, respectively, in order of appearance.

[0078] **Fig. 22** shows synergy between nalidixic acid and PNAs for *E. coli* MG1655 treated cells measuring OD vs. time measured at 600 nm for 10 μ M P-PNAs.

[0079] **Fig. 23** shows the effect of 6 nucleotide PNA molecules on *E. coli* MG1655 cells measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate.

[0080] **Fig. 24** shows the effect of 6 nucleotide PNA molecules compared to 8 nucleotide sequences on *E. coli* MG1655 cells measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate.

[0081] **Fig. 25** shows the effect of PNA molecules on gram-positive *Staphylococcus* cells measured as OD at 600 nm vs. time for bacteria grown in a 96 well plate.

[0082] **Fig. 26** shows RT-PCR of mRNA in *E. coli*. Lane 1 is the result for a ladder. Lanes 2-5 are the results for RT_PCR with primers corresponding to mRNAs of *ftsZ*, *mreB*, *rodZ*, and *rrsH* in treated cells. Lanes 6-10 are the results for *ftsZ*, *mreB*, *rodZ*, and *rrsH* mRNAs in untreated cells.

[0083] **Fig. 27** OD vs time measured at 600 nm for 10 micromolar concentrations of 6 nt PNA GCGGGT (dash-dot) and GGTGGT (long dash-small dash), and a combination of both (small dash).

DETAILED DESCRIPTION

[0084] Approaches that target a sequence that is present in multiple bacteria essential genes would be advantageous in overcoming the development of antibiotic resistance and in treating bacterial infections. The methods and compositions described herein are based, in part, on the discovery that oligonucleotides with a modified nucleic acid backbone, conjugated to a cell penetrating peptide and that are substantially complementary to 5'-GGTGGTGG-3' are effective antibacterial agents, including against antibiotic resistant bacteria. Further aspects of the technology are described in detail herein below.

[0085] Methods of inhibiting antibiotic resistant bacteria are provided, in some embodiments, that can be carried out *in vitro*, *ex vivo*, or *in vivo*. It is understood that any reference to uses of compounds throughout the description contemplates use of the compound in preparation of a pharmaceutical composition or medicament for use in the treatment of a condition (e.g., bacterial infection). Thus, as one non-limiting example, this aspect of the disclosure includes use of such single stranded oligonucleotides in the preparation of a medicament for use in the treatment of disease.

[0086] The following includes considerations to facilitate one of skill in the art to make and perform the compositions and methods described herein.

Antisense Oligonucleotides

[0087] Embodiments of the compositions and methods described herein relate to the use of antisense oligonucleotides. Antisense technology is an effective means for modulating the expression of one or more specific gene products and is useful in a number of therapeutic, diagnostic, and research applications. Antisense oligonucleotides (also referred to herein as “ASOs”) are commonly designed such that they have sequence complementary or substantially complementary to a target sequence for which one wishes to modulate expression. Provided herein are antisense nucleic acid compounds useful for modulating bacterial gene expression via interactions with bacterial nucleic acids. Target sequence for a given ASO, or a sequence with which an ASO as described herein can interact (e.g., via hybridization or other interaction), can occur in one or more genes or gene transcripts in a given bacterium. In one aspect, antisense compounds provided herein modulate genes of divisome proteins and proteins that maintain cell shape in certain bacteria, including, but not limited to populations of antibiotic resistant bacteria.

[0088] Antisense oligonucleotides are single-stranded nucleic acid molecule that are complementary to a sequence on a nucleic acid transcript, such as that of 5'-GGTGGTGG-3' or that of other sequences within a few mismatches of 5'-GGTGGTGG-3'. Oligonucleotides are chosen that are sufficiently complementary to the 5'-GGTGGTGG-3' to give the desired effect. For example, an antisense oligonucleotide can comprise at least 6, at least 7, at least 8, at least 9, or at least 10 bases complementary to a portion of a sequence containing 5'-GGTGGTGG-3'. Some non-limiting examples of ASOs targeting 5'-GGTGGTGG-3' are provided in Table 1.

[0089] Antisense oligonucleotides can be obtained from cells or produced in vitro via enzymatic or chemical synthetic methods. However, chemically modified oligonucleotides as described herein are generally produced synthetically. The modifications can advantageously change properties including, but not limited to stability from degradation (in vivo or in vitro), solubility, ability to cross biological membranes, and stability of base paired hybridization to target sequences.

[0090] As used herein, the term “nucleic acid” or “nucleic acid sequence” refers to any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one nucleic acid strand of a denatured double-stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA or a DNA analog. In another aspect, the nucleic acid can be RNA or an RNA analog. Suitable nucleic acid molecules are DNA, including, but not limited to genomic DNA or cDNA. Other suitable nucleic acid molecules are RNA, including mRNA. Further suitable nucleic acid molecules are peptide nucleic acids (PNA), locked nucleic acids (LNA), and/or bridged nucleic acids (BNA).

[0091] As used herein, the term “nucleotide” refers to an organic molecule that serve as the monomer unit for forming the nucleic acid polymers deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleotides are the building blocks of nucleic acids and are composed of three subunit molecules: a nitrogenous base, a five-carbon sugar, and at least one phosphate group. Nucleotides can be modified. The preparation of modified nucleic acids, backbones, and nucleobases described above are well known in the art. The nucleic acids described herein may be synthesized and/or modified by methods well established in the art, such as those described in “Current protocols in nucleic acid chemistry,” Beaucage, S.L. et al. (Eds.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference.

[0092] Oligonucleotides are generally comprised of nucleotides joined by phosphodiester bonds. A nucleotide comprises a nitrogenous base (generally adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), but others, e.g., inosine, etc. are contemplated) or modified version(s) thereof, linked to a five-carbon pentose sugar (generally ribose or deoxyribose) and at least one phosphate group. A nucleotide serves as the monomer unit for forming the natural nucleic acid polymers deoxyribonucleic acid (DNA) and ribonucleic acid

(RNA). The so-called “backbone” of a nucleic acid molecule is a polymer made up of the sugar moiety of one nucleotide monomer, linked via a phosphodiester bond to the sugar moiety of a successive nucleotide.

[0093] Oligonucleotides as described herein can be DNA or RNA oligonucleotides. An RNA oligonucleotide is a polymer of nucleosides that include the sugar ribose as a component. A DNA oligonucleotide is a polymer of nucleosides that include the sugar deoxyribose as a component. Oligonucleotides with a combination of ribose and deoxyribose sugars are specifically contemplated for use in the compositions and methods described herein.

[0094] Nucleic acid modifications can include addition of chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and functionality to the individual nucleic acid bases, to the nucleic acid backbone, or to the nucleic acid as a whole. Modifications include, for example, (a) end modifications, e.g., 5' end modifications (phosphorylation, conjugation, inverted linkages, etc.) 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, e.g., replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases, (c) sugar modifications (e.g., at the 2' position or 4' position) or replacement of the sugar, as well as (d) backbone modifications, including modification or replacement of the phosphodiester linkages. Nucleic acid modifications are known in the art, see, e.g., US20160367702A1; US20190060458A1; U.S. Pat. No. 8,710,200; and US Pat No. 7,423,142, which are incorporated herein by reference in their entireties.

[0095] Modifications to the sugar-phosphate backbone include, for example, modifications to the sugar moieties that often change the flexibility or range of conformations the molecule can assume, as well as changes or replacements in the phosphate groups linking the nucleotide monomers. For example, phosphorothioate-modified oligonucleotides replace a non-bridging oxygen atom in a phosphodiester linkage with a sulfur atom. Such modifications improve nuclease stability and pharmacokinetics of ASOs comprising them. Exemplary sugar modifications include, but are not limited to, 2'-Fluoro, 3'-Fluoro, 2'-OMe, 3'-OMe, and acyclic nucleotides, e.g., peptide nucleic acids (PNA), locked nucleic acid (LNA), bridged nucleic acid (BNA), unlocked nucleic acids (UNA), or glycol nucleic acid (GNA).

[0096] Peptide nucleic acid (PNA) have been shown to have excellent hybridization properties. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to atoms of the amide portion of the backbone.

[0097] A locked nucleic acid (LNA) is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids has been shown to increase molecule stability in serum, and to reduce off-target effects (Elmen, J. et al., (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. et al., (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. et al., (2003) *Nucleic Acids Research* 31(12):3185-3193).

[0098] The term 'BNA' refers to bridged nucleic acid, and is often referred as constrained or inaccessible RNA. BNA can contain a 5-, 6- membered, or even a 7-membered bridged structure with a "fixed" C_{3'}-endo sugar puckering. The bridge is typically incorporated at the 2'-, 4'-position of the ribose to afford a 2', 4'-BNA nucleotide (e.g., LNA, or ENA).

[0099] Other useful nucleic acid derivatives incorporate nucleotides having modified carbohydrate moieties, such as 2'-O-alkylated residues or 2'-O-methyl ribosyl derivatives and 2'-O-fluoro ribosyl derivatives or 2'-O,4'-constrained 2'-ethyl nucleoside or 2'-O, 4'-ethylene nucleoside. The nucleotide bases may also be modified. Any modified base useful for inhibiting or interfering with the expression of a target sequence may be used. For example, halogenated bases, such as 5-bromouracil and 5-iodouracil can be incorporated. The bases may also be alkylated, for example, 7-methylguanosine can be incorporated in place of a guanosine residue. Non-natural bases that yield successful inhibition can also be incorporated. Such modifications are known to one skilled in the art and are described, for example, in Braasch et al., *Biochemistry*, 42: 7967-7975, 2003; Morita et al., *Bioorg Med Chem Lett*. 12(1); 2002.

[00100] Modified nucleotides can include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are

also included. Modified RNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; others having mixed N, O, S and CH₂ component parts, and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)--O--CH₂--[known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --N(CH₃)--CH₂--CH₂--[wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--].

[00101] Exemplary modified nucleobases include, but are not limited to, thymine (T), inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, and substituted or modified analogs of adenine, guanine, cytosine and uracil, such as 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, N⁶, N⁶-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N³-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-acetyl cytosine, 2-thiocytosine, N⁶-methyladenine, N⁶-isopentyladenine, 2-methylthio-N⁶-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia of Polymer Science and Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and

those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613. Further methods of generating synthetic nucleic acids are disclosed by Devine K.G., et al., Life (Basel), 2020, 10(12): 346 and Duffy, K., et al., BMC Biology, 2020, 18, 112.

[00102] As discussed herein, bacterial target gene expression can be reduced by administering antisense oligonucleotides (ASOs) complementary to one or more region(s) of a target transcript. While antisense oligonucleotides generally work by complementary base pairing with a target gene or target gene transcript, it is not necessary to know the exact target gene or transcript with which a given oligonucleotide interacts for it to be useful in inhibiting bacterial cell metabolism or growth, e.g., knocking down essential genes involved in cell division and cell wall maintenance in antibiotic resistant bacteria. In one aspect, oligonucleotides substantially complementary to 5'-GGTGGTGG-3' are provided for inhibiting growth or replication of a bacterium. In one embodiment, oligonucleotides that differ somewhat from this sequence yet retain the ability to hybridize with bacterial sequences involved in growth or metabolism can also be used. For example, oligonucleotides with base pair mismatches (e.g., 1 or two mismatches), which can be located at either terminus of the oligonucleotide or internally, but that permit hybridization to bacterial sequences involved in growth or metabolism can permit effective bacterial inhibition. In one embodiment, mismatch includes one additional nucleotide on either its 5' or 3' end relative to 5'-GGTGGTGG-3'. In another embodiment the mismatch includes a one nucleotide deletion. In some embodiments, oligonucleotides that contain mismatches but are substantially complementary to 5'-GGTGGTGG-3' retains hybridization energy predicted to permit hybridization using, e.g. S-Fold 2.2 under conditions specified above.

[00103] In some embodiments, an oligonucleotide as described herein, reduces expression of a 1 gene by hybridizing to 5'-GGTGGTGG-3'. In another embodiment, an oligonucleotide as described here in reduces the expression of 2 genes, of 3 genes, of 4 genes, of 5 genes, of 6 genes, of 7 genes, of 8 genes, of 9 genes, of 10 genes, or more by hybridizing to 5'-GGTGGTGG-3'. In another embodiment one or more oligonucleotides as described herein are used in combination to reduce the expression of 1 gene, of 2 genes, of 3 genes, of 4 genes, of 5 genes, of 6 genes, of 7 genes, of 8 genes, of 9 genes, of 10 genes, or more.

[00104] In general, antisense oligonucleotides (ASOs) described herein include 6-10 nucleotide (nt) sequences designed to bind to 5'-GGTGGTGG-3' targets. ASOs can inhibit transcription, RNA folding, or can cause cleavage by RNase in regions where the ASO binds

to target nucleic acids. ASOs can also act as a simple steric-blocker with regard to sequences they hybridize to. In some embodiments of any of the aspects, the antisense oligomer consists of from 6 to 10 nucleotides, 6 to 9 nucleotides, 6 to 8 nucleotides, 7 to 10 nucleotides, 7 to 9 nucleotides, 7 to 8 nucleotides, 8 to 10 nucleotides, or 8 to 9 nucleotides.

[00105] In some embodiments, the oligonucleotide that contains a sequence complementary to 5'GGTGGTGG-3' is 6 nucleotides long, 7 nucleotides long, 8 nucleotides long, 9 nucleotides long, 10 nucleotides long, 11 nucleotides long, 12 nucleotides long, 13 nucleotides long, 14 nucleotides long, 15 nucleotides long, 16 nucleotides long, 17 nucleotides long, 18 nucleotides long, 19 nucleotides long, 20 nucleotides long, 21 nucleotides long, 22 nucleotides long, 23 nucleotides long, 24 nucleotides long, 25 nucleotides long, 26 nucleotides long, 27 nucleotides long, 28 nucleotides long, 29 nucleotides long, 30 nucleotides long, or longer.

[00106] In other embodiments the oligonucleotide that contains a sequence complementary to 5'GGTGGTGG-3' is not longer than 30 nucleotides long, not longer than 29 nucleotides long, not longer than 28 nucleotides long, not longer than 27 nucleotides long, not longer than 26 nucleotides long, not longer than 25 nucleotides long, not longer than 24 nucleotides long, not longer than 23 nucleotides long, not longer than 22 nucleotides long, not longer than 21 nucleotides long, not longer than 20 nucleotides long, not longer than 19 nucleotides long, not longer than 18 nucleotides long, not longer than 17 nucleotides long, not longer than 16 nucleotides long, not longer than 15 nucleotides long, not longer than 14 nucleotides long, not longer than 13 nucleotides long, not longer than 12 nucleotides long, not longer than 11 nucleotides long, or not longer than 10 nucleotides long.

[00107] Whether a given antisense oligonucleotide will hybridize to a given bacterial target sequence is determined by the degree of complementarity between the oligonucleotide and the target. The degree of complementarity needed for a given nucleic acid to hybridize or form a hydrogen-bonded, base-paired duplex with another under physiological conditions depends upon the length and specific nucleotide makeup (e.g., %GC vs %AT or AU content) of the nucleic acid. A calculation of the free energy of binding of a nucleic acid with its complement or with a molecule with at least partial complementarity can provide a prediction of whether a given sequence will hybridize to another under given conditions. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). Furthermore,

calculations and/or predictions of hybridization energy can be determined using software tools or modeling known in the art, including but not limited to S-Fold, available on the world wide web at sfold.wadsworth.org; PFRED, available on the world wide web at ncbi.nlm.nih.gov/pms/articles/PMC7822268; OligoEvaluator from Sigma available on the world wide web at oligoevaluator.com/oligocalcservlet; OligoAnalyzer from IDT available on the world wide web at idtdna.com/pages/tools/oligoanalyzer; see also, e.g., Wang et al., 2022, *Plos One*. 17(5), and Tulpan et al., 2010 *BMC Bioinformatics* 105.

[00108] In some embodiments, an antisense oligonucleotide is complementary over its full length to the 5'-GGTGGTGG-3' sequence (e.g., 5'-CACCACC-3'). In other embodiments, as discussed above, antisense oligonucleotides include one or more mismatches relative to the 5'-GGTGGTGG-3' sequence but are predicted to hybridize using, e.g. S-Fold 2.2 under conditions specified above.

[00109] Table 1: Table listing sequences of ASOs (SEQ ID NOs: 2-18) targeting genes including 5'-GGTGGTGG-3'. ASO sequences with "P-" are conjugated to cell-penetrating peptides. ASOs contain a modified nucleic acid backbone wherein PNA is a peptide-nucleic acid, BNA is a bridged nucleic acid, and LNA is a locked nucleic acid.

Name of Oligo with peptide	Oligo Sequence (5' to 3')
P-PNA _{GGT}	5'-CCACCACC-3'
P-PNA _{MM1T}	5'-CCACCAAC-3'
P-PNA _{MM1C}	5'-CCACCAGC-3'
P-PNA _{MM1A}	5'-CCACCATC-3'
P-BNA _{MM1C}	5'-CCACCAGC-3'
PNA _{MM1C}	5'-CCACCAGC-3'
LNA _{MM1C}	5'-CCACCAGC-3'
P-PNA _{MM2}	5'-CCAGCGCC-3'
P-PNA _{LF}	5'-CTAGTGGA-3'
P-BNA _{LF}	5'-CTAGTGGA-3'
P-PNA _{HF}	5'-CGCTGGCG-3'
P-PNA _{Chicomp}	5'-GCTGGTGG-3'
P-PNA _{10GC}	5'-CCACCAGCGC-3' (SEQ ID NO: 14)
P-PNA _{10MrEB}	5'-ACCACCACCG-3' (SEQ ID NO: 15)
P-PNA _{10ftsZ}	5'-ACCACCACCC-3' (SEQ ID NO: 16)
P-PNA _{GGT6}	5'-ACCACC-3'
P-PNA _{MM1C6}	5'-ACCAGC-3'

[00110] In one embodiment, the ASO comprises an oligonucleotide of 6 to 10 linked nucleotides in length, which comprises a sequence at least 80% identical to a nucleotide

sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and/or SEQ ID NO: 18.

[00111] In another embodiment, the ASO comprises an oligonucleotide of 6 to 12 linked nucleotides in length, which comprises a sequence 100% identical to a nucleotide sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and/or SEQ ID NO: 18.

[00112] In some embodiments, any one or more thymidine (T) nucleotides (or modified nucleotide thereof) or uridine (U) nucleotides (or a modified nucleotide thereof) in a sequence provided herein, including a sequence provided in the sequence listing, may be replaced with any other nucleotide suitable for base pairing (e.g., via a Watson-Crick base pair) with an adenosine nucleotide. In some embodiments, any one or more thymidine (T) nucleotides (or modified nucleotide thereof) or uridine (U) nucleotides (or a modified nucleotide thereof) in a sequence provided herein, including a sequence provided in the sequence listing, may be suitably replaced with a different pyrimidine nucleotide or vice versa. In some embodiments, any one or more thymidine (T) nucleotides (or modified nucleotide thereof) in a sequence provided herein, including a sequence provided in the sequence listing, may be suitably replaced with a uridine (U) nucleotide (or a modified nucleotide thereof) or vice versa.

Cell Penetrating Peptides

[00113] ASOs as described herein are conjugated to a cell-penetrating peptide (CPP). CPPs permit transmembrane delivery of the ASOs, which generally target intracellular nucleic acids. CPPs are a class of small cationic peptides of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 15, or at least 20, or at least 25, or at least 30 amino acids that facilitate transmembrane drug delivery through various forms of endocytosis for low-molecular weight compounds, including drugs, imaging agents, oligonucleotides, peptides and proteins. CPPs are also known as ‘protein transduction domains’.

[00114] It is known that certain peptides have the ability to penetrate a lipid bilayer (e.g., cell membranes) and translocate an attached cargo across the cell membrane. This is referred to herein as "translocation activity". Without wishing to be bound by theory, the CPP internalization mechanism has been reported as a caveolae, clathrin-dependent endocytosis and macropinocytosis. Järver et al., 35 Biochem. Soc. Trans. 770-74 (2007); Richard et al., 278 J. Biol. Chem. 585-90 (2003); Ferrari et al., 8 Mol. Ther. 284-94 (2003); Holm et al., 1 Nat. Protoc. 1001-05 (2006); Lundberg & Langel, 12 Intl. J. Pept. Res. Ther. 105-14 (2006). The cellular uptake has also been reported to be independent of endocytotic pathways and to occur through transient pore formation. Vivès et al., 1786 Biochim. Biophys. Acta. 126-38 (2008); Deshayes et al., 1667 Biochim. Biophys. Acta. 141-47 (2004); Deshayes, 43 Biochem. 1449-57 (2004); El-Andaloussi et al., 8 J. Gene. Med. 1262-73 (2006); Abes et al., 35 Biochem. Soc. Trans. 53-55 (2007). Additionally, it has been suggested that CPPs may simultaneously utilize different mechanisms of endocytosis and uptake occurs by an additional rapid translocation process. Duchardt et al., 8 Traffic 848-66 (2007). CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or have sequences that contain an alternating pattern of polar, charged amino acids and non-polar, hydrophobic amino acids. Methods to synthesize such peptides are well known to one of ordinary skill in the art. Non-limiting examples of CPPs are set out in Table 2.

Table 2: Cell-penetrating peptides from literature

Cell penetrating peptides (from provided examples)			
Annotation	Sequence	SEQ ID NO:	Reference
KFF	KFFKFFKFFK	19	Popella et al 2021
RXR	RXRRXRXRXR	20	Popella et al 2021
TAT	GRKKRRQRRYK	21	Popella et al 2021
ANT	RQIKIWFQNRRMKWKK	22	Popella et al 2021
SAP	VRLPPPVRLLPPP	23	Popella et al 2021
SAPE	VELPPPVELPPPVELPPP	24	Popella et al 2021
(KFF) ₃ **	H-KFFKFFKFF-K*-NH ₂	25	Inoue et al 2021
K3	H-KKK-K*-NH ₂	26	Inoue et al 2021
K6	H-KKKKKK-K*-NH ₂	27	Inoue et al 2021
K9	H-KKKKKKKKK-K*-NH ₂	28	Inoue et al 2021
K12	H-KKKKKKKKKKKKK-K*-NH ₂	29	Inoue et al 2021
R3	H-RRR-K*-NH ₂	30	Inoue et al 2021
R6	H-RRRRRR-K*-NH ₂	31	Inoue et al 2021
R9	H-RRRRRRRRR-K*-NH ₂	32	Inoue et al 2021
R12	H-RRRRRRRRRRRRR-K*-NH ₂	33	Inoue et al 2021

ϵK_9	H- $\epsilon K \epsilon K \epsilon K \epsilon K \epsilon K \epsilon K \epsilon K \epsilon K$ -K*-NH ₂	34	Inoue et al 2021
Orn6	H-Orn ₆ -K*-NH ₂		Inoue et al 2021
Orn9	H-Orn ₉ -K*-NH ₂		Inoue et al 2021
Orn12	H-Orn ₁₂ -K*-NH ₂		Inoue et al 2021
Dab3	H-Dab ₃ -K*-NH ₂		Inoue et al 2021
Dab6	H-Dab ₆ -K*-NH ₂		Inoue et al 2021
Dab9**	H-Dab ₉ -K*-NH ₂		Inoue et al 2021
Dab12	H-Dab ₁₂ -K*-NH ₂		Inoue et al 2021
Dap3	H-Dap ₃ -K*-NH ₂		Inoue et al 2021
Dap6	H-Dap ₆ -K*-NH ₂		Inoue et al 2021
2,3-diaminopropionic acid nonamer (Dap9)	H-Dap ₉ -K*-NH ₂		Inoue et al 2021
Dap12	H-Dap ₁₂ -K*-NH ₂		Inoue et al 2021

[00115] Additional cell penetrating peptides include, for example, the homeodomain of antennapedia, a *Drosophila* transcription factor (Wang et al., (1995) PNAS USA., 92, 3318-3322); a fragment representing the hydrophobic region of the signal sequence of Kaposi fibroblast growth factor with or without NLS domain (Antopolsky et al. (1999) Bioconj. Chem., 10, 598-606); a signal peptide sequence of caiman crocodylus Ig(5) light chain (Chaloin et al. (1997) Biochem. Biophys. Res. Comm., 243, 601-608); a fusion sequence of HIV envelope glycoprotein gp4114, (Morris et al. (1997) Nucleic Acids Res., 25, 2730-2736); a transportan A -achimeric 27-mer consisting of N-terminal fragment of neuropeptide galanine and membrane interacting wasp venom peptide mastoporan (Lindgren et al., (2000), Bioconjugate Chem., 11, 619-626); a peptide derived from influenza virus hemagglutinin envelop glycoprotein (Bongartz et al., 1994, Nucleic Acids Res., 22, 468 1 4688); RGD peptide; and a peptide derived from the human immunodeficiency virus type-1 ("HIV-1"). Purified HIV-1 TAT protein is taken up from the surrounding medium by human cells growing in culture (Frankel and Pabo, (1988) Cell, 55, pp. 1189-93). TAT protein trans-activates certain HIV genes and is essential for viral replication. The full-length HIV-1 TAT protein has 86 amino acid residues. The HIV tat gene has two exons. TAT amino acids 1-72 are encoded by exon 1, and amino acids 73-86 are encoded by exon 2. The full-length TAT protein is characterized by a basic region which contains two lysines and six arginines (amino acids 47-57) and a cysteine-rich region which contains seven cysteine residues (amino acids 22-37). The basic region (i.e., amino acids 47-57) is thought to be important for nuclear localization and cell penetration (Ruben et al., J. Virol. 63: 1-8 (1989); Hauber et al., J. Virol. 63 1181-1187 (1989); Rudolph et al. (2003) 278(13):11411). The cysteine-rich region mediates the formation of metal-linked dimers in vitro (Frankel et al., Science 240: 70-73 (1988); Frankel, et al., Proc. Natl. Acad. Sci USA 85: 6297-6300 (1988)) and is essential for its activity as a

transactivator (Garcia et al., EMBO J. 7:3143 (1988); Sadaie. et al., J. Virol. 63: 1 (1989)). As in other regulatory proteins, the N-terminal region can be involved in protection against intracellular proteases (Bachmair et al., Cell 56: 1019-1032 (1989). See also, e.g., Morris, M. C. et al., Nature Biotechnol. 19:1173-1176 (2001); Dupont, A. J. and Prochiantz, A., CRC Handbook on Cell Penetrating Peptides, Langel, Editor, CRC Press, (2002); Chaloin, L. et al., Biochemistry 36(37):11179-87 (1997); and Lundberg, P. and Langel, U., J. Mol. Recognit. 16(5):227-233 (2003); all publications incorporated herein by reference.

[00116] In another embodiment the present invention, the cell penetrating peptides comprise cationic peptides with membrane translocation activity. Cationic amino acids include for example, but are not limited to, arginine, lysine, and ornithine. Active peptides with arginine rich sequences are present in the Grb2 binding protein, having the sequence RRWRRWRRWRRWRR (SEQ ID NO: 35) (Williams, E. J. et al., J. Biol. Chem. 272:22349-22354 (1997)) and polyarginine heptapeptide RRRRRRR (SEQ ID NO: 36) (7R) (Chen, L. et al., Chem. Biol. 8:1123-1129 (2001); Futaki, S. et al., J. Biol. Chem. 276:5836-5840 (2001); and Rothbard, J. B. et al., Nat. Med. 6(11):1253-7 (2000) which are incorporated herein in their entirety by reference). Also useful are branched cationic peptides capable of translocation across membranes, including by way of example and not limitation, (KKKK)₂GGC (“KKKK” disclosed as SEQ ID NO: 37), (KWKK)₂GCC (“KWKK” disclosed as SEQ ID NO: 39), and (RWRR)₂GGC (“RWRR” disclosed as SEQ ID NO: 41) (Plank, C. et al., Human Gene Ther. 10:319-332 (1999) which are incorporated herein in their entirety by reference.

[00117] Even though most CPPs have been studied and utilized in mammalian cells, several CPPs have shown similar properties when used in prokaryotes and eukaryotes. For example, the CPPs derived from the HIV Tat sequence work well in both *E. coli* and mammalian cells. Examples of CPP delivery efficiency being translatable can be found in the art, see, for example, Lee, H-M, et al., Communication Biology. 4, 205 (2021), which is incorporated herein in their entirety by reference.

[00118] Conjugation or attachment of the CPP to the ASO can be by means of linkers, chemical modification, peptide linkers, chemical linkers, covalent or non-covalent bonds, or by other means known to one skilled in the art, see, for example Klabenkova K, et. al., Chemistry of Peptide-Oligonucleotide Conjugates: A Review, *Molecules* 26(17): 5420 (2021); and Gayraud, F, et al., Recent Advances and Trends in Chemical CPP-Drug Conjugation Techniques.

Molecules 26(6): 1591 (2021), which are incorporated herein in their entirety by reference. The peptide can be conjugated to the 5' (peptide-PNA) or 3' end of the ASO (PNA-peptide). An O-linker can be added between the peptide and PNA as a spacer. PNA-conjugated oligos are commercially available from, e.g., PNA-Bio, Thousand Oaks, CA. The joining can be permanent or reversible. In some embodiments, several linkers can be included in order to take advantage of desired properties of each linker in the conjugate. Flexible linkers and linkers that increase the solubility of the conjugates are contemplated for use alone or with other linkers.

Target Bacteria and Antibiotics

[00119] The ASOs described herein target nucleic acids including sequence substantially similar to 5'-GGTGGTGG-3'. In some embodiments, the compositions and methods described herein target sequences in genes of the divisome, which code for proteins that underlie cell elongation and cell division in bacteria. ASOs that target these genes are effective against bacteria but are less likely to harm eukaryotic cells. In some embodiments, the ASOs inhibit growth of bacteria, including e.g., antibiotic resistant bacteria.

[00120] Examples of infectious bacterial organisms include, but are not limited to *Acetobacter aurantius*, *Acinetobacter baumannii*, *Actinomyces israelii*, *Agrobacterium radiobacter*, *Agrobacterium tumefaciens*, *Anaplasma phagocytophilum*, *Azorhizobium caulinodans*, *Azotobacter vinelandii*, *viridans streptococci*, *Bacillus anthracis*, *Bacillus brevis*, *Bacillus cereus*, *Bacillus fusiformis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacteroides fragilis*, *Bacteroides gingivalis*, *Bacteroides melaninogenicus*, *Prevotella melaninogenica*, *Bartonella henselae*, *Bartonella quintana*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Calymmatobacterium granulomatis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Campylobacter pylori*, *Chlamydia*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae*, *Chlamydia pneumoniae*, *Chlamydophila psittaci*, *Chlamydia psittaci*, *Citrobacter freundii*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium welchii*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Corynebacterium fusiforme*, *Coxiella burnetii*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Eikenella corrodens*, *Enterobacter cloacae*, *Enterococcus avium*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus maloratus*, *Escherichia coli*, *Fusobacterium necrophorum*,

Fusobacterium nucleatum, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Haemophilus pertussis*, *Haemophilus vaginalis*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactococcus lactis*, *Legionella pneumophila*, *Leishmania donovani*, *Leptospira interrogans*, *Leptospira noguchii*, *Listeria monocytogenes*, *Methanobacterium extroquens*, *Microbacterium multifforme*, *Micrococcus luteus*, *Moraxella catarrhalis*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium diphtheriae*, *Mycobacterium intracellulare*, *Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Mycoplasma fermentans*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Mycoplasma penetrans*, *Mycoplasma pneumoniae*, *Mycoplasma mexican*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*, *Pasteurella tularensis*, *Peptostreptococcus*, *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *Bacteroides melaninogenicus*, *Pseudomonas aeruginosa*, *Rhizobium radiobacter*, *Rickettsia prowazekii*, *Rickettsia psittaci*, *Rickettsia quintana*, *Rickettsia*, *Rickettsia trachomae*, *Rochalimaea henselae*, *Rochalimaea quintana*, *Rothia dentocariosa*, *Salmonella enteritidis*, *Salmonella typhi*, *Salmonella typhimurium*, *Serratia marcescens*, *Shigella dysenteriae*, *Spirillum volutans*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Streptococcus*, *Streptococcus agalactiae*, *Streptococcus avium*, *Streptococcus bovis*, *Streptococcus cricetus*, *Streptococcus faecium*, *Streptococcus faecalis*, *Streptococcus ferus*, *Streptococcus gallinarum*, *Streptococcus lactis*, *Streptococcus mitior*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus rattus*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Streptococcus sobrinus*, *Treponema*, *Ureaplasma urealyticum*, *Vibrio cholerae*, *Vibrio comma*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Yersinia enterocolitica*, *Yersinia pestis* and *Yersinia pseudotuberculosis*. It is contemplated that these bacteria may be targeted with ASOs as described herein, especially to the extent that they share divisome- and/or cell elongation-regulating genes with similarity to those of the species demonstrated herein to be inhibited by such ASOs.

[00121] In some embodiments of the compositions and methods described herein, the ASOs inhibit the growth of antibiotic resistant bacteria, or render such bacteria sensitive to one or more antibiotics.

[00122] Antibiotic resistance can be assessed by a skilled practitioner using anti-microbial susceptibility assays. Antimicrobial susceptibility testing is used to determine the effectiveness

of particular antimicrobials against particular microbes, whether the microbes are resistant to selected antimicrobials, and/or to identify antimicrobial resistance patterns.

[00123] In some embodiments of the compositions and methods described herein, the ASOs inhibit the growth of Enterobacteriaceae. In some embodiments, the ASOs inhibit the growth of bacteria expressing extended-spectrum beta-lactamases (ESBLs), including, but not limited to *Escherichia coli*, *E. coli* MG1655, *Citrobacter freundii*, and *Haemophilus influenzae*.

Antibiotics and Antibiotic Resistance

[00124] Bacterial strains that are resistant to multiple antibiotics are now widespread, and bacteria as a whole have developed at least one mechanism of resistance (and frequently many more) to every single antibiotic class. Despite this, there are relatively few new antibacterial agents in the pharmaceutical pipeline. Instead, the majority of antibiotics developed in the last decade are molecules re-engineered from existing antibiotic classes for which underlying resistance mechanisms are already present. Therefore, effective new therapeutic options for treatment of infections caused, particularly those caused by multi-drug resistant bacteria are urgently needed.

[00125] In some embodiments, the bacterium to be treated with the ASO described herein exhibits non-specific resistance to antibiotics, for example, by the formation of biofilms. The main mechanisms of resistance to antimicrobial agents are: limiting uptake of a drug, modification of a drug target, inactivation of a drug, and active efflux of a drug. Types of resistance to antimicrobial agents are known in the art, see, e.g., Reygaert W. C. et al., *AIMS Microbiol.* 4(3): 482-501 (2018) and Jani, S. et al., *Biomedicines*, 9, 416 (2021), which are incorporated herein by reference.

[00126] In some embodiments, the bacterium exhibits specific resistance to particular antibiotics (e.g., quinolone, penicillin, tetracycline). The specific resistance in the bacteria can be either innate or acquired. In some embodiments, the bacterium exhibits both specific and non-specific resistance to one or more antibiotics.

[00127] In some embodiments, the bacterium to be treated with an ASO as described herein is also treated with an antimicrobial agent. In some embodiments, an antimicrobial agent included in the composition can be an antibiotic. As used herein, the term "antibiotic" is art recognized and includes, as non-limiting examples, antimicrobial agents naturally produced by

microorganisms such as bacteria (including *Bacillus* species), actinomycetes (including *Streptomyces*) or fungi that inhibit growth of or destroy other microbes, whether isolated from such natural source or from genetically-engineered versions or variants thereof. Substances of similar structure and mode of action can be synthesized chemically, or natural compounds can be modified to produce semi-synthetic antibiotics. Exemplary classes of antibiotics include, but are not limited to, (1) β -lactams, including the penicillins, cephalosporins monobactams, methicillin, and carbapenems; (2) aminoglycosides, *e.g.*, gentamicin, kanamycin, neomycin, tobramycin, netilmycin, paromomycin, and amikacin; (3) tetracyclines, *e.g.*, doxycycline, minocycline, oxytetracycline, tetracycline, and demeclocycline; (4) sulfonamides (*e.g.*, mafenide, sulfacetamide, sulfadiazine and sulfasalazine) and trimethoprim; (5) quinolones, *e.g.*, ciprofloxacin, norfloxacin, and ofloxacin; (6) glycopeptides (*e.g.*, vancomycin, telavancin, teicoplanin); (7) macrolides, which include for example, erythromycin, azithromycin, and clarithromycin; (8) carbapenems (*e.g.*, ertapenem, doripenem, meropenem, and imipenem); (9) cephalosporins (*e.g.*, cefadroxil, cefepime, and ceftobiprole); (10) lincosamides (*e.g.*, clindamycin, and lincomycin); (11) monobactams (*e.g.*, aztreonam); (12) nitrofurans (*e.g.*, furazolidone, and nitrofurantoin); (13) Penicillins (*e.g.*, amoxicillin, and Penicillin G); (14) polypeptides (*e.g.*, bacitracin, colistin, and polymyxin B); and (15) other antibiotics, *e.g.*, ansamycins, polymycins, carbacephem, chloramphenicol, lipopeptide, and drugs against mycobacteria (*e.g.*, the ones causing diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*), and any combinations thereof.

[00128] In some embodiments, the antibiotic is selected from quinolone antibiotics. Non-limiting examples of quinolone antibiotics include nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid, ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin, balofloxacin, grepafloxacin, levofloxacin, pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.

[00129] In some embodiments, the antibiotic is selected from penicillins. Non-limiting examples of penicillins include amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.

[00130] In some embodiments, the antibiotic is selected from tetracyclins. Non-limiting examples of tetracyclins include tetracycline, chlortetracycline, oxytetracycline,

demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.

[00131] Additional exemplary antimicrobial agent can include, but are not limited to, antibacterial agents, antifungal agents, antiprotozoal agents, antiviral agents, and any mixtures thereof.

[00132] Exemplary antibacterial agents include, but are not limited to, Acrosoxacin, Amifloxacin, Amoxycillin, Ampicillin, Aspoxicillin, Azidocillin, Azithromycin, Aztreonam, Balofloxacin, Benzylpenicillin, Biapenem, Brodimoprim, Cefaclor, Cefadroxil, Cefatrizine, Cefcapene, Cefdinir, Cefetamet, Cefmetazole, Cefprozil, Cefroxadine, Ceftibuten, Cefuroxime, Cephalexin, Cephalonium, Cephaloridine, Cephamandole, Cephazolin, Cephradine, Chlorquinaldol, Chlortetracycline, Ciclacillin, Cinoxacin, Ciprofloxacin, Clarithromycin, Clavulanic Acid, Clindamycin, Clofazimine, Cloxacillin, Danofloxacin, Dapsone, Demeclocycline, Dicloxacillin, Difloxacin, Doxycycline, Enoxacin, Enrofloxacin, Erythromycin, Fleroxacin, Flomoxef, Flucloxacillin, Flumequine, Fosfomycin, Isoniazid, Levofloxacin, Mandelic Acid, Mecillinam, Metronidazole, Minocycline, Mupirocin, Nadifloxacin, Nalidixic Acid, Nifurtoinol, Nitrofurantoin, Nitroxoline, Norfloxacin, Ofloxacin, Oxytetracycline, Panipenem, Pefloxacin, Phenoxymethylpenicillin, Pipemidic Acid, Piromidic Acid, Pivampicillin, Pivmecillinam, Prulifloxacin, Rufloxacin, Sparfloxacin, Sulbactam, Sulfabenzamide, Sulfacytine, Sulfametopyrazine, Sulphacetamide, Sulphadiazine, Sulphadimidine, Sulphamethizole, Sulphamethoxazole, Sulphanilamide, Sulphasomidine, Sulphathiazole, Temafloxacin, Tetracycline, Tetroxoprim, Tinidazole, Tosufloxacin, Trimethoprim, and pharmaceutically acceptable salts or esters thereof.

[00133] In some embodiments, ASO compositions as described herein can be used, either alone or in combination with one or more antimicrobial agents to treat and/or prevent an infection caused by a microbe that is resistant to at least one, at least two, at least three, at least four or more antimicrobial agents known in the art or described herein. In one embodiment, the composition can be used to treat and/or prevent an infection caused by a microbe that is resistant to at least one, at least two, at least three, at least four or more antibiotics described herein. For example, in one embodiment, the composition can be used to treat and/or prevent an infection caused by methicillin-resistant *S. aureus*. In another embodiment, the composition can be used to treat and/or prevent an infection caused by *Enterobacteriaceae*.

[00134] Exemplary antimicrobial applications and/or products: The ASO compositions described herein can be formulated or configured for different applications and/or products such as antimicrobial products. In some embodiments, the ASO compositions described herein can be formulated as pharmaceutical compositions as described below, *e.g.*, for therapeutic treatment.

Formulations

[00135] In some embodiments, the oligonucleotides described herein can be administered as a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises an ASO composition as described herein (including an oligonucleotide with a modified nucleic acid backbone, conjugated to a cell penetrating peptide, wherein the oligonucleotide is substantially complementary to 5'-GGTGGTGG-3') and pharmaceutically acceptable carrier. In other embodiments, the oligonucleotide described herein is administered with an antibiotic agent. In some embodiments, the oligonucleotide and the antibiotic agent are in the same pharmaceutical composition. In other embodiments, the oligonucleotide and the antibiotic agent are administered in different compositions.

[00136] Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. Some non-limiting examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C₂-C₁₂ alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting

agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein

[00137] Pharmaceutical compositions or formulations comprising the antisense oligonucleotide of the described compositions and for use in any of the described methods can be prepared according to conventional techniques well known in the pharmaceutical industry and described in the published literature. In some embodiments, a pharmaceutical composition or formulation for treating a subject comprises an effective amount of any antisense oligomer as described herein, or a pharmaceutically acceptable salt, solvate, hydrate or ester thereof, and a pharmaceutically acceptable diluent. The antisense oligomer of a pharmaceutical formulation can further comprise a pharmaceutically acceptable excipient or carrier.

[00138] Pharmaceutically acceptable salts are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, etc., and are commensurate with a reasonable benefit/risk ratio. (See, e.g., S. M. Berge, et al., J. Pharmaceutical Sciences, 66: 1-19 (1977), incorporated herein by reference for this purpose) The salts can be prepared in situ during the final isolation and purification of the compounds, or separately by reacting the free base function with a suitable organic acid. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other documented methodologies such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic

ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate and aryl sulfonate.

[00139] In some embodiments, the compositions are formulated into any of many possible dosage forms including, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. In some embodiments, the compositions are formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension can also contain stabilizers. In some embodiments, a pharmaceutical formulation or composition applicable to the compositions and methods described herein includes, but is not limited to, a solution, emulsion, microemulsion, foam or liposome-containing formulation (e.g., cationic or noncationic liposomes).

[00140] In some embodiments, the antisense oligonucleotides applicable in the compositions and methods described herein are chemically linked to one or more moieties or conjugates, e.g., a targeting moiety or other conjugate that enhances the activity or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, a lipid moiety, e.g., as a cholesterol moiety, a cholesteryl moiety, an aliphatic chain, e.g., dodecandiol or undecyl residues, a polyamine or a polyethylene glycol chain, or adamantane acetic acid. Oligonucleotides comprising lipophilic moieties, and preparation methods have been described in the published literature. In some embodiments, the antisense oligonucleotide is conjugated with a moiety including, but not limited to, an abasic nucleotide, a polyether, a polyamine, a polyamide, a peptides, a carbohydrate, e.g., N-acetylgalactosamine (GalNAc), N—Ac-Glucosamine (GluNAc), or mannose (e.g., mannose-6-phosphate), a lipid, or a polyhydrocarbon compound. Conjugates can be linked to one or more of any nucleotides comprised by the antisense oligonucleotide at any of several positions on the sugar, base or phosphate group, as understood in the art and described in the literature, e.g., using a linker. Linkers can include a bivalent or trivalent branched linker. In some embodiments, the conjugate is attached to the 3' end of the antisense oligonucleotide. Methods of preparing oligonucleotide conjugates are described, e.g., in U.S. Pat. No. 8,450,467, "Carbohydrate conjugates as delivery agents for oligonucleotides," incorporated by reference herein.

[00141] The pharmaceutical composition or formulation for use in the methods as described herein can comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients as appropriate and well known to those of skill in the art or described in the published literature. In some embodiments, cell penetrating peptides (CPP) are conjugated to the oligonucleotides. CPP comprised by ASO compositions described herein can be conjugated, for example, to the 3' end of the oligonucleotide or to the 5' end of the oligonucleotide. In other embodiments, liposomes are conjugated to the oligonucleotides. Examples of the use of liposomes for delivery to bacteria, can be found in the art, see, for example, Ferreira M., et. al., Liposomes as Antibiotic Delivery Systems: A Promising Nanotechnological Strategy against Antimicrobial Resistance. 26(7): 2047 (2021). Liposomes also include sterically stabilized liposomes, e.g., liposomes comprising one or more specialized lipids. These specialized lipids result in liposomes with enhanced circulation lifetimes. In some embodiments, a sterically stabilized liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. In some embodiments, a surfactant is included in the pharmaceutical formulation or compositions. The use of surfactants in drug products, formulations and emulsions is well known in the art. In some embodiments, the methods and compositions described herein employ a penetration enhancer to effect the efficient delivery of the antisense oligonucleotide, e.g., to aid diffusion across cell membranes and/or enhance the permeability of a lipophilic drug. In some embodiments, the penetration enhancer is a cell penetrating peptide, surfactant, fatty acid, bile salt, chelating agent, or non-chelating nonsurfactant.

[00142] Further description of methods for producing stable polynucleotide or oligonucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are described in, e.g., WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci.*, USA 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984, which are incorporated by reference in their entirety. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, e.g., Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986, which is incorporated by

reference in its entirety). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984, which is incorporated by reference in its entirety).

[00143] Liposomes that are pH-sensitive or negatively-charged entrap nucleic acid molecules rather than complex with them. Since both the nucleic acid molecules and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid molecules are entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al., Journal of Controlled Release*, 19, (1992) 269-274, which is incorporated by reference in its entirety).

[00144] One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

[00145] Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

[00146] Cationic liposomes may also be used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used, e.g., to deliver ASOs to macrophages.

[00147] Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation

of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

[00148] A positively charged synthetic cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of ASOs (see, *e.g.*, Felgner, P. L. *et al.*, Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA, which are incorporated by reference in their entirety).

[00149] A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane (“DOTAP”) (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

[00150] Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoeoylamide (“DOGS”) (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide (“DPPE”) (see, *e.g.*, U.S. Pat. No. 5,171,678).

[00151] Another cationic lipid conjugate includes derivatization of the lipid with cholesterol (“DC-Chol”) which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., Biochim. Biophys. Res. Commun. 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, Biochim. Biophys. Acta 1065:8, 1991, which is

incorporated by reference in its entirety). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

[00152] Liposomes that include oligonucleotide and/or ASOs described herein can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transfersomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include oligonucleotide and/or ASOs described herein can be delivered, for example, subcutaneously by injection in order to deliver ASOs to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transfersomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

[00153] Other formulations amenable to the present invention are described in United States provisional application serial nos. 61/018,616, filed January 2, 2008; 61/018,611, filed January 2, 2008; 61/039,748, filed March 26, 2008; 61/047,087, filed April 22, 2008 and 61/051,528, filed May 8, 2008. PCT application no PCT/US2007/080331, filed October 3, 2007 also describes formulations that are amenable to the present invention.

[00154] Another example of a liposome is a lipid nanoparticle (LNP). As used herein, the term "LNP" refers to a stable nucleic acid-lipid particle. LNPs contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). LNPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site).

[00155] In some embodiments, the pharmaceutical composition comprises both an ASO composition as described herein (including an oligonucleotide with a modified nucleic acid

backbone, conjugated to a cell penetrating peptide, wherein the oligonucleotide is substantially complementary to 5'-GGTGGTGG-3') and an antibiotic agent. In other embodiments, the oligonucleotide and the antibiotic agent are administered in different compositions.

Dosage and Administration

Dosage

[00156] The dosage of an oligonucleotide composition as described herein can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. With respect to duration and frequency of treatment, it is typical for skilled clinicians to monitor subjects in order to determine when the treatment is providing therapeutic benefit, and to determine whether to administer further doses, discontinue treatment, resume treatment, or make other alterations to the treatment regimen. The dosage should not be so large as to cause undue adverse side effects. Generally, the dosage will vary with the age, condition, and sex of the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

[00157] Typically, the dosage ranges are between 0.1 µg/kg body weight to 1 g/kg body weight, inclusive. In some embodiments, the dosage range is from 0.1 µg/kg body weight to 0.5 g/kg body weight, from 0.1 µg/kg body weight to 0.1 g/kg body weight, from 0.1 µg/kg body weight to 50 mg/kg body weight, from 0.1 µg/kg body weight to 25 mg/kg body weight, from 0.1 µg/kg body weight to 10 mg/kg body weight, from 0.1 µg/kg body weight to 5 mg/kg body weight, from 0.1 µg/kg body weight to 1 mg/kg body weight, from 0.1 µg/kg body weight to 0.1 mg/kg body weight, from 0.1 µg/kg body weight to 0.005 mg/kg body weight, from 0.1 µg/kg body weight to 0.001 mg/kg body weight, from 0.1 µg/kg body weight to 0.5 µg/kg body weight. Alternatively, in some embodiments the dosage range is from 0.001 g/kg body weight to 1 g/kg body weight, from 0.005 g/kg body weight to 5 g/kg body weight, from 1 g/kg body weight to 1 g/kg body weight, from 0.01 g/kg body weight to 5 g/kg body weight, from 2 g/kg body weight to 1 g/kg body weight, from 0.05 g/kg body weight to 5 g/kg body weight, from 3 g/kg body weight to 1 g/kg body weight, from 0.1 g/kg body weight to 5 g/kg body weight, from 4 g/kg body weight to 1 g/kg body weight, from 0.5 g/kg body weight to 5 g/kg body weight, from 0.8 g/kg body weight to 1 g/kg body weight. In one embodiment, the dose range is from 5 µg/kg body weight to 30 µg/kg body weight. In another embodiment, the dose range is from 0.1 µg/kg body weight to 10 µg/kg body weight, from 0.1 µg/kg body weight to 10 µg/kg body weight, from 0.1 µg/kg body weight to 10 µg/kg body weight, from 0.2 µg/kg body weight

to 10µg/kg body weight, from 0.3µg/kg body weight to 10µg/kg body weight, from 0.4µg/kg body weight to 10µg/kg body weight, from 0.5µg/kg body weight to 10µg/kg body weight, from 0.6µg/kg body weight to 10µg/kg body weight, from 0.7µg/kg body weight to 10µg/kg body weight, from 0.8µg/kg body weight to 10µg/kg body weight, from 0.9µg/kg body weight to 10µg/kg body weight, from 1µg/kg body weight to 10µg/kg body weight, from 2µg/kg body weight to 10µg/kg body weight, from 3µg/kg body weight to 10µg/kg body weight, from 4µg/kg body weight to 10µg/kg body weight, from 5µg/kg body weight to 10µg/kg body weight, from 6µg/kg body weight to 10µg/kg body weight, from 7µg/kg body weight to 10µg/kg body weight, from 8µg/kg body weight to 10µg/kg body weight, or from 9µg/kg body weight to 10µg/kg body weight. Alternatively, the dose range will be titrated to maintain serum levels between 0.1µg/mL and 30µg/mL.

[00158] In some embodiments, the pharmaceutical compositions can conveniently be presented in unit dosage form. Pharmaceutical preparations can be prepared in unit dosage form according to standard procedures of pharmaceutical formulation. The quantity of active compound per unit dose can be varied according to the nature of the active compound and the intended dosage regime. A unit dosage form will typically be adapted to one or more specific routes of administration of the pharmaceutical composition. "Unit dosage form" as the term is used herein refers to a dosage suitable for one administration. By way of example, a unit dosage form can be an amount of therapeutic disposed in a delivery device, e.g., a syringe or intravenous drip bag. In one embodiment, a unit dosage form is administered in a single administration. In another embodiment more than one unit dosage form can be administered simultaneously.

[00159] In some embodiments, the unit dosage form is adapted for administration by inhalation. In some embodiments, the unit dosage form is adapted for administration by a vaporizer. In some embodiments, the unit dosage form is adapted for administration by a nebulizer. In some embodiments, the unit dosage form is adapted for administration by an aerosolizer. In some embodiments, the unit dosage form is adapted for oral administration, for buccal administration, or for sublingual administration. In some embodiments, the unit dosage form is adapted for intravenous, intramuscular, or subcutaneous administration. In some embodiments, the unit dosage form is adapted for intrathecal or intracerebroventricular administration. In some embodiments, the pharmaceutical composition is formulated for topical administration. The amount of active ingredient which can be combined with a carrier

material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

[00160] Liquid dosage forms include solutions, suspensions and emulsions. Liquid form preparations may be administered by intravenous, intracerebral, intraperitoneal, parenteral or intramuscular injection or infusion. Sterile injectable formulations may comprise a sterile solution or suspension of the active agent in a non-toxic, pharmaceutically acceptable diluent or solvent. Suitable diluents and solvents include sterile water, Ringer's solution and isotonic sodium chloride solution, etc. Liquid dosage forms also include solutions or sprays for intranasal administration.

Administration

[00161] In some aspects, the methods described herein for treating a subject comprise administering an ASO. The agents described herein can be administered to a subject in need thereof by any appropriate route which results in an effective treatment in the subject. One aspect of the technology described herein includes a method of treating a bacterial infection, the method comprising administering to a subject in need thereof, a therapeutically effective amount of an antisense oligonucleotide (ASO) with substantial complementarity to 5'-GGTGGTGG-3', thereby treating the bacterial infection.

[00162] The pharmaceutical composition is preferably administered in an amount effective to modulate the expression of a targeted bacterial gene or genes by at least 1%, by at least 3%, by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 30%, by at least 40%, by at least 50%, by at least 60%, by at least 70%, by at least 80%, by at least 90% or by 100%. It is preferred that the amount administered is an amount effective to maximize the modulation of the expression of the targeted gene or genes while minimizing toxicity.

[00163] Administration of an agent described herein (e.g., an ASO) can be performed in a variety of manners, for example, in a single dose, in reoccurring multiple doses, via continuous infusion, or via pulsed administration. In one embodiment, an agent described herein can be administered to a subject at least once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours; or every 1, 2, 3, 4, 5, 6, or 7 days; or every 1, 2, 3, or 4 weeks; or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months, or more. It is specifically contemplated herein that the dosing of an agent described herein is determined based on the half-life of the agent, e.g., such that the effect of the agent described herein is maintained at a

level that provides continuous, or nearly continuous, effect in the subject until a bacterial infection is controlled or eliminated.

[00164] In one embodiment, an agent as described herein is administered at least once. In one embodiment, an agent as described herein is administered at least twice. For example, an agent as described herein can be administered at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more times.

[00165] In some embodiments, the methods described herein comprise administering an effective amount of an agent as described herein to a subject in order to alleviate at least one symptom of a given disease. As used herein, "alleviating at least one symptom of a given disease" refers to ameliorating a condition or symptom associated with that disease. As compared with an equivalent untreated control, such reduction or amelioration is by at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, 99% or more as measured by any standard technique. A variety of means for administering an agent as described herein to subjects are known to those of skill in the art. In one embodiment, the agent is administered systemically or locally (e.g., to an affected organ). In one embodiment, the agent is administered intravenously. In one embodiment, the agent is administered continuously, in intervals, or sporadically. The route of administration of the agent will be optimized for the type of agent being delivered (e.g., an ASO composition as described herein), and can be determined by a skilled practitioner.

[00166] In one embodiment, the agent is administered continuously (e.g., at constant levels over a period of time). Continuous administration of an agent can be achieved, e.g., by epidermal patches, continuous release formulations, or on-body injectors.

[00167] Effective amounts, toxicity, and therapeutic efficacy can be evaluated by standard pharmaceutical procedures in cell cultures or experimental animals. The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index, and can be expressed as the ratio LD₅₀/ED₅₀. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (*i.e.*, the concentration of the agent, which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography.

The effects of any particular dosage can be monitored by a suitable bioassay, e.g., measuring bacterial load or metabolite(s), or blood work, among others. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

[00168] Exemplary modes of administration of the ASOs disclosed herein or pharmaceutical compositions comprising them include oral, rectal, transmucosal, intranasal, inhalation (e.g., via an aerosol), buccal (e.g., sublingual), vaginal, intrathecal, intraocular, transdermal, intraendothelial, in utero (or in ovo), parenteral (e.g., intravenous, subcutaneous, intradermal, intracranial, intramuscular (including administration to skeletal, diaphragm and/or cardiac muscle), intrapleural, intracerebral, and intraarticular), topical (e.g., to both skin and mucosal surfaces, including airway surfaces, and transdermal administration), intralymphatic, and the like, as well as direct tissue or organ injection (e.g., to liver, eye, skeletal muscle, cardiac muscle, diaphragm muscle or brain), e.g., to directly access a site of infection.

[00169] Aerosol preparations suitable for inhalation can include solutions and solids in powder form, which can be combined with a pharmaceutically acceptable carrier, such as an inert compressed gas. Use of a long-term sustained release implant can be particularly suitable for treatment of chronic infection. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 5 days, for at least 10 days, for at least 15 days, for at least 20 days, for at least 30 days, for at least 40 days, for at least 50 days or for at least 60 days. Long-term sustained release implants and their design and preparation are known to those of ordinary skill in the art.

[00170] Advantageously, the administration of an ASO can be coupled with other treatment methodologies. The ASO-containing agents described herein can also be used in combination in order to achieve the desired therapeutic effect. Certain combinations of agents can act cooperatively, additively or synergistically, when co-administered or when administered sequentially. The antisense treatment can be applied before, after, or in combination with other treatments.

[00171] Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid or limit the frequency of repeated administrations of the therapeutic agents described herein, increasing convenience to the subject and the physician. Many types of delayed or extended release delivery systems are available and known to those of ordinary skill in the art. They include, for example, polymer based systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones,

polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of such polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the therapeutic agent(s) of described herein are contained in a form within a matrix such as those described in U.S. Pat. Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

[00172] The most suitable route in any given case will depend on the nature and severity of the condition being treated, ameliorated, and/or prevented and on the nature of the particular ASO or ASO formulation being used. Additionally, ASOs permit one to administer more than one ASO e.g. multiple ASOs (e.g. an ASO cocktail).

Efficacy

[00173] The efficacy of a composition in, e.g. the treatment of a condition described herein, or to induce a response as described herein can be determined by the skilled clinician. However, a treatment is considered "effective treatment," as the term is used herein, if one or more of the signs or symptoms of a condition is altered in a beneficial manner, other clinically accepted symptoms are improved or ameliorated, or a desired response is induced e.g., by at least 10% following treatment according to the methods described herein. Efficacy can be assessed, for example, by measuring a marker, indicator, symptom, and/or the incidence of a condition treated according to the methods described herein or any other measurable parameter appropriate. Efficacy can also be measured by a failure of an individual to worsen as assessed by hospitalization, or need for medical interventions (i.e., progression of the infection is halted). Methods of measuring these indicators are known to those of skill in the art and/or are described herein. Treatment includes any treatment of a bacterial infection in an individual or an animal (some non-limiting examples include a human or an animal) and includes: (1) inhibiting the infection, e.g., preventing a worsening of symptoms (e.g. pain, local redness, fever, or fatigue); or (2) relieving the severity of the infection, e.g., causing regression of

symptoms. An effective amount for the treatment of an infection means that amount which, when administered to a subject in need thereof, is sufficient to result in effective treatment as that term is defined herein, for that infection. Efficacy of an agent can be determined by assessing physical indicators of a condition or desired response. It is well within the ability of one skilled in the art to monitor efficacy of administration and/or treatment by measuring any one of such parameters, or any combination of parameters. Efficacy can be assessed in animal models of a condition described herein. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change in a marker is observed.

[00174] In some embodiments, evaluation of efficacy can comprise monitoring a change in the bacterial load in a sample after administration of an ASO-containing formulation as described herein. In some embodiments, the total amount of the bacterial load in the subject contacted with the ASO is decreased about 1.1 to about 10-fold, about 1.5 to about 10-fold, about 2 to about 10-fold, about 3 to about 10-fold, about 4 to about 10-fold, about 1.1 to about 5-fold, about 1.1 to about 6-fold, about 1.1 to about 7-fold, about 1.1 to about 8-fold, about 1.1 to about 9-fold, about 2 to about 5-fold, about 2 to about 6-fold, about 2 to about 7-fold, about 2 to about 8-fold, about 2 to about 9-fold, about 3 to about 6-fold, about 3 to about 7-fold, about 3 to about 8-fold, about 3 to about 9-fold, about 4 to about 7-fold, about 4 to about 8-fold, about 4 to about 9-fold, at least about 1.1-fold, at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 3.5-fold, at least about 4-fold, at least about 5-fold, or at least about 10-fold, relative to the total amount of the bacterial load produced in a control subject.

[00175] In some embodiments, evaluation of efficacy can comprise monitoring susceptibility of the target bacterium to one or more antibiotics. In vitro and animal model assays permit the assessment of a given dose of a composition and the susceptibility to additional antibiotics.

[00176] In some embodiments, the ASOs can be used in both veterinary and medical applications.

[00177] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely

for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00178] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00179] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A composition comprising an oligonucleotide with a modified nucleic acid backbone wherein the oligonucleotide is conjugated to a cell penetrating peptide, and wherein the oligonucleotide is substantially complementary to 5'-GGTGGTGG-3'.
2. The composition of paragraph 1, wherein the modified nucleic acid backbone is a peptide-nucleic acid (PNA), a locked nucleic acid (LNA), or a bridged nucleic acid (BNA) backbone.
3. The composition of paragraph 1 or 2, wherein the modified nucleic acid backbone is a PNA backbone.
4. The composition of any of paragraphs 1-3, wherein the cell penetrating peptide is selected from those listed in Table 1.

5. The composition of any of paragraphs 1-4, wherein the oligonucleotide has a sequence of 6 to 10 nucleotides.
6. The composition of any of paragraphs 1-5, wherein the oligonucleotide sequence is complementary to 5'-GGTGGTGG-3'.
7. The composition of any of paragraphs 1-5, wherein the oligonucleotide sequence is complementary to 5'-GXTGGTGG-3', and wherein the X is A, T, or G.
8. The composition of any of paragraphs 1-5, wherein the oligonucleotide sequence is substantially complementary to 5'-GGTGGTGG-3', and wherein one nucleotide differs from the complementary sequence.
9. The composition of any of paragraphs 1-5, wherein the oligonucleotide sequence differs from the sequence complementarity with 5'-GGTGGTGG-3' by one nucleotide, and wherein that different nucleotide is A, T, G, or C.
10. The composition of any one of paragraphs 1-9, further comprising an antibiotic.
11. The composition of paragraph 10, wherein the antibiotic is selected from the group consisting of: nalidixic acid, ampicillin, and tetracycline.
12. A pharmaceutical formulation comprising a composition of any one of paragraphs 1-11 and a pharmaceutically-acceptable carrier.
13. A method of inhibiting growth or replication of a bacterium, the method comprising contacting the bacterium with a composition of any one of paragraphs 1-12.
14. The method of paragraph 13, wherein the bacterium is an antibiotic resistant bacterium.
15. The method of paragraph 13 or 14, wherein the bacterium is selected from enterobacteriaceae.
16. The method of paragraph 13 or 14, wherein the bacterium is selected from the group consisting of: bacteria expressing extended-spectrum beta-lactamases (ESBLs), *Escherichia coli*, *E. coli* MG1655, *Citrobacter freundii*, and *Haemophilus influenzae*.
17. The method of paragraph 13 or paragraph 14, further comprising contacting the bacterium with an antibiotic.
18. The method of paragraph 17, wherein the antibiotic is selected from quinolone antibiotics.
19. The method of paragraph 18, wherein the quinolone antibiotic is selected from nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid, ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin balofloxacin, grepafloxacin, levofloxacin,

- pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.
20. The method of paragraph 17, wherein the antibiotic is selected from penicillins.
 21. The method of paragraph 20, wherein the penicillin is selected from amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.
 22. The method of paragraph 17, wherein the antibiotic is selected from tetracyclins.
 23. The method of paragraph 22, wherein the tetracyclin antibiotic is selected from tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.
 24. A method of treating a bacterial infection, the method comprising administering a composition of any of paragraphs 1-12 to a subject in need thereof.
 25. The method of paragraph 20, wherein the infection is an infection with an antibiotic resistant bacterium.
 26. The method of paragraph 24 or 25, wherein the bacterium is selected from enterobacteriaceae.
 27. The method of any one of paragraphs 24-26, further comprising administering an antibiotic.
 28. The method of paragraph 27, wherein the antibiotic is selected from quinolone antibiotics.
 29. The method of paragraph 28, wherein the quinolone antibiotic is selected from nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid, ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin balofloxacin, grepafloxacin, levofloxacin, pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.
 30. The method of paragraph 27, wherein the antibiotic is selected from penicillins antibiotics.
 31. The method of paragraph 30, wherein the quinolone antibiotic is selected from amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.
 32. The method of paragraph 27, wherein the antibiotic is selected from tetracyclins.

33. The method of paragraph 32, wherein the tetracyclin is selected from tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.

[00180] The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

EXAMPLES

EXAMPLE 1: Adding single-stranded nucleic acids analogs complementary to sequences that appear in several genes can offer a robust mutation resistant antibacterial strategy

[00181] **Abstract:** New antibiotics are urgently needed to counteract the increasing prevalence of infections caused by bacteria resistant to currently available antibiotics. Recent research has demonstrated bacterial inhibition using single-stranded nucleic acids and nucleic acid analogs that bind to a target mRNA and inhibit the function of the essential gene encoded by that mRNA. Here, it is demonstrated that a peptide-nucleic acid complementary to GGTGGTGG (P-PNA_{GGT}) is an effective antibacterial agent against bacteria carrying extended-spectrum beta-lactamases (ESBLs) antibiotic resistance genes. P-PNA_{GGT} also inhibits growth of *E. coli* MG1655, *Citrobacter freundii*, and *Haemophilus influenzae*, but not *Klebsiella pneumoniae*. Cells treated with P-PNA_{GGT} do not divide appropriately and have irregular shapes. Furthermore, the octamer GGTGGTGG appears in several divisome genes as well as genes responsible for shape maintenance. Peptide-nucleic acid analogs that target sequences that differ from GGTGGTGG by only one or two bases show some cell distortion and growth inhibition, but less than that shown by P-PNA_{GGT}; however, other peptide-bound sequences show little or no inhibition in both *E.coli* and *C.freundii*, even though their target sequences are more frequent in genomes and occur in more essential genes than GGTGGTGG.

Introduction

[00182] Antibiotic resistance is approaching a crisis as the efficacy of existing antibiotics wanes and the discovery of new antibiotics stagnates. Short oligonucleotides could offer high efficacy in antibacterial treatments with higher specificity, lower toxicity, and fewer secondary

effects than many other approaches. [1] Short oligonucleotides can target complementary RNA [2-10] or DNA sequences via complementary Watson-Crick pairing [11]. Such pairing can disrupt transcription and translation and thus essential cellular processes as diverse as fatty acid synthesis and cell division [12]. To be effective, the short oligonucleotides need to efficiently enter the cells and bind to the target sequence with high specificity and affinity. Antisense oligonucleotides (ASOs) target mRNAs or rRNAs to inhibit translation of the corresponding gene through steric blockage [2-10] or degradation of the resulting product [13-15]. Targeting genes that promote virulence or antibiotic resistance can also improve clinical outcomes [13].

[00183] A variety of DNA analogs can bind to single-stranded nucleic acid targets in cells. DNA in water has a negatively charged backbone that inhibits uptake of single-stranded DNA (ssDNA) into cells, but some DNA analogs have uncharged backbones. For example, peptide-like nucleic acids (PNA) have uncharged backbones, which allows them to enter cells more readily than ssDNA. In addition, DNA analogs can bind peptides that promote transport of oligonucleotides through cell membranes [16]. Furthermore, DNA analogs such as LNA (locked nucleic acids) and the related analog BNA (bridged nucleic acids) can provide higher binding affinity than ssDNA.

[00184] For a single-stranded DNA oligonucleotide or DNA analog to be an effective antibacterial agent the following properties are essential: 1. The single-stranded analog must enter the cell. 2. The single-stranded analog must bind to a target or targets in the cell with a binding affinity that is high enough to impede target function. To be effective at a reasonable dosing level the following properties are useful: 1. The target or targets in the cell should have a significant probability of being unpaired so they are readily available to pair with the oligonucleotide. 2. the single-stranded analog be fairly stable against degradation by DNA nucleases 3. the probability of stably binding to sequences other than the target or targets should be much lower than the probability of binding stably to the target or targets.

[00185] Previous work has emphasized targets near the start codon because those regions are likely to offer single-stranded regions that are available to bind the single-stranded nucleic acid analog [2, 4, 17-22]; however, other regions of the target mRNA may also offer single-stranded targets [14, 23-25]. Furthermore, a drawback to targeting regions near start codons is that they have diverse sequences, so it is difficult for a single oligonucleotide to target the same start codon region in different essential genes; however, it may be desirable to target multiple

genes because the development of antibiotic resistance is more difficult if there are multiple different mRNA targets in the cell that can bind to the same DNA analog.

[00186] The sequence GGTGGTGG appears in genes of divisome proteins and proteins that maintain cell shape. Proteins that underlie cell elongation and cell division have long been antibacterial targets [18, 20, 26-30] since they are effective against bacteria but less likely to harm eukaryotic cells. Thus, as described herein, GGTGGTGGT is a target of antisense oligos. Also studied is a P-PNA that targets the Chi sequence, GCTGGTGG in *E. coli* because the Chi sequence is highly overrepresented in the genome and plays vital roles in DNA repair. Targeting the Chi sequence does inhibit growth, but less than the P-PNA that targets the sequence GGTGGTGG.

[00187] Thus, demonstrated herein P-PNA_{GGT}, a peptide-PNA conjugate that targets the GGTGGTGG sequence in *E. coli* is an effective antibacterial agent against ESBL *E. coli*. P-PNA_{GGT} also inhibits growth of *E. coli* MG1655, but less effectively than in ESBL *E. coli*. In *E. coli* MG1655, P-PNA_{GGT} shows synergistic effects in combination with nalidixic acid and less synergy with tetracycline. Importantly, the morphological defects in the treated cells are consistent with a reduction of function in *ftsZ*, *mreB*, and *rodZ*, and RT-PCR suggests that mRNA levels in cells are altered when cells are treated with P-PNA_{GGT}. In contrast, peptide-PNA conjugates targeting other sequences inhibited growth less, and without a bound peptide no inhibition was observed regardless of the sequence of the PNA. Furthermore, the sequences considered also show antibacterial effects in *Haemophilus influenzae* and *Citrobacter freundii*, indicating that the effects are not confined to *E. coli*. Finally, little effect is seen in *Klebsiella quasipneumoniae* (*K. pneumoniae* ATCC 700603), but S-fold suggests that PNA_{GGT} does not bind favorably to the mRNAs corresponding to divisome and cell shape proteins for *Klebsiella*. The selection of ASOs and formulation in a manner similar to that described herein is specifically contemplated for ASOs that bind favorably to mRNAs for divisome and cell shape proteins expressed by *Klebsiella* or other species. 10 nt and 6 nt sequences that targeted sequence regions in *ftsZ*, *mreB*, and *rodZ* were also tried, but the 8 nt sequence inhibited growth more effectively.

[00188] In sum, PNA_{GGT} strongly inhibits the growth of ESBL *E. coli* in a manner consistent with interfering with the function of *ftsZ*, *mreB*, and *rodZ*.

Materials and methods

Strains and reagents

[00189] *E. coli* MG1655 was obtained from VG. The permeable *E. coli* strain AS19 was obtained from P. Nielsen (Univ. of Copenhagen) and M. Tolmasky (Univ. of South California). ESBL *E. coli* was clinically isolated, and *Haemophilus influenzae* (ATCC 10211), *Citrobacter freundii* (ATCC 13316), and *Klebsiella pneumoniae subsp. pneumoniae* (Schroeter) Trevisan (ATCC 700603) were obtained from ATCC.

[00190] Isogenic strains V66 (rec+), V73, V2624, V2909 and V2831 (Δ recBCD2731) [Amundsen et al., G&D 2007; PMID 18079176] were provided by Prof G. Smith

[00191] Cells were grown on Luria-Bertani (LB) agar plates at 37°C overnight. Before each microplate experiment, cells from a few colonies were suspended in LB broth at an initial OD 600 nm of 0.1-0.2 and incubated with shaking at 37°C until the OD reached 0.45-0.5.

[00192] The PNA peptides (PNA bio) were obtained conjugated to a cell penetrating peptide (KFF)3K-O (SEQ ID NO: 43)- at the 5' end; the sequences are shown in Table 1. The BNA peptide was N-terminal of peptide-(RXR)₄XB-Cys-SMCC-C6 amino-5'-C+CA +C+C+A +GC-3' (SEQ ID NO: 44) (the "+" before the base indicates the modified base; R, arginine; X, 6-aminohexanoic acid; B, alanine; SMCC, sulfosuccinimidyl-trans-4-(N-maleimidomethyl) cyclohexane-1-carboxylate) [31]. The modified oligonucleotides were obtained in lyophilized form and dissolved in water to a final 1 mM concentration.

[00193] Nalidixic, sodium salt (MP Biomedicals), Tetracycline hydrochloride USP (VWR), NADA-green (biotechnie/Tocris), ProLong™ glass antifade mountant with NucBlue™ stain (Invitrogen), LB broth (Teknova), and LB plates (Sigma-Aldrich) were used as received.

[00194] Primers for RT-PCT were obtained from IDT (Integrated DNA Technology), and after amplification each product was loaded onto a 3% agarose gel (Invitrogen, UltraPure agarose) using a gel loading dye (New England Biolabs), a low molecular weight ladder (New England Biolabs), and TBE buffer (Corning).

[00195] Primers used in RT-PCR: for *ftsZ* mRNA 5'-TGACCGTTGCTGTCGTCAC-3' (SEQ ID NO: 45) (f) and 5'-CAGCGATACCTTGACAGCG-3' (SEQ ID NO: 46) (r) yielding 216 bp amplicon, for *mreB* mRNA, 5'-AGCGACCGGTTCTATGGTGG-3' (SEQ ID NO: 47) (f) and 5'-TACGTTCTGCGGTGGCTTCA-3' (SEQ ID NO: 48) (r) yielding 182 bp amplicon, for *rodZ* mRNA, 5'-CCCGCCGATCTTGCTTCAAC-3' (SEQ ID NO: 49) (f) and 5'-CTCTGCATCGGCGCAACTTT-3' (SEQ ID NO: 50) (r) yielding 140 bp amplicon, and for

rrsH, 5'-CCTGGTCTTGACATCCACAGAAC-3' (SEQ ID NO: 51) (f) and 5'-GCTCGTTGCGGGACTTAACC-3' (SEQ ID NO: 52) (r) yielding 124 bp amplicon.

Measurements of cell growth in the presence of modified oligonucleotides

[00196] Cell cultures were started by introducing 2-3 colonies in LB broth for an initial OD = 0.15- 0.2. The cells were incubated with continuous shaking at 37°C till the OD increased to 0.45- 0.5. Serial dilutions were prepared and aliquots of 5x10⁴ cells/ml were transferred to a low-binding microplate (96-well cell culture microplate, µClear, black, Cellstar®, cell repellent surface (Greiner Bio-One)) and mixed with 1 mM PNA solution. Typically, 2 µL of 1 mM PNA were added to 194 µL cells and 5 µL water so the final concentration of PNA was 10 µM. A control with the same volume of cells and 6 µL water was included in each experiment. Experiments were done in duplicate. Plates were incubated at 37°C with double orbital shaking and turbidity was recorded at 600 nm every 10 minutes for 20-24 hours using a Biotek Epoch 2 plate reader (Agilent) with Biotek Gen5 software.

Microscopy measurements with and without dyes

[00197] Slides were prepared with 10⁵ cell/ml aliquots treated with or without PNA in microplate wells for 2-3 hours. Usually, aliquots from two wells were combined and washed three times with PBS buffer. Cells labeled with NADA-green were obtained by growing the cells in the microplate in the presence of 500 µM NADA-green. Hoescht was incorporated into the cells by using Prolong glass antifade mountant with NucBlue. After washing the cells, they were resuspended in 20 µL of water, transferred to glass bottom microwell dishes (Mat Tek Corp.), and imaged by SR-SIM using a Plan-Apochromat 63x/1.4 oil objective, in an Elyra microscope (Zeiss), using 3% 488 nm laser for NADA-green and 4 % 405 nm laser for Hoescht. Images were acquired using a sCMOS camera (exposure time 300 ms) and reconstructed using ZEN software (black edition).

[00198] Unstained cells were placed onto a thin film of 1 % agarose mounted on a microscopy slide. Images were taken on a Nikon Eclipse TE2000-U inverted microscope with a Plan Apo TIRF 100x-oil objective, Roper Scientific Coolsnap HQ2 Firewire digital camera.

[00199] *E.coli* MG1655 was subbed on LB- agar plates and grown overnight. Two or three colonies were resuspended in LB broth and grown to an OD₆₀₀ of about 0.5. This suspension was diluted to obtain a cell concentration of 10⁵ cells/ml. Aliquots of this dilution were distributed in 20 wells of a microplate and PNA was added to ten wells to a final concentration

of 30 μ M. An equal volume of sterile water was added to other 20 wells and used as a negative control (untreated cells). The plate was incubated at 37°C for 2-3 hours, and the cells were subsequently collected in separate tubes and mixed with RNAprotect bacteria (Qiagen) following manufacture's protocol. After a 5-minute incubation at room temperature, the cells were centrifuged for 10 minutes at 5000 $\times$ g. The supernatant was carefully removed by inverting the tube, and the resulting pellets were used right away or stored at -80C and processed the next day for RNA isolation and purification using the RNeasy Mini kit (Qiagen) and protocol 4 from the user's manual. The pellets were initially incubated with 15 mg/ml lysozyme in TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing proteinase K for 10 minutes and vortexed every 2 minutes for 10 s. After addition of RLT buffer supplemented with β -mercaptoethanol and ethanol, total RNA was purified using RNAeasy Mini spin columns (Qiagen). Finally, the OD was measured to quantify the concentration of RNA from treated and untreated cells.

[00200] RT-PCR was performed using the OneStep Ahead RT-PCR kit (Qiagen) and based on the user's manual instructions. The RT-PCR cycling conditions were those for amplicons < 1kb. The reverse transcription-PCR was done in separate tubes for each set of primers corresponding to unique 200-300 bp regions in mRNAs of *ftsZ*, *mreB*, and *rodZ*. As a control, a 124 bp region in *rrsH* mRNA was used. The primers were designed using NCBI Primer-Blast (Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. PrimerBLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012;13:134). Finally, 12 μ L aliquots from each reaction tube were loaded on to a 3% agarose gel containing 1:10000 SybrSafe stain (Invitrogen) and run at 80 V for about 2 hours. The bands were visualized with a midrange UV trans-illuminator and recorded with a camera or using a gel documentation system Azure c200 and EPI Blue LED at 470 nm. The bands were quantified using ImageJ and normalized using the intensity of the *rrsH* mRNA amplicon band which typically showed little difference in intensity between the treated and untreated cells.

Results

PNA 5'-CCACCACC-3' complementary to the sequence 5'-GGTGGTGG-3' inhibits growth of ESBL (extended-spectrum beta-lactamases) producing *E. coli* but less effective on *E.coli* MG1655

[00201] The effectiveness of antibacterial treatments was measured by monitoring the optical density (OD) at 600 nm as a function of time for samples in a 96-well plate array. Figure

1 shows results for an ESBL (extended-spectrum beta-lactamase) producing *E. coli* and *E. coli* MG1655. Growth curves for ampicillin treated ESBL *E. coli* cells show that this strain produces beta-lactamase and is therefore not affected by high concentrations (3xMIC) of this antibiotic (Figure 4).

[00202] Figures 1A and 1B show results for an ESBL *E. coli* and *E. coli* MG1655 when treated with peptide-PNA with several different sequences. The solid curve shows the results for untreated cells. The initial cell concentration $\sim 5 \times 10^4$ cells/mL was below the OD detection limit = 0.01 for $\sim 5 \times 10^6$ cells/mL, but after ~ 3 hours the cells entered the visible exponential growth phase. Increasing delay indicates increasing growth inhibition, as noted below. The long dash-small dash curve shows results for a peptide nucleic acid (PNA) with sequence 5'-CTAGTGGA-3' designed to bind 5'-TCCACTAG-3'. The target occurs only five times in the *E. coli* MG1655 genome. Since the frequency of that sequence in the genome is low, this peptide-PNA conjugate is referred to as P-PNA_{LF}. The long dash-small dash curve shows that with 10 μ M P-PNA_{LF} exponential growth is not delayed compared with the control. The long dash curve also shows no delay.

[00203] Also tested were a peptide-PNA that targets the Chi sequence: 5'-GCTGGTGGT-3' so the PNA has the sequence 5'-ACCACCAGC-3', and three other peptide-PNAs that have the same sequence as Chi except for the second base from the 5' end (Table 1). The long dash-double dot curves show the result for 10 μ M P-PNA_{GGT}, a peptide-PNA analog with sequence 5'-CCACCACC-3' complementary to the sequence 5'-GGTGGTGG-3'. For ESBL *E. coli*, no growth is observed within 16 hours and growth remains strongly suppressed for more than 24 hours. Furthermore, for 10 μ M P-PNA_{GGT} *E. coli* MG1655 growth is delayed by ~ 5 hours and for 20 μ M P-PNA_{GGT} no growth is observed within 24 hours.

[00204] The long dash-double small dash, dash-dot, and small dash curves show results for 10 μ M peptide-PNA conjugates targeting sequences with a one base mismatch with respect to 5'-GGTGGTGG-3'. P-PNA_{MMIC}, P-PNA_{MMIA}, and P-PNA_{MMIT} all show fairly similar results with an approximately 5-hour delay for *E. coli* MG1655 and delays between 6 and 8 hours for ESBL-*E. coli*. As expected, the effects of the peptide-PNA depend on the PNA concentration (Figure 1 and Fig. 5).

Exploring mechanisms that might underlie inhibition

[00205] The PNA sequences that were effective are strongly overrepresented in the genomes (Figure 18), so genome frequency or the frequency of occurrences in essential genes was probed for whether they were relevant to the observed effect (Figs. 6 and 7). Some sequences that are more strongly represented in genes or in essential genes have less effect, so the inhibition that was observed is not simply due to prevalence in genomes.

[00206] The target GGTGGTGG has one base mismatch with respect to the Chi sequence, so part of the effect that was observed might be due to interfering with the RecBCD repair pathway; however, growth of RecBCD mutants is inhibited as effectively as growth of WT cells (Figs. 8 and 9) suggesting that the inhibition is not dominantly due to targeting the RecBCD pathway.

PNAs that inhibit growth cause significant morphology changes

[00207] To gain insight into the mechanism or mechanisms underlying inhibition, images of the treated cells were taken and compared with untreated bacteria. Figure 2A shows typical rod-shaped cells with hemispherical caps when *E.coli* MG1655 is incubated in broth. Figures 2B and 2C show cells incubated with P-PNAGGT. Though the untreated cells are uniform cylinders with rounded ends, the treated cells show highly distorted morphologies.

[00208] Cells grown in the presence of NADA-green (Fig. 10) a green-fluorescent alanine derivative that can be incorporated into peptidoglycans in the cell membrane, show green-fluorescent contours, but only a few show stained septa. The fixed cells were covered with mountant containing Hoescht that stains the DNA before observation. In contrast cells treated with P-PNAGGT are highly elongated, their shapes are no longer rod-shaped, and no green-fluorescent septa can be seen. Controls with low frequency PNA showed no cell distortions (not shown). In sum, Figure 2 and Figure 10 indicate that treating cells with peptide conjugate P-PNAGGT strongly distorts cell morphology and cell division, and that the extent of the distortion depends on the sequence of the PNA.

Effect on mRNA

[00209] Gel assays were performed in which the concentrations of mRNAs of *ftsZ*, *mreB*, and *rodZ* were measured in treated cells and in untreated cells. The total RNA in treated and untreated cells was extracted and subsequently the corresponding mRNAs was reverse amplified using several different primer sets. The results are consistent with P-PNA treatment

reducing the levels of *ftsZ* and *mreB* mRNAs, but the differences between the positive results and the controls were not sufficient to unambiguously claim that the *ftsZ* levels were reduced (Fig. 26). In contrast, *rodZ* mRNA concentration was either unaffected or slightly larger in the treated samples.

Synergies

[00210] To probe whether this treatment has a synergistic relationship with antibiotics, *E. coli* MG1655 cells were treated with low doses of nalidixic acid in combination with low doses of peptide-PNA. A very strong synergistic effect between nalidixic acid and peptide-PNA was seen (Fig. 11). There is also synergy with tetracycline, but that synergy is less effective (Fig. 12). Finally, there is a synergy with ampicillin; however, interestingly, in the ESBL *E. coli* there is no synergy with ampicillin (Fig. 13).

Factors that may limit growth inhibition on *E. coli* MG1655

[00211] It is important to consider what can limit the effectiveness of treatments. To evaluate whether cell penetration was a limiting factor, Results in *E. coli* MG1655 were compared to results in *E. coli* AS19, a strain known to have permeable cell membranes [1, 31, 32]. Indeed, the dose response in *E. coli* AS19 is much larger than in *E. coli* MG1655, suggesting that penetration of the P-PNA into the cells may be limiting the effectiveness of inhibition (Figure 3).

[00212] Similarly, to determine whether binding affinity is affecting the results, we treated cells using peptide conjugate bridged nucleic acids, which are known to yield higher melting temperatures when bound to DNA. The P-BNAMM1C had no effect on *E. coli* MG1655 cells, but in *E. coli* AS19 cells much more inhibition that P-PNAMM1C can be observed (Figure 3 and Fig. 14). This is consistent with binding affinity limiting the treatment effectiveness since the more tightly bound BNA is more effective than the PNA; however, the charge on the BNA backbones decreases membrane penetration into the cells.

[00213] Increasing the binding affinity by increasing the length of the P-PNA to 10 nt was also tried, but the longer single strands did not show enhanced inhibition, and in some cases showed reduced inhibition. This could be due either to a decrease in cell penetrability with length or increased off-target binding. Experiments with *E. coli* AS19 hint that the effect is dominantly due to off-target binding since longer single strands do improve inhibition even in the highly permeable *E. coli* AS19 cells (Fig. 14).

[00214] To further probe how PNA length influences inhibition, two 6 nt sequences were considered. The sequences targeted were GGTGGT and GCTGGT. Neither sequence complements inhibited cell growth as effectively as the 8-nt sequences GGTGGTGG and GCTGGTGG, but the 6-nt sequence that targeted GCTGGT showed larger inhibition than the sequence that targeted GGTGGT (Fig. 27)

[00215] It has been shown that oligonucleotide analogs can bind to dsDNA forming triple helical structures. [33-35] Thus, an experiment was designed using FRET to evaluate the interaction between a rhodamine labeled PNA and a fluorescein labeled dsDNA. The fluorophores were placed on the complementary strands as shown in Fig. 15. If the rhodamine labeled PNA binds close to the fluorescein label, there should be a significant decrease in the signal due to FRET between these two fluorophores. Although a small decrease after the addition of the PNA was observed, a control where buffer is added to the dsDNA showed a similar decrease, suggesting there is no triplex formation (Fig. 15).

[00216] Finally, whether the inhibition effects extend beyond *E. coli* was considered by using another Gram-negative bacteria, *Citrobacter freundii* (Fig. 16). *C. freundii* also shows growth inhibition that depends on the P-PNA sequence, and the deformation of the treated cells also shows morphological distortions similar to those seen in *E. coli* MG1655 (Fig. 17). Comparison of growth inhibition with other Gram-negative bacteria correlates well with favorable binding to divisome and cell shape maintenance genes (Fig. 18 and Fig. 19).

Discussion

[00217] These results indicate that the inhibition observed is not a consequence of replication blocking since blocking P-PNA_{GGT} inhibits more effectively than peptide conjugate PNAs that target sequences P-PNA_{HF} and P-PNA_{MG} that occur much more frequently in the *E. coli* MG1655 genome and more frequently in genes. P-PNA_{HF} and P-PNA_{MG} also have higher binding affinities than P-PNA_{GGT} (Table 1), so the results do not depend simply on the binding energy between the PNA and target sequences. In addition, the peptide conjugate PNAs are not binding to dsDNA (Figure 27), suggesting the oligos are binding to a single-stranded nucleic acid. Furthermore, the effectiveness of peptide bound dsDNA that target sequences other than 5'-GGTGGTGG-3' became decreasingly effective as their sequence departs from 5'-GGTGGTGG-3' and the morphological changes due to P-PNAGTT and P-PNAM1C are similar though the changes were less marked for P-PNAM1C. Thus, the inhibition results

dominantly from interactions in which the peptide conjugate PNA binds to single stranded targets with sequences that are close to 5'-GGTGGTGG-3'.

[00218] The binding of P-PNAGGT to single-stranded nucleic acids could decrease the effectiveness of those proteins either by inhibiting transcription, RNA folding, or by causing cleavage by RNase in regions where the PNA binds to target ssDNA. Previous antibacterial strategies have used anti-sense oligos to target mRNA associated with essential genes. Typically, those strategies targeted sequence regions near the start codon of essential genes. [1]. The sequence 5'-GGTGGTGG-3' appears in essential genes involved in cell division and cell wall maintenance including ftsA, ftsW, ftsZm mreB, and murE; however, the targets do not appear in regions in the start codons. In addition, the mfold algorithm (found on the world wide web at unafold.org/mfold/applications/rna-folding-form-v2.php) suggests that the target regions in those genes are not accessible; however, we note that in vitro measurements of RNA folding and in silico calculations of RNA folding may not accurately capture all features of RNA folding in vivo [36]. Calculating binding energies using Sfold algorithms shows that for a few genes the binding energy is favorable.

Oligo Name	Oligo Sequence	Target Sequence	Mismatches from GGTGGTGG	Targets on Given	Targets on Comp	Total targets
P-PNA _{GGT}	5'-CCACCACC-3'	5'-GGTGGTGG-3'	0	332	104	436
P-PNA _{MM1T}	5'-CCACCAAC-3'	5'-GTTGGTGG-3'	1	122	89	211
P-PNA _{MM1C} P-BNA _{MM1C} PNA _{MM1C} LNA _{MM1C}	5'-CCACCAGC-3'	5'-GCTGGTGG-3'	1	499	509	1008
P-PNA _{MM1A}	5'-CCACCATC-3'	5'-GATGGTGG-3'	1	196	185	365
P-PNA _{MM2}	5'-CCAGCGCC-3'	5'-GGCGCTGG-3'	2	1157	219	1376
P-PNA _{LF}	5'-CTAGTGGA-3'	5'-TCCACTAG-3'	6	3	2	5
P-PNA _{HF}	5'-CGCTGGCG-3'	5'-CGCCAGCG-3'	6	380	1063	1443
P-PNA _{10GC}	5'-CCACCAGCGC-3'	5'-GCGCTGGTGG-3'	NA	62	76	138

Table 3 (Table discloses SEQ ID NOS 14 and 38, respectively in order of appearance): List of the oligonucleotides used in the experiments, the number of occurrences of the target sequence on each strand, and the number of genes in the Watson strand that contain the target sequence. The table also include a row showing the expected distribution of an 8 bp sequence in a

randomly chosen sequences with the same length as the *E.coli* MG1655 genome. This is also approximately the number of occurrences of a randomly chosen 8 bp sequence in the *E.coli* genome. The distributions for the sequences considered in this work are very different than the results for a randomly chosen 8 bp sequence.

Oligo Sequence	Target Sequence	Mismatches from GGTGGTGG	Delay with MG1655	Delay with AS19 mutant <i>E. coli</i> with permeable cell walls	Delay with MG1655 + nalidixic	Delay with ESBL ampicillin resistant <i>E. coli</i>
5'-CCACCACC-3'	5'-GGTGGTGG-3'	0	10 micromolar 8 hours a 20 micromolar > 17 hours k	10 micromolar 8 hours g	10 micromolar ~> 17 hours a	10 micromolar >17 hours h
5'-CCACCAAC-3'	5'-GTTGGTGG-3'	1	10 micromolar ~ 2 hours a		10 micromolar ~> 17 hours a	10 micromolar ~5 hours h
5'-CCACCAGC-3'	5'-GCTGGTGG-3'	1	10 micromolar ~ 2 hours a	10 micromolar 8 hours SI 5	10 micromolar ~> 17 hours a	10 micromolar ~4 hours h
5'-CCACCATC-3'	5'-GATGGTGG-3'	1	10 micromolar ~ 2 hours a		10 micromolar ~> 17 hours a	10 micromolar ~2 hours h
5'-CCACCAGC-3'	5'-GCTGGTGG-3'	1	10 micromolar < 1 hour b	1 micromolar >17 hours j	10 micromolar < 1 hour i	
5'-CCAGCGCC-3'	5'-GGCGCTGG-3'	2	10 micromolar ~ 1 hour c			
5'-CTAGTGGA-3'	5'-TCCACTAG-3'	6	10 micromolar < 1 hour a		10 micromolar < 1 hour a	10 micromolar <1 hours h
5'-CTAGTGGA-3'	5'-TCCACTAG-3'	6		10 micromolar < 1 hour g		
5'-CGCTGGCG-3'	5'-CGCCAGCG-3'	6	10 micromolar < 1 hour e			
5'-GCTGGTGG-3'	5'-CCACCAGC-3'	7	10 micromolar < 1 hour a			10 micromolar <1 hours h
5'-CCACCAGCGC-3'	5'-GCGCTGGTGG-3'	1	10 micromolar ~ 1 hour c			
5'-ACCACCACCG-3'	5'-CGGTGGTGGT-3'	NA	10 micromolar < 1 hour d	10 micromolar ~ 1.5 hours g		
5'-ACCACCACCC-3'	5'-GGGTGGTGGT-3'	NA	10 micromolar < 1 hour d	10 micromolar ~ 1.5 hours g		
5'-ACCACC-3'	5'-GGTGGT-3'	NA	30 micromolar ~ 1 hour f			
5'-ACCAGC-3'	5'-GCTGGT-3'	NA	30 micromolar ~ 7.5 hours f			

Table 4 (Table discloses SEQ ID NOS 14, 38, 15, 40, 16 and 42, respectively in order of appearance): List of the oligonucleotides tested in the experiments and their effectiveness. a-k represent separate trials.

[00219] References

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CLAIMSWhat is claimed:

1. A composition comprising an oligonucleotide with a modified nucleic acid backbone wherein the oligonucleotide is conjugated to a cell penetrating peptide, and wherein the oligonucleotide is substantially complementary to 5'-GGTGGTGG-3'.
2. The composition of claim 1, wherein the modified nucleic acid backbone is a peptide-nucleic acid (PNA), a locked nucleic acid (LNA), or a bridged nucleic acid (BNA) backbone.
3. The composition of claim 1 or 2, wherein the modified nucleic acid backbone is a PNA backbone.
4. The composition of any of claims 1-3, wherein the cell penetrating peptide is selected from those listed in Table 1.
5. The composition of any of claims 1-4, wherein the oligonucleotide has a sequence of 6 to 10 nucleotides.
6. The composition of any of claims 1-5, wherein the oligonucleotide sequence is complementary to 5'-GGTGGTGG-3'.
7. The composition of any of claims 1-5, wherein the oligonucleotide sequence is complementary to 5'-GXTGGTGG-3', and wherein the X is A, T, or G.
8. The composition of any of claims 1-5, wherein the oligonucleotide sequence is substantially complementary to 5'-GGTGGTGG-3', and wherein one nucleotide differs from the complementary sequence.
9. The composition of any of claims 1-5, wherein the oligonucleotide sequence differs from the sequence complementarity with 5'-GGTGGTGG-3' by one nucleotide, and wherein that different nucleotide is A, T, G, or C.
10. The composition of any one of claims 1-9, further comprising an antibiotic.
11. The composition of claim 10, wherein the antibiotic is selected from the group consisting of: nalidixic acid, ampicillin, and tetracycline.
12. A pharmaceutical formulation comprising a composition of any one of claims 1-11 and a pharmaceutically-acceptable carrier.
13. A method of inhibiting growth or replication of a bacterium, the method comprising contacting the bacterium with a composition of any one of claims 1-12.
14. The method of claim 13, wherein the bacterium is an antibiotic resistant bacterium.

15. The method of claim 13 or 14, wherein the bacterium is selected from enterobacteriaceae.
16. The method of claim 13 or 14, wherein the bacterium is selected from the group consisting of: bacteria expressing extended-spectrum beta-lactamases (ESBLs), *Escherichia coli*, *E. coli* MG1655, *Citrobacter freundii*, and *Haemophilus influenzae*.
17. The method of claim 13 or claim 14, further comprising contacting the bacterium with an antibiotic.
18. The method of claim 17, wherein the antibiotic is selected from quinolone antibiotics.
19. The method of claim 18, wherein the quinolone antibiotic is selected from nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid, ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin, balofloxacin, grepafloxacin, levofloxacin, pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.
20. The method of claim 17, wherein the antibiotic is selected from penicillins.
21. The method of claim 20, wherein the penicillin is selected from amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.
22. The method of claim 17, wherein the antibiotic is selected from tetracyclins.
23. The method of claim 22, wherein the tetracyclin antibiotic is selected from tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.
24. A method of treating a bacterial infection, the method comprising administering a composition of any of claims 1-12 to a subject in need thereof.
25. The method of claim 20, wherein the infection is an infection with an antibiotic resistant bacterium.
26. The method of claim 24 or 25, wherein the bacterium is selected from enterobacteriaceae.
27. The method of any one of claims 24-26, further comprising administering an antibiotic.
28. The method of claim 27, wherein the antibiotic is selected from quinolone antibiotics.
29. The method of claim 28, wherein the quinolone antibiotic is selected from nalidixic acid, amfonelic acid, oxolinic acid, rosoxacin, piromidic acid, pipemidic acid,

ciprofloxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, enoxacin balofloxacin, grepafloxacin, levofloxacin, pazufloxacin, sparfloxacin, temafloxacin, clinafloxacin, gatifloxacin, moxifloxacin, sitafloxacin, prulifloxacin, besifloxacin, and delafloxacin.

30. The method of claim 27, wherein the antibiotic is selected from penicillins antibiotics.
31. The method of claim 30, wherein the quinolone antibiotic is selected from amoxicillin, ampicillin, benzylpenicillin, benzathine benzylpenicillin, dicloxacillin, flucloxacillin, phenoxymethylpenicillin, and piperacillin.
32. The method of claim 27, wherein the antibiotic is selected from tetracyclins.
33. The method of claim 32, wherein the tetracyclin is selected from tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, doxycycline, tigecycline, eravacycline, sarecycline, and omadacycline.

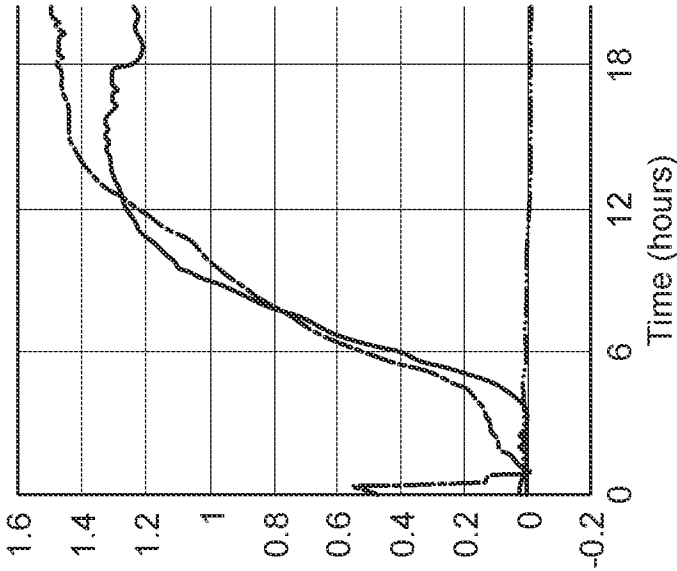


FIG. 1C

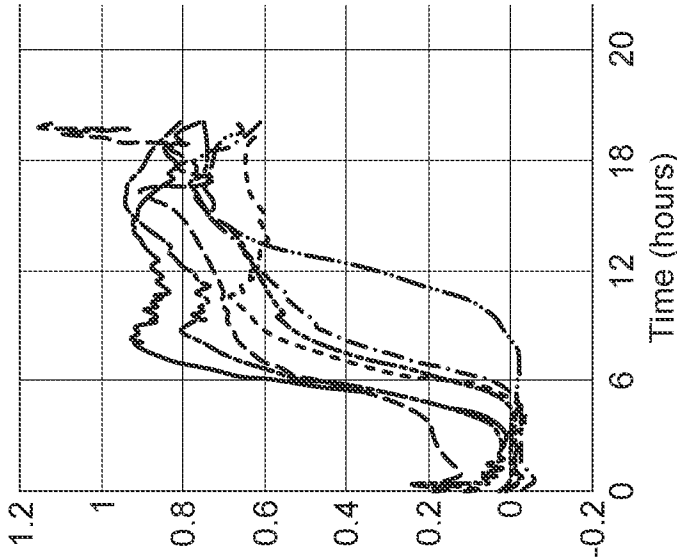


FIG. 1B

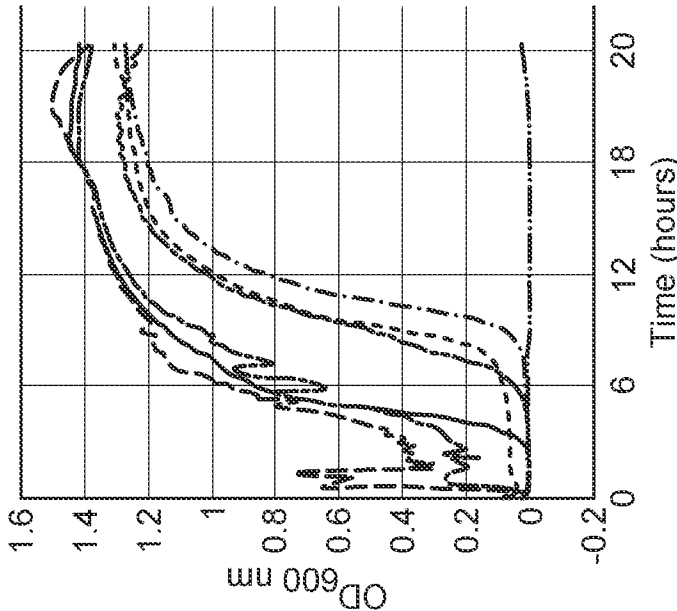


FIG. 1A

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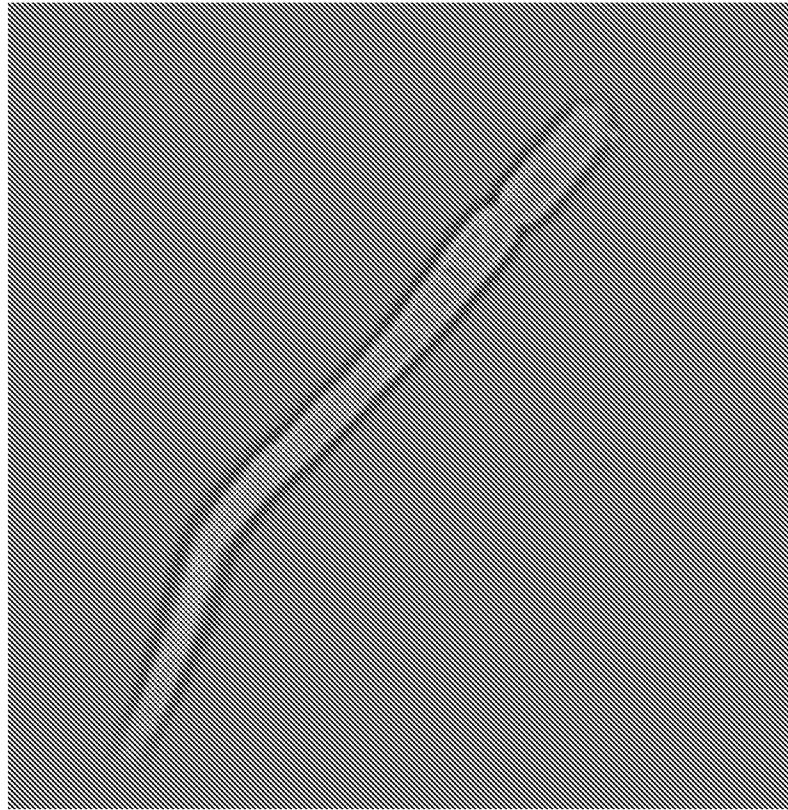


FIG. 2C

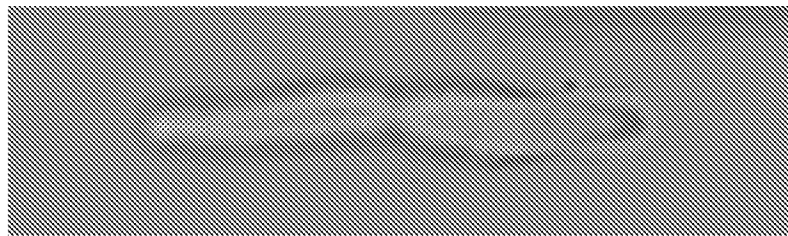


FIG. 2B



FIG. 2A

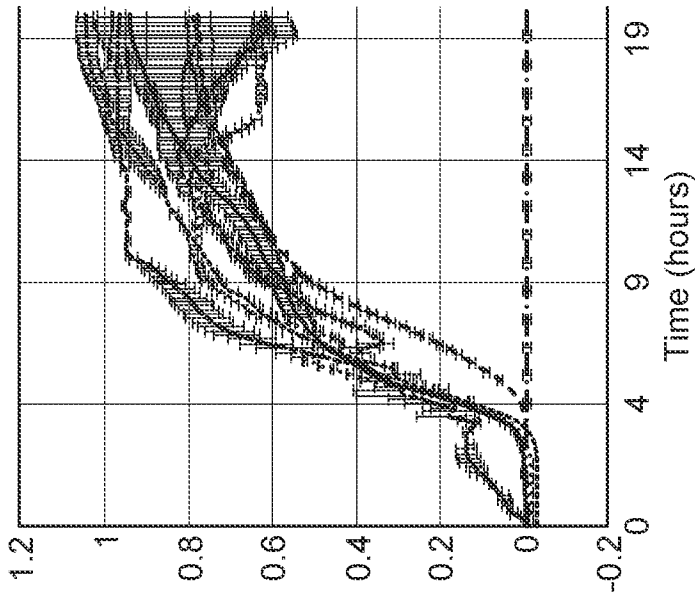


FIG. 3B

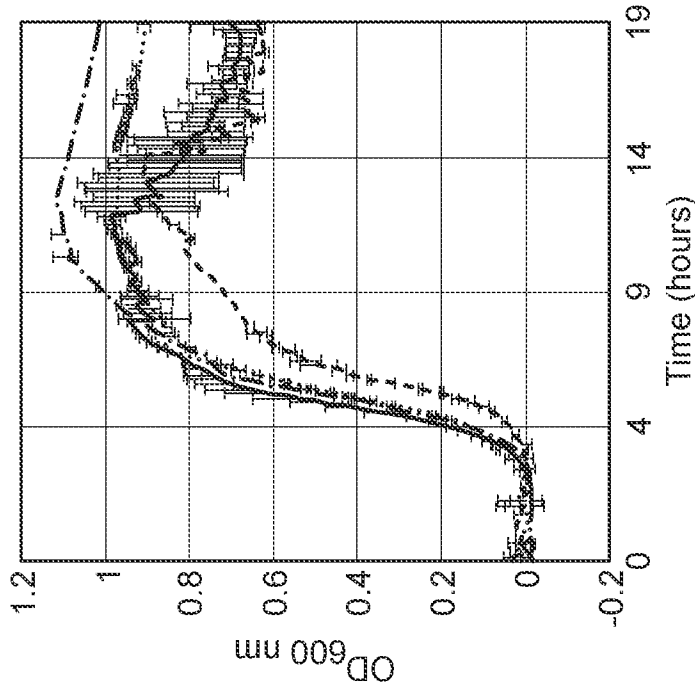


FIG. 3A

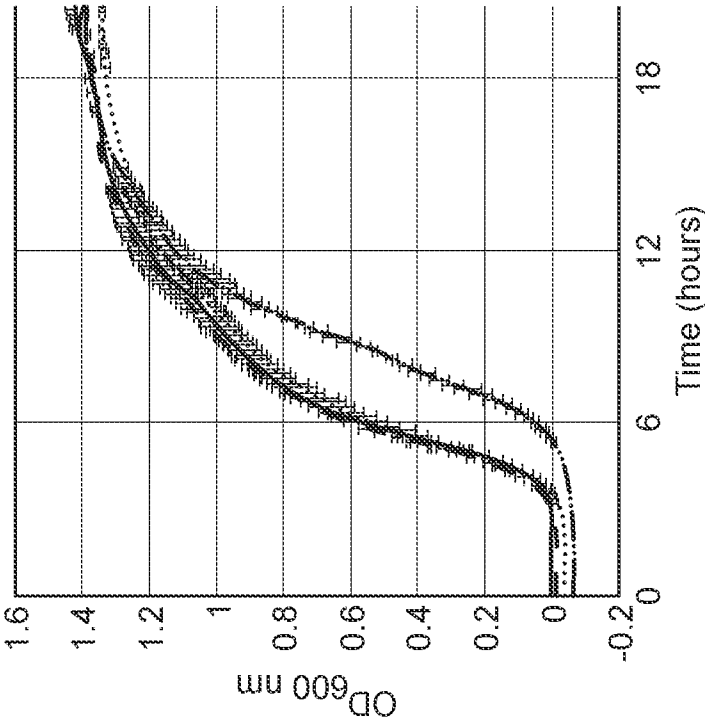


FIG. 5

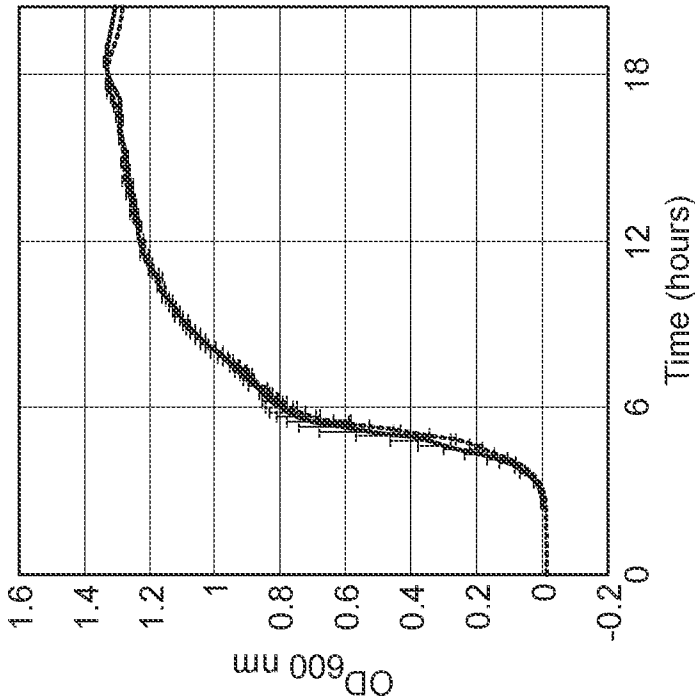


FIG. 4

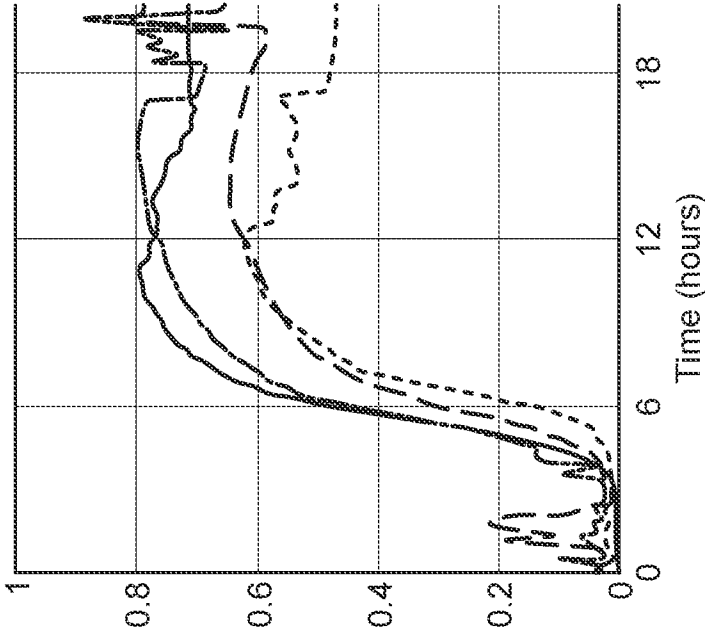


FIG. 6B

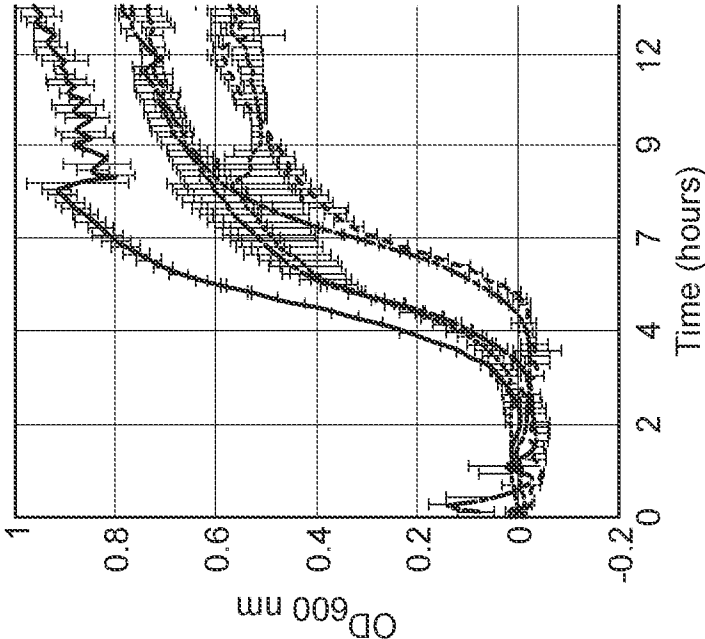


FIG. 6A

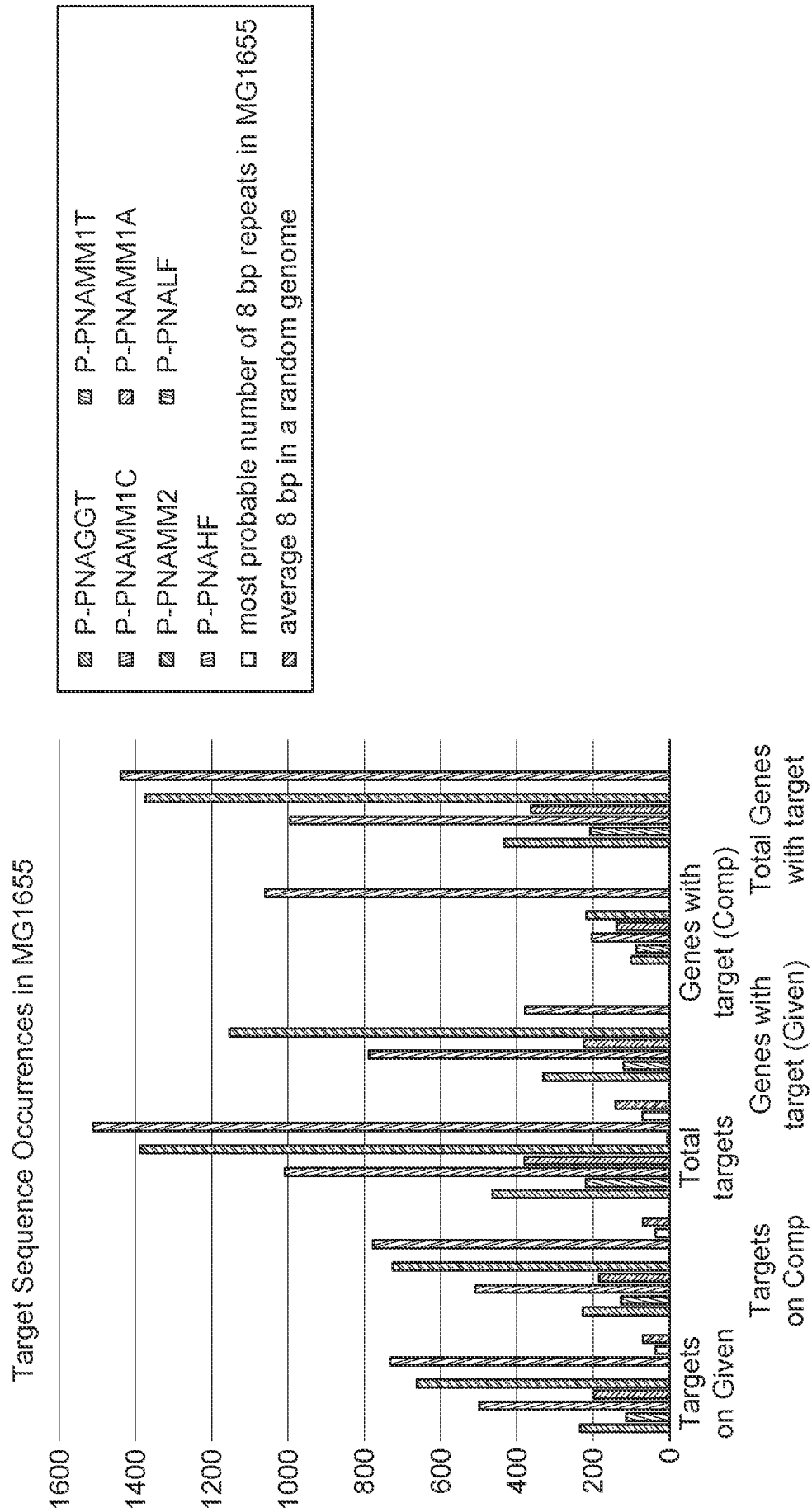


FIG. 7A

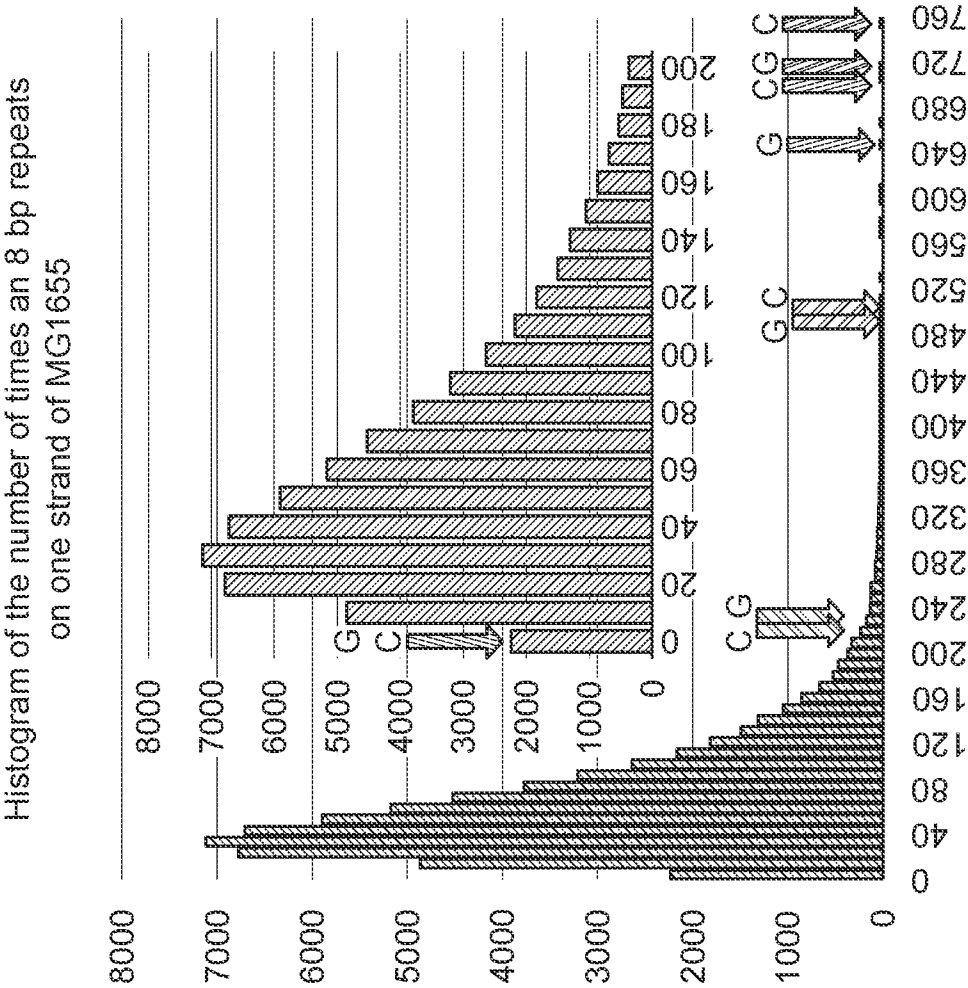


FIG. 7B

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Number of appearances on each strand	Sequence on Given Strand	Sequence on Comp Strand	Oligo name in the paper
486	CTTCCAGC	GCTGGAAG	
489	GCCAGCAG	CTGCTGGC	
490	ATCGCCAG	CTGGCGAT	
492	AGCGCCAG	CTGGCGCT	
492	CTGGCGCA	TGCGCCAG	
494	CACCAGCG	CGCTGGTG	
496	CTGGCGGC	GCCGCCAG	
498	CGCCAGTT	AACTGGCG	
499	GCTGGTGG	CCACCAGC	MM1C=Chi
509	CCACCAGC	GCTGGTGG	MM1C=Chi
518	GCTGGCGA	TCGCCAGC	
521	TCGCCAGC	GCTGGCGA	
523	GCCGCCAG	CTGGCGGC	
528	TGCGCCAG	CTGGCGCA	
548	TGGCGCTG	CAGCGCCA	
582	GCGCCAGC	GCTGGCGC	
590	GCCAGCGC	GCGCTGGC	
591	GCGCTGGC	GCCAGCGC	
599	GCTGGCGC	GCGCCAGC	
612	GCTGGCGG	CCGCCAGC	
628	CAGCGCCA	TGGCGCTG	
629	TGCTGGCG	CGCCAGCA	
662	GGCGCTGG	CCAGCGCC	MG
663	CCGCCAGC	GCTGGCGG	
668	CGCCAGCA	TGCTGGCG	
726	CCAGCGCC	GGCGCTGG	MG
734	CGCCAGCG	CGCTGGCG	HF
778	CGCTGGCG	CGCCAGCG	HF

FIG. 7C

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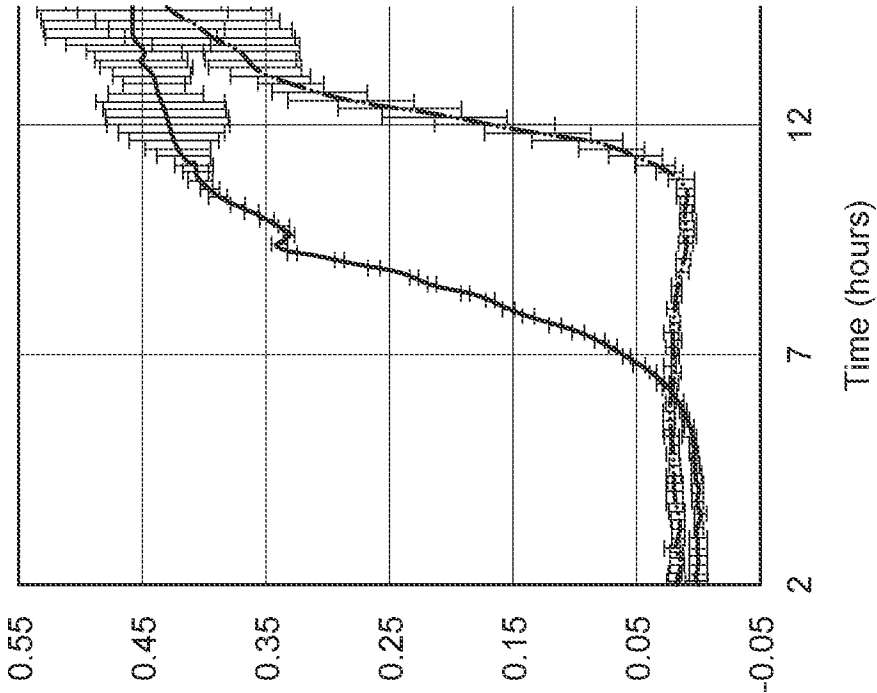


FIG. 8A

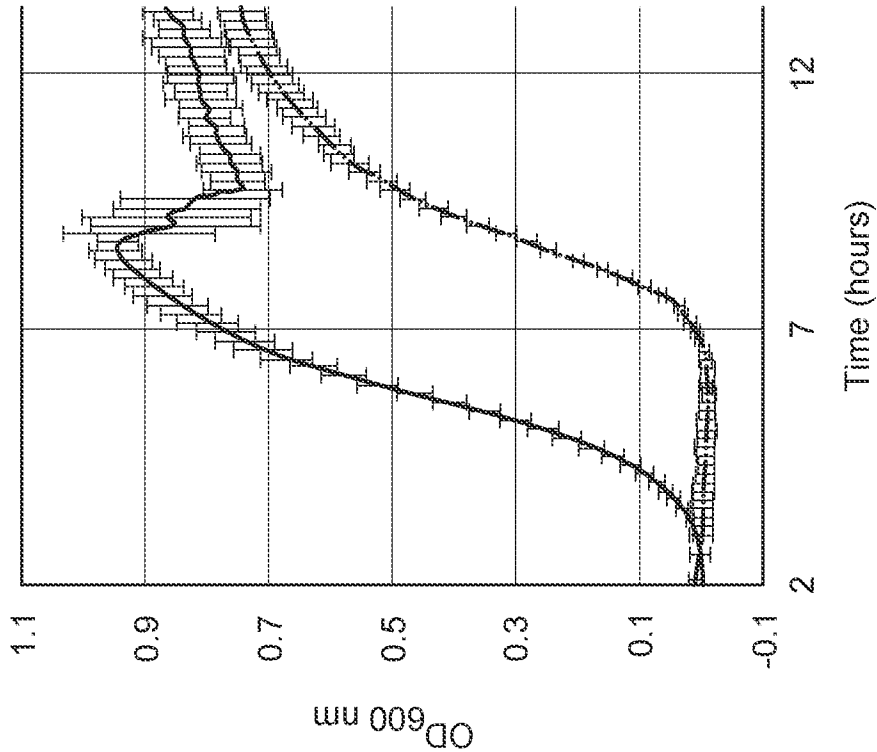


FIG. 8B

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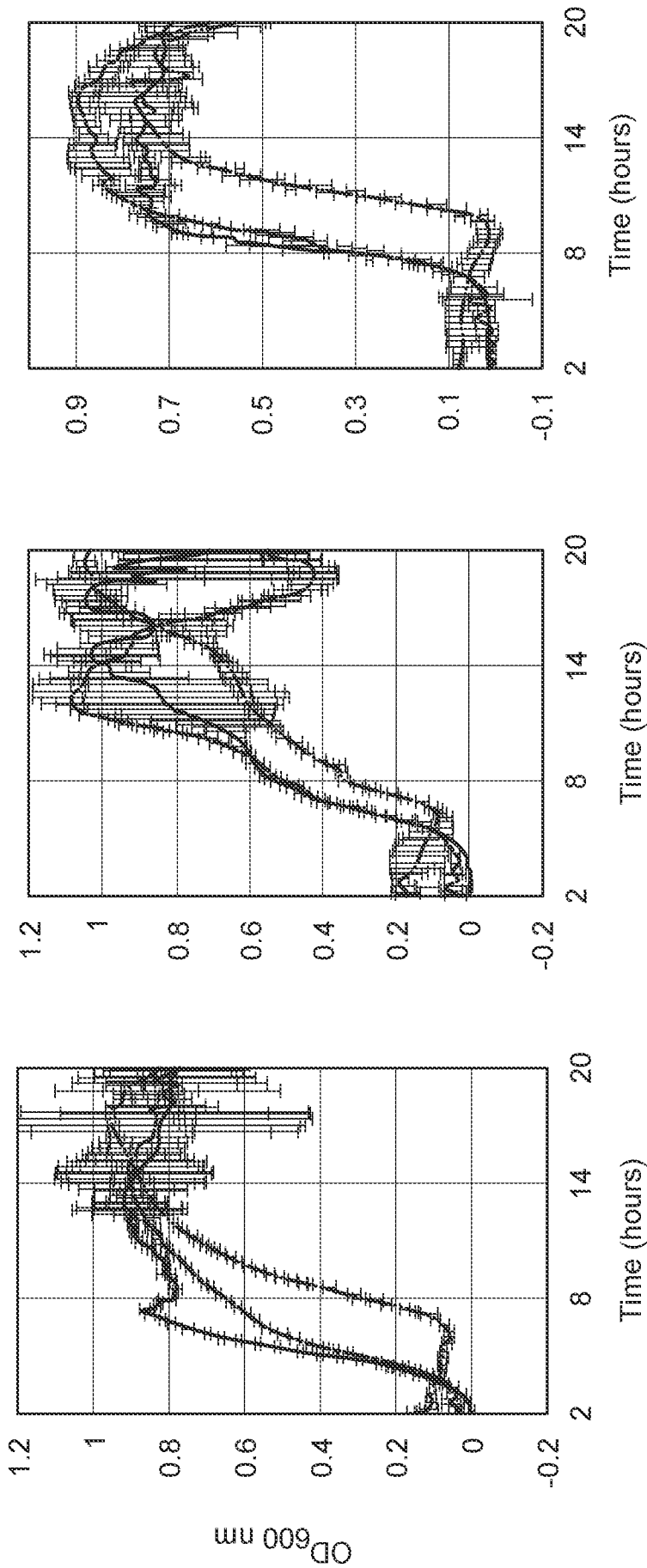


FIG. 9C

FIG. 9B

FIG. 9A

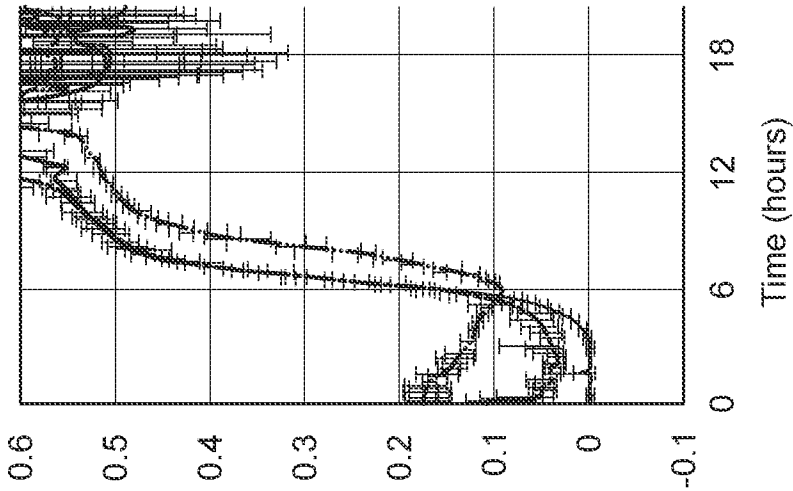


FIG. 9E

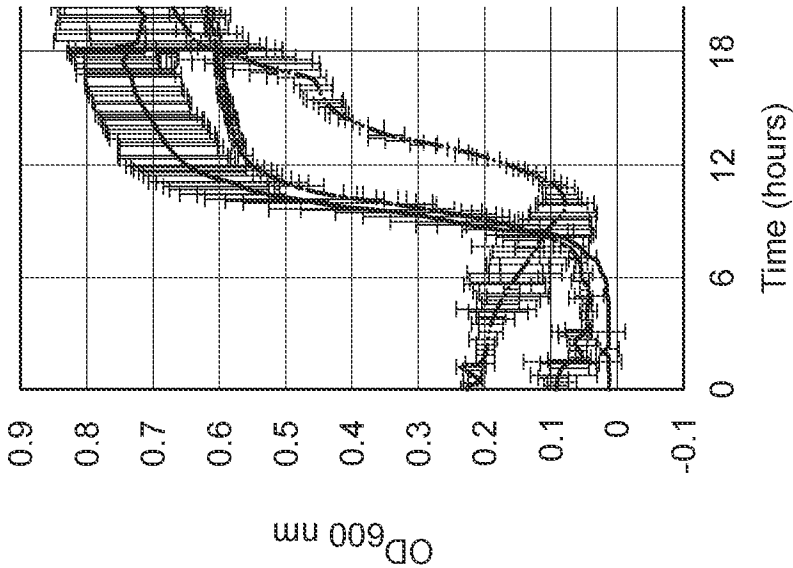


FIG. 9D

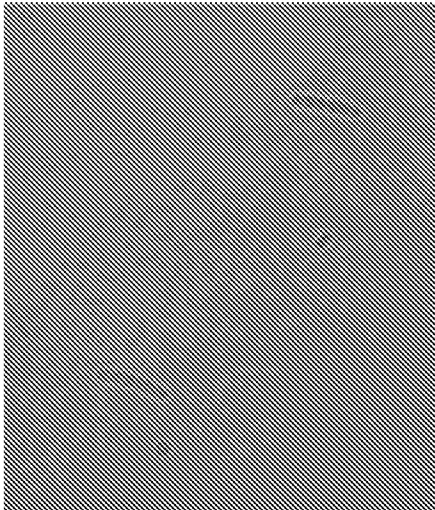
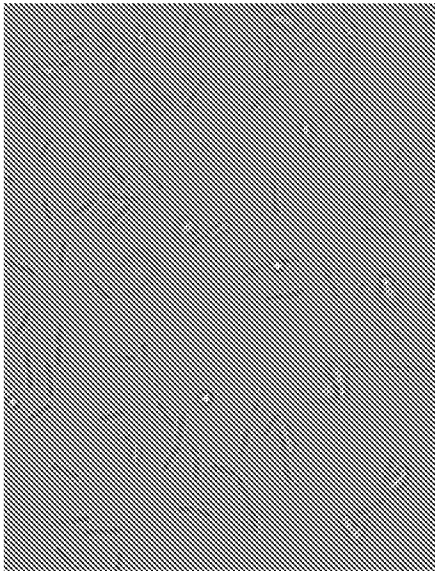


FIG. 10A

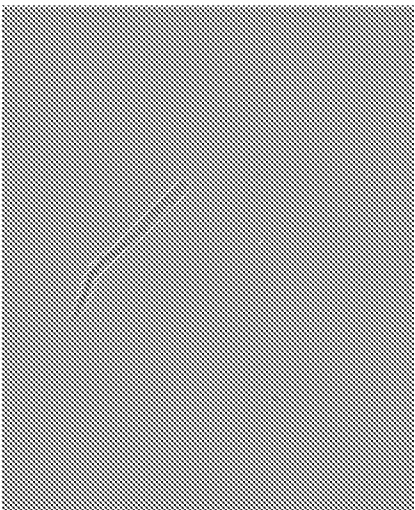
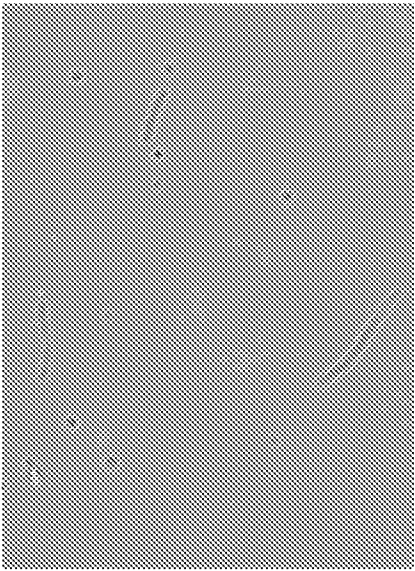


FIG. 10B

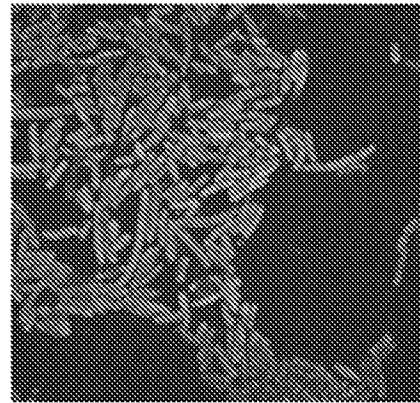
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FIG. 10C



FIG. 10D



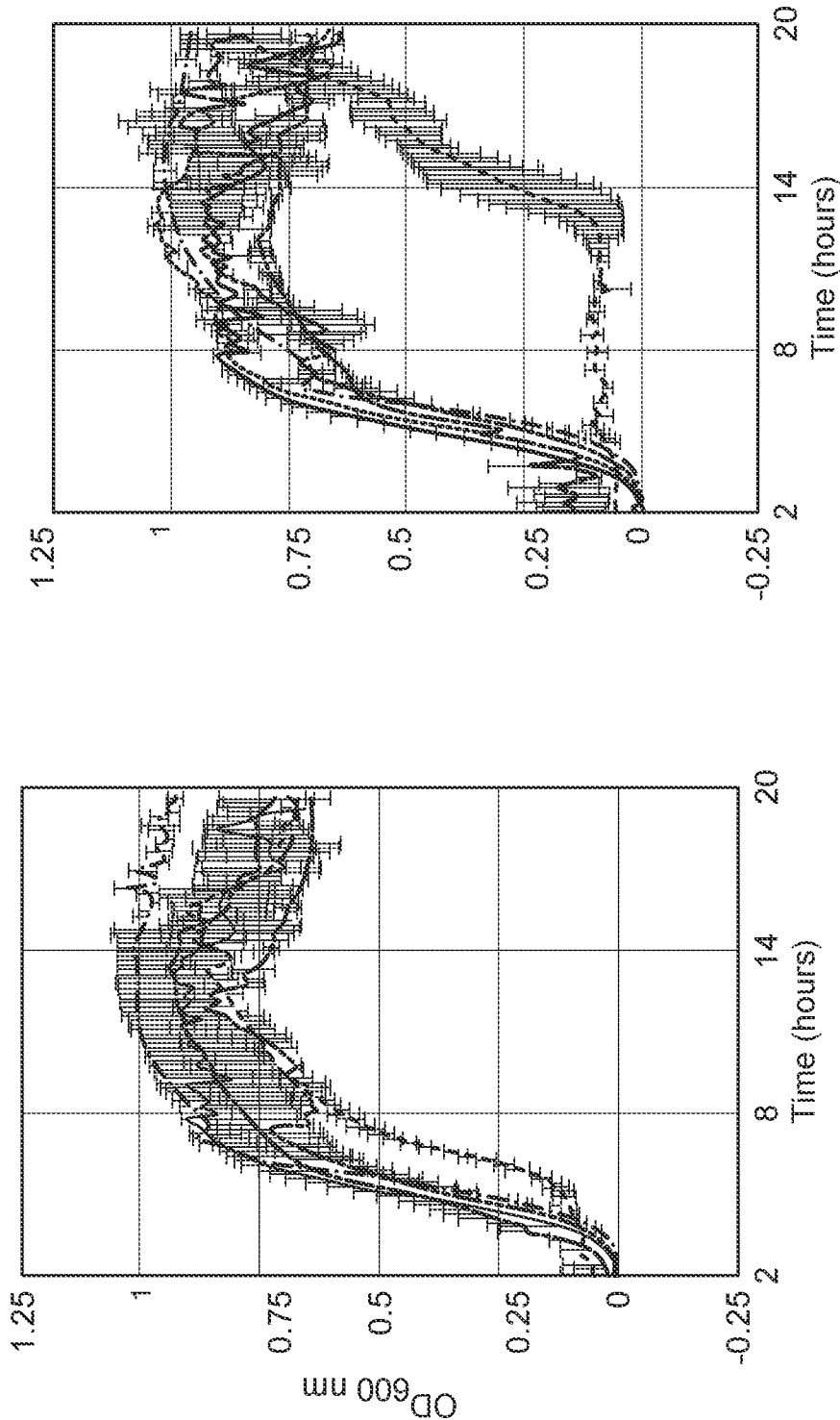


FIG. 11B

FIG. 11A

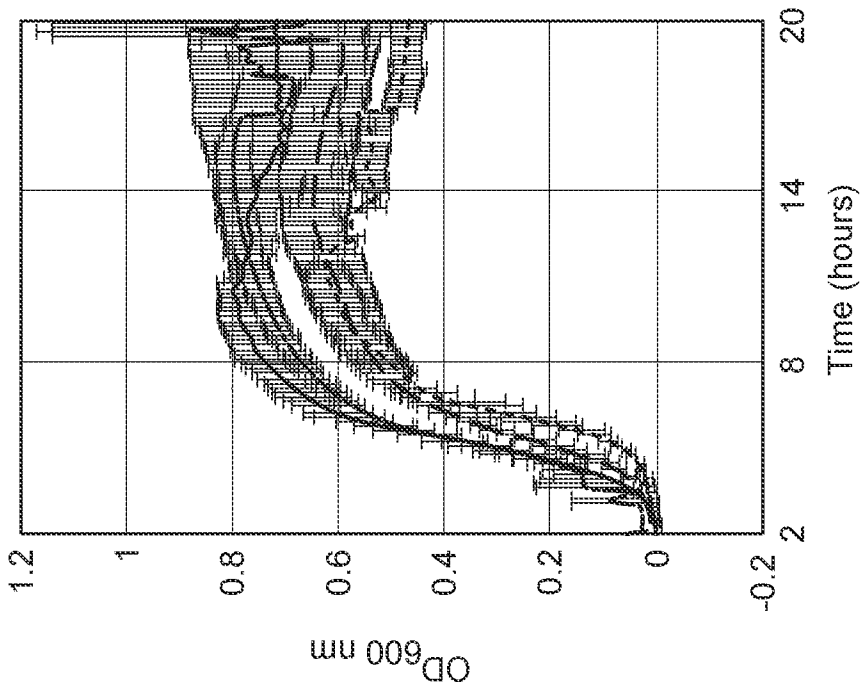


FIG. 12A

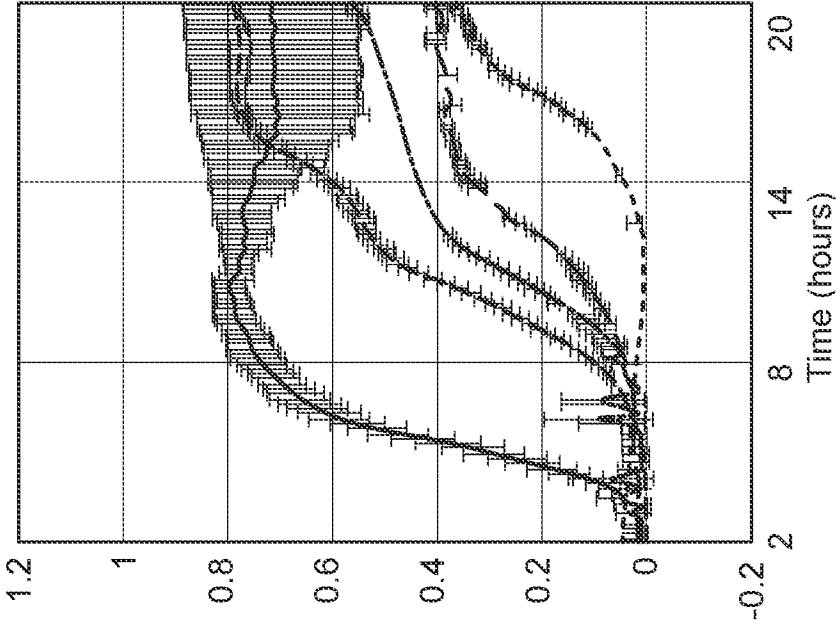


FIG. 12B

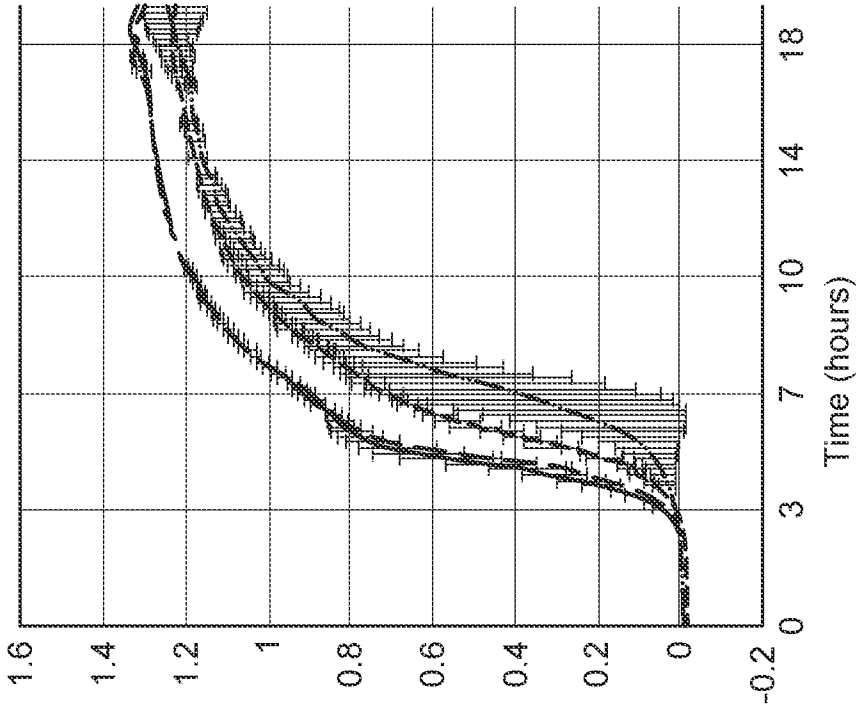


FIG. 13B

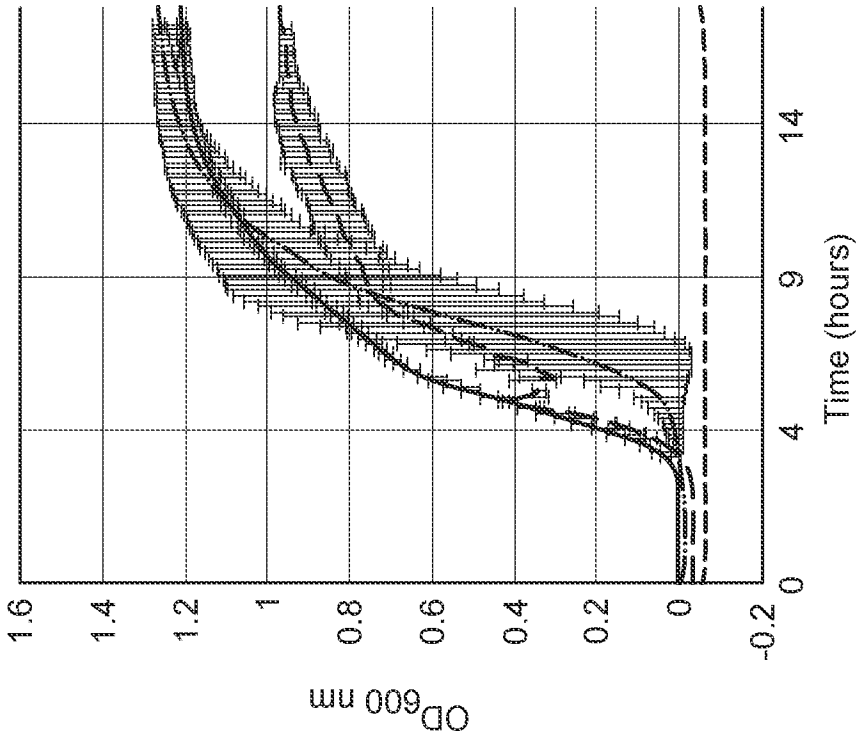


FIG. 13A

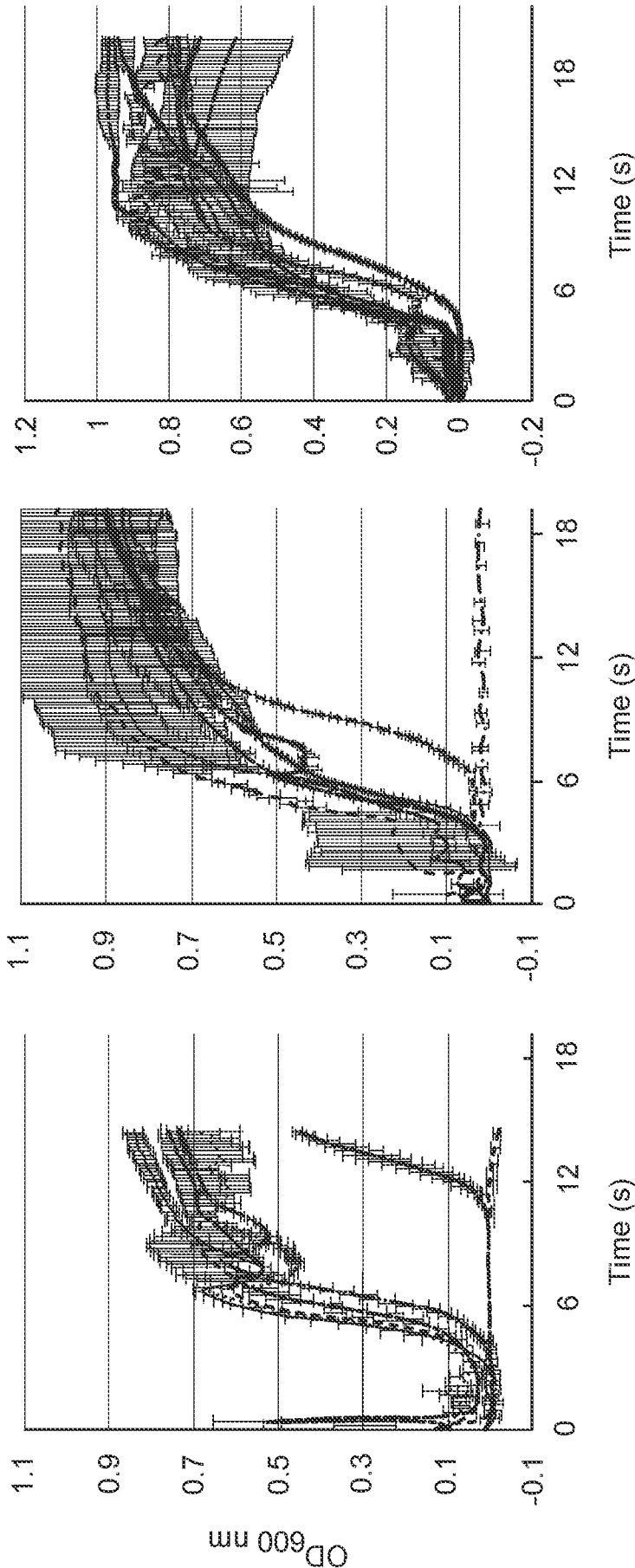


FIG. 14C

FIG. 14B

FIG. 14A

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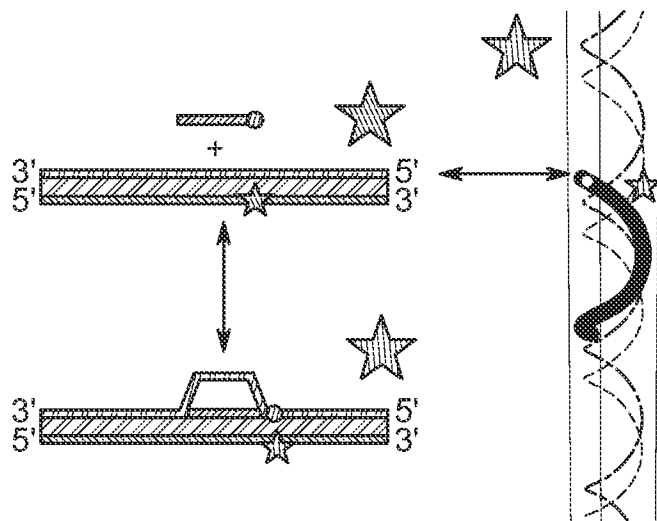
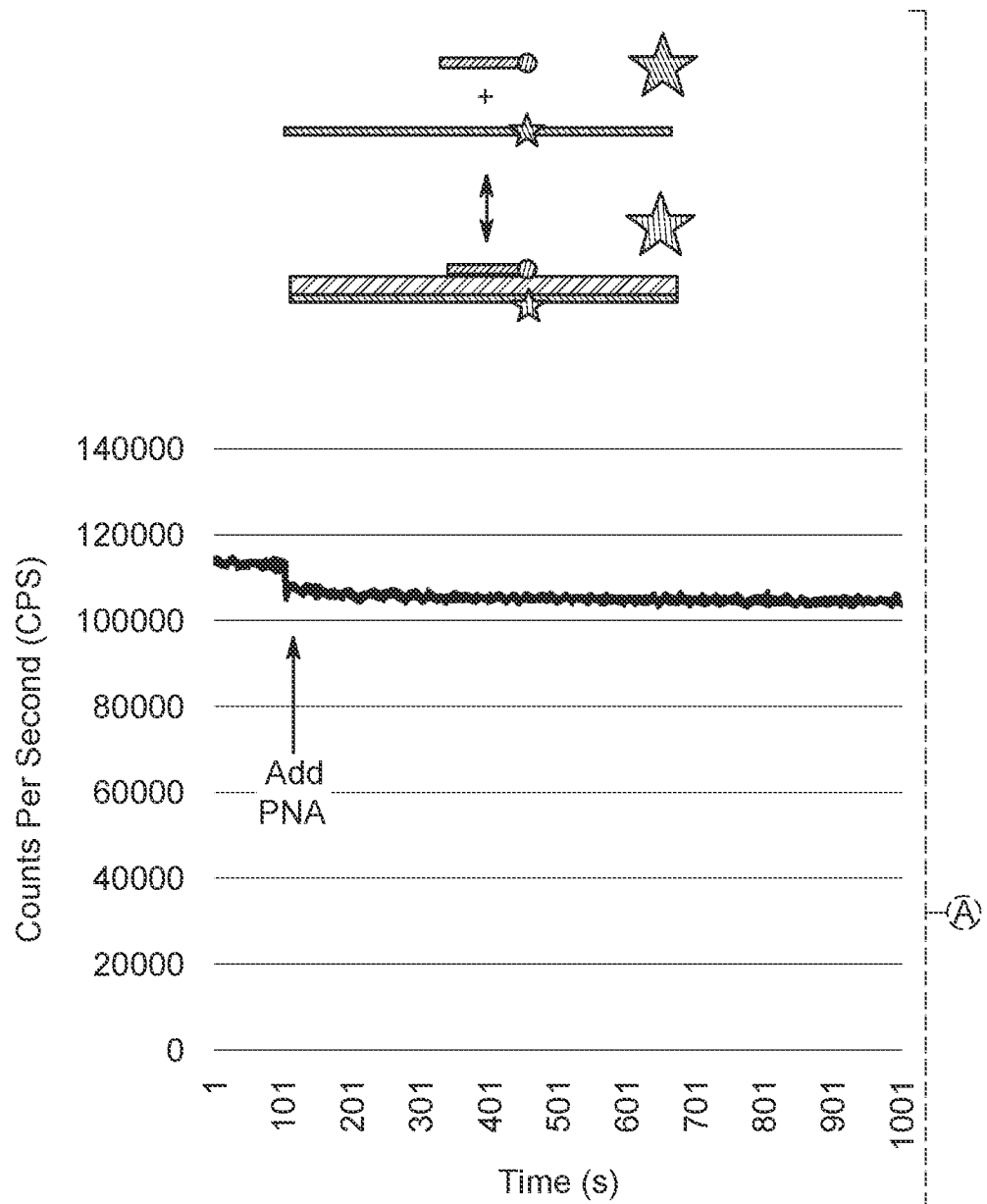


FIG. 15

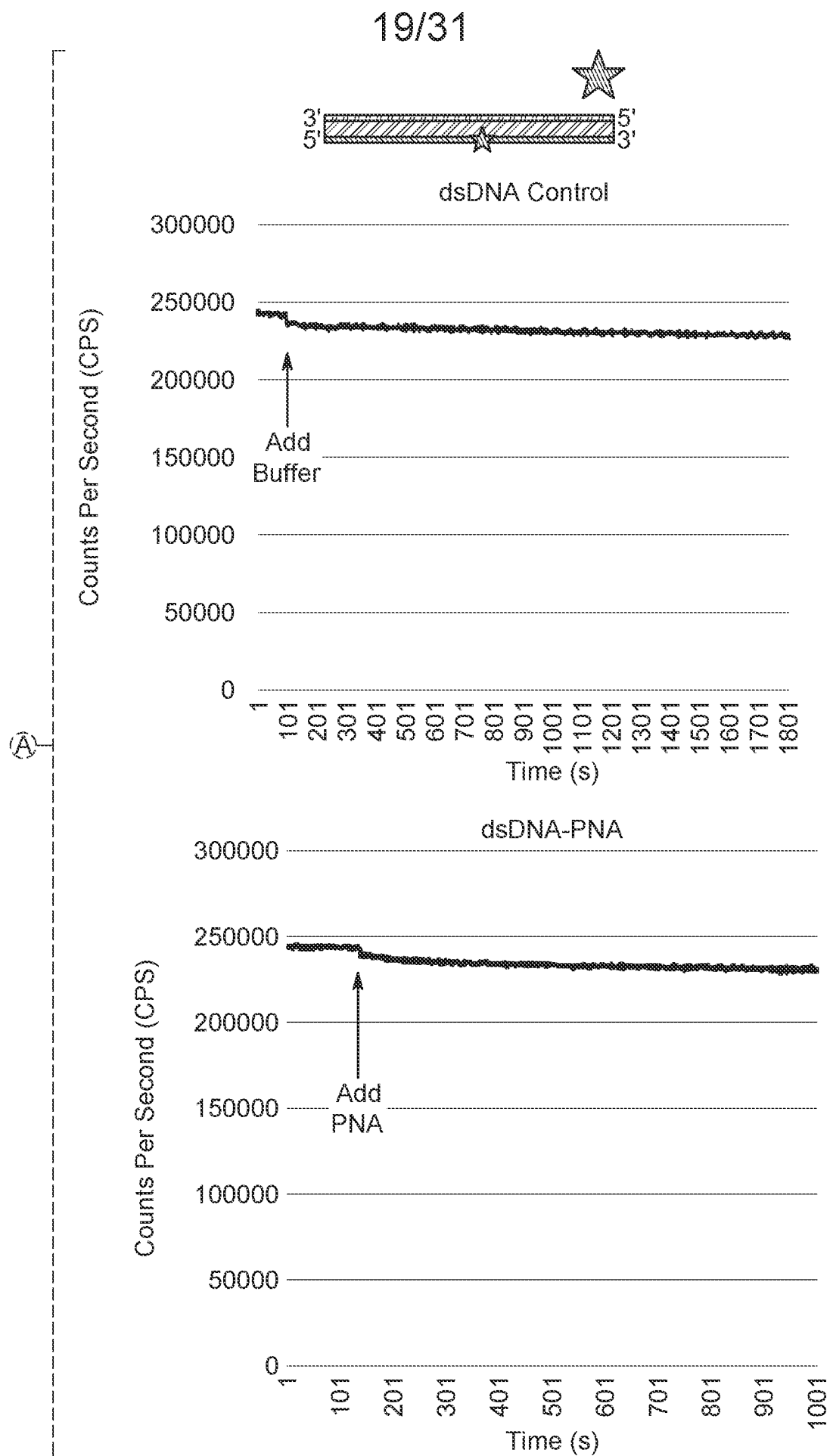


FIG. 15 (Continued)

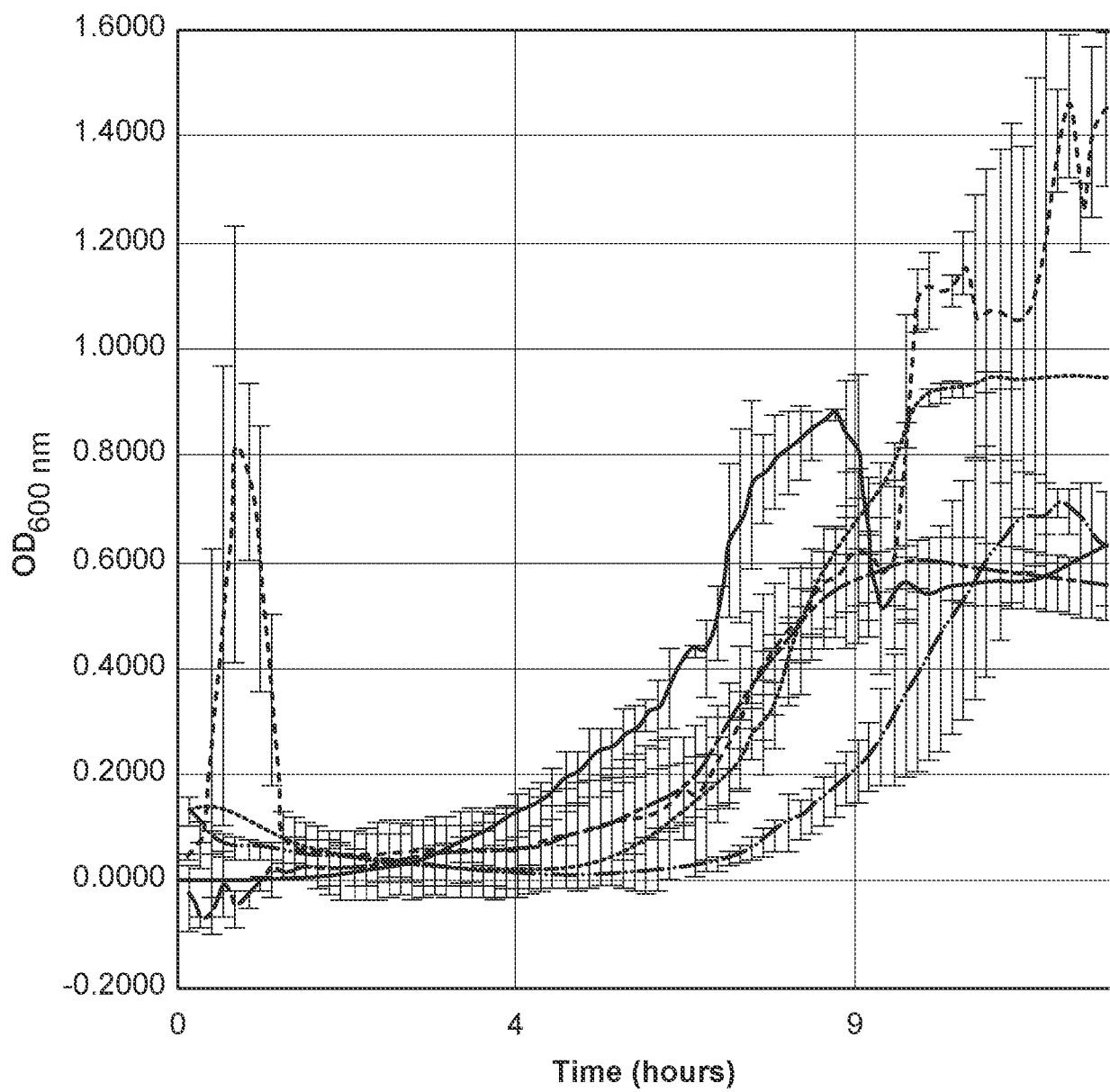


FIG. 16

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FIG. 17B



FIG. 17A

GGTGGTGG	<i>E.coli</i> Antisense binding energy (kcal/mol)	<i>K.quasipneumoniae</i> Antisense binding energy (kcal/mol)	<i>H.influenzae</i> Antisense binding energy (kcal/mol)	<i>C.freundii</i> Antisense binding energy (kcal/mol)
Sfold mreB	-0.3; -1.5	0.5	2.8	1.2
Sfold flsZ	-0.7	Sequence not in gene	Sequence not in gene	Sequence not in gene
Sfold rodZ	-5.0	0.4	-5.1	-0.8
Sfold flsA	1.0	1.9	-0.1; 2	-0.9

FIG. 18

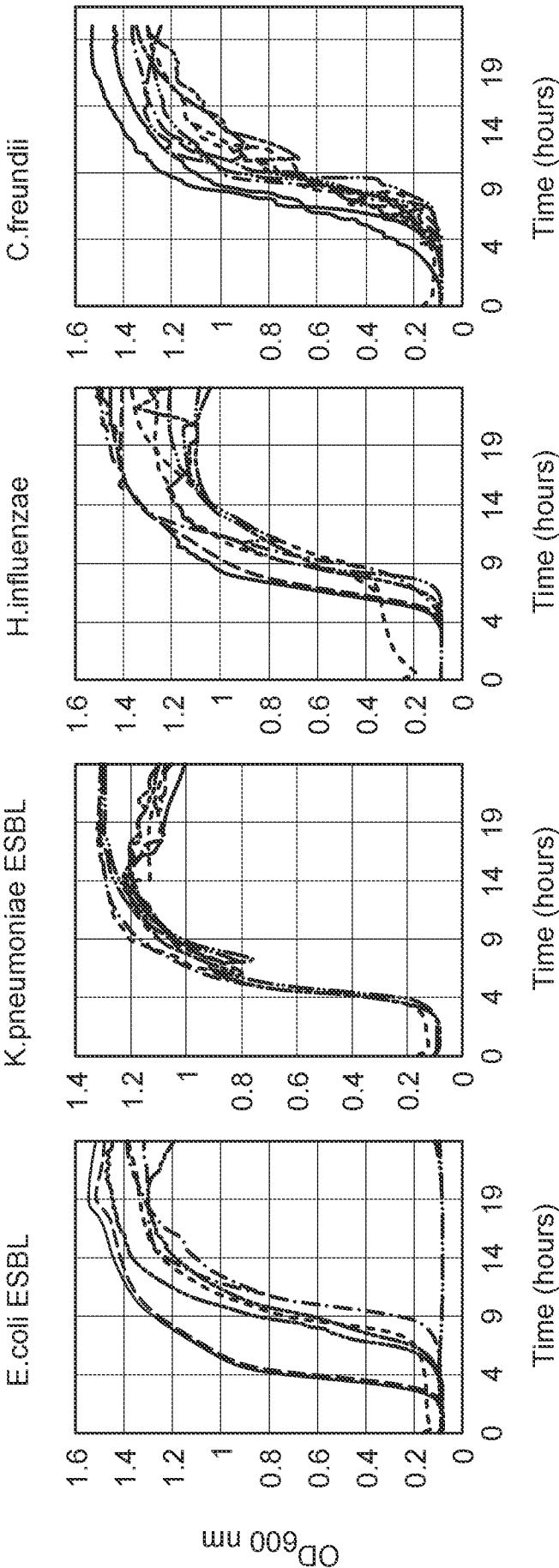


FIG. 19

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c MG1655 with 10 nt Ave Subtract

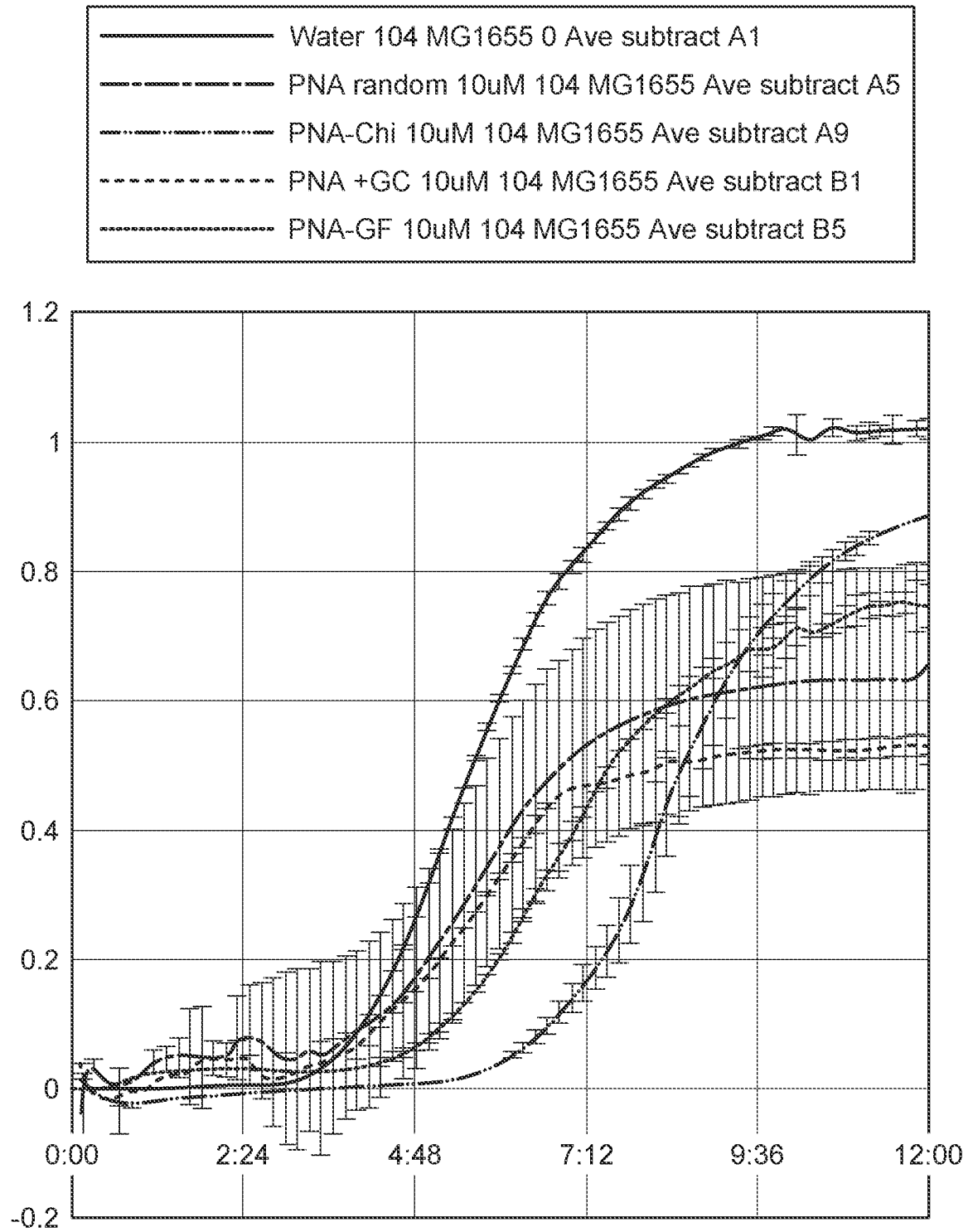


FIG. 20

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g 10 nt AS19

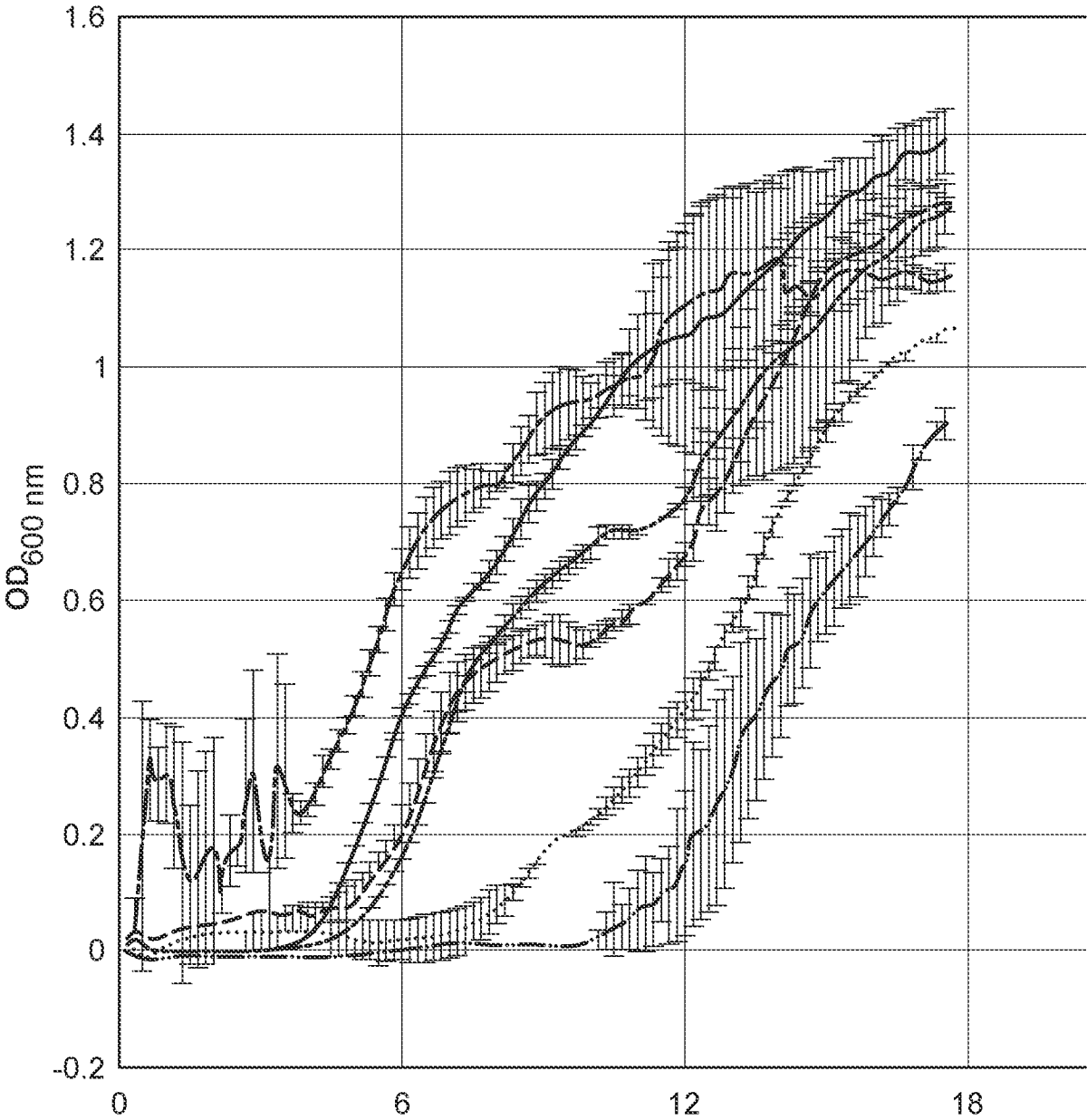
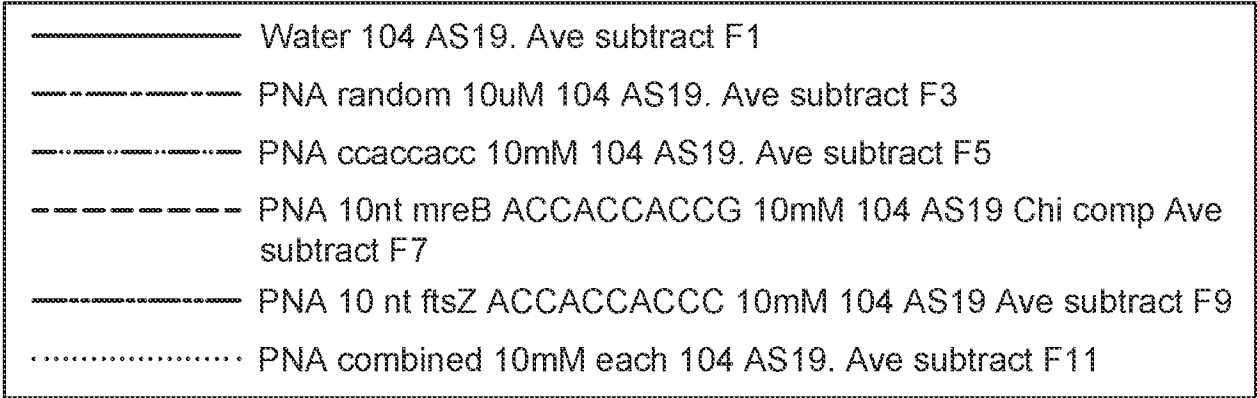


FIG. 21

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a MG1655 PNA synergy subtract Ave

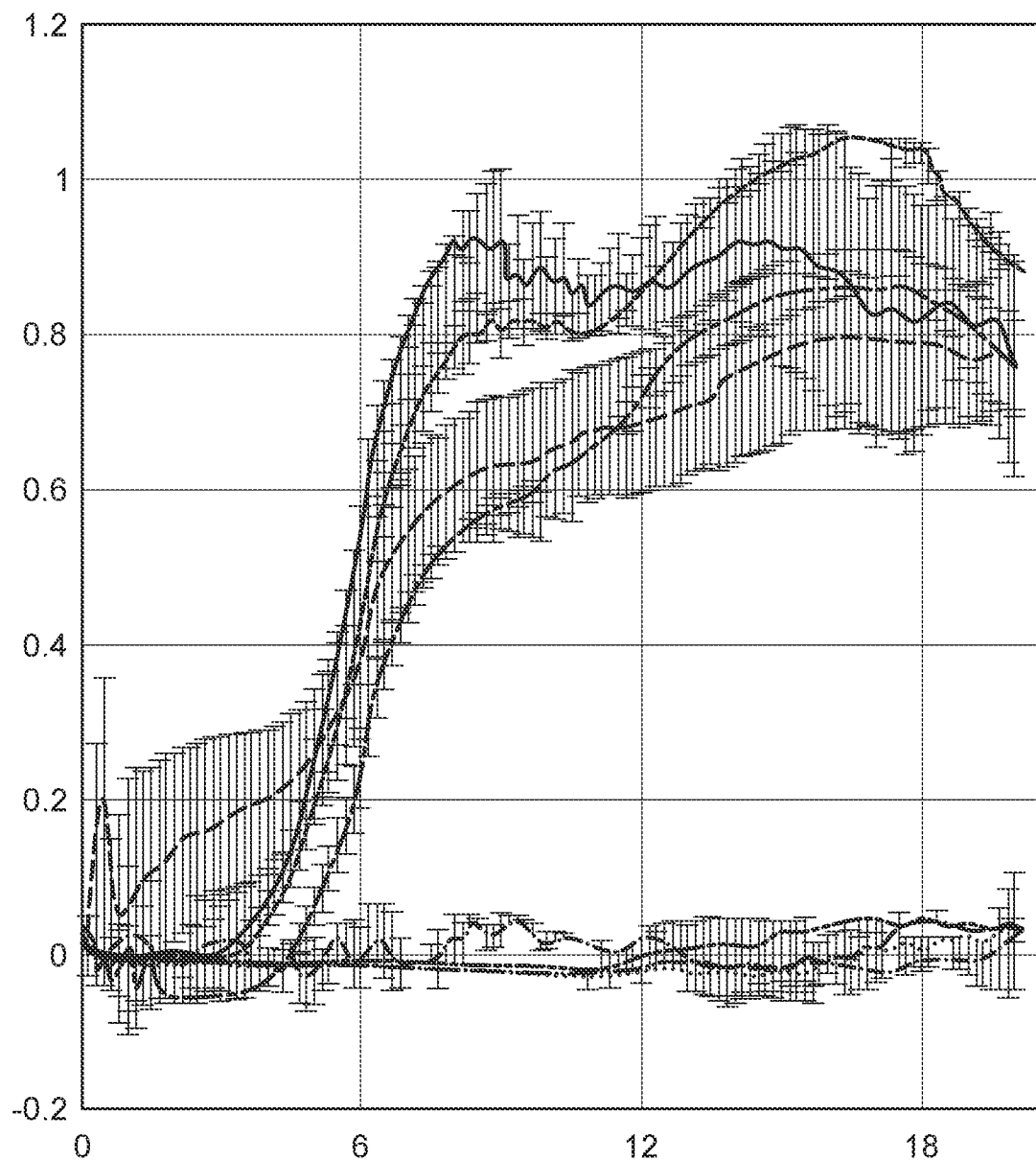
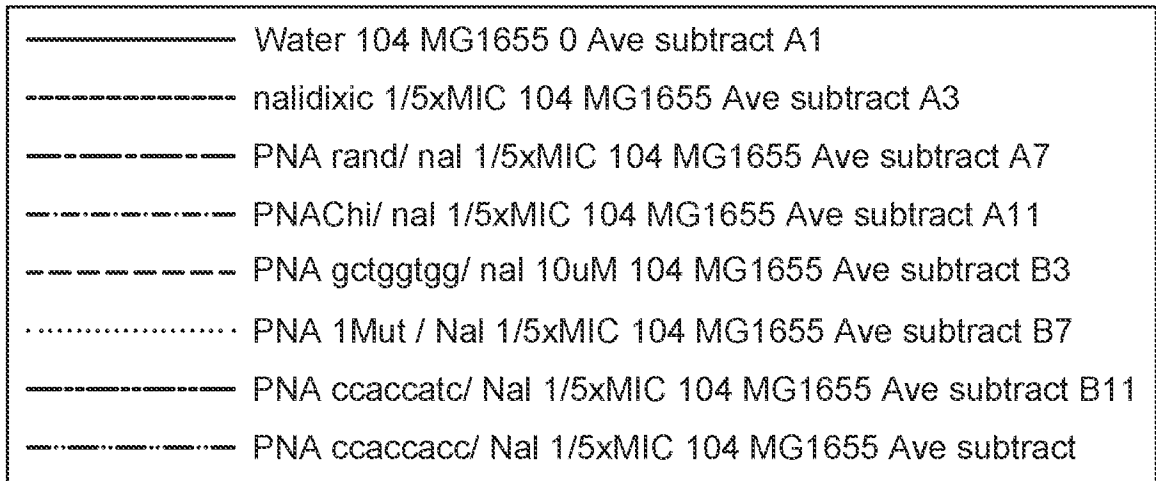


FIG. 22

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f 6 nt sequences 10 micromolar and 30
micromolar concentrations subtract Ave

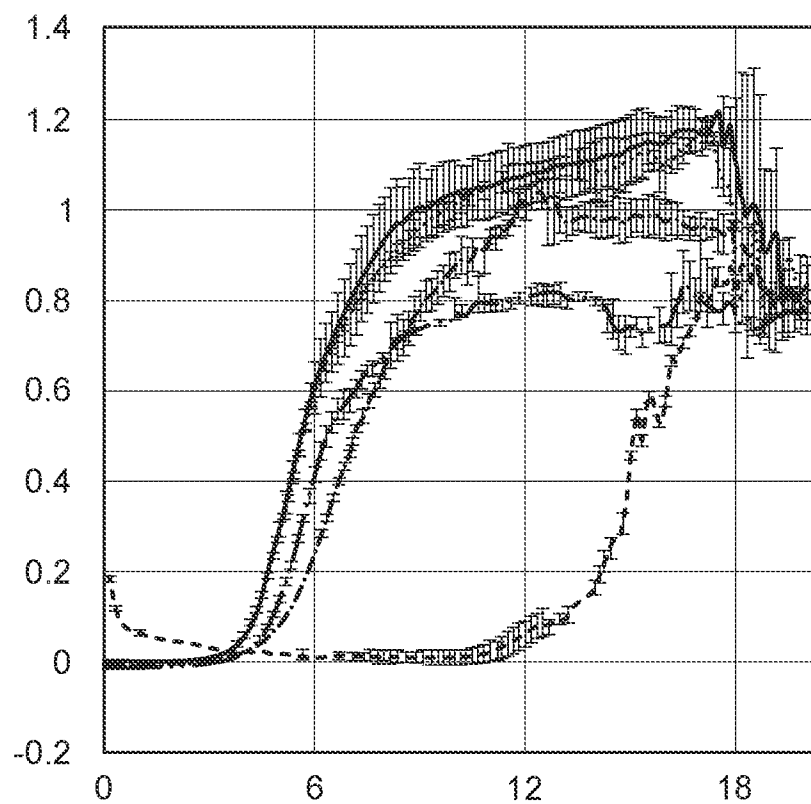
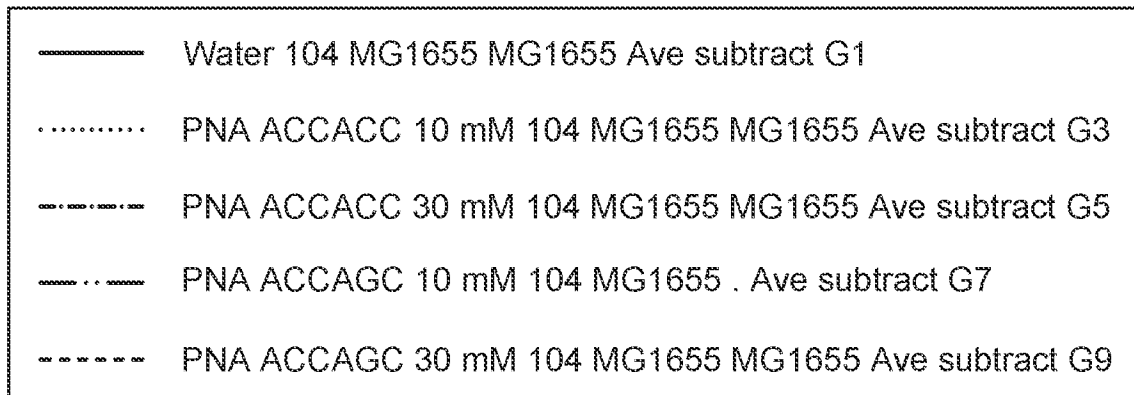


FIG. 23

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f 6 nt sequences (solid lines) with a 8 nt sequences
(dashed line) 10 micromolar subtract Ave

- Water 104 MG1655 MG1655 Ave subtract G1
- PNA ACCACC 10 mM 104 MG1655 MG1655 Ave subtract G3
- PNA ACCAGC 10 mM 104 MG1655 . Ave subtract G7
- Water 104 MG1655 0 Ave subtract A1
- PNA random 10uM 104 MG1655 Ave subtract A5
- PNA-Chi 10 uM 104 MG1655 Ave subtract A9
- PNA ccaccacc GGTGGTGG 10mM 104 MG1655 0 44721 Ave subtract C1

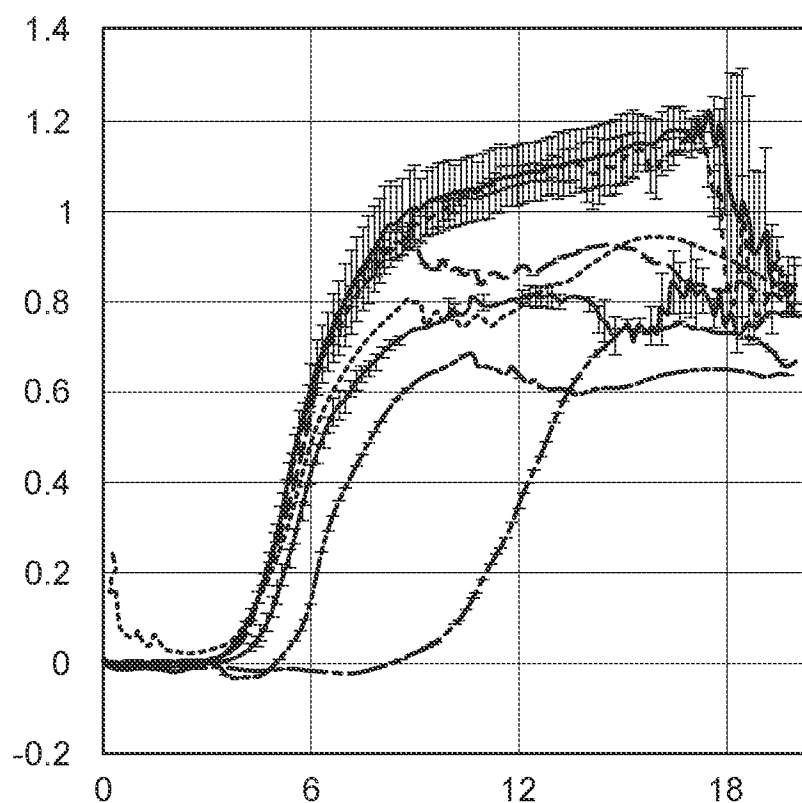


FIG. 24

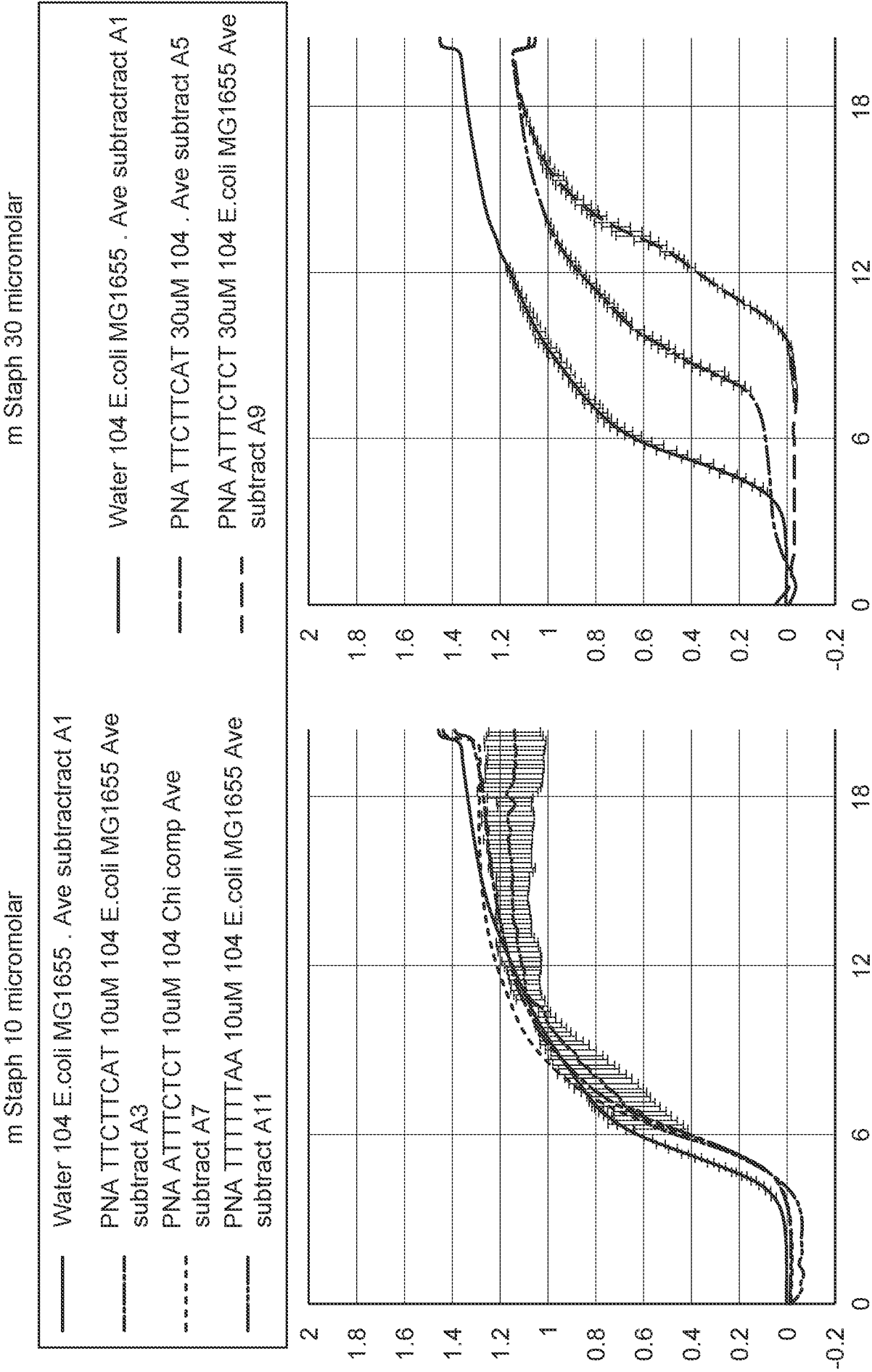


FIG. 25

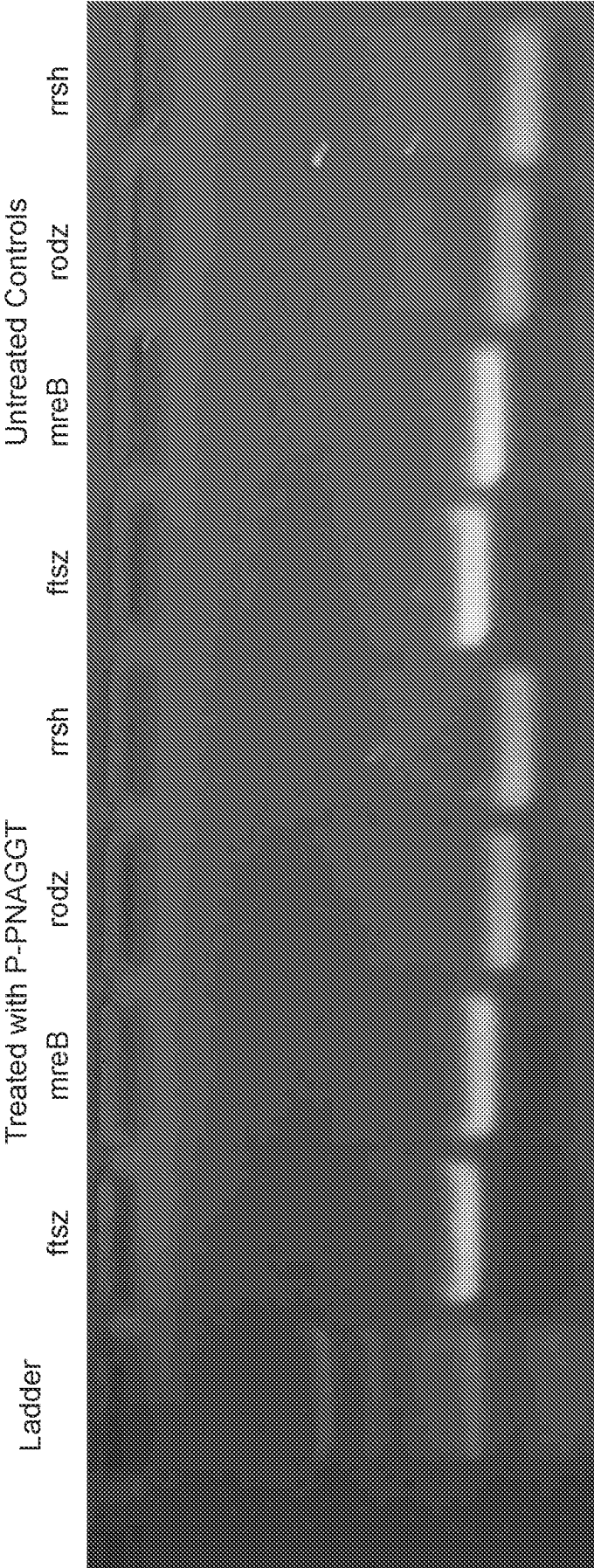


FIG. 26

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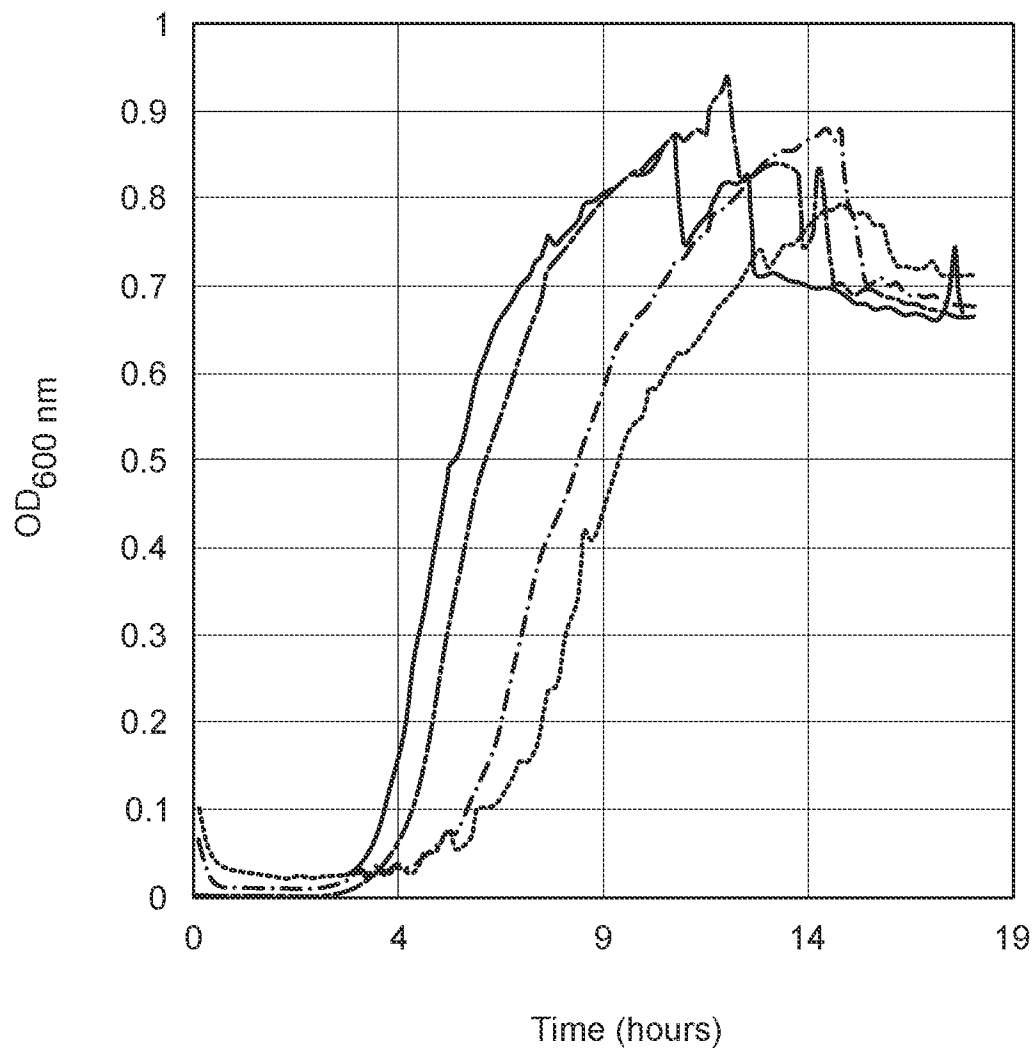


FIG. 27