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(54) Title: COMPOSITIONS COMPRISING COCKTAILS OF ANTIBACTERIAL PHAGES AND USES THEREOF FOR THE TREATMENT OF BACTERIAL INFECTIONS

(57) Abstract: The present invention is directed to the field of phage therapy for the treatment and control of bacterial infections, in particular diabetic foot infections. More specifically, the present invention is directed to novel cocktails of bacteriophage strains F44/10, F1 25/10, F770/05, F510/08, F1 245/05, and/or variants thereof; and methods of using same in the treatment and prevention of bacterial infections, including cutaneous ulcers associated with diabetic foot infections, caused by, e.g., Staphylococcus aureus, Pseudomonas aeruginosa, and/or Acinetobacter baumannii. The cocktails are used as pharmaceutical compositions either alone or in further combination with other therapies, e.g., antibiotics, growth factors, or other standard, as well as non-standard, therapies for diabetic foot infections.



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COMPOSITIONS COMPRISING COCKTAILS OF ANTIBACTERIAL PHAGES AND USES THEREOF FOR THE TREATMENT OF BACTERIAL INFECTIONS

1. SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 14, 2012, is named 16395105.txt and is 574,104 bytes in size.

2. FIELD OF THE INVENTION

[0002] The present invention is directed to the field of phage therapy for the treatment and control of bacterial infections, in particular chronic ulcers such as diabetic foot infections. More specifically, the present invention is directed to novel cocktails of bacteriophage strains F44/10, F125/10, F770/05, F510/08, F1245/05, other phage, and/or variants thereof; and methods of using same in the treatment and prevention of bacterial infections, including cutaneous ulcers associated with diabetic foot infections, caused by, e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and/or *Acinetobacter baumannii*. The cocktails are used as pharmaceutical compositions either alone or in further combination with other therapies, e.g., antibiotics, growth factors, or other standard and non-standard therapies for chronic ulcers .

3. BACKGROUND

[0003] Diabetic foot infections (DFIs) are a frequent and serious complication of diabetes mellitus (DM) and are the world leading cause of non-traumatic lower limb amputation (Jeffcoate WJ, et al. 2003. *Lancet* 361:1545-1551). In current clinical practice, the treatment of DFIs includes debridement and systemic antibiotics (see, e.g., Lipsky BA, et al. 2004. *Clin Infect Dis.* 39:885-910). Nonetheless, because of deficient vascularization and the local microenvironment, antibiotic concentrations are many times sub-therapeutic (Lipsky BA, et al. 2009. *Clin Infect Dis.* 49:1541-1549). Moreover, the increasing incidence of multidrug resistant organisms, such as methicillin-resistant *Staphylococcus aureus*, as well as pan-drug-resistant non-fermenting negative bacilli, is threatening the outcome in increasing numbers of community and hospitalized patients (Mendes JJ, et al. 2012. *Diabetes Res Clin Pract.* 95(1):153-161; Tascini C, et al. 2011. *Diabetes Res Clin Pract* 94 (1):133-139). Accordingly, there remains a need to identify new strategies for the treatment, control, and management of DFIs.

[0004] Topical treatment provides the advantages of avoiding systemic adverse effects, providing increased target site concentration, and allowing the use of agents not available for systemic therapy. Mechanical debridement improves topical treatment because it reduces the bio-burden of bacteria present and also opens a time-dependent therapeutic window for topical antimicrobial therapy (TAT) (Wolcott RD, et al. 2010. J Wound Care 19:320-328). Nevertheless, to date, no TAT agent has been proven to be effective for treating DFI (Nelson EA, et al. 2006. Diabet Med 23:348-359).

[0005] Bacteriophage (phage) are viruses that specifically infect and lyse bacteria. Phage therapy, a method of using whole phage viruses for the treatment of bacterial infectious diseases, was introduced in the 1920s by Felix d'Herelle. With the development of antibiotics in the 1940s, however, interest in phage-based therapeutics declined in the Western world. One of the most important factors that contributed to this decline was the lack of standardized testing protocols and methods of production. The failure to develop industry wide standards for the testing of phage therapies interfered with the documentation of study results, leading to a perceived lack of efficacy, as well as problems of credibility, regarding the value of phage therapy. Another problem in phage production related to the purity grade of commercial preparations of phage, with preparations containing undesired bacterial components, e.g., endotoxins. Accordingly, adverse events were often associated with the preparations, particularly in patients receiving them intravenously.

[0006] Nevertheless, in Eastern Europe and the former Soviet Union, where access to antibiotics was limited, the development and use of phage therapy continued jointly with, or in place of, antibiotics. Further, with the rise of antibiotic resistant strains of many bacteria, interest in phage-based therapeutics has returned in the Western world. That is, even though novel classes of antibiotics may be developed, the prospect that bacteria will eventually develop resistance to the new drugs has intensified the search for non-chemotherapeutic means for controlling, preventing, and treating bacterial infections.

[0007] Lytic bacteriophage, especially when complemented by adequate mechanical debridement, offer a solution to treating DFIs, e.g., for use as novel TAT agents. Lytic bacteria can offer the advantages of specificity and efficiency in lysing pathogenic bacteria, even those associated with multidrug resistance (Rossney AS, et al. 1994. J Hosp Infect 26:219-234.) Further advantages can include absence of pathogenicity to man and animals (Burrowes B, et al.

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2011. *Expert Rev Anti Infect Ther* 9:775-785), antibacterial activity against bacteria in biofilms, and activity in microaerophilic environments, even with high bacterial load (Azeredo J, et al. 2008. *Curr Pharm Biotechnol* 9:261-266), as well as the generally accepted safety of bacteriophage therapy in some parts of the world (Sulakvelidze A, et al., 2001, *Antimicrob Agents Chemother.* 45(3): 649-659). Recent animal trials of bacteriophage therapy have demonstrated its potential to heal or improve skin bacterial diseases, both in internal (McVay CS, et al. 2007. *Antimicrob Agents Chemother* 51:1934-1938) and external applications (Soothill JS. 1994. *Burns* 20:209-211; Wills QF, et al. 2005. *Antimicrob Agents Chemother* 49:1220-1221). However, there is little published evidence supporting the use of bacteriophage to cure infections established for longer than a few hours (Ryan EM, et al. 2011 *J Pharm Pharmacol* 63:1253-1264).

[0008] In particular, phage cocktails may provide advantages to the use of phages individually, e.g., to increase the lytic activity against a particular bacterial strain, and to decrease the possibility of emergence of bacteria resistant to an individual bacteriophage. That is, different bacteriophage can be mixed as cocktails to broaden their properties, preferably resulting in a collectively greater antibacterial spectrum of activity e.g., an expanded host range, to which development of resistance is less likely. Nonetheless, to date, few phage cocktails exist with antimicrobial activity against different bacteria, possibly because of the difficulty in combining different specificities of bacteriophage while maintaining storage stability.

[0009] There is therefore a need to develop novel phage products as therapeutic and/or prophylactic agents for use in vivo against pathogenic bacteria. There also is a need for better treatments, particularly topical treatments, for DFIs. In particular, there is a need for bacteriophage cocktails capable of lysing bacteria responsible for DFIs, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and/or *Acinetobacter baumannii*. This application addresses this and other needs.

[0009a] Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment, or any form of suggestion, that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be ascertained, understood and regarded as relevant by a person skilled in the art.

[0009b] As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude other additives, components, integers or steps.

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4. SUMMARY OF THE INVENTION

[0009c] Embodiments of the invention are defined by each of the accompanying claims. For example, in one embodiment, the invention relates to a composition comprising a first and a second purified strain of bacteriophage, each of said strains having a genome which comprises at least 95% sequence identity to the nucleotide sequence selected from the group of consisting of SEQ ID NO:1 and SEQ ID NO:2, and showing antibacterial activity against *Staphylococcus aureus*; a third purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:3 and showing antibacterial activity against *Pseudomonas aeruginosa*; a fourth purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:4, and showing antibacterial activity against *Pseudomonas aeruginosa*; and a fifth purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:5 and showing antibacterial activity against *Acinetobacter baumannii* as defined by claim 1.

[0010] In one aspect, the present invention is directed to compositions comprising phage cocktails. In some embodiments, the invention provides compositions comprising at least two different isolated strains of bacteriophage, each having a genome that comprises a nucleic acid sequence selected from the group of consisting of SEQ ID NO:1 (F44/10), SEQ ID NO:2 (F125/10), SEQ ID NO:3 (F770/05), SEQ ID NO:4 (F510/08), and SEQ ID NO:5 (F1245/05),

or a variant thereof, the variant having at least 95% sequence identity to the corresponding nucleic acid sequence and showing antibacterial activity against at least one of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and/or *Acinetobacter baumannii*. In some embodiments, one of the at least two bacteriophage strains is the strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2, or the variant thereof. In some embodiments, the at least two bacteriophage strains are the strains having genomes that comprise the nucleic acid sequences of SEQ ID NO:1 and SEQ ID NO:2, or variants thereof. In some embodiments, the composition further comprises at least a third bacteriophage strain, the third strain having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or the variant thereof. In some embodiments, the composition further comprises at least a third and a fourth bacteriophage strain, the third and fourth strains each having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or the variant thereof. In some embodiments, one of the at least two bacteriophage strains is the strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:3 or SEQ ID NO:4, or the variant thereof. In some embodiments, the at least two bacteriophage strains are the strains having genomes that comprise the nucleic acid sequences of SEQ ID NO:3 and SEQ ID NO:4 or the variant thereof. In some embodiments, the composition further comprises at least a third bacteriophage strain, the third strain having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:5, or the variant thereof. In some embodiments, the composition further comprises at least third and fourth bacteriophage strains, the third and fourth strains each having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:5, or the variant thereof. In some embodiments, one of the at least two bacteriophage strains is the strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:5, or the variant thereof. In some embodiments, the composition further comprises at least a third bacteriophage strain, the third strain having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4, or the variant thereof. In some embodiments, the composition further comprises at least third and fourth bacteriophage strains, the third and fourth strains each having

a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4, or the variant thereof.

[0011] In some preferred embodiments, the invention is directed to a composition comprising at least five isolated bacteriophage strains, the strains having genomes that comprise nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or a variant thereof, where the variant has at least 95% sequence identity to the corresponding nucleic acid sequence and shows antibacterial activity against at least one of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and/or *Acinetobacter baumannii*. In some embodiments, the bacteriophage strains having genomes that comprise the nucleic acid sequences of SEQ ID NOs:1, 2, 4, and 5, or the variants thereof, are each present in the composition in an amount corresponding to about 10 times that of the bacteriophage strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:3, or the variant thereof. In some embodiments, the composition comprises bacteriophage strains having genomes that comprise nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

[0012] In another aspect, the instant invention is directed to pharmaceutical compositions comprising phage cocktails, specifically, pharmaceutical compositions comprising any of the compositions described above and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition is formulated for topical application. In some embodiments, the pharmaceutical composition comprises a sterile buffer, e.g., a buffer comprising about 0.05 M Tris-HCl, about 0.1M NaCl, and about 10mM MgSO₄·7H₂O. In some embodiments, the pharmaceutical composition is contained in an ampoule.

[0013] In some embodiments, the pharmaceutical composition further comprises an additional agent, e.g., an agent selected from the group consisting of an antibiotic agent, an anti-inflammatory agent, an antiviral agent, a local anesthetic agent, a growth factor, and a corticosteroid. In some embodiments, the additional agent is an antibiotic agent, e.g., an antibiotic agent having antibacterial activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and/or *Staphylococcus aureus*; or an antibiotic agent having antibacterial activity against bacteria other than *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. More specifically, in some embodiments, the additional agent is an antibiotic agent having antibacterial activity against *Staphylococcus aureus* or an antibiotic agent having antibacterial activity against bacteria other than *Staphylococcus aureus*. In some

embodiments, the additional agent is an antibiotic agent having antibacterial activity against *Pseudomonas aeruginosa* or an antibiotic agent having antibacterial activity against bacteria other than *Pseudomonas aeruginosa*. In some embodiments, the additional agent is an antibiotic agent having antibacterial activity against *Acinetobacter baumannii* or an antibiotic agent having antibacterial activity against bacteria other than *Acinetobacter baumannii*. In some embodiments, administration of the antibiotic agent comprises systemic administration.

[0014] In some embodiments, the composition is for use in treating a bacterial infection associated with an area of non-intact skin, and each of the phage strains is present in the composition in an amount corresponding to 10^3 to 10^{13} phage particles/cm² of the area. In some embodiments, each of the phage strains is present in the composition in an amount corresponding to 10^7 to 10^9 phage particles/cm² of the area.

[0015] Another aspect of the instant invention is directed to methods of treating or preventing a bacterial infection in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the pharmaceutical composition according to the invention. In some embodiments, the bacterial infection is an infection by one or more of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. In some embodiments, the pharmaceutical composition is administered topically. In some embodiments, the subject is a mammal, e.g., a human. In some embodiments, the bacterial infection is diabetic foot infection. In some embodiments, the diabetic foot infection comprises a cutaneous ulcer. In some embodiments, the bacterial infection is associated with an area of non-intact skin selected from a sore associated with cellulitis, an erysipelas lesion, a burn wound, a chronic ulcer, a decubitus ulcer, and a pressure sore. In some embodiments, the treatment comprises topically administering the pharmaceutical composition to a cutaneous ulcer associated with diabetic foot infection. In some preferred embodiments, administration follows mechanical debridement of the ulcer. In some embodiments, administration comprises use of at least one of a dressing, an instillation device, and a negative pressure wound therapy device.

[0016] In some embodiments, the pharmaceutical composition is administered every 4 hours or every 6 hours for an initial 24 hours. In some embodiments, following the initial 24 hours, the pharmaceutical composition is administered every 12 hours or every 24 hours for at least 3 additional days. In some embodiments, the pharmaceutical composition is administered every 12 hours or every 24 hours for at least 4 additional days.

[0017] In some embodiments, the method is used in combination with a standard therapy for diabetic foot infection, e.g., a standard therapy selected from the group consisting of extracellular matrix replacement therapy, moist wound therapy, negative pressure wound therapy, arterial re-vascularization therapy, hyperbaric oxygen therapy, administration of an antibiotic agent, and administration of a growth factor. In some embodiments, the moist wound therapy comprises use of an adhesive-backing film, a silicone-coated foam, a hydrogel, and/or a hydrocolloid. In some embodiments, the extracellular matrix replacement therapy comprises use of bio-engineered tissue. In some embodiments, administration of the antibiotic agent comprises systemic administration. In some embodiments, the growth factor is at least one selected from the group consisting of platelet-derived growth factor, granulocyte colony-stimulating factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, and vascular endothelial growth factor. In some embodiments, administration of the growth factor comprises topical administration. In some embodiments, the method is used in combination with a non-standard therapy for diabetic foot infection, e.g., where diabetic foot infection is refractory to a standard therapy.

4.1 DEFINITIONS

[0018] As used herein, the term “isolated” in the context of nucleic acid molecules refers to a first nucleic acid molecule which is separated from other nucleic acid molecules which are present in the natural source of the first nucleic acid molecule. Moreover, an “isolated” nucleic acid molecule, such as a cDNA molecule, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized and may be free of other cDNA or other genomic DNA molecules, e.g., where it has been isolated from other clones in a nucleic acid library. Further, “isolated” genomic DNA is substantially free of other viral cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized, and may be free of other cDNA or other genomic DNA molecules, e.g., where it has been isolated from preparations containing more than bacteriophage and/or bacterial strain.

[0019] The term “purified” with respect to a bacteriophage means that the phage has been measurably increased in concentration by any purification process, including but not limited to, isolation from the environment or culture, e.g., isolation from culture following propagation

and/or amplification, centrifugation, etc., thereby partially, substantially, nearly completely, or completely removing impurities, such as host cells and host cell components. One of skill in the art will appreciate the amount of purification necessary for a given use. For example, an isolated phage meant for use in therapeutic compositions intended for administration to humans ordinarily must be of high purity in accordance with regulatory standards and good manufacturing processes.

[0020] The term “purified” means that the peptide, polypeptide, fusion protein, or nucleic acid molecule has been measurably increased in concentration by any purification process, including but not limited to, column chromatography, HPLC, precipitation, electrophoresis, etc., thereby partially, substantially, nearly completely, or completely removing impurities, such as precursors or other chemicals involved in preparing the peptide, polypeptide, fusion protein, or nucleic acid molecule. One of skill in the art will appreciate the amount of purification necessary for a given use. For example, isolated genomic DNA meant for use in therapeutic compositions intended for administration to humans ordinarily must be of high purity in accordance with regulatory standards and good manufacturing processes.

[0021] As used herein, the term “variant” in the context of nucleic acid sequences refers to a nucleic acid sequence that comprises or consists of a nucleic acid sequence having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with a reference nucleic acid sequence. A variant may be selected that maintains one or more function of the reference nucleic acid sequence. For example, a variant bacteriophage may exhibit at least one biological activity, e.g., antimicrobial or antibacterial activity (e.g., lytic killing activity), of the bacteriophage from which it is derived.

[0022] As used herein, the term “host cell” refers to the particular subject cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell that contain the nucleic acid molecule or chromosomally integrated version thereof. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome. For the generation of bacteriophage, the host cell may or may not be of the same species or strain from which the bacteriophage was isolated or cultured.

[0023] As used herein, the term “in combination” or “in further combination” or “further in combination” refers to the use of an additional prophylactic and/or therapeutic agent as well as a phage cocktail of the invention. The use of the term “in combination” does not restrict the order in which prophylactic and/or therapeutic agents are administered to a subject. A first prophylactic or therapeutic agent can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second prophylactic or therapeutic agent (different from the first prophylactic or therapeutic agent) to a subject.

[0024] As used herein, the terms “prophylactic agent” and “prophylactic agents” refer to an agent, such as a bacteriophage cocktail of the invention, which can be used in the prevention, management, or control of one or more symptoms of a disease or disorder, in particular, a disease or disorder associated with a bacterial infection, such as diabetic foot infection.

[0025] As used herein, the terms “therapeutic agent” and “therapeutic agents” refer to an agent, such as a bacteriophage cocktail of the invention, that can be used in the treatment, management, or control of one or more symptoms of a disease or disorder, in particular, a disease or disorder associated with a bacterial infection, such as diabetic foot infection.

[0026] As used herein, the terms “treat”, “treatment” and “treating” refer to obtaining a therapeutic benefit in a subject receiving a pharmaceutical composition. With respect to achieving a therapeutic benefit, the object is to eliminate, lessen, decrease the severity of, ameliorate, or slow the progression of the symptoms or underlying cause (e.g., bacterial infection) associated with the pathological condition or disorder. A “therapeutically effective amount” refers to that amount of a therapeutic agent, such as a phage cocktail pharmaceutical composition of the invention, sufficient to achieve at least one therapeutic benefit in a subject receiving the pharmaceutical composition.

[0027] As used herein, the terms “prevent”, “prevention” and “preventing” refer to obtaining a prophylactic benefit in a subject receiving a pharmaceutical composition. With respect to achieving a prophylactic benefit, the object is to delay or prevent the symptoms or underlying

cause (e.g., bacterial infection) associated with the pathological condition or disorder. A “prophylactically effective amount” refers to that amount of a prophylactic agent, such as a phage cocktail pharmaceutical composition of the invention, sufficient to achieve at least one prophylactic benefit in a subject receiving the pharmaceutical composition.

[0028] As used herein, the terms “antibacterial activity” and “antimicrobial activity”, with reference to a bacteriophage (or variant or fragment thereof) or bacteriophage product, are used interchangeably to refer to the ability to kill and/or inhibit the growth or reproduction of a microorganism, in particular, the bacteria of the species or strain that the bacteriophage infects. In certain embodiments, antibacterial or antimicrobial activity is assessed by culturing bacteria, e.g., Gram-positive bacteria (e.g., *S. aureus*), Gram-negative bacteria (e.g., *A. baumannii*, *E. coli*, and/or *P. aeruginosa*) or bacteria not classified as either Gram-positive or Gram-negative, according to standard techniques (e.g., in liquid culture or on agar plates), contacting the culture with a bacteriophage or variant thereof of the invention and monitoring cell growth after said contacting. For example, in a liquid culture, the bacteria may be grown to an optical density (“OD”) representative of a mid-point in exponential growth of the culture; the culture is exposed to one or more concentrations of one or more bacteriophage of the invention, or variants thereof, and the OD is monitored relative to a control culture. Decreased OD relative to a control culture is representative of a bacteriophage exhibiting antibacterial activity (e.g., exhibits lytic killing activity). Similarly, bacterial colonies can be allowed to form on an agar plate, the plate exposed to one or more bacteriophage of the invention, or variants thereof, and subsequent growth of the colonies evaluated related to control plates. Decreased size of colonies, or decreased total numbers of colonies, indicate a bacteriophage with antibacterial activity.

5. BRIEF DESCRIPTION OF THE FIGURES

[0029] FIG. 1 illustrates the preparation of an exemplary phage cocktail composition in accordance with the instant invention.

[0030] FIG. 2 illustrates the study protocol used for demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0031] FIG. 3 illustrates the study protocol used for demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0032] FIG. 4 illustrates results of lytic studies evaluated against *Staphylococcus aureus* 743/06, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention.

[0033] FIG. 5 illustrates results of lytic studies evaluated against *Pseudomonas aeruginosa* 433/07, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention.

[0034] FIG. 6 illustrates results of lytic studies evaluated against *Acinetobacter baumannii* 1305/05, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention.

[0035] FIG. 7 illustrates results of microbial load analyses for control (C) and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0036] FIG. 8 illustrates results of wound closure analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0037] FIG. 9 illustrates results of histological analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0038] FIG. 10 illustrates results of microbial load analyses for control (C) and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0039] FIG. 11 illustrates results of wound closure analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0040] FIG. 12 illustrates results of histological analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and

Acinetobacter baumannii-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0041] FIG. 13 illustrates diabetic foot infection classifications and application of phage therapy thereto using exemplary phage cocktail compositions in accordance with the instant invention.

[0042] FIG. 14 illustrates a clinical study design for exemplary phage cocktail compositions in accordance with the instant invention for use in therapy for diabetic foot ulcers.

5.1 DETAILED DESCRIPTION

[0043] The present invention is directed to phage therapy for the treatment and control of bacterial infections, in particular, diabetic foot infections. In one aspect, the invention relates to novel cocktail compositions of different bacteriophage strains. The “cocktail” may comprise at least two different isolated strains of bacteriophage, for example, two, three, four, five, six, seven, eight, nine, ten, or more different isolated bacteriophage strains. The cocktail may be used alone or in further combination with other therapies, e.g., antibiotic agents and/or growth factors.

[0044] Phage cocktails provide advantages to the use of phages individually, e.g., to increase the lytic activity against a particular bacterial strain and/or to decrease the possibility of emergence of bacteria resistant to an individual bacteriophage. Different bacteriophage can be mixed as cocktails to broaden their properties, preferably resulting in a collectively greater antibacterial spectrum of activity. However, few phage cocktails exist with antimicrobial activity against different bacteria, probably because of the difficulty in combining different specificities of bacteriophage strains, while maintaining infecting ability and/or lytic activity of the individual bacteriophage in the presence of distinct bacteriophage strains.

[0045] In some particularly preferred embodiments, the instant invention provides a cocktail composition comprising the five isolated bacteriophage strains F44/10, F125/10, F770/05, F510/08, and F1245/05, where the cocktail composition is formulated as a topical formulation and finds use in the treatment and/or prevention of diabetic foot infections.

[0046] The instant invention, in some embodiments, provides cocktail compositions comprising at least two different isolated bacteriophage strains, with antibacterial activity against the same or different bacterial species or strains. In preferred embodiments, the therapeutic components of the cocktail target two or more species or strains of bacteria. In some embodiments, the phage

cocktail comprises at least 2 phage strains, at least 3 phage strains, at least 4 phage strains, at least 5 phage strains, at least 6 phage strains, at least 7 phage strains, at least 8 phage strains, at least 9 phage strains, at least 10 phage strains, or more. In some embodiments, the phage cocktail comprises 2-20 phage strains, 2-15 phage strains, 2-10 phage strains, 3-8 phage strains, or 4-6 phage strains. In more preferred embodiments, the combination does not impair or reduce (or does not substantially or significantly impair or reduce) infecting ability and/or lytic activity of the individual bacteriophage in the presence of distinct bacteriophage strains

[0047] In some embodiments, at least one phage strain of the cocktail is a strain with antibacterial activity against at least one Gram-negative bacterium, including but not limited to *Acinetobacter baumannii* and *Pseudomonas aeruginosa*; and/or against at least one Gram-positive bacteria including but not limited to *Staphylococcus aureus*. In some embodiments, the cocktail composition comprises at least two different isolated bacteriophage strains where the strains show antibacterial activity against at least one of *S. aureus*, *P. aeruginosa*, and/or *A. baumannii*. In some preferred embodiments, the cocktail composition shows antibacterial activity against at least two of *S. aureus*, *P. aeruginosa*, and/or *A. baumannii*. In some even more preferred embodiments, the cocktail composition shows antibacterial activity against each of *S. aureus*, *P. aeruginosa*, and *A. baumannii*.

[0048] In some embodiments, the cocktail composition comprises at least one phage strain showing antibacterial activity against *Staphylococcus aureus*. *S. aureus* is a Gram-positive spherical facultative anaerobe, which grows as grape-like clusters with a characteristic golden color, and the most common cause of staph infections. It is frequently part of the flora of human skin and responsible for a range of infections, including pimples, carbuncles, scalded skin syndrome, pneumonia, gastroenteritis, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. It also is frequently involved in diabetic foot infections, including but not limited to cutaneous ulcers. Such cutaneous ulcers also are referred to herein as “diabetic foot ulcers.”

[0049] Of particular concern are the methicillin-resistant *Staphylococcus aureus* strains (MRSA). MRSA remained an uncommon occurrence in hospital setting until the 1990's, when there was an explosion in MRSA prevalence in hospitals. MRSA now is considered endemic to hospitals, especially in the UK (Johnson AP et al. 2001 J. Antimicrobial Chemotherapy 48(1): 143-144). Moreover, MRSA presents a new threat in diabetic foot infections (Retrieved January

17, 2009, from CDC: Centers for Disease Control and Prevention Web site). The ulcers and open sores that can occur in diabetic feet put patients at risk for contracting MRSA, and recent studies show evidence of MRSA impairing healing when present in the diabetic wound (Bowling FL, et al. 2009 Curr Diab Rep 9(6):440-444). See also, Kosinski, MA, et al. 2010. Expert Rev AntiInfect Ther. 8(11):1293-1305.

[0050] In some embodiments, the invention provides a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:1. A specific example in accordance with this embodiment is the isolated bacteriophage F44/10, which targets a number of strains of Staphylococcus species, including *S. aureus*. Strain F44/10 was deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bears accession number 41867. In some embodiments, the invention provides a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:2. A specific example in accordance with this embodiment is the isolated bacteriophage F125/10, which also targets a number of strains of Staphylococcus species, including *S. aureus*. Strain F125/10 was deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bears accession number 41866. In some embodiments, the cocktail composition includes at least both F44/10 and F125/10 phage strains. In certain embodiments, the phage cocktail comprises at least one phage strain exhibiting antibacterial activity against one or more strains of *S. aureus*. (e.g., F44/10 and/or F125/10) and at least one phage strain exhibiting antibacterial activity against a different bacteria. For example, in some embodiments, the phage cocktail comprises a phage strain having a genome comprising or consisting of SEQ ID NOs:1 or 2, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:3, 4, or 5, or a variant thereof.

[0051] In some embodiments, the cocktail composition comprises at least one phage strain showing antibacterial activity against *Pseudomonas aeruginosa*. *P. aeruginosa* is a common Gram-negative rod-shaped bacterium found in soil, water, skin flora and most man-made environments. It thrives not only in normal atmospheres, but also with little oxygen as a facultative anaerobe, and can infect damaged tissues or immunocompromised individuals,

including diabetic patients. Indeed, *P. aeruginosa* frequently causes severe tissue damage in diabetic foot ulcers and a major problem with *P. aeruginosa* infection is that this pathogen exhibits a high degree of resistance to a broad spectrum of antibiotics (Murugan, S. et al. 2010 Intl J of Microbiol Res 1(3):123-128). For example, in the Murugan et al. study, 100% of *P. aeruginosa* isolates from diabetic foot ulcers were found to be resistant to meropenem and over 71% were found to be resistant to imipenem.

[0052] In some embodiments, the invention provides a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:3. A specific example in accordance with this embodiment is the isolated bacteriophage F770/05, which targets a number of strains of *Pseudomonas* species, including *P. aeruginosa*. See also, International Application Publication WO 2010/090542, disclosing said bacteriophage strain. Strain F770/05 was deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bears accession number 41864. In some embodiments, the invention provides a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:4. A specific example in accordance with this embodiment is the isolated bacteriophage F510/08, which also targets a number of strains of *Pseudomonas* species, including *P. aeruginosa*. Strain F510/08 was deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bears accession number 41868. In some embodiments, the cocktail composition includes at least both F770/05 and F510/08 phage strains. In certain embodiments, the phage cocktail comprises at least one phage strain exhibiting antibacterial activity against one or more strains of *P. aeruginosa* (e.g., F770/05 and/or F510/08) and at least one phage strain exhibiting antibacterial activity against different bacteria. For example, in some embodiments, the phage cocktail comprises a phage strain having a genome comprising or consisting of SEQ ID NOs:3 or 4, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:1, 2, or 5, or a variant thereof.

[0053] In some embodiments, the cocktail composition comprises at least one phage strain showing antibacterial activity against *Acinetobacter baumannii*. *A. baumannii* is a species of bacteria that causes a number of severe clinical infections, particularly in individuals with

compromised immune systems, including diabetic patients. For example, *A. baumannii* has been isolated from diabetic patients with lower extremity infection (Colayco, CAS, et al 2002 *Phil J Microbiol Infect Dis* 31(4):151-106). *A. baumannii* is a pleomorphic aerobic gram-negative bacillus that often enters the body through open wounds, such as diabetic foot ulcers. It also is known to be resistant to multiple antibiotics and the number of nosocomial infections caused by *A. baumannii* has increased in recent years. See also Browne AC, et al. 2001 *Ostomy Wound Management* 47(10):44-49 (discussing the occurrence of *S. aureus*, *P. aeruginosa*, and *Acinetobacter* species in diabetic foot ulcers). *Acinetobacter baumannii* is a colonizer that generally appears later in the process of wound infection. Accordingly, in certain embodiments, it is important that the phage cocktail composition comprise bacteriophage that infect *Acinetobacter baumannii*.

[0054] In some embodiments, the invention provides a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:5. A specific example in accordance with this embodiment is the isolated bacteriophage F1245/05, which targets a number of strains of *Acinetobacter* species, including *A. baumannii*. See also, International Application Publication WO 2010/090542, disclosing said bacteriophage strain. Strain F1245/05 was deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bears accession number 41865. In certain embodiments, the phage cocktail comprises at least one phage strain exhibiting antibacterial activity against one or more strains of *A. baumannii* (e.g., F1245/05) and at least one phage strain exhibiting antibacterial activity against different bacteria. For example, in some embodiments, the phage cocktail comprises a phage strain having a genome comprising or consisting of SEQ ID NO:5, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:1, 2, 3, or 4, or a variant thereof.

[0055] In certain embodiments, the cocktail of the invention comprises a bacteriophage that is a variant of any of the nucleic acid sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, which variant bacteriophage exhibits at least one biological activity, e.g., antimicrobial or antibacterial activity (e.g., lytic killing activity), of one or more of bacteriophage strains F44/10, F125/10, F770/05, F510/08, and F1245/05. In some preferred embodiments, a variant of bacteriophage strains F44/10 or F125/10 maintains antimicrobial or

antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Staphylococcus* species, more preferably including *S. aureus*. In some preferred embodiments, the cocktail comprises a variant of bacteriophage strains F770/05 or F510/08 that maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Pseudomonas* species, more preferably including *P. aeruginosa*. In some preferred embodiments, the cocktail comprises a variant of bacteriophage strain P1245/05 that maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Acinetobacter* species, more preferably including *A. baumannii*.

[0056] A variant bacteriophage strain may comprise or consist of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and/or SEQ ID NO: 5, which bacteriophage exhibits at least one biological activity, e.g., antimicrobial or antibacterial activity (e.g., lytic killing activity), of bacteriophage F44/10, F125/10, F770/05, F510/08, and F1245/05, respectively. In some preferred embodiments, a variant of bacteriophage strain F44/10 comprises or consists of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO:1 and maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Staphylococcus* species, more preferably including *S. aureus*. In some preferred embodiments, a variant of bacteriophage strain F125/10 comprises or consists of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO:2 and maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Staphylococcus* species, more preferably including *S. aureus*. In some preferred embodiments, a variant of bacteriophage strain F770/05 comprises or consists of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO:3 and maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Pseudomonas* species, more preferably including *P. aeruginosa*. In some preferred embodiments, a variant of bacteriophage strain F510/08 comprises or consists of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO:4 and maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against

one or more of strains of *Pseudomonas* species, more preferably including *P. aeruginosa*. In some preferred embodiments, a variant of bacteriophage strain P1245/05 comprises or consists of a genome having a sequence identity of at least 80%, 85%, 90%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the nucleic acid sequence of SEQ ID NO:5 and maintains antimicrobial or antibacterial activity (e.g., lytic killing activity) against one or more of strains of *Acinetobacter* species, more preferably including *A. baumannii*.

[0057] Alternatively, or in addition, the cocktail of the invention comprises a variant that has a genome comprising a functional fragment of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and/or SEQ ID NO: 5, which variant bacteriophage exhibits at least one biological activity, e.g., antimicrobial or antibacterial activity (e.g., lytic killing activity), of bacteriophage F44/10, F125/10, F770/05, F510/08, and F1245/05, respectively, preferably as described above.

[0058] In some embodiments, the invention provides a cocktail composition comprising at least two different isolated strains of bacteriophage, each strain having a genome that comprises a nucleic acid sequence selected from the group of consisting of SEQ ID NO:1 (F44/10), SEQ ID NO:2 (F125/10), SEQ ID NO:3 (F770/05), SEQ ID NO:4 (F510/08), and SEQ ID NO:5 (F1245/05), or a variant thereof, as described above. In some preferred embodiments, the cocktail composition comprises at least one of the bacteriophage strains having a genome that comprises the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2, or a variant thereof. In some more preferred embodiments, the cocktail composition comprises at least both bacteriophage strains having genomes that comprise the nucleic acid sequences of SEQ ID NO:1 and SEQ ID NO:2, or variants thereof. In some still more preferred embodiments, the cocktail composition comprises at least a third bacteriophage strain, said third strain having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or a variant thereof. In some even more preferred embodiments, the cocktail composition comprises at least a third and a fourth bacteriophage strain, said third and fourth strains each having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or a variant thereof.

[0059] In some preferred embodiments, the cocktail composition comprises at least one of the bacteriophage strains having a genome that comprises the nucleic acid sequence of SEQ ID

NO:3 or SEQ ID NO:4, or a variant thereof. In some more preferred embodiments, the cocktail composition comprises at least both bacteriophage strains having genomes that comprise the nucleic acid sequences of SEQ ID NO:3 and SEQ ID NO:4, or variant(s) thereof. In some still more preferred embodiments, the cocktail composition comprises at least a third bacteriophage strain, said third strain having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:5, or variant(s) thereof. In some even more preferred embodiments, the cocktail composition comprises at least a third and a fourth bacteriophage strain, said third and fourth strains each having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:5, or variant(s) thereof.

[0060] In some preferred embodiments, the cocktail composition includes the bacteriophage strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:5, or a variant thereof. In some more preferred embodiments, the cocktail composition comprises the bacteriophage strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:5, or a variant thereof, along with one, two, or three additional bacteriophage strains, each having a genome that comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4, or a variant thereof.

[0061] In a particularly preferred embodiment, the invention provides a cocktail composition comprising at least five isolated bacteriophage strains, said strains having genomes that comprise nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or a variant thereof. In some such embodiments, the variant selected for any one of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 has at least 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the corresponding nucleic acid sequence, and shows antibacterial activity against at least one of *S. aureus*, *P. aeruginosa*, and *A. baumannii*. Particularly preferred embodiments combine antibacterial activities against all three bacterial strains. In some embodiments, the cocktail composition further comprises one or more additional phage strains, said additional phage strain having antibacterial activity against at least one of *S. aureus*, *P. aeruginosa*, and *A. baumannii*, and/or against other bacteria.

[0062] In a particularly preferred embodiment, the invention provides a cocktail composition comprising five isolated bacteriophage strains, said strains having genomes that comprise nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID

NO:5, or a variant thereof, further in combination with at least one additional phage strain. In some preferred embodiments, the additional phage strain is selected from the group consisting of bacteriophage strain F168/08 having antibiotic activity against one or more strains of *E. faecalis* and/or *E. faecium* (as disclosed in WO 2011/065854 and US Patent Application Publication No. 2012/0052048), bacteriophage strain F170/08 having antibiotic activity against one or more strains of *E. faecalis* and/or *E. faecium* (as disclosed in WO 2011/065854 and US Patent Application Publication No. 2012/0052048), bacteriophage strain F197/08 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F86/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F87s/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F91a/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F391/08 having antibacterial activity against one or more strains of *Klebsiella pneumoniae* (as disclosed in US Provisional Application No. 61/384,015), bacteriophage strain F394/08 having antibacterial activity against one or more strains of *Acinetobacter baumannii* (as disclosed in US Provisional Application No. 61/384,01), bacteriophage strain F488/08 having antibacterial activity against one or more strains of *Escherichia coli* (as disclosed in US Provisional Application No. 61/384,01), and bacteriophage strain F387/08 having antibacterial activity against one or more strains of *Klebsiella pneumoniae* (as disclosed in US Provisional Application No. 61/384,015) (the contents of each are hereby incorporated by reference in their entireties). The contents of U.S. Provisional Application No. 61/384,015, filed on September 17, 2010, and International Application PCT/PT2011/000031, filed on September 19, 2011, also are hereby incorporated by reference in their entireties

[0063] In some embodiments, the invention provides a composition comprising at least five isolated bacteriophage strains, said strains having genomes that comprise nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or a variant thereof, where the bacteriophage strains having genomes that comprise the nucleic acid sequences of SEQ ID NOs:1, 2, 4, and 5, or said variants thereof, are each present in said

composition in higher amounts compared to that of said bacteriophage strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:3, or said variant thereof. In some preferred embodiments, the bacteriophage strains having genomes that comprise the nucleic acid sequences of SEQ ID NOs:1, 2, 4, and 5, or variants thereof, are each present in a cocktail composition in an amount corresponding to about 2 times, about 5 times, about 8 times, about 9, times, about 10 times, about 11 times, about 12 times, about 15 times, or about 20 times, that of the bacteriophage strain having a genome that comprises the nucleic acid sequence of SEQ ID NO:3, or a variant thereof.

[0064] In some embodiments, the phage cocktail composition may or may not involve phage selected for increased in vivo half-life, e.g., as disclosed in US 5,688,501, the contents of which are incorporated herein by reference. In some embodiments, the cocktail is administered in the absence of an isolated polypeptide, such as in the absence of a lyase.

[0065] The invention also provides for isolated bacteria infected with one or more of the bacteriophage of the invention. In certain embodiments, the invention provides isolated *S. aureus* infected with a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:1 and/or 2, or a variant thereof. In certain embodiments, the invention provides isolated *P. aeruginosa* infected with a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:3 and/or 4, or a variant thereof. In certain embodiments, the invention provides isolated *A. baumannii* infected with a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:5, or a variant thereof.

[0066] The bacteriophage for use in the phage cocktails of the invention can be produced and/or isolated by any methods known in the art and/or disclosed herein. For example, the skilled artisan can use one or more methods to produce and/or isolate a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, as well of variants thereof. A method of producing and/or isolating a bacteriophage having a genome that comprises or consists of the nucleic acid sequence of SEQ ID NO: 1 and/or SEQ ID NO: 2, and/or a variant of either, may comprise (i) obtaining a culture of *S. aureus*; (ii) infecting it with the bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:1 and/or SEQ ID NO:2, and/or a variant of either; (iii) culturing until significant lysis of the culture is observed; and (iv)

isolating from the culture the bacteriophage. The host cell used may be any bacterial strain, for example, any *S. aureus* strain, susceptible to infection by the bacteriophage and that can be used to replicate same. In some embodiments, the host cell used is *S. aureus* strain 743/06, deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) with accession number NCIMB 41862. A method of producing and/or isolating a bacteriophage having a genome that comprises or consists of the nucleic acid sequence of SEQ ID NO:3 and/or SEQ ID NO:4, and/or a variant of either, may comprise (i) obtaining a culture of *P. aeruginosa*, (ii) infecting it with the bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:3 and/or SEQ ID NO:4, and/or a variant of either; (iii) culturing until significant lysis of the culture is observed; and (iv) isolating from the culture the bacteriophage. The host cell used may be any bacterial strain, for example, any *P. aeruginosa* strain, susceptible to infection by the bacteriophage and that can be used to replicate same. In some embodiments, the host cell used may be, for example, *P. aeruginosa* strain 433/07, deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) with accession number NCIMB 41861. A method of producing and/or isolating a bacteriophage having a genome that comprises or consists of the nucleic acid sequence of SEQ ID NO:5 and/or a variant thereof, may comprise (i) obtaining a culture of *A. baumannii*; (ii) infecting it with the bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:5 and/or a variant thereof; (iii) culturing until significant lysis of the culture is observed; and (iv) isolating from the culture the bacteriophage. The host cell used may be any bacterial strain, for example, any *A. baumannii* strain, susceptible to infection by the bacteriophage and that can be used to replicate same. In some embodiments, the host cell used may be, for example, *A. baumannii* strain 1305/05, deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) with accession number NCIMB 41863.

[0067] Bacteriophage may be isolated from a bacterial sample using any method described herein or known in the art (see, e.g., Carlson, "Working with bacteriophage: common techniques and methodological approaches," In, Kutter and Sulakvelidze (Eds) *Bacteriophage: Biology and Applications*, 5th ed. CRC Press (2005); incorporated herein by reference in its entirety).

Specific bacterial strains that may be used include, e.g. *Staphylococcus aureus* 743/06 strain (e.g., for isolating phage F44/10 and F125/10), *Pseudomonas aeruginosa* 433/07 strain (e.g., for isolating phage F770/05 and F510/08), and *Acinetobacter baumannii* strain 1305/05 (e.g., for isolating phage F1245/05). *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05 strains were deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bear accession numbers NCIMB 41862, NCIMB 41861, and NCIMB 41863, respectively. Bacteriophage also may be isolated from any other bacterial strain susceptible to infection by one or more of the bacteriophage, and in which the bacteriophage replicate.

5.2 ANTIBIOTIC COMPOSITIONS

[0068] The phage cocktails of the present invention are incorporated into a pharmaceutical composition for the use in treatment and/or prevention of bacterial infections (e.g., diabetic foot infections) caused by bacteria including, but not limited to, *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*. A cocktail of different phage strains, e.g., as disclosed herein, may be combined with a pharmaceutically acceptable carrier, such as an excipient or stabilizer. Examples of pharmaceutically acceptable carriers, excipients, and stabilizers include, but are not limited to, buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin and gelatin; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™. The pharmaceutical compositions of the present invention (e.g., antibacterial compositions) can also include a lubricant, a wetting agent, a sweetener, a flavoring agent, an emulsifier, a suspending agent, and a preservative, e.g., in addition to the above ingredients.

[0069] The bacteriophage cocktail compositions of the present invention may also be combined with one or more non-phage therapeutic and/or prophylactic agents, useful for the treatment and/or prevention of bacterial infections, as described herein and/or known in the art (e.g. one or more antibiotic agents). Other therapeutic and/or prophylactic agents that may be used in

combination with the phage cocktails of the invention include, but are not limited to, antibiotic agents, anti-inflammatory agents, antiviral agents, local anesthetic agents, growth factors, and corticosteroids. In some preferred embodiments, the pharmaceutical composition is formulated for treatment and/or prevention of diabetic foot infections and comprises one or more additional therapeutic and/or prophylactic agents selected from antibiotic agents, local anesthetic agents, and growth factors. In some embodiments, the phage cocktail is administered in the absence of an antibiotic agent.

[0070] Standard antibiotics that may be used with pharmaceutical compositions comprising a phage cocktail of the invention include, but are not limited to, amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin, apramycin, rifamycin, naphthomycin, mupirocin, geldanamycin, ansamitocin, carbacephems, imipenem, meropenem, ertapenem, faropenem, doripenem, panipenem/betamipron, biapenem, PZ-601, cephalosporins, cefacetrile, cefadroxil, cefalexin, cefaloglycin, cefalonium, cefaloridine, cefalotin, cefapirin, cefatrizine, cefazaflur, cefazedone, ceftazolin, cefradine, cefroxadine, ceftazole, cefaclor, cefonicid, cefprozil, cefuroxime, cefuzonam, cefmetazole, cefotetan, cefoxitin, cefcapene, cefdaloxime, cefdininir, cefditoren, cefetamet, cefixime, cefmenoxime, cefteram, ceftibuten, ceftiofur, ceftiolene, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime latamoxef, cefclidine, cefepime, ceftuprenam, cefoselis, cefozopran, cefpirome, cefquinome, flomoxef, ceftobiprole, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, aztreonam, penicillin and penicillin derivatives, actinomycin, bacitracin, colistin, polymyxin B, cinoxacin, flumequine, nalidixic acid, oxolinic acid, piromidic acid, pipemidic acid, rosoxacin, ciprofloxacin, enoxacin, fleroxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, balofloxacin, gatifloxacin, grepafloxacin, levofloxacin, moxifloxacin, pazufloxacin, sparfloxacin, temafloxacin, tosufloxacin, clinafloxacin, garenoxacin, gemifloxacin, stifloxacin, trovafloxacin, prulifloxacin, acetazolamide, benzolamide, bumetanide, celecoxib, chlorthalidone, clopamide, dichlorphenamide, dorzolamide, ethoxzylamide, furosemide, hydrochlorothiazide, indapamide, mafendide, mefruside, metolazone, probenecid, sulfacetamide, sulfadimethoxine, sulfadoxine, sulfanilamides, sulfamethoxazole, sulfasalazine, sultiame, sumatriptan, xipamide, tetracycline, chlortetracycline, oxytetracycline, doxycycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, methicillin, nafcillin, oxacilin, cloxacillin, vancomycin, teicoplanin,

clindamycin, co-trimoxazole, flucloxacillin, dicloxacillin, ampicillin, amoxicillin and any combination thereof in amounts that are effective to additively or synergistically enhance the therapeutic and/or prophylactic effect of a phage cocktail of the invention for a given infection.

[0071] In some preferred embodiments, the pharmaceutical composition of the invention comprises an antibiotic agent having antibacterial activity against one or more of *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*. In some more preferred embodiments, the pharmaceutical composition of the invention comprises an antibiotic agent having antibacterial activity against *A. baumannii*, *P. aeruginosa*, and *S. aureus*. In some other embodiments, the pharmaceutical composition of the invention comprises an antibiotic agent having antibacterial activity against bacteria other than *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*.

[0072] In some embodiments, the pharmaceutical composition of the invention is formulated for use in treating and/or preventing bacterial infections caused by *Staphylococcus* species, such as *S. aureus*. In some such embodiments, the pharmaceutical composition comprises a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:1, such as the isolated bacteriophage F44/10, which targets a number of strains of *Staphylococcus* species, including *S. aureus*. In some embodiments, the pharmaceutical composition comprises a cocktail comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:2, such as isolated bacteriophage F125/10, which targets a number of strains of *Staphylococcus* species, including *S. aureus*. In some embodiments, the pharmaceutical composition comprises a cocktail including at least both F44/10 and F125/10 phage strains. In certain embodiments, the pharmaceutical composition comprises a cocktail including at least one phage strain exhibiting antibacterial activity against one or more strains of *S. aureus*. (e.g., F44/10 and/or F125/10) and at least one phage strain exhibiting antibacterial activity against a different bacteria. For example, in some embodiments, the pharmaceutical composition comprises a cocktail including a phage strain having a genome comprising or consisting of SEQ ID NOs:1 or 2, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:3, 4, or 5, or a variant thereof. In some embodiments, the pharmaceutical composition may further comprise an additional agent, e.g., an antibiotic agent having antibacterial activity against *S. aureus*; and/or an antibiotic agent having antibacterial activity against bacteria other than *S. aureus*.

[0073] In some embodiments, the pharmaceutical composition of the invention is formulated for use in treating and/or preventing bacterial infections caused by *Pseudomonas* species, such as *P. aeruginosa*. In some such embodiments, the pharmaceutical composition comprises a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:3, such as the isolated bacteriophage F770/05, which targets a number of strains of *Pseudomonas* species, including *P. aeruginosa*. In some embodiments, the pharmaceutical composition comprises a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:4, such as the isolated bacteriophage F510/08, which also targets a number of strains of *Pseudomonas* species, including *P. aeruginosa*. In some embodiments, the pharmaceutical composition comprises a cocktail including at least both F770/05 and F510/08 phage strains. In certain embodiments, the pharmaceutical composition comprises a cocktail including at least one phage strain exhibiting antibacterial activity against one or more strains of *P. aeruginosa* (e.g., F770/05 and/or F510/08) and at least one phage strain exhibiting antibacterial activity against different bacteria. For example, in some embodiments, the pharmaceutical composition comprises a cocktail including a phage strain having a genome comprising or consisting of SEQ ID NOs:3 or 4, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:1, 2, or 5, or a variant thereof. In some embodiments, the pharmaceutical composition may further comprise an additional agent, e.g., an antibiotic agent having antibacterial activity against *P. aeruginosa*; and/or an antibiotic agent having antibacterial activity against bacteria other than *P. aeruginosa*.

[0074] In some embodiments, the pharmaceutical composition of the invention is formulated for use in treating and/or preventing bacterial infections caused by *Acinetobacter* species, such as *A. baumannii*. In some such embodiments, the pharmaceutical composition comprises a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NO:5, such as the isolated bacteriophage F1245/05, which targets a number of strains of *Acinetobacter* species, including *A. baumannii*. In certain embodiments, the pharmaceutical composition comprises a cocktail including at least one phage strain exhibiting antibacterial activity against one or more strains of *A. baumannii* (e.g., F1245/05) and at least one phage strain exhibiting antibacterial activity against different bacteria. For example, in some embodiments, the pharmaceutical composition comprises a cocktail

including a phage strain having a genome comprising or consisting of SEQ ID NO:5, or a variant thereof, in combination with at least one other phage strain having a genome comprising or consisting of SEQ ID NOs:1, 2, 3, or 4, or a variant thereof. In some embodiments, the pharmaceutical composition may further comprise an additional agent, e.g., an antibiotic agent having antibacterial activity against *A. baumannii*; and/or an antibiotic agent having antibacterial activity against bacteria other than *A. baumannii*.

[0075] Local anesthetics that may be formulated for use with pharmaceutical compositions of the invention include, but are not limited to, tetracaine, tetracaine hydrochloride, lidocaine hydrochloride, dimethisoquin hydrochloride, dibucaine, dibucaine hydrochloride, butambenpicrate, and pramoxine hydrochloride. An exemplary concentration of local anesthetic is about 0.025% to about 5% by weight of the total composition. In some preferred embodiments, the anesthetic agent is formulated with a cocktail of the invention in a pharmaceutical composition that is a topical formulation.

[0076] Corticosteroids that may be used with pharmaceutical compositions of the invention include, but are not limited to, betamethasone, dipropionate, fluocinolone, actinide, betamethasone valerate, triamcinolone actinide, clobetasol propionate, desoximetasone, diflorasone diacetate, amcinonide, flurandrenolide, hydrocortisone valerate, hydrocortisone butyrate, and desonide. An exemplary concentration of corticosteroid is about 0.01% to about 1% by weight of the total composition.

[0077] Growth factors that may be used with pharmaceutical compositions of the invention include, but are not limited to, platelet-derived growth factor, granulocyte colony-stimulating factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, and vascular endothelial growth factor.

[0078] In some preferred embodiments, growth factors used in the treatment and control of diabetic foot ulcers can be used also in combination with a phage cocktail pharmaceutical composition of the invention. For example, platelet-derived growth factor (PDGF) and granulocyte colony-stimulating factor (GCSF) are important growth factors in the treatment and control of diabetic foot ulcers (Papanas et al. 2007. *Lower Extremity Wounds* 6(1):37-53). PDGF is believed to aid macrophage migration to the ulcer, as well as stimulate collagen synthesis, thus improving healing (see, e.g., Meyer-Ingold W et al. 1995 *Cell Biol Int* 19:389-398). PDGF is available commercially. Commercially available forms include Procurin

(Curative Technologies In., New York), which comprises a solution of all platelet-associated growth factors suspended in a collagen base; and becaplermin (Regranex gel, Ortho-McNeil Pharmaceutical, Inc., Titusville, NJ), which comprises a recombinant homodimeric PDGF. GCSF is believed to enhance bactericidal and phagocytic activity of neutrophils, activities that may be impaired in the diabetic patient (Roilides E et al 1991 J Infect Dis 163:579-583). PDGF also is available commercially. Commercially available forms include filgrastim (nonglycosylated GCSF; Neupogen, Amgen Inc., Thousand Oaks, CA) and lenograstim (glycosylated GCSF; Granocyte, Sanofi Aventis Inc., Paris France).

[0079] Additional growth factors used in the treatment and control of diabetic foot ulcers include, but are not limited to, epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF). EGF is believed to promote collagen synthesis, epithelialization, and angiogenesis (Brown GL et al 1986 J Exp Med 163:1319-1342; and Brown GL et al 1991 Plast Reconstr Surg 88:189-194). FGF also is believed to promote collagen synthesis, epithelialization, and angiogenesis, as well as aiding fibroblast proliferation (Sasaki T 1992 J Dermatol 19:664-666; and Aurinia A et al 1998 Scand J Plast Reconstr Hand Surg 32:9-18). NGF is believed to promote healing by stimulating keratinocyte growth and new vessel formation (Generini S et al 2004 Exp Clin Endocrinol Diabetes 112:542-544). VEGF also has been shown to accelerate cutaneous healing and is believed to mobilize vascular progenitors and endothelial cells from bone marrow (Galiano RD et al. 2004 Am J Pathol 164:1935-1947). One or more of the growth factors disclosed herein and/or known in the art may be used in combination with a pharmaceutical composition comprising a phage cocktail of the invention.

[0080] Pharmaceutical compositions comprising a phage cocktail of the present invention can be formulated in a unit dose or multi-dose formulation. Preferred formulations are formulations that can be topically applied, e.g., formulations selected from ointments, solutions, and sprays. Other suitable formulations include suspensions, emulsions, extracts, powders, or granules; and additionally may include a dispersing agent or a stabilizing agent.

[0081] The pharmaceutical compositions of the invention preferably are administered topically (e.g., in the form of a lotion, solution, cream, ointment, or dusting powder), or epi- or transdermally (e.g., by use of a skin patch). In addition or alternatively, the pharmaceutical compositions of the invention can be administered by inhalation, in the form of a suppository or

pessary, orally (e.g., as a tablet, which may contain excipients such as starch or lactose, as a capsule, ovule, elixir, solution, or suspension, each optionally containing flavoring, coloring agents, and/or excipients), or they can be injected parenterally (e.g., intravenously, intramuscularly or subcutaneously). For parenteral administration, the compositions may be used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration, the compositions may be administered in the form of tablets or lozenges, which can be formulated in a conventional manner. In a preferred embodiment, a phage cocktail of the present invention is formulated for topical administration, either as a single agent, or in combination with other therapeutic and/or prophylactic agents, as described herein or known in the art.

[0082] In particularly preferred embodiments, the pharmaceutical compositions of the instant invention are formulated for topical administration, e.g., to an area of non-intact skin. Non-intact skin can include, but is not limited to, skin lesions, vesicles, chronic ulcers, cysts, blisters, bullae, open sores such as decubitus ulcers (bed sores) and other pressure sores, cellulitis sores, erysipelas lesions, wounds, burn wounds, carbuncles, cutaneous ulcers, e.g., cutaneous ulcers associated with diabetic foot infections, or other conditions where the skin is damaged, broken, cracked, breached and/or otherwise compromised. Topical formulations generally include a sterile buffer, such as a sterile PBS, water, or saline buffer, or a sterile SM buffer. One particular SM buffer suitable for use in certain embodiments of the instant invention comprises Tris-HCl, NaCl, and/or $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, e.g., about 0.05 M Tris-HCl (pH 7.4-7.5), about 0.1 M NaCl, and/or about 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. In other embodiments, the formulation further comprises an SM buffer and 10 mM MgCl_2 . In still other embodiments, the formulation further comprises an SM buffer and about 20% to about 30% ethanol.

[0083] For topical application to the skin, the pharmaceutical compositions of the present invention may be combined with one or a combination of carriers for topical formulations, which can include, but are not limited to, an aqueous liquid, an alcohol base liquid, a water soluble gel, a lotion, an ointment, a nonaqueous liquid base, a mineral oil base, a blend of mineral oil and petrolatum, lanolin, liposomes, proteins carriers such as serum albumin or gelatin, powdered cellulose carmel, and combinations thereof.

[0084] Carriers for topical formulations may comprise semi-solid and/or gel-like vehicles, which may include a polymer thickener, water, preservatives, active surfactants, emulsifiers, and/or a solvent or mixed solvent system. U.S. Patent No. 5,863,560 discloses a number of different carrier combinations that can aid in the exposure of skin to a medicament, and its contents are incorporated herein by reference. The carrier may or may not involve a controlled-release formulation, e.g., as disclosed in US 2008/0260697, the contents of which are incorporated herein by reference. The carrier may or may not involve phage adsorbed on a matrix, e.g., as described in any one of US 2008/0038322, US 2008/0138311, US 2009/0130196, EP 1 812 025, EP 1 817 043, and EP 1 833 497, the contents of which are incorporated herein by reference. In some embodiments, the carrier may or may not involve a viscous formulation, e.g., a gel, e.g., as disclosed in US 2009/0191254, the contents of which are incorporated herein by reference.

[0085] In some particularly preferred embodiments, topical pharmaceutical compositions of the invention are provided in a hermetically sealed container. The container may be a vial, tube, bottle, ampoule, or the like; and may comprise or consist of glass, plastic, or other suitable material. Ampoules, for example, generally are produced industrially from short lengths of glass tubing, shaped by heating with gas torches and gravity. Computer vision techniques often are employed, e.g., for quality control. The filling and sealing of ampoules may be done by automated machinery. Blank ampoules can be purchased from scientific glass supply houses and sealed, e.g., with a small gas torch, preferably under inert atmospheres. In some embodiments, the container also may be filled with an inert gas, in addition to the pharmaceutical composition. In some embodiments, the phage cocktail composition is provided in an ampoule, or other suitable container, and transferred for use to a vehicle suitable for direct contact with non-intact skin, e.g., a patch, wipe, bandage, dressing, as described below.

[0086] The topical mode of delivery may include a smear, a spray, a bandage, a time-release patch, a liquid-absorbed wipe, and combinations thereof. In some particularly preferred embodiments, the phage cocktail composition of the invention is provided, either directly or in a carrier(s), in a patch, wipe, bandage, dressing, or other vehicle suitable for direct contact with the skin, in particular, non-intact skin.

[0087] In some embodiments, topical administration of a pharmaceutical composition of the invention comprises use of a dressing. The pharmaceutical composition comprising a phage cocktail of the invention may be incorporated into a dressing and/or applied separately along

with the use of a dressing. A dressing promotes healing by keeping a wound moist, creating a barrier against infection, and/or keeping the surrounding skin dry.

[0088] In some embodiments, the dressing comprises a moist wound dressing. Moist wound therapy, comprising use of moist wound dressings, represents a standard therapy in the treatment and control of non-healing wounds, including diabetic foot ulcers, for example. In moist wound therapy, wounds are dressed with materials that offer protection from outside contaminants, prevent wound desiccation, and provide an environment conducive to wound closure. The degree of moisture in a wound is to be considered when treating a diabetic ulcer. High levels of exudate warrant the choice of a moisture-absorbing material, including but not limited to alginates, foams, collagen-alginate combinations, carboxymethylcellulose materials, or gauze. Low exudate and desiccated wounds generally respond well to hydrogels. Hydrogel sheets often comprise three-dimensional networks of cross-linked hydrophilic polymers. Amorphous hydrogels are similar in composition to hydrogel sheets but lack the cross-linking. The gel also may comprise additional ingredients, such as collagens, alginate, or complex carbohydrates.

[0089] Standard dressing care for the treatment of diabetic foot ulcers in the US is still the use of wet-to-dry or wet-to-moist saline gauze dressings. Alginate dressings often comprise calcium or calcium-sodium salts of natural polysaccharides derived from brown seaweed. When the alginate material comes into contact with sodium-rich wound exudates, an ion exchange takes place, producing a hydrophilic gel.

[0090] Additional dressing choices include but are not limited to films including adhesive-backing films, gels, and foams including silicone-coated foams, hydrocolloids, collagen-based dressings, absorbent polymers, and the like. Hydrocolloid dressings often comprise adhesive, absorbent, and elastomeric components. Carboxymethylcellulose, for example, is a common absorptive ingredient. Hydrofiber dressing also often comprise carboxymethylcellulose, for example, sodium carboxymethylcellulose. Foam dressings often comprise a polymer, often polyurethane, with small, open cells that are able to hold fluids. Some varieties of foam dressings have a waterproof film covering the top surface and may have an adhesive coating on the wound contact side or on the wound border. Film dressings often comprise a single thin transparent sheet of polyurethane coated on one side with an adhesive. The sheet is permeable to gases and water vapor but impermeable to wound fluids. Hydrofiber dressings often comprise sodium carboxymethylcellulose fibers. Collagen-based dressings often comprise purified

collagen derived from bovine, porcine, equine, or avian sources. Collagen-based dressings are believed to aid wound healing e.g., by stimulating fibroblast production.

[0091] In some embodiments, topical administration of a pharmaceutical composition of the invention comprises instillation. The pharmaceutical composition comprising a phage cocktail of the invention may be incorporated into an instillation and/or applied separately along with the use of an instillation. Instillation refers to administration by introduction of the fluid pharmaceutical composition gradually, e.g., drop by drop of the fluid. Typical instillation therapy instills fluid into a wound under a low positive pressure. Devices for use in instillation include, e.g., Kritter-type instillation catheters (see, e.g., Brent H. et al. 2005. Wounds 17(2):37-48). Techniques known in the art to improve instillation and distribution of the fluid include, but are not limited to, filling a wound with instillation fluid, applying porous wound fillers, and/or combining with negative pressure wound therapy.

[0092] In some embodiments, topical administration of a pharmaceutical composition of the invention comprises negative pressure wound therapy. Negative pressure wound therapy (NPWT) refers to use of reduced pressure in proximity to a wound, or other area of non-intact skin, to augment and/or accelerates the growth of new tissue. The therapy involves controlled application of sub-atmospheric pressure to the area, using a sealed wound dressing connected to a vacuum pump. “Negative pressure wound therapy” may also be referred to as “reduced pressure therapy” or “vacuum therapy”. Typically, reduced pressure is applied to the area of non-intact skin through a porous pad. The porous will contain pores capable of distributing the reduced pressure to the area and/or channeling fluids drawn out.

[0093] A number of devices can be used in NPWT. NPWT devices often comprise a vacuum pump, drainage tubing, and/or a dressing set. The pump may be stationary or portable, rely on AC or battery power, and/or allow for regulation of the suction strength. The dressing sets may comprise foam or gauze dressing, e.g., to be placed on the wound, and an adhesive film drape for sealing the area. The drainage tubes may come in a variety of configurations depending on the dressings used or wound to be treated. Once the dressing is sealed, the vacuum pump can be set to deliver continuous or intermittent pressures, with levels of generally varying between -125 and -75 mmHg. NPWT may be used for administration of a pharmaceutical composition of the invention, e.g., where the NPWT device used allows for delivery of fluids, such as a fluid pharmaceutical composition. (See, e.g., Gerry R, et al. 2007. Ann Plast Surg 59(1):58-62).

[0094] Modes of administration described herein and/or known in the art may be used to deliver desired dosages of the phage cocktails of the invention and in accordance with suitable dosage regimens. Dosages and dosage regimens may vary depending on the particular formulation, route of administration, condition being treated, and other factors. Animal experiments can provide reliable guidance for the determination of effective doses in human therapy, e.g., as within the skill of the ordinary physician. Interspecies scaling of effective doses can be performed by one of ordinary skill in the art following the principles described, e.g., by Mordenti, J. et al. "The use of interspecies scaling in toxicokinetics" in *Toxicokinetics and New Drug Development*, Yacobi et al., Eds., Pergamon Press, New York 1989, pp 42-96.

[0095] The pharmaceutical compositions of the invention can be administered according to a dosage regimen. In the treatment of chronic ulcers and diabetic foot infections, e.g., including but limited to the treatment of cutaneous ulcers associated therewith, a first dosage regimen may be followed initially, e.g., during an induction phase, and a second dosage regimen may be followed after, e.g., during a maintenance phase. In some embodiments, an induction phase dosage regimen is followed over an initial about 12 hours of treatment, or over an initial about 18 hours, about 24 hours, about 36 hours, or about 48 hours. In some preferred embodiments, the induction phase dosage regimen comprises administration of a pharmaceutical composition of the invention about every hour, about every 2 hours, 4 hours, 6 hours, 8 hours, 10 hours, or 12 hours. In more preferred embodiments, the induction phase dosage regimen comprises administration of a pharmaceutical composition of the invention about every 4 hours or about every 6 hours, e.g., over an initial 24 hours. In even more preferred embodiments, the pharmaceutical composition is administered topically in accordance with an induction phase dosage regimen.

[0096] In some embodiments, the induction phase is followed by a maintenance phase, e.g., where a different dosage regimen may be followed. The maintenance phase may continue for a number of days, weeks, months, or longer, following initial treatment. In some embodiments, the maintenance phase continues for about 1, 2, 3, 4, 5, 6, or 7 days following the induction phase. In some embodiments, the pharmaceutical composition is administered for 2, 3, or 4 weeks; 2, 4, 6, 8, 10, or 12 months; or 2, 3, 4, 5 or more years. In still some embodiments, the pharmaceutical composition according to the invention is administered chronically, e.g. for several years or over the life of the patient.

[0097] In some embodiments, the maintenance phase dosage regimen comprises administration of a pharmaceutical composition of the invention at a lower frequency of doses compared to the induction phase dosage regimen. For example, in some preferred embodiments, the pharmaceutical composition is administered about every 6 hours, 8 hours, 10 hours, 12 hours, 16 hours, 20 hours, 24 hours, 30 hours, 36 hours, 42 hours, or 48 hours. In more preferred embodiments, the maintenance phase dosage regimen comprises administration of a pharmaceutical composition of the invention about every 12 hours or about every 24 hours, e.g., for at least about 3 or 4 additional following the induction phase. In even more preferred embodiments, the pharmaceutical composition is administered topically in accordance with a maintenance phase dosage regimen.

5.3 THERAPEUTIC USE

[0098] Another aspect of the instant invention relates to the use of phage cocktail compositions in preventing and/or treating bacterial infections. In specific embodiments, the subject receiving a pharmaceutical composition of the invention is a mammal (e.g., bovine, ovine, caprine, equid, primate (e.g., human), rodent, lagomorph or avian (e.g., chicken, duck, goose)). In preferred embodiments, the subject receiving a pharmaceutical composition of the invention is a human, and particularly a diabetic patient that suffers from or is at risk of suffering from chronic ulcers, including diabetic foot infections. In the context of the present invention, “treatment” refers to obtaining a therapeutic benefit in a subject receiving the pharmaceutical composition. With respect to achieving a therapeutic benefit, the object is to eliminate, lessen, manage, decrease the severity of, prevent worsening, ameliorate, or slow the progression of the symptoms or underlying cause (e.g., bacterial infection) associated with the pathological condition or disorder. It is also contemplated that phage cocktails of the invention, in certain embodiments, may act as a prophylactic or preventative measure, preventing the onset of infection caused by one or more bacteria. “Prevention” refers to obtaining a prophylactic benefit in a subject receiving the pharmaceutical composition. With respect to achieving a prophylactic benefit, the object is to delay or prevent the symptoms or underlying cause (e.g., bacterial infection) associated with the pathological condition or disorder.

[0099] The phage cocktails of the present invention have activity against a plurality of bacterial strains. In some preferred embodiments, the phage cocktails have activity against a plurality of strains of *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*. Accordingly, another aspect of the

invention provides methods of treating and/or preventing infections associated with *A. baumannii*, *P. aeruginosa*, and/or *S. aureus* in both humans and animals using a phage cocktail composition. In other aspects, the invention provides methods of treating and/or preventing infections associated with related species or strains of these bacteria. In some particularly preferred embodiments, the bacterial infection is an infection associated with diabetic lower extremity infections, such as diabetic foot infections.

[00100] *A. baumannii*, *P. aeruginosa*, and *S. aureus* are responsible for many severe opportunistic infections, particularly in individuals with compromised immune systems, including diabetic patients. The pharmaceutical compositions of the present invention are contemplated for treating and/or preventing any infection associated with *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*, or associated with other species or strains of bacteria, including, but not limited to, infections of the skin, infections in and around wounds, chronic ulcers, ulcers associated with burn wounds, post-operative infections, infections associated with catheters and surgical drains, and infections of the blood. In preferred embodiments, the pharmaceutical compositions of the invention find use in treating and/or preventing bacterial infections associated with areas of non-intact skin. Infections associated with areas of non-intact skin include, but are not limited to, infections associated with cutaneous ulcers, such as diabetic foot ulcers, skin lesions, vesicles, cysts, blisters, bullae, open sores such as decubitus ulcers (bed sores) and other pressure sores, chronic ulcers, cellulitis and sores associated therewith, erysipelas and lesions associated therewith, wounds, burns and wounds associated therewith, carbuncles, or other conditions where the skin is damaged, cracked, broken, breached, and/or otherwise compromised.

[0101] In particularly preferred embodiments, the phage cocktail compositions of the instant invention find use in treating chronic ulcers. Chronic ulcers may arise from wounds caused by a variety of factors, especially in patients with impaired blood circulation, for example, caused by cardiovascular issues or external pressure from a bed or a wheelchair. More than 8 million patients are diagnosed with chronic skin ulcers each year in the United States alone (Harsha, A. et al., 2008, *Journal of Molecular Medicine*, 86(8): 961–969), which costs more than 10 billion dollars per year (Margolis, DJ, et al., 2002, *Journal of the American Academy of Dermatology* 46(3): 381–386). Chronic ulcers may develop in the mouth, throat, stomach, and skin. Chronic skin ulcers include diabetic ulcers, venous ulcers, radiation ulcers, and pressure ulcers, the three

major categories of chronic skin ulcers being diabetic ulcers, venous stasis ulcers, and pressure ulcers. Chronic ulcers can cause the loss of the integrity of large portions of the skin, even leading to morbidity and mortality.

[0102] In even more particularly preferred embodiments, the phage cocktail compositions of the instant invention find use in treating diabetic lower extremity infections, such as diabetic foot infections. Diabetic foot infection is one of the major complications of diabetes mellitus, occurring in about 15% of all diabetic patients and resulting in about 85% of all lower leg amputations. (Brem, et al., J. Clinical Invest., 2007, 117(5):1219–1222). Diabetes mellitus impedes the normal steps of the wound healing process, such that diabetic foot infections can become associated with non-healing, chronic cutaneous ulcers.

[0103] A chronic wound represents a failure of the normal processes of acute wound healing. Wound healing has traditionally been divided into three distinct phases: inflammation, proliferation and remodeling. The inflammatory phase of wound healing begins at the time of injury by forming a clot via a platelet plug, thereby initiating a response from neutrophils and macrophages. Neutrophils initially clear the wound of bacteria and debris by releasing a variety of proteases and reactive oxygen free radicals. Macrophages are then attracted to the wound site by chemoattractants and subsequently release their own chemoattractants to stimulate fibroblasts and more macrophages. During the proliferation phase, fibroblasts initiate epithelialization, angiogenesis, and collagenation. Epithelialization generally occurs from the basement membrane if it remains intact and from the wound margins if not intact. Fibroblasts synthesize type III collagen during this phase and transform into myofibroblasts, which help to stimulate wound contraction. During the remodeling phase, type III collagen begins to be replaced by type I collagen. Collagen is woven into an organized, cross-linked network whose strength approaches 80% of the original uninjured tissue.

[0104] There are many factors that can stall the three-phase healing process and convert an acute wound into a chronic wound. These may include a low proliferative capacity of the fibroblasts, downregulation of receptors, reduced growth factors, or the absence of a suitable protein matrix in the dermis. Further, poor perfusion and/or nutrition can cause a wound to halt in the inflammatory phase and lead to excessive build-up of exudate in the wound. A chronic ulcer can be considered to be a non-healing area of non-intact skin, such as an area of non-intact skin that fails to follow the normal processes of wound healing, e.g., as described above, and/or that fails

to respond, or fails to respond appropriately, to initial treatment. A chronic ulcer on the skin may be characterized as a wound lesion lasting more than four weeks, without remarkable healing tendency or as a frequently recurrent wound (Fonder, M. et al., 2012, Journal of the American Academy of Dermatology 58(2): 185–206). A chronic wounds may appear with red granulation and yellow pus, a dim purple skin around granular tissues, or gray-white and swelling granulation. Standard care procedures for chronic skin ulcer include, e.g., the following: removal of necrotic or infected tissue; establishment of adequate blood circulation; maintenance of a moist wound environment; management of wound infection; wound cleansing; and nutritional support, including blood glucose control for subjects with diabetic ulcers. For example, in the diabetic patient, poor control of blood glucose levels allows bacteria to grow more rapidly in a wound; further still, neural degeneration in diabetes means the condition may not be painful and thus go undetected, at least initially. Chronic ulcers, including diabetic foot ulcers, often become further infected with opportunistic bacteria, leading to exacerbation of the condition. *A. baumannii*, *P. aeruginosa*, and *S. aureus* are associated with such infections.

[0105] *A. baumannii*, *P. aeruginosa*, and *S. aureus* also are associated with infections that involve organ systems that have a high fluid content, and it is contemplated that the phage cocktails of the invention have therapeutic and/or prophylactic use with respect to such infections. For example, the pharmaceutical compositions of the invention may be used for the prevention or treatment of infections of the respiratory tract, of the cerebrospinal fluid, of peritoneal fluid, and of the urinary tract. The compositions of the invention may also be used to prevent and/or treat nosocomial pneumonia, infections associated with continuous ambulatory peritoneal dialysis (CAPD), catheter-associated bacteremia, and nosocomial meningitis. In some embodiments, a phage cocktail composition of the invention is used prophylactically, e.g., in a hospital setting. For example, a phage cocktail composition of the instant invention may find use in preventing infections associated with wounds or damaged skin, e.g., due to catheterization and any other medical procedures or devices.

[0106] In some preferred embodiments, the pharmaceutical composition of the invention is formulated for use in methods of treating and/or preventing bacterial infections caused by *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*. In some more preferred embodiments, the pharmaceutical composition of the invention is formulated for use in methods of treating and/or preventing bacterial infections caused by *A. baumannii*, *P. aeruginosa*, and *S. aureus*. In some

other embodiments, the pharmaceutical composition of the invention is formulated for use in methods of treating and/or preventing bacterial infections caused by bacteria other than *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*.

[0107] In some preferred embodiments, the pharmaceutical composition of the invention is formulated for use in methods of treating and/or preventing bacterial infections caused by *Staphylococcus* species, such as *S. aureus*; *Pseudomonas* species, such as *P. aeruginosa*; and *Acinetobacter* species, such as *A. baumannii*. In some such embodiments, the pharmaceutical composition comprises a cocktail composition comprising a bacteriophage having a genome comprising or consisting of the nucleic acid sequence of SEQ ID NOs:1, 2, 3, 4, and 5, or a variant thereof, such as the isolated bacteriophage strains F44/10, F125/10, F770/05, F510/08, and F1245/05, or variants thereof. In some particularly preferred embodiments, the pharmaceutical cocktail composition is used in the treatment, prevention, control, and/or management of chronic ulcers, such as diabetic foot infections and cutaneous ulcers associated therewith.

[0108] In some embodiments, the invention provides methods of treating and/or preventing chronic ulcers, comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition of the instant invention. In preferred embodiments, administration comprises topical administration to the area of non-intact skin associated with the chronic ulcer. In more preferred embodiments, topical administration follows debridement of the area to be treated

[0109] In some embodiments, the invention provides methods of treating and/or preventing diabetic foot infections, comprising administering to a subject in need thereof a therapeutically or prophylactically effective amount of a pharmaceutical composition of the instant invention. In preferred embodiments, administration comprises topical administration to the area of non-intact skin associated with the diabetic foot infection, e.g., a cutaneous ulcer. In more preferred embodiments, topical administration follows debridement of the area to be treated.

[0110] Debridement can be accomplished by a number of approaches. Surgical debridement involves cutting away dead tissues of the wound or other area of non-intact skin. Mechanical debridement uses various methods to loosen and remove wound debris, such as a pressurized irrigation device, a whirlpool water bath, or specialized dressings. Autolytic debridement enhances the body's natural process of recruiting enzymes to break down dead tissue, for

example, using an appropriate dressing that keeps the wound moist and clean. Enzymatic debridement uses chemical enzymes and appropriate dressings to further aid in the break down dead tissues at the site of a wound or other area of non-intact skin.

[0111] Debridement improves topical treatment because it reduces the bio-burden of bacteria present and also opens a time-dependent therapeutic window for topical antimicrobial therapy (TAT) (Wolcott RD, et al. 2010. J Wound Care 19:320-328). Regarding the timing for debridement, early or immediate debridement is preferred to delayed debridement once this treatment option is chosen in the management of a wound. Further, multiple debridements during wound management may be indicated (Wolcott RD, et al. 2009. J Wound Care 18(2):54-6). For example, in some embodiments, debridement precedes topical application of a phage cocktail composition of the instant invention, and is repeated before every administration of the cocktail composition. In some embodiments, debridement is performed only before every other administration of the cocktail composition, or only before every 3rd, 4th, 5th, or 6th administration of the cocktail composition. In some embodiments, whether or not wound debridement is performed before topical administration of a cocktail composition of the instant invention is within the clinical judgement of a health care practitioner treating the wound, e.g., the physician, physician's assistant, or emergency medical personnel.

[0112] Phage cocktail compositions of the present invention can find use in the treatment, management, control, and/or prevention of infections associated with chronic ulcers, including diabetic foot infections and cutaneous ulcers associated therewith. In other embodiments, phage cocktail compositions of the present invention find use in the treatment, management, control, and/or prevention of bacterial infections associated with other areas of non-intact skin, such as a cellulites sore, an erysipelas lesion, a decubitus ulcer, a burn wound, and a pressure sore. In some such embodiments, the composition used may be a topical composition, formulated for topical administration, e.g., for direct application to an area of non-intact skin, such as described above.

[0113] Phage cocktail compositions of the present invention also find use in the treatment, management, control, and/or prevention of decubitus ulcers. Decubitus ulcers, also called pressure sores or pressure ulcers, are injuries to the skin and underlying tissues resulting from prolonged pressure on the area. For example, bedsores most often develop on skin that covers bony areas of the body, such as the heel, ankles, hips or buttocks.

[0114] Bedsores fall into one of four stages based on their severity. Stage I is the beginning stage of a pressure sore while the skin still is intact. The skin may appear red, ashen, bluish or purple, and fails to blanch when touched. Stage II often involves an open wound of non-intact skin. At this stage, the outer layer of skin (epidermis) and part of the underlying layer of skin (dermis) has been damaged or lost. The ulcer may appear as a shallow, pinkish-red, basin-shaped wound. In stage III, the ulcer is a deep wound, where the loss of skin may expose some amount of fat, and the ulcer has a crater-like appearance. The bottom of the wound also may have some yellowish dead tissue (slough). A Stage IV ulcer exhibits large-scale loss of tissue, where the wound may expose muscle, bone and tendons. The bottom of the wound will likely contain slough or dark, crusty, dead tissue (eschar).

[0115] As in the treatment of diabetic foot ulcers, debridement may be used to remove damaged, dead, or infected tissue from the wound, facilitating proper healing, e.g., as described herein and/or otherwise known in the art. In some embodiments, administration of a pharmaceutical composition of the invention follows debridement. For example, a pharmaceutical composition comprising a phage cocktail disclosed herein may be topically administered to a decubitus ulcer following surgical, mechanical, autolytic, or enzymatic debridement thereof.

[0116] Phage cocktail compositions of the present invention also find use in the treatment, management, control, and/or prevention of cellulitis and/or erysipelas, including but not limited to sores and lesions associated with cellulitis and erysipelas. Cellulitis and erysipelas are skin infections that develop as a result of bacterial entry via breaches in the protective barrier of the skin. For example, cracks in the skin, cuts, blisters, burns, insect bites, spider bites, tattoos, surgical wounds, intravenous drug injection, or sites of intravenous catheter insertion may provide a means of entry for bacteria. Group A Streptococcus and Staphylococcus are the most common bacteria involved in cellulitis. Cellulitis is observed most frequently among middle-aged and elderly individuals, while erysipelas occurs in young children and the elderly (Ellis Simonsen SM et al. 2006. Epidemiol Infect. 134(2):293; and Eriksson B. et al. 1996 Clin Infect Dis 23:1091). Also, people with immune deficiency, diabetes, alcoholism, fungal infections, and impaired lymphatic drainage are at increased risk. Diabetics are especially prone to cellulitis in the feet, because the disease causes impairment of blood circulation in the legs. The lower extremities are the most common site of infection for both erysipelas and cellulitis (Ellis

Simonsen SM et al. 2006. *Epidemiol Infect.* 134(2):293; Chartier C et al 1996 *Int J Dermatol* 35:779).

[0117] Cellulitis and erysipelas often coexist and generally manifest as areas of skin erythema, edema, and warmth. They differ in that erysipelas involves the upper dermis and superficial lymphatics, whereas cellulitis involves the deeper dermis and subcutaneous fat. Accordingly, erysipelas has more distinctive anatomic features than cellulitis -- erysipelas lesions may be raised above the level of surrounding skin with a clear line of demarcation between involved and uninvolved tissue (Bisno AL et al. 1996 *N Engl J Med* 334:240). The lesion may appear red, swollen, warm, hardened, and/or as a rash similar in consistency to an orange peel. Erysipelas may appear on the face, for example, in a “butterfly” pattern. More severe infections can result in vesicles, bullae, and petechiae, with possible skin necrosis. In addition, patients with erysipelas tend to have acute onset of symptoms with systemic manifestations, including fever and chills.

[0118] Patients with cellulitis tend to have a more gradual course of development, with symptoms appearing over a few days’ time. Various forms of cellulitis include periorbital cellulitis, abdominal wall cellulitis (in morbidly obese individuals), buccal cellulitis (due to *Streptococcus pneumoniae*), Ludwig’s angina (cellulitis within the submandibular space), and perianal cellulitis (due to group A beta-hemolytic streptococcus) (Barzilai A, et al, 1998 *Pediatr Infect Dis J.* 17(4):358; Thorsteinsdottir B, et al. 2005 *Scand J Infect Dis.* 37(8):605). Cellulitis also can result in influenza-like symptoms, with high temperatures and shaking.

[0119] In some embodiments, treatment of cellulitis or erysipelas further comprises administration of an antibiotic agent. For example, a pharmaceutical composition according to the invention may be topically administered to an erysipelas lesion, in combination with an antibiotic agent selected from the group consisting of penicillin, clindamycin, and erythromycin. As another example, a pharmaceutical composition according to the invention may be topically administered to a sore associated with cellulitis, in combination with an antibiotic agent selected from the group consisting of flucloxacillin, dicloxacillin, penicillins, ampicillin, and amoxicillin. The antibiotic may be administered orally, intravenously, or topically, e.g., along with topical administration of a cocktail of the instant invention.

[0120] Phage cocktail compositions of the present invention also find use in the treatment, management, control, and/or prevention of infections associated with burn wounds. A burn

wound is any area of non-intact skin caused, directly or indirectly, from a burn. A burn is a type of injury to the skin that can be caused by heat, as well as electricity, chemicals, light, radiation or friction. Burns may affect only the skin (epidermal tissue), but in some cases also injure deeper tissues, such as muscle, bone, and blood vessels. Burns can be classified by mechanism of injury, depth, extent and associated injuries, and comorbidities. Burns conventionally are described based on the depth of injury to the dermis, being loosely classified as first, second, third, and fourth degree burns. Walls et al., 2009, Rosen's Emergency Medicine: Expert Consult Premium Edition (Rosen's Emergency Medicine: Concepts & Clinical Practice (2v)). Important characteristics of a burn wound include its cause (thermal, chemical, electrical), anatomic location, depth (full or partial thickness), duration, and extent (percent total body surface area). Patient characteristics that affect burn wound healing include age, nutritional status, underlying medical conditions, and concomitant injury (e.g., head trauma, inhalation injury, bone fractures).

[0121] Infections among burn patients are a major problem, with the reported incidence of nosocomial infections varying at 63-240 per 100 patients and 53-93 per 1000 patient days, mainly depending on the definitions used (Chim H, et al, 2007, Burns 33:1008-1014; and Wibbenmeyer L, et al., 2006, J Burn Care Res 27:152-60). Moreover, bacterial infection of burn wounds are associated with adverse outcomes and mortality. In a series of 175 patients with severe burns, for example, infections preceded multiorgan dysfunction in 83% of patients and were considered the direct cause of death in 36% of patients who did not survive (Fitzwater J, et al., 2003, J Trauma 54:959-66). Burn wounds may become infected from multiple sources. Burn wounds may become initially infected with Gram positive bacteria, mainly staphylococci, that are normal deep inhabitants of the sweat glands and hair follicles exposed by the burn (Sharma BR., 2007, Infect Dis Clin North Am 21:745-59;ix). The moist, vascular burn eschar further may foster microbial growth. Gram negative bacterial infections may result from translocation from the colon, for example, due to reduced mesenteric blood flow at the time of burn and subsequent insults (Herndon DN, et al., 2000, Crit Care Med 28:1682-3). Furthermore, burns patients may develop immune deficits, including impaired cytotoxic T lymphocyte response, myeloid maturation arrest causing neutropenia, impaired neutrophil function, and decreased macrophage production (Sharma BR., 2007, Infect Dis Clin North Am 21:745-59;ix; Gamelli RL, et al., 2000, J Burn Care Rehabil 21:64-9; Hunt JP, et al., 1998, J Surg Res 80:243-51; and Shoup M, et al., 1998, Ann Surg 228:112-22). Finally, burns patients are susceptible to

hospital acquired infections, common to other patients in intensive care units, including intravascular catheter related infections and ventilator associated pneumonia, with an overall incidence of infection higher than that of other patients in intensive care units (Chim H, et al., 2007, Burns 33:1008-14; and Wibbenmeyer L, et al., 2006, J Burn Care Res 27:152-60). Indeed, most episodes of bloodstream infection in burn patients after the first week are caused by hospital-type multidrug resistant bacteria (Wibbenmeyer L, et al., 2006, J Burn Care Res 27:152-60; and Ressler RA, et al., 2008, J Am Coll Surg 206:439-44).

[0122] Convention treatment of burns includes debridement and excision, applying dressings the wound, wound closure, skin grafting, fluid resuscitation, management of wound infection such as administering antibiotics, pain control, nutritional support, and/or measures to inhibit excessive scar formation. A burn may be covered with a clean and dry sheet or dressing (such as cling film). Early cooling with cool water, within 30 minutes of the burn, reduces burn depth and pain. Debridement, cleaning, and dressings are important aspects of burn wound care.

[0123] In some embodiments, treatment of a burn wound further comprises administration of an antibiotic agent. It has been shown that antibiotic prophylaxis may reduce mortality, bacteraemia, and ventilator associated pneumonia among patients in intensive care units (Silvestri L, et al, 2007, J Hosp Infect 65:187-203; and De Smet AM, et al., 2009, N Engl J Med 360:20-31). In burns patients, the skin is an additional source of infection (Avni T, et al., 2010, BMJ 340: c241). In some embodiments, treatment of a burn wound further comprises administration of an agent for managing pain. A pharmaceutical composition according to the invention may be topically administered to an burn wound, in combination with an agent for pain management selected from the group consisting of a simple analgesic, ibuprofen, acetaminophen, and a narcotic. The antibiotic agent and/or agent for managing pain may be administered orally, intravenously, or topically, e.g., along with topical administration of a cocktail of the instant invention. One or more other aspects of conventional treatment of burns also may be used in combination with a phage cocktail composition of the instant invention.

[0124] In some embodiments, the agent for pain management for use in combination with a phage cocktail composition of the invention includes one or more agents selected from the group consisting of: paracetamol (acetaminophen), a non steroidal anti-inflammatory drug, ibuprofen, ketoprofen, piroxicam, hydrocodone, morphine, hydromorphone, oxymorphone, fentanyl, oxycodone, diamorphine, methadone, buprenorphine, meperidine, pentazocine, dextromoramide,

dipipanone, amitriptyline, dilaudid, tapentadol, and methadone. The agent for pain management may include any other agent for pain described herein and/or known in the art.

[0125] In some preferred embodiments, the agent for pain management is one that can be applied topically, such as a topical anesthetic agent. A topical anesthetic agent is a local anesthetic agent that is used to numb the surface of a body part, such as any area of the skin, the front of the eyeball, the inside of the nose, ear or throat, the anus, or the genital area. In some embodiments, the agent for pain management for use in combination with a phage cocktail composition of the invention includes one or more topical anesthetic agents selected from the group consisting of benzocaine, butamben, dibucaine, lidocaine, oxybuprocaine, pramoxine, prilocaine, proparacaine, proxymetacaine, and tetracaine (amethocaine). Topical anesthetic agents are available in creams, ointments, aerosols, sprays, lotions, and jellies. In further embodiments, the topical anesthetic agent may be used with one or more additional agents for pain management, such as another topical anesthetic agent, or a different agent for pain management, such as any other agent(s) for pain management described herein and/or known in the art.

[0126] Phage cocktail compositions of the invention will comprise a therapeutically and/or prophylactically effective amount of one of more phage strains, as described herein. A therapeutically and/or prophylactically effective amount refers to an amount required to bring about a therapeutic and/or prophylactic benefit, respectively, in a subject receiving said amount. A therapeutically and/or prophylactically effective amount will depend on the particular formulation, route of administration, condition being treated, whether other agents or therapies are used in combination with methods of the invention, and other factors.

[0127] In some specific embodiments, the phage cocktail compositions of the instant invention are formulated as pharmaceutical compositions for use in treating and/or preventing bacterial infections associated with areas of non-intact skin. The therapeutically and/or prophylactically effective amount will depend on the area of non-intact skin and the pharmaceutical compositions can be formulated to reflect same. For example, in some preferred embodiments, the pharmaceutical composition comprises phage strains where each is present in an amount corresponding to about 10^3 to about 10^{13} phage particles/cm² of said area. In some more preferred embodiments, the therapeutic and/or prophylactic amount may correspond to at least about 10^4 , at least about 10^5 , at least about 10^6 , at least about 10^7 , at least about 10^8 , or at least

about 10^9 , phage particles/cm² of the area of non-intact skin to be treated. In some more preferred embodiments, the therapeutic and/or prophylactic amount may correspond to less than about 10^{13} , less than about 10^{12} , less than about 10^{11} , less than about 10^{10} , less than about 10^9 , or less than about 10^8 phage particles/cm² of the area of non-intact skin to be treated. In still more preferred embodiments, each phage strain is present in the pharmaceutical composition in an amount corresponding to 10^7 to 10^9 phage particles/cm² of the non-intact skin area.

[0128] In some embodiments, administration of a therapeutically effective amount of a phage cocktail composition, in accordance with the instant invention, results in improved wound closure, such as a reduction in the area of non-intact skin (wound area) compared to the area before initiation of treatment. Wound area can be expressed as a percentage of the initial wound area, at one or more time points after initiation of treatment. For example, in some preferred embodiments, wound area decreases by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%; or at least about 90% over a course of treatment with a phage cocktail composition of the invention. In some specific embodiments, the decrease in wound area occurs at least by day 1 after treatment initiation (t1), day 2 after treatment initiation (t2), day 3 after treatment initiation (t3), day 4 after treatment initiation (t4), day 5 after treatment initiation (t5), day 6 after treatment initiation (t6), day 7 after treatment initiation (t7), day 8 after treatment initiation (t8), day 9 after treatment initiation (t9), day 10 after treatment initiation (t10), day 12 after treatment initiation (t12), day 15 after treatment initiation (t15), day 20 after treatment initiation (t20), day 25 after treatment initiation (t25), or day 30 after treatment initiation (t30). In a particularly preferred embodiment, wound area is reduced by about 30% to about 40%, by at least day 9 after treatment initiation (t9). In a further particularly preferred embodiment, wound area is reduced by about 40% to about 50%, by at least day 9 after treatment initiation (t9). In still a further particularly preferred embodiment, wound area is reduced by about 50% to about 60%, by at least day 9 after treatment initiation (t9). In an even more particularly preferred embodiment, wound area is reduced by about 60% to about 70%, by at least day 9 after treatment initiation (t9).

[0129] In some embodiments, administration of a therapeutically effective amount of a phage cocktail composition, in accordance with the instant invention, results in improved wound healing, such as an improvement in ulcer grade based on the PEDIS classification compared to the ulcer grade before initiation of treatment. PEDIS is a routinely used, validated classification

system for infections associated with wounds that has been developed by the International Working Group on the Diabetic Foot (IWGDF). IWGDF specifically developed a system for classifying wounds associated with diabetic foot infections that uses the acronym PEDIS, which stands for perfusion, extent (size), depth (tissue loss), infection, sensation (neuropathy). The classification originally was developed as a research tool (Schaper NC., 2004, Diabetes Metab Res Rev 20(Suppl 1):S90–5), and offers a semi-quantitative gradation for the severity of each of the categories. Specifically, PEDIS Grade 1 corresponds to no symptoms or signs of infection; Grade 2 corresponds to a local infection involving only the skin and subcutaneous tissue (without involvement of deeper tissues and without systemic signs), while any erythema involved must be between 0.5 cm and 2 cm; Grade 3 corresponds to a local infection, as described for Grade 2, but involving an erythema of greater than 2 cm or involving structures deeper than skin and subcutaneous tissues (e.g., abscess, osteomyelitis, septic arthritis, fasciitis), but without any systemic inflammatory response signs; and Grade 4 corresponds to a local infection, as described for Grades 2 and 3, but with the signs of systemic inflammatory response syndrome, as manifested by more than two of the following: a temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; a heart rate >90 beats/min; a respiratory rate >20 breaths/min or partial pressure of arterial carbon dioxide <32 mm Hg; and a white blood cell count $>12\,000$ or <4000 cells/ μL or $\geq 10\%$ immature (band) forms (see, e.g., Lipsky, BA, et al., 2012, CID 54:e132-e173). Another classification system has been developed by the IDSA (the Infectious Diseases Society of America), which rates the infection severity of infected wounds, in particular, diabetic foot infections. Specifically, the IDSA rates PEDIS Grades 1-4 as “uninfected”, “mild”, “moderate”, and “severe”, respectively (see, again, Lipsky, BA, et al., 2012, CID 54:e132-e173).

[0130] In some preferred embodiments, the PEDIS grade decreases from Grade 4 to Grade 3, Grade 2, or Grade 1, over a course of treatment with a phage cocktail composition of the invention. In other preferred embodiments, the PEDIS grade decreases from Grade 3 to grade 2 or Grade 1, over a course of treatment with a phage cocktail composition of the invention. In still other preferred embodiments, the PEDIS grade decreases from Grade 2 to Grade 1 over a course of treatment with a phage cocktail composition of the invention. In some specific embodiments, the decrease in ulcer grade occurs by at least day 1 after treatment initiation (t1), day 2 after treatment initiation (t2), day 3 after treatment initiation (t3), day 4 after treatment initiation (t4), day 5 after treatment initiation (t5), day 6 after treatment initiation (t6), day 7 after

treatment initiation (t7), day 8 after treatment initiation (t8), day 9 after treatment initiation (t9), day 10 after treatment initiation (t10), day 12 after treatment initiation (t12), day 15 after treatment initiation (t15), day 20 after treatment initiation (t20), day 25 after treatment initiation (t25), or day 30 after treatment initiation (t30).

[0131] In certain embodiments, a phage cocktail composition of the invention is used as a single agent for treating or preventing infections caused by *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*, such as diabetic foot infections. In other embodiments, a phage cocktail the invention is used in further combination with other agents, including other bacteriophage (for example, that target a different species or strain of bacteria involved in diabetic foot infections), or with antibiotics that target the same or different kinds of bacteria, including bacteria selected from any gram-positive bacteria, any gram-negative bacteria, and any other groups of bacteria that is not classified as gram-positive or gram-negative. The compositions of the invention may also be used in combination with any other means of treating bacterial infection known to one of skill in the art, in particular, any other means of treating diabetic foot ulcers.

[0132] In some embodiments, the cocktail composition according to the invention is used in combination with at least one additional phage strain against the same or a different bacteria species. In some preferred embodiments, the cocktail composition according to the invention is used in combination with at least one additional phage strain selected from the group consisting of bacteriophage strain F168/08 having antibiotic activity against one or more strains of *E. faecalis* and/or *E. faecium* (as disclosed in WO 2011/065854 and US Patent Application Publication No. 2012/0052048), bacteriophage strain F170/08 having antibiotic activity against one or more strains of *E. faecalis* and/or *E. faecium* (as disclosed in WO 2011/065854 and US Patent Application Publication No. 2012/0052048), bacteriophage strain F197/08 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F86/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F87s/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F91a/06 having antibacterial activity against one or more strains of *Staphylococcus aureus* (as disclosed in US Patent Application Publication No. 2012/0052048), bacteriophage strain F391/08 having

antibacterial activity against one or more strains of *Klebsiella pneumoniae* (as disclosed in US Provisional Application No. 61/384,015), bacteriophage strain F394/08 having antibacterial activity against one or more strains of *Acinetobacter baumannii* (as disclosed in US Provisional Application No. 61/384,01), bacteriophage strain F488/08 having antibacterial activity against one or more strains of *Escherichia coli* (as disclosed in US Provisional Application No. 61/384,01), and bacteriophage strain F387/08 having antibacterial activity against one or more strains of *Klebsiella pneumoniae* (as disclosed in US Provisional Application No. 61/384,015) (the contents of each are hereby incorporated by reference in their entireties). The contents of U.S. Provisional Application No. 61/384,015, filed on September 17, 2010, and International Application PCT/PT2011/000031, filed on September 19, 2011, also are hereby incorporated by reference in their entireties.

[0133] As used herein, the term “in combination” or “in further combination” or “further in combination” refers to the use of an additional prophylactic and/or therapeutic agent as well as a phage cocktail of the invention. The use of the term “in combination” does not restrict the order in which prophylactic and/or therapeutic agents are administered to a subject. A first prophylactic or therapeutic agent can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second prophylactic or therapeutic agent (different from the first prophylactic or therapeutic agent) to a subject.

[0134] In some embodiments, the invention provides methods of treating and/or preventing diabetic foot infections comprising administering a phage cocktail of the invention in combination with a standard and/or non-standard therapy for diabetic foot infections. Standard therapies for diabetic foot infections, including but not limited to cutaneous ulcers associated therewith, includes extracellular matrix replacement therapy, moist wound therapy, negative pressure wound therapy, arterial re-vascularization therapy, hyperbaric oxygen therapy, administration of an antibiotic agent, and administration of a growth factor (Blume et al. 2008 *Diabetes Care* 31: 631-636).

[0135] In some embodiments, the phage cocktail composition of the invention is administered topically, e.g., to the site of a diabetic foot ulcer, while an additional agent is administered systemically. For example, in some preferred embodiments, a phage cocktail composition of the invention is administered topically, e.g., to the site of a diabetic foot ulcer, while an antibiotic agent is administered systemically. In still more preferred embodiments involving the treatment of diabetic foot ulcers, the systemically administered antibiotic agent has antibacterial activity against *A. baumannii*, *P. aeruginosa*, and/or *S. aureus*. In some embodiments, the phage cocktail composition of the invention is administered topically, e.g., to the site of a diabetic foot ulcer, along with an additional agent, also being administered topically. For example, in some preferred embodiments, the phage cocktail pharmaceutical composition of the invention is administered topically along with a growth factor, e.g., to the site of a diabetic foot ulcer.

[0136] Extracellular matrix therapy is used in the treatment, management, control, and/or prevention of non-healing areas of non-intact skin, and is a standard therapy for diabetic foot ulcers. The synthesis of the extracellular matrix (ECM) is a key feature in wound healing, especially when there has been a significant loss of tissue. Extracellular matrix therapy is designed to reduce protease levels in wound fluids by providing a competitive substrate (collagen) for the proteases, thereby reducing proteolytic destruction of essential extracellular matrix (ECM) components and promotes healing. For example, ECM therapy may comprise administration of agents that reduce proteolytic destruction of fibronectin and/or platelet-derived growth factors (PDGFs); as well as reduce the synthesis of matrix metalloproteinases (MMPs), such as a mixture of metal cations. ECM therapy also may comprise administration of amelogenin, an ECM protein with biological activity in the regeneration and repair of skin (Romanelli M. 2010 Wounds - Clinical Review 6(2):47-52).

[0137] ECM therapy also may comprise use of bio-engineered tissue, e.g., to replace the lost ECM. Bio-engineered tissues, also called “skin-replacement products” or “skin substitutes”, often comprise biologic matrices, either with or without living cells (Brian DL et al. 2011. Expert Rev Dermatol. 6(3):255-262). Most bio-engineered tissues can be divided into living tissue substitutes versus bioactive adjuncts. The bio-engineered tissue may look like a thin, circular piece of real skin and can be placed directly on an area of non-intact skin. While the precise mechanism of healing is not completely understood, it is believed that bioengineered tissues improve healing by filling the wound with extracellular matrix proteins, and possibly also

expressing additional growth factors and cytokines that facilitate healing. Particular examples of bioengineered tissues used in the treatment of diabetic foot ulcers include Apligraf and Dermagraft, which are commercially available.

[0138] Arterial revascularization therapy (ART) also is used in the treatment, management, control, and/or prevention of non-healing areas of non-intact skin, and also is a standard therapy for diabetic foot ulcers. A preferred approach in treating diabetic foot infections includes the percutaneous method of ART, which involves percutaneous balloon angioplasty, possibly with stenting. Other revascularization approaches include aortoiliac reconstruction with aortofemoral bypass and femoral-popliteal-tibial bypass using the saphenous vein.

[0139] Hyperbaric oxygen therapy (HBOT) also is used in the treatment, management, control, and/or prevention of non-healing areas of non-intact skin, and also is a standard therapy for diabetic foot ulcers. HBOT refers to intermittent treatment of the entire body at greater than normal atmospheric pressures, often with increased oxygen content compared to that of normal air. For example, using a hyperbaric oxygen chamber, pressure may be increased up to two times normal atmospheric pressure. Also, the patient may be exposed to oxygen at a concentration of up to 100%. The increased pressure, combined with the increase in oxygen content, dissolves oxygen in the blood plasma, body cells, tissues, and fluids, which in turn aids the wound-healing process. It is believed that HBOT can stimulate the growth of new blood vessels to locations with reduced circulation, improving blood flow to areas with arterial blockage.

[0140] In some other embodiments, the invention provides methods of treating and/or preventing diabetic foot infections comprising administering a phage cocktail composition of the invention in combination with a non-standard therapy for diabetic foot infections. Non-standard therapies generally are used where the diabetic foot ulcer is refractory to one or more standard therapies.

6. EXAMPLES

[0141] It is understood that the following examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

[0142] Unless otherwise indicated, specific bacteriophage disclosed herein were isolated, processed and analyzed according to the following methods. Further, the study described below

was approved locally by the Animal Ethics Committee of the Instituto de Medicina Molecular and approved nationally by the Portuguese General Directorate of Veterinary Services (Direcção Geral de Veterinária), in accordance with Portuguese law. All animals in the study were maintained in accordance with European Directive 86/609/EC (Council of the European Communities. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Off J Eur Communities L358:1-28), Portuguese law (Portaria 1005/92) (Portuguese Agricultural Ministry. Portaria no. 1005/92 of 23 October on the protection of animals used for experimental and other scientific purposes. Diário da República I – Série B245:4930-4942), and the Guide for the Care and Use of Laboratory Animals (NRC 2011) (Institute for Laboratory Animal Research. 2011. Guide for the care and use of laboratory animals. Washington (DC): National Academies Press.).

[0143] One aim of this study was to investigate the antimicrobial activity and wound-healing capability of topically delivered bacteriophage solutions against wounds with chronic *S. aureus*, *P. aeruginosa*, and *A. baumannii* infections in two animal models of DM (rat and porcine).

6.1.1 PREPARATION OF BACTERIAL STRAINS

[0144] *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07 and *Acinetobacter baumannii* 1305/05 strains were isolated from human clinical skin wound samples collected from patients and identified in hospitals from the Lisbon area. The *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07 and *Acinetobacter baumannii* 1305/05 strains were deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bear accession numbers NCIMB 41862, NCIMB 41861, and NCIMB 41863, respectively. Each isolate was streaked onto tryptone soy agar media plates (TSA, Biokar Diagnostics, PantinCedex, France) and incubated at 37°C for 18h. All host strains were stored in tryptone soy broth (TSB, Biokar Diagnostics, Pantin Cedex, France) with 15% glycerol (w/v) at -70°C until needed.

[0145] Cryopreserved strains at -70°C were grown overnight on TSA at 37°C.

[0146] For in vitro experiments, single colonies were grown overnight in TSB at 37°C with agitation. Another bacterial suspension (a dilution of the overnight culture) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase

(optical density at 600 nm 0.3-0.5). An inoculum of approximately 2.0×10^7 cfu/ml was used for the growth curves.

[0147] For in vivo experiments, single colonies were grown overnight on tryptone soy agar (TSA, Biokar Diagnostics) at 37°C. After 24-h incubation, a bacterial suspension was prepared in saline (NaCl 0.9%, Applichem, Darmstadt, Germany) and compared with a McFarland Standard, that is, adjusted to McFarland's scale (bioMérieux, Craponne, France), with a subsequent 1:10 dilution, producing a final solution concentration of 2.0×10^7 cfu/mL. A single dose of 2.0×10^6 cfu of the clinical strains was used to inoculate the wounds.

6.1.2 PREPARATION OF BACTERIOPHAGE STRAINS

[0148] *Staphylococcus aureus* F44/10 and F125/10, *Pseudomonas aeruginosa* F770/05 and F510/08 and *Acinetobacter baumannii* F1245/05 lytic bacteriophage were isolated from sewage water from the Lisbon area and amplified in *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05 clinical strains, respectively. Standard methods (Adams M. Bacteriophages. New York: Interscience Publishers, Inc., 1959) for bacteriophage isolation and amplification were employed using the host strains described above. To produce bacteriophage stocks in sufficient quantities for experiments, a previously described protocol of amplification, concentration by high-speed centrifugation, and purification on a cesium chloride gradient was used. (Miller H., 1987, Methods Enzymol. 152: 145-70). Final concentrations were determined with double agar overlay plaque assays (Kropinski et al., 2009, In: Clokie M, Kropinski A, editors. Bacteriophages Methods and Protocols, volume 1: isolation, characterization, and interactions. New York: Humana Press, Springer Science + Business Media, 69-76). The phage strains F44/10, F125/10, F770/05, F510/08, and F1245/05 were deposited on September 16, 2011, under the terms of the Budapest Treaty at NCIMB Limited (Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland UK) and bear accession numbers NCIMB 41867, NCIMB 41866, NCIMB 41864, NCIMB 41868, and NCIMB 41865, respectively.

[0149] To isolate lytic bacteriophage against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, three clinical strains were used (indicator strains). Sewage water from different origins of the Lisbon urban area was concentrated by high speed centrifugation, before being used in the double agar overlay plaque assay to determine the presence of bacteriophage.

[0150] Water samples, 50 ml, were centrifuged at 8000xg for 10 minutes at 4°C. Supernatants were filtered with 0.45 µm Millex filters (Millipore, Massachusetts, USA) and centrifuged at 17000 rpm (Beckman J2-21M/E, rotor JA-20) for 3 hours at 4°C. The pellet was eluted in 5 ml of SM buffer (0.05 M Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM MgSO₄·7H₂O, 0.03 % gelatin) and allowed to elute overnight at 4°C. The re-suspended pellet was stored at 4°C until needed.

[0151] Water samples were also enriched to increase the chance of bacteriophage isolation (Van Twest and Kropinski 2009). One single colony of *Staphylococcus aureus* 743/06 indicator strain was inoculated in 5 ml tryptone soy broth supplemented with 0.5% yeast extract (TSBY, Biokar Diagnostics, PantinCedex, France) and incubated overnight at 37°C with agitation. A culture with 5 ml of TSBY, 50 µl of the overnight bacterial culture, 100 µl of the concentrated water, and 5mM of CaCl₂ and MgCl₂ was prepared and incubated overnight at 37°C with agitation. Before centrifuging the culture at 8000xg for 10 minutes at 4°C, chloroform was added and incubated for 5 to 10 minutes at room temperature to lyse the cells and free the intracellular bacteriophage in the medium. The supernatant was filtered with 0.45 µm Millex filters (Millipore, Massachusetts, USA) and stored at 4°C until needed.

[0152] After concentration and/or enrichment, the sewage water samples were tested for the presence of bacteriophage with the ability to infect *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07 and *Acinetobacter baumannii* 1305/05 clinical strains by the double agar overlay plaque assay. Briefly, bacterial indicator strains were grown overnight in TSB or TSBY (for the isolation of *Staphylococcus aureus* bacteriophage) at 37°C with agitation. Another bacterial suspension (a dilution of the overnight culture: 1:200 for *Staphylococcus aureus* indicator strain, 1:50 for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* indicator strains) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase (optical density at 600 nm 0.3-0.5). Each culture was supplemented with CaCl₂ and/or MgCl₂ (*Staphylococcus aureus* strain with 5 mM CaCl₂ and MgCl₂, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl₂) and placed in glass tubes (200 µl for *Staphylococcus aureus*, 400 µl for *Pseudomonas aeruginosa* and 150 µl for *Acinetobacter baumannii* strains) with the concentrated and/or enriched water samples (100 µl for the isolation of *Staphylococcus aureus* bacteriophage and 50 µl for the isolation of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteriophage). The mixture was incubated at 37°C for 30 minutes, after which 3 ml of soft agar was added (0.35% for the

isolation of *Staphylococcus aureus* bacteriophage and 0.7% for the isolation of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteriophage) pre-equilibrated at 50°C. After a brief vortex, the agar-water-bacterial suspension was overlaid onto tryptone soy agar supplemented with 0.5% yeast extract (TSAY, Biokar Diagnostics, Pantin Cedex, France) for the isolation of *Staphylococcus aureus* bacteriophage or TSA plates 1.5% (for the isolation of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteriophage), allowed to solidify at room temperature and incubated at 37°C. After 18 to 24 hours, the plates were checked for bacteriophage (clearing zones) within the bacterial lawn, indicating the presence of bacteriophage. Bacteriophage plaques were picked using sterile pipette tips, transferred to 100 µl of SM buffer and stored at 4°C.

6.1.3 PHAGE PROPAGATION AND CHARACTERIZATION

[0153] The isolated bacteriophage were subject to a process of propagation, amplification, and purification (3 consecutive elutions) in the indicator strains, before evaluation of its host range. Sensibility of 30 bacterial isolates against a particular bacteriophage was performed using the small drop plaque assay system (Mazzocco A, et al. 2009. Bacteriophages, methods and protocols vol. 1 chapter 9 Humana Press.). Briefly, bacterial indicator strains were grown overnight in TSB at 37°C with agitation. A new bacterial suspension (a dilution of the overnight culture: 1:200 for *Staphylococcus aureus* indicator strains, 1:50 for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* indicator strains) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase (optical density at 600 nm 0.3-0.5). Each culture was supplemented with CaCl₂ and/or MgCl₂ (*Staphylococcus aureus* strains with 5 mM CaCl₂ and MgCl₂, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl₂) and placed in glass tubes (200 µl for *Staphylococcus aureus*, 400 µl for *Pseudomonas aeruginosa* and 150 µl for *Acinetobacter baumannii* strains) to which was added 3 ml of soft agar (0.35% for the isolation of *Staphylococcus aureus* bacteriophage and 0.7% for the isolation of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteriophage) pre-equilibrated at 50°C. After a brief vortex, the agar-bacterial suspension was overlaid onto TSA plates 1.5% and allowed to solidify at room temperature. A small volume (5 µl) of each of the newly-isolated bacteriophage was dropped onto the freshly prepared bacterial lawns and plates were allowed to dry at room temperature before incubation overnight at 37°C. The sensibility of

30 bacterial isolates against a particular bacteriophage was determined by observing the appearance of a lytic zone in the spot area.

[0154] The bacteriophage with the best percentage of infection in the host range were selected and passed to a new process of amplification, concentration by high speed centrifugation, purification in Cesium chloride (CsCl) gradient, extraction of bacteriophage genomic DNA, and restriction. The process was repeated on a host range with 100 bacterial isolates, until final selection of bacteriophage was made, and their genomes sequenced.

6.1.4 PHAGE COCKTAILS IN VITRO EFFICACY

[0155] In vitro assays were performed to study the lytic activity of *Staphylococcus aureus* F44/10 and F125/10, *Pseudomonas aeruginosa* F770/05 and F510/08, and *Acinetobacter baumannii* F1245/05 bacteriophage, each individually or combined in liquid cultures, against *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07 and *Acinetobacter baumannii* 1305/05 indicator strains.

[0156] Bacterial indicator strains were grown overnight in TSB at 37°C with agitation. A fresh bacterial suspension (a dilution of the overnight culture: 1:200 for *Staphylococcus aureus* indicator strains, 1:50 for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* indicator strains) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase (optical density at 600 nm 0.3-0.5). Each culture was supplemented with CaCl₂ and/or MgCl₂ (*Staphylococcus aureus* strains with 5 mM CaCl₂ and MgCl₂, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl₂).

[0157] For each bacterium, three liquid cultures of 10ml TSB were prepared and tested simultaneously. A control culture of bacteria was inoculated with medium and 2.0×10^7 cfu/ml of the respective indicator strain (*Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05) in the exponential growth phase. A control culture of bacteriophage was inoculated with medium and the bacteriophage to be tested against the indicator strain, at a predetermined multiplicity of infection (F44/10 MOI = 10, F125/10 MOI = 10, F770/05 MOI = 1, F510/08 MOI = 10, and F1245/05 MOI = 10). A test culture was inoculated with medium, the bacteriophage to be tested (F44/10 MOI = 10, F125/10 MOI = 10, F770/05 MOI = 1, F510/08 MOI = 10 and F1245/05 MOI = 10) and 2.0×10^7 cfus/ml of the respective indicator strain (*Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05) in the exponential growth phase. All cultures were

supplemented with CaCl_2 and/or MgCl_2 (*Staphylococcus aureus* strains with 5 mM CaCl_2 and MgCl_2 , *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl_2). Cultures were incubated at 37°C with low agitation. Samples of 100 μl aliquots were taken from each culture at 1 hour intervals for a 24 hour-incubation period and used for serial dilutions.

[0158] Viable bacteria counts were quantified by the 10-fold serial dilution method (Murray PR, et al. 2003. Manual of clinical microbiology. Washington, DC: ASM Press.). For the control cultures of bacteria and test cultures, 100 μl of each dilution was inoculated onto the respective selective media plates: Chapman mannitol salt agar (Biokar diagnostics, PantinCedex, France) for *Staphylococcus aureus*, cefrimide agar (Merck Chemical, Darmstadt, Germany) for *Pseudomonas aeruginosa*, and CHROmagar *Acinetobacter* (CHROmagar, Paris, France) for *Acinetobacter baumannii*. The plates were incubated under aerobic conditions at 37°C for 24 hours, after which colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *Staphylococcus aureus*, based on colony morphology and mannitol salt agar fermentation (Chapman GH. 1946. J Bacteriol 51:409-410). The isolates grown on cefrimide agar were presumptively identified as *Pseudomonas aeruginosa*, based on colony morphology (Brown VI, et al. 1965. J Clin Pathol 18:752-756). The isolates grown on CHROmagar *Acinetobacter* were presumptively identified as *Acinetobacter baumannii*, based on colony red color (Wareham DW, et al. 2011. J Clin Pathol 64:164-167).

[0159] For the control cultures of bacteriophage, 100 μl aliquots were taken at time point (t_0) and immediately diluted to determine the initial titre of each bacteriophage by the double agar overlay plaque assay. Briefly, bacterial indicator strains were grown overnight in TSB at 37°C with agitation. A fresh bacterial suspension (a dilution of the overnight culture: 1:200 for *Staphylococcus aureus* indicator strain, 1:50 for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* indicator strains) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase (optical density at 600 nm 0.3-0.5). Each culture was supplemented with CaCl_2 and/or MgCl_2 (*Staphylococcus aureus* strain with 5 mM CaCl_2 and MgCl_2 , *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl_2) and placed in glass tubes (200 μl for *Staphylococcus aureus*, 400 μl for *Pseudomonas aeruginosa*, and 150 μl for *Acinetobacter baumannii* strains) with 100 μl of the bacteriophage culture dilution. The mixture was incubated at 37°C during 30 minutes, after which 3 ml of soft agar was added (0.35% for *Staphylococcus aureus* and 0.7% for *Pseudomonas aeruginosa* and

Acinetobacter baumannii cultures) pre-equilibrated at 50°C. After a brief vortex, the agar-bacteriophage-bacterial suspension was overlaid onto TSA plates 1.5%, allowed to solidify at room temperature, and incubated at 37°C. After 18 to 24 hours, the bacteriophage titer was determined by enumeration of the plaque forming units (pfus).

6.1.5 PHAGE COCKTAIL PREPARATION

[0160] FIG. 1 illustrates the preparation of an exemplary phage cocktail composition in accordance with the instant invention. After the *in vitro* assays of F44/10, F125/10, F770/05, F510/08, and F1245/05 bacteriophage, individually and combined, the lytic activity of the five bacteriophage was tested together in a single bacteriophage cocktail. Three primary cocktails (an *S. aureus* cocktail, a *P. aeruginosa* cocktail, and an *A. baumannii* cocktail) and one final cocktail were prepared using different concentrations and relative proportions of purified bacteriophages. The bacteriophage cocktail was prepared in saline with each bacteriophage present at predetermined MOIs (F44/10 MOI = 10, 10^{10} pfu/mL; F125/10 MOI = 10, 10^{10} pfu/mL; F770/05 MOI = 1, 10^9 pfu/mL; F510/08 MOI = 10, 10^{10} pfu/mL; and F1245/05 MOI = 10, 10^{10} pfu/mL).

[0161] Each culture was performed as previously described for individual bacteriophage testing. Control cultures of bacteria, control culture of the bacteriophage cocktail, and test cultures were prepared for *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05 indicator strains. Cultures were incubated at 37°C with low agitation and 100 µl aliquots were taken at 1 hour intervals for 24 hours and used for serial dilutions.

[0162] Viable bacteria counts were quantified by the 10-fold serial dilution method (Murray PR, et al. 2003. Manual of clinical microbiology. Washington, DC: ASM Press.). For the control cultures of bacteria and test cultures, 100 µl of each dilution was inoculated onto the respective selective media plates: Chapman mannitol salt agar for *Staphylococcus aureus*, cetrimide agar for *Pseudomonas aeruginosa*, and CHROmagar *Acinetobacter* for *Acinetobacter baumannii*. The plates were incubated at 37°C for 24 hours, after which colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *Staphylococcus aureus*, based on colony morphology and mannitol salt agar fermentation (Chapman GH. 1946. J Bacteriol 51:409-410). The isolates grown on cetrimide agar were presumptively identified as *Pseudomonas aeruginosa*, based on colony morphology (Brown VI, et al. 1965. J Clin Pathol 18:752-756). The isolates grown on CHROmagar *Acinetobacter* were presumptively identified

as *Acinetobacter baumannii*, based on colony red color (Wareham DW, et al. 2011. J Clin Pathol 64:164-167).

[0163] The initial bacteriophage titre was determined by the double agar overlay plaque assay. Samples of 100 µl aliquots were taken at time point (t0) and immediately diluted. Bacterial indicator strains were grown overnight in TSB at 37°C with agitation. A fresh bacterial suspension (a dilution of the overnight culture: 1:200 for *Staphylococcus aureus* indicator strain, 1:50 for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* indicator strains) was prepared, incubated at 37°C with agitation, and harvested when it reached the exponential growth phase (optical density at 600 nm 0.3-0.5). Each culture was supplemented with CaCl₂ and/or MgCl₂ (*Staphylococcus aureus* strain with 5 mM CaCl₂ and MgCl₂, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains with 10 mM MgCl₂) and placed in glass tubes (200 µl for *Staphylococcus aureus*, 400 µl for *Pseudomonas aeruginosa*, and 150 µl for *Acinetobacter baumannii* strains) with 100 µl of the bacteriophage cocktail culture dilution. The mixture was incubated at 37°C for 30 minutes, after which 3 ml of soft agar was added (0.35% for *Staphylococcus aureus* and 0.7% for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* cultures) pre-equilibrated at 50°C. After a brief vortex, the agar-bacteriophage-bacterial suspension was overlaid onto TSA plates 1.5%, allowed to solidify at room temperature, and incubated at 37°C. After 18 to 24 hours, the bacteriophage titer was determined by pfu (plaque-forming unit) enumeration.

6.1.6 PHAGE COCKTAIL IN VIVO EFFICACY IN A RAT MODEL

[0164] FIG. 2 illustrates the study protocol used for demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition, in accordance with the instant invention. A previously optimized rodent wound infection model in chemically-induced diabetic Wistar mice was used (Mendes JJ, et al. 2012. Comp Med 62:1-12).

[0165] Animals

[0166] Specific pathogen-free male Wistar rats [CrI:WI(Han)], weighing 250-350 g (8 to 10 weeks old) were obtained from Charles River Laboratories (L'Arbresle, Cedex, France). The animals were hosted in an approved animal care center under the following conditions: housing in microisolators in a room with controlled humidity (50-70%) and temperature (20-22°C), a 14-hour light and 10-hour dark cycle, and free access to pelleted rodent chow and filter-sterilized water. The animals were initially housed in groups of two. After hair removal and subsequent

procedures, they were housed individually to preserve skin and, later, dressing integrity. All surgical procedures were performed in a sanitized surgery room using autoclave-sterilized instruments.

[0167] Induction of DM

[0168] DM was chemically induced as described by Wu et al. (Wu K, et al. 2008. Curr Protoc Pharmacol 40:5.47.1-5.47.14). After a 12 hour fast, animals were given a single intraperitoneal (i.p.) injection of streptozotocin (65 mg/kg; Merck Chemical, Darmstadt, Germany) freshly prepared in 0.1 M citrate buffer (pH 4.5). A blood glucose measurement was performed on tail-vein blood using a glucometer 8 days later. Rats showing fasting blood glucose levels higher than 250 mg/dL were considered diabetic.

[0169] Hair removal

[0170] After DM confirmation (eight days later), 42 diabetic rats were anesthetized by i.p. injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (25 mg/kg), and their dorsal surface hair was trimmed with an electric clipper while the remaining hair was waxed thoroughly using cold wax strips (Veet cold wax strips, Reckitt Benckiser, West Ryde, Australia). The dorsum of the animals was then rinsed with a 10 % povidone-iodine solution and, after drying and cleansing, a liquid film-forming acrylate (Cavilon Skin Cleanser, 3M Health Care, Saint Paul, MN) was applied evenly to cover the hair removal area.

[0171] Wounding, splinting, first photograph, and dressing

[0172] Four days after hair removal, the animals were again anesthetized with the same protocol, and the dorsum skin was thoroughly washed with sterile saline followed by disinfection with 10% povidone-iodine and washing with 70% isopropyl alcohol after 10 minutes of povidone-iodine contact time. A round wound was created by making one full-thickness incision extending through the panniculus carnosus muscle in the interscapular region of the upper back of each rat using a punch biopsy instrument (diameter, 6 mm; Accu-Punch, Acuderm, Fort Lauderdale, FL, USA), and the skin flap was excised using Iris scissors. An oval-shaped silicone splint was adapted from a self-adhesive corn cushion (Comforsil, Toledo, Spain). An immediately bonding cyanoacrylate glue in a disposable single-dose package (Loctite, Henkel Corporation, Westlake, OH) was used to fix the splint to the skin, followed by interrupted 3-0 nylon sutures to ensure its position. Before dressing, wounds were photographed from a standard height (a 1.5-cm distance) using a mounted digital microscope (SuperEyes 200× USB

Digital Microscope, Shenzhen Tak and Assistive Technology, Shenzhen, China). Liquid film-forming acrylate was then applied to the epilated area, and the wound and the surrounding area were covered with a previously tailored, semi-occlusive, non-woven polyester dressing (Fixomull Stretch, BSN Medical, Hamburg, Germany). The splint and dressing were maintained in place throughout the entire course of the experiment using a jacket made from adhesive tape (Leukoplast surgical tape, BSN Medical, Hamburg, Germany).

[0173] Group randomization

[0174] After applying the dressing, and with the animals still anesthetized, the animals were randomly divided into 7 groups: negative control (n = 6), *Staphylococcus aureus*-inoculated control (n = 6) and test (n = 6), *Pseudomonas aeruginosa*-inoculated control (n = 6) and test (n = 6), and *Acinetobacter baumannii*-inoculated control (n = 6) and test (n = 6).

[0175] Wound infection

[0176] The wounds of the animals in the negative control group were injected with 100 μ L of sterile saline, whereas the wounds of the inoculated groups (test and control) were respectively inoculated with 100 μ L of the cultured *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Acinetobacter baumannii* (approximately 2.0×10^6 cfu) re-suspended in sterile saline by inserting a 27G/19-mm needle attached to a 1-mL disposable syringe through the silicon splint at a 45° angle.

[0177] Debridement

[0178] On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off and the wound debrided, in all animals. Debridement consisted of the simple mechanical removal of the scab, defined as a crust of dried blood, serum, and exudate, using strict aseptic technique.

[0179] Bacteriophage treatment protocol

[0180] The bacteriophage treatment protocol was divided into an induction phase and a maintenance phase and performed in all test groups. The induction phase was carried out after the first debridement (post-wounding day 4) and consisted of six (every four hours) 100 μ L primary bacteriophage solution administrations. The maintenance phase was carried out from day 5 to day 8 and consisted of twice daily (every 12 hours) 100 μ L primary bacteriophage solution administrations. If debridement was performed, the bacteriophage administration followed. The control groups received 100 μ L sterile saline with the same frequency.

[0181] Microbiological analysis

[0182] On days 4, 5, and 8 post-wounding and after debridement, a liquid Amies elution swab (eSwab Collection and Preservation System, Copan, Corona, CA) was used to collect and transport swab cultures. Bacteria collection was performed using the one-point method described by Sullivan et al. (Sullivan PK et al. 2004 Wounds 16:115-123). Briefly, using the sterile swab, the center surface of each wound was scrubbed by rotating the swab 3 times clockwise with enough manual pressure to produce a small amount of exudate. The swab was then inserted into the tube and transported to the laboratory for immediate processing. The swab collection tube was vortexed (with the swab inside) for 5 seconds, and a 100- μ L aliquot of the resulting suspension was used for serial dilutions.

[0183] Quantification was performed using the 10-fold serial dilution method (Murray PR et al. 2003. Manual of clinical microbiology. Washington, DC: ASM Press). In the infected/innoculated groups, 100 μ L of each dilution was plated onto the respective selective media plates: Chapman mannitol salt agar (Biokar diagnostics, Pantin Cedex, France) for *Staphylococcus aureus*, cetrimide agar (Merck Chemical, Darmstadt, Germany) for *Pseudomonas aeruginosa*, and CHROmagar *Acinetobacter* (CHROmagar, Paris, France) for *Acinetobacter baumannii*. In *Acinetobacter baumannii*-infected groups, 100 μ L of each dilution was simultaneously inoculated onto tryptone soy agar media plates (TSA, Biokar Diagnostics, Pantin Cedex, France). In the negative control group, 100 μ L of each dilution was inoculated onto tryptone soy agar media plates. The plates were incubated under aerobic conditions at 37°C for 24 hours, after which colony counts were performed. The isolates grown on Chapman mannitol salt agar were presumptively identified as *Staphylococcus aureus*, based on colony morphology and mannitol salt agar fermentation (Chapman GH. 1946. J Bacteriol 51:409-410). The isolates grown on cetrimide agar were presumptively identified as *Pseudomonas aeruginosa*, based on colony morphology (Brown VI, et al. 1965. J Clin Pathol 18:752-756). The isolates grown on CHROmagar *Acinetobacter* were presumptively identified as *Acinetobacter baumannii*, based on colony red color (Wareham DW, et al. 2011. J Clin Pathol 64:164-167).

[0184] Wound closure kinetics (planimetry)

[0185] On post-wounding day 9, before sacrifice, wounds were photographed from a 1.5 cm standard height using a mounted digital microscope as previously described. Wound kinetics was quantified using image-processing software (ImageJ, US National Institutes of Health,

Bethesda, MD) to measure the wound area by planimetry; wound area was expressed as a percentage of the initial wound area.

[0186] Histological analysis

[0187] All animals were sacrificed by i.p. injection of pentobarbital (200 mg) on day 9 post-wounding and each ulcer, including a 0.5-cm skin border, was entirely harvested using sterile surgical scissors and placed in a tube. The sample was fixed in 10% buffered formalin solution and, after overnight fixation, the tissue was trimmed and cut through at the widest margin, embedded in paraffin, and sectioned in 3- μ m increments. Sections were made perpendicular to the anterior-posterior axis and perpendicular to the surface of the wound.

[0188] For each wound, two serial sections were placed on a slide and stained with hematoxylin and eosin. Under light microscopy, the sections were photographed using a motorized inverted bright-field microscope (Zeiss Axiovert 200M, Göttingen, Germany) equipped with a color camera (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany) at 50 $\times$ magnification. Panoramic cross-sectional digital images of each wound were prepared using microscopy automation software (MetaMorph, MDS Analytical Technologies, Sunnyvale, CA) and processed using an image-processing software (ImageJ, US National Institutes of Health, Bethesda, MD). The images were analyzed for epithelial gap (EG) and dermal gap (DG) using the same image-processing software.

[0189] EG was defined as the distance between the advancing edges of clear, multiple-layer neoepidermis (Galiano RD, et al. 2004. *Wound Repair Regen* 12:485-492; Scherer SS, et al. 2008. *Wounds* 20:18-28), and its size was measured in mm, with an EG of zero representing a completely re-epithelialized wound. DG was defined as the distance between uninjured dermis on both sides of the wound (Galiano RD, et al. 2004. *Wound Repair Regen* 12:485-492; Scherer SS, et al. 2008. *Wounds* 20:18-28) and was measured in mm. All wound kinetics and histological measurements were performed with the investigator blinded as to sample origin (test or control).

6.1.7 PHAGE COCKTAIL IN VIVO EFFICACY IN A PIG MODEL

[0190] FIG. 3 illustrates the study protocol used for demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention. A previously optimized pig wound infection model in animal with chemically induced DM, as described by Hirsch et al. (Hirsch T, et al. 2008. *BMC Surg* 8:5), was modified to fit the needs of

the instant study. Three animals (negative control, inoculated-control, and inoculated-test) with a total of 48 excisional wounds (12 negative control wounds, 12 *Pseudomonas aeruginosa*-inoculated wounds, 12 *Staphylococcus aureus*-inoculated wounds, and 12 *Acinetobacter baumannii*-inoculated wounds) were used in this study.

[0191] Animals

[0192] Three female Yorkshire pigs (Farm) weighting ± 60 kg at arrival were allowed to acclimatize for 1 week prior to initiation of the experiment. Animals were housed singly in a cage, had free access to water, and were fed twice daily with a standard diet. During procedures, pigs were kept in a containment device.

[0193] Induction and control of DM

[0194] Pigs were fasted for 12 hours before DM induction. On the day of the procedure, the animals were weighed and given induction intramuscular anesthesia with xylazine hydrochloride and ketamine hydrochloride. While the animals were under anesthesia, a 21-Gauge intravenous (i.v.) catheter was inserted into an ear vein. Streptozotocin was prepared at a dose of 150 mg/kg body weight diluted in 10mL/g sterile saline, sterilized by filtration, and administered through the catheter over 1 minute. After recovering from anesthesia, post-procedural anti-emetic therapy with metoclopramide was given. Pigs were continuously observed for the first 3 hours and then food was offered ad libitum, in order to avoid hypoglycemia. Blood glucose was measured on a daily basis during the experiment. To keep the blood glucose concentration between 250 and 400 mg/dL, pigs received daily injections of 16 IU of pre-mixed neutral suspension of neutral (30%) and isophane insulin (70%) (Mixtard 30, Novo Nordisk, Bagsværd, Denmark) subcutaneously.

[0195] Hair removal, wounding, first photograph, and infection

[0196] Fourteen days after induction of DM, pigs received induction anesthesia as previously described. After induction, they underwent endotracheal intubation and were mechanically ventilated with a volume-limited, time-cycled BIRD ventilator (Mark 9; Bird Corporation, Palm Springs, CA) on a mixture of room air and titrated isoflurane (0.5% to 1.5%). The tidal volume was set at 12 mL/kg and ventilator rate at 12 breaths per minute. Prior to surgery, the dorsal surface hair was trimmed with an electric clipper and the remaining hair was waxed thoroughly using cold wax strips, the paraspinal area was thoroughly disinfected using 10% povidone iodine paint, and then washed with 70% isopropanol after 15 minutes of contact time.

[0197] For the inoculated-control and inoculated-test pigs, nine full-thickness excisional wounds (measuring 6 mm in diameter and with a depth of 6 mm) were created in each side of the paraspinal area (eighteen in total) using a 6 mm diameter biopsy punch. For the negative control pig, only 6 excisional wounds were created in each side of the paraspinal area (twelve in total). Subsequently, sterile forceps and a surgical blade were used to remove the full-thickness skin flap, and sterile gauze was utilized to cleanse the wounds of any coagulated blood and to control bleeding. Before covering with the adhesive chamber, wounds were photographed from a standard height using a mounted digital microscope. A modified adhesive chamber, made of a colostomy bag (Two Piece 35-mm Ostomy, Hollister Incorporated, Libertyville, IL) covered by a semi-occlusive non-woven polyester dressing, was placed over each wound and secured in place with surgical staples (Manipler AZ, B. Braun, Tuttlingen, Germany) and adhesive bandages.

[0198] In the inoculated-control and inoculated-test animals, wounds were divided into three subgroups: *Staphylococcus aureus* (2 x 6 ulcers); *Pseudomonas aeruginosa* (2 x 6 ulcers); and *Acinetobacter baumannii* (2 x 6 ulcers). To immerse the enclosed surface, wounds were respectively inoculated with 2×10^6 *Staphylococcus aureus* cfu in 100 μ L total solution (sterile 0.9% saline), 2×10^6 *Pseudomonas aeruginosa* cfu in 100 μ L total solution (sterile 0.9% saline), and 2×10^6 *Acinetobacter baumannii* cfu in 100 μ L total solution (sterile 0.9% saline). In the negative control group (12 ulcers), wounds were injected with 100 μ L of sterile saline. After recovering from anesthesia, post-procedural anesthesia (buprenorphine 0.005 mg/kg) and anti-emetic therapy was given every 12 hours for 48 hours.

[0199] Debridement

[0200] On days 4, 5, and 8 post-wounding, the semi-occlusive dressing was cut off and the wound debrided. Debridement consisted of the simple mechanical removal of the scab, defined as a crust of dried blood, serum, and exudate, using strict aseptic technique, as described for the rodent model.

[0201] Bacteriophage treatment protocol

[0202] A bacteriophage treatment protocol divided into an induction phase and a maintenance phase, similar to the rodent model, was used. The induction phase was carried out after the first debridement (post-wounding day 4) and consisted of six (every four hours for 24 hours) 100 μ L bacteriophage solution administrations, using the final bacteriophage cocktail. The maintenance phase was carried from day 5 to day 8 and consisted of twice daily (every 12 hours) 100 μ L

bacteriophage solution administrations, using the final bacteriophage cocktail. If debridement was performed, the bacteriophage administration followed. The control group received 100 µL sterile saline with the same frequency.

[0203] Microbiological analysis

[0204] A microbiological analysis protocol similar to the rodent study was used. On days 4, 5, and 8 post-wounding, and after debridement, a liquid Amies elution swab was used to collect and transport swab cultures. Bacteria collection was performed using the one-point method described by Sullivan et al. (Sullivan PK, et al. 2004. Wounds 16:115-123), as previously described. The swab was then inserted into the tube and transported to the laboratory for immediate processing. Quantification was performed using the 10-fold serial dilution method (Murray PR, et al. 2003. Manual of clinical microbiology. Washington, DC: ASM Press).

[0205] In the infected/innoculated groups, 100 µL of each dilution was plated onto the respective selective media plates: Chapman mannitol salt agar, cefrimide agar, and CHROmagar Acinetobacter. In the negative control group, 100 µL of each dilution was inoculated onto tryptone soy agar media plates. The plates were incubated under aerobic conditions at 37°C for 24 hours, after which colony counts were performed. The isolates were presumptively identified as previously described. In the negative control group, ulcers with more than 10³ cfu/swab on any given day were considered to be critically colonized, and excluded from further analysis.

[0206] Wound closure kinetics (planimetry)

[0207] On post-wounding day 9, after sacrifice, wounds were photographed from a standard height using a mounted digital microscope as previously described. Wound kinetics were quantified using image-processing software, as for the rodent study. Wound area was expressed as a percentage of the initial wound area.

[0208] Histological analysis

[0209] All animals were sacrificed by i.v. injection of pentobarbital on day 9 post-wounding and each ulcer, including a 0.5-cm skin border, was entirely harvested using sterile surgical scissors and placed in a tube. The samples were processed and photographed as described for the rodent study. The images were analyzed for epithelial gap (EG) using the same methods as in the rodent study.

[0210] Statistical analysis

[0211] All quantitative microbiological results are presented as the mean with the respective standard deviation and expressed as logarithm-transformed values [log(cfu/swab) for swab samples and log(cfu/ulcer) for tissue samples]. The data were compared using a logarithmic scale owing to the wide variations in cfu/swab among cultures. Comparisons between groups were performed using two-tailed Student t-tests, and a p value < 0.05 was considered significant. All planimetric results are expressed as the mean with the respective standard deviation of the percentage in area of the original wound size. Comparisons between groups were performed using two-tailed Student t-tests, and a p value < 0.05 was considered significant. Histological measures results are presented as the mean values with the respective standard deviation. Comparisons between groups were performed using two-tailed Student t-tests, and a p value < 0.05 was considered significant. All data was entered into a spreadsheet program (Excel, Microsoft, Redmond, WA) for statistical analysis. Analytical statistics were performed by Analyse-it version 2.21 Excel 12+ (Analyse-it Software, Leeds, UK), a statistical add-in program for the spreadsheet program.

6.1.8 RESULTS

[0212] Preclinical Studies - Pharmacology/Proof of Concept

[0213] To overcome the problem of the emergence of resistance to an individual bacteriophage strain being used, the instant inventors have developed bacteriophage cocktail compositions using more than one distinct bacteriophage strains, each having high lytic activity, despite previous difficulties in combining different specificities of bacteriophage, such as storage instability. The instant cocktails surprisingly demonstrate superior combined efficacy than when the phage are used individually, as illustrated in FIGs. 4, 5, and 6 kill curves for the in vitro assays. The decrease observed, for example, using cultures of F770/05 and F510/08 combined, in comparison with the use of cultures of the individual bacteriophage strains, demonstrates the advantage of using more than one bacteriophage strain (i.e., using bacteriophage cocktails in accordance with the instant invention) to increase the lytic activity against *Pseudomonas aeruginosa* strains while decreasing the emergence of bacteria resistant to the bacteriophage strains. Here, the inventors measured the capacity of infection of each of the selected bacteriophage in 100 different strains of bacteria -- F510/08 has a host range of 80%, F770/05 has a host range of 55%, F44/10 and F125/10 both have a host range of 100%, and F1245/05 has a host range of 75%.

[0214] Development of phage cocktail

[0215] The lytic activities of the newly isolated and characterized *Staphylococcus aureus* bacteriophage strains F44/10 and F125/10, *Pseudomonas aeruginosa* bacteriophage strain F770/05 and F510/08, and *Acinetobacter baumannii* bacteriophage strain F1245/05 were evaluated against *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05 strains, respectively, in order to develop a bacteriophage cocktail for application in wound infections. In vitro use of the bacteriophage cocktail (red line) lead to a significant reduction in *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* bacterial counts, as demonstrated in FIGs. 4, 5, and 6. The results of the in vitro use of the bacteriophage cocktail showed no inhibition of the bacteriophage strain's infecting ability due to the presence of different bacteriophage strains of distinct bacteria and further demonstrate that the cocktail actually enhances the lysis of bacteria in some cases (e.g, in the case of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteria).

[0216] Conventional growth curves were performed under controlled conditions using a previously determined bacterial inoculum. A preliminary study was conducted to determine the mass load of bacteria in a four day infection with *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, or *Acinetobacter baumannii* 1305/05 strains in the rat model. The cfu determination indicated a bacteria load of approximately 2.0×10^7 cfu/wound. This was the inoculum used in the in vitro assays.

[0217] Before evaluating a bacteriophage cocktail composition, each bacteriophage was tested individually and in combination, with different MOIs (data not shown) to screen their efficacy for potential therapeutic and experimental use in the animal models. Viable bacteria counts were monitored at 1-hour intervals for 24 hours.

[0218] FIG. 4 illustrates results of lytic studies evaluated against *Staphylococcus aureus* 743/06, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention. Bacteriophage F44/10 was tested individually at MOI equal to 10 to infect *Staphylococcus aureus* 743/06. Within the first 3 hours viable bacteria counts were reduced by approximately 4 log units compared with the control culture of bacteria. Afterwards, bacteria began to increase and 6 hours post-infection of the culture, viable bacteria were at 2.1×10^6 cfu/ml. Although there was a reduction of 97% when compared to the control culture of bacteria (at 24-hour incubation, viable counts were at 9.9×10^9 cfu/ml), bacteriophage F44/10 failed to eliminate completely the host cells. Similar results were observed for the 5 bacteriophage when assayed individually and probably were the result of the appearance of less susceptible bacteria to the bacteriophage infection.

[0219] Bacteriophage F125/10 was used at MOI equal to 10 to infect *Staphylococcus aureus* 743/06 (as illustrated in FIG. 4) and, within one hour, reduced the viable counts of bacteria by approximately 3 log units when compared with the control culture of bacteria 743/06. At 6-hour incubation, viable bacteria were at 3.6×10^7 cfu/ml (lower than the values of F44/10 culture) and at the end of the incubation period (24-hours), viable bacteria were at 6.6×10^9 cfu/ml. The distinct behavior, seen in the variations during the three initial hours of incubation of bacteriophage F44/10 and F125/10 activities, reflects the differences in their adsorption rates, latent periods, and burst sizes (data not shown).

[0220] It was expected that the combination of the two bacteriophage strains (F44/10 and F125/10) to *Staphylococcus aureus* 743/06 would reduce further the bacterial growth compared

to that observed individually. Bacteriophage F44/10 and F125/10 early lysed the bacteria reaching a 5 log unit reduction (viable bacteria at 1.9×10^4 cfu/ml) when compared with the control culture of bacteria. A low level of viable bacteria counts was maintained for four hours, however, despite the decrease when compared with the control culture of bacteria. At 24-hours incubation, viable bacteria counts had reached 4.3×10^9 cfu/ml (a 56.6% reduction of the bacterial counts).

[0221] FIG. 5 illustrates results of lytic studies evaluated against *Pseudomonas aeruginosa* 433/07, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention. A similar trend as seen with *Staphylococcus aureus* 743/06 was observed for bacteriophage F770/05 and F510/08 infecting *Pseudomonas aeruginosa* 433/07.

[0222] Bacteriophage F770/05 was tested individually at MOI equal to 1 against *Pseudomonas aeruginosa* 433/07, as illustrated in FIG. 5. Within the first 2 hours of incubation, viable bacteria counts were reduced approximately 3 log units compared with the control culture of bacteria 433/07, reaching 4.6×10^5 cfu/ml. Within 3 hours of incubation, bacteria started to grow exponentially, and at 6 hours of incubation, viable bacteria were at 7.3×10^7 cfu/ml. At the end of the culture incubation period (24 hours), bacteria counts were 2.8×10^9 cfu/ml, a 41.6% reduction when compared with the control culture of bacteria with 4.8×10^9 cfu/ml.

[0223] Bacteriophage F510/08 was also tested individually at MOI equal to 10 against *Pseudomonas aeruginosa* 433/07, as illustrated in FIG. 5, before its use when combined with F770/05 was tested. Within 2 hours of incubation, bacteriophage F510/08 had lysed the bacteria reaching approximately a 5 log unit reduction in bacteria counts when compared with the control culture of bacteria. At 6 hours of incubation, viable bacteria were at 1.7×10^7 cfu/ml, reaching the initial inoculum of approximately 2.0×10^7 cfu/ml. After 24 hours, the culture of F510/08 presented bacterial counts similar to those of the control culture of bacteria, with 2.8×10^9 cfu/ml, equivalent to a 41.6% reduction.

[0224] The previous assays with bacteriophage F770/05 and F510/08 with different MOIs demonstrated a suitable multiplicity of infection for use in a combination of the two bacteriophage: use of F770/05 with MOI equal to 1 and F510/08 with MOI equal to 10 was more effective in infecting *Pseudomonas aeruginosa* 433/07.

[0225] Bacteriophage F770/05 and F510/08 with MOI equal to 1 and 10, respectively, were tested against *Pseudomonas aeruginosa* 433/07, also as illustrated in FIG. 5. Within 3 hours of incubation, the bacteria counts presented a 4 log unit reduction when compared with the control culture of bacteria, and a 1 log unit reduction when compared with the culture of F770/05 alone. At this time point, F510/08 appeared to be more effective alone. At 6 hours of incubation, that is, 6 hours post-infection of the culture, the viable bacteria counts were at 1.4×10^8 cfu/ml; while at the end of the incubation period (24 hours), viable bacteria were at 1.7×10^8 cfu/ml. This represents a 96.5% reduction when compared with the control culture of bacteria. This decrease observed using both F770/05 with F510/08, compared to the use of the individual cultures of each bacteriophage, demonstrates the advantage to using more than one bacteriophage (as in bacteriophage “cocktails”), e.g., as discussed herein.

[0226] FIG. 6 illustrates results of lytic studies evaluated against *Acinetobacter baumannii* 1305/05, demonstrating in vitro efficacy of an exemplary phage cocktail composition in accordance with the instant invention. Bacteriophage F1245/05 also was selected to be included in the exemplary bacteriophage cocktail composition illustrated here, and was tested individually at MOI equal to 10 against *Acinetobacter baumannii* 1305/05, as illustrated in FIG. 6. At this MOI, bacteriophage F1245/05 caused a rapid decrease in viable bacteria, such that in only 1 hour, counts were reduced from 2.0×10^7 cfu/ml to 6.8×10^4 cfu/ml. Low cfu values were maintained for approximately 3 hours, when compared with the control culture of bacteria. After 6 hours of incubation, viable bacteria counts were at 1.1×10^8 cfu/ml, and at the end of the incubation period (24 hours), cells had grown to 2.2×10^9 cfu/ml. Bacteriophage F1245/05 individually thus showed high lytic activity against *Acinetobacter baumannii* 1305/05, achieving at 24 hours of incubation, a 76.3% reduction of the bacterial counts when compared with the control culture.

[0227] The results indicate that use of individual bacteriophage strains eventually may lead to the emergence of resistance to the particular bacteriophage strain, and one way to avoid or minimize this is to develop compositions comprising more than one bacteriophage strains, preferably each having high lytic activity against a specific bacteria.

[0228] Exemplary bacteriophage cocktail

[0229] The purpose of this study was to produce an exemplary bacteriophage cocktail against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* strains using

bacteriophage strains that display broad activity against a range of these bacteria and that may be used in the management of a wound infection.

[0230] After testing individual activities of certain bacteriophage strains against the bacterial strains, a bacteriophage cocktail was prepared having the following composition: F44/10 in a MOI = 10, F125/10 in a MOI = 10, F770/05 in a MOI = 1, F510/08 in a MOI = 10, and F1245/05 in a MOI = 10. See FIG. 1.

[0231] This bacteriophage cocktail was tested in vitro against *Staphylococcus aureus* 743/06, *Pseudomonas aeruginosa* 433/07, and *Acinetobacter baumannii* 1305/05 strains. The viable bacteria counts were determined for each bacterium individually in growth curves at 1 hour intervals for 24 hours. In vitro application of the bacteriophage cocktail lead to a significant reduction of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* bacterial counts (See FIGs. 4-6, respectively).

[0232] A single inoculation of the bacteriophage cocktail was sufficient to reduce *Staphylococcus aureus* 743/06 by 5 log units when compared with the control culture of bacteria, as illustrated in FIG. 4. A similar decrease had been observed for the activity of the two *Staphylococcus aureus* bacteriophage strains combined. During the second and sixth hour of incubation, the efficacy of the cocktail was lower than the two bacteriophage strains F44/10 and F125/10 together; however 24 hours later, the difference between the two cultures was significant. Bacterial counts started to decrease and at the end of the incubation period (24 hours), viable bacteria were at 6.8×10^8 cfu/ml (a reduction of 93.1% when compared with the control culture).

[0233] The bacteriophage cocktail was also tested against *Pseudomonas aeruginosa* 433/07, as illustrated in FIG. 5, presenting a reduction of the bacterial counts of almost 4 log units at the 2-hour incubation period. At the end of the culture incubation time (24 hours), bacteria counts were at 3.9×10^8 cfu/ml and, while slightly higher than when both bacteriophage strains were tested together, this represents a 91.9% reduction of viable bacteria when compared with control culture of bacteria *Pseudomonas aeruginosa* 433/07. This difference was not sufficient to associate an inhibitory effect of the cocktail on *Pseudomonas aeruginosa* bacteriophage strains F770/05 and F510/08 lytic activity.

[0234] The bacteriophage cocktail also was tested against *Acinetobacter baumannii* 1305/05 strain, as illustrated in FIG. 6. At 2 hours of incubation, the bacteriophage cocktail had reduced

the bacteria counts by approximately 4 log units when compared with the control culture of bacteria 1305/05. At the end of the culture incubation period (24 hours), bacterial counts had reached 8.6×10^8 cfu/ml, which represents a reduction of 92.8% when compared with the control culture. These results indicate a better performance of this bacteriophage in the cocktail.

[0235] The results of the in vitro application of the bacteriophage cocktail indicated that there was no inhibition of each bacteriophage's infecting ability due to the presence of different bacteriophage of distinct bacteria.

[0236] Results using the Rodent Model

[0237] To recap, studies conducted in rodents were approved locally by the Animal Ethics Committee of the Instituto de Medicina Molecular and nationally by the Portuguese General Directorate of Veterinary Services (Direcção Geral de Veterinária), in accordance with Portuguese law. All animals were maintained in accordance with European Directive 86/609/EC, Portuguese law (Portaria 1005/92), and the Guide for the Care and Use of Laboratory Animals (NRC 2011). A previously optimized rodent wound infection model in chemically-induced diabetic Wistar mice was used (Mendes JJ, et al. 2012. Comp Med 62:1-12).

[0238] As noted above, FIG. 2 illustrates the study protocol. Briefly, after induction and establishment of diabetes, treatment was administered based on the lysis curves of the bacteriophage strains and similar to antibiotic posology, i.e., every 4 hours during the first 24 hours and then once a day for 5 days. Doses were based on the results obtained in an epidemiological study done previously in a range of patients collected from several Portuguese hospitals (data not shown). In this study, it was concluded that the concentration of bacteria that infects diabetic foot ulcers ranges from 10^6 to 10^8 -- the in vitro assays indicated that the MOI is 1 or 10, depending on the bacteriophage strain, such that the phage concentration was 10^7 to 10^9 per cm^2 of ulcer.

[0239] The choice of an infected chronic wound model was based on the fact that bacteriophage only replicate in their specific live bacterial host, thus it did not make sense to study models where infection would not be chronic. The primary endpoint was microbiological decrease in the wounds. Wound closure also was measured although differences in wound closure do not always reflect the real decrease in the dermal and epidermal gap, as when comparing histopathology to microbiology results.

[0240] To overcome problems of bacteriophage waste and the appearance of bacterial resistance, which might occur in the animals models associated with environmental conditions and the extension of treatment, larger numbers of bacteriophage were used in the in vivo experiments, that is, to inoculate the wounds and treat the animals. The number of bacteriophage particles present in the exemplary cocktail for in vivo use was increased by 1 log.

[0241] Microbiology analysis

[0242] FIG. 7 illustrates results of microbial load analyses for control (C) and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0243] After induction therapy (t1), there was a statistically significant difference in colony counts in selective media between control and test subgroups in *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated groups. At day four after treatment initiation (t4), there was a statistically significant difference in colony count in selective media between control and test subgroups. From t0 to t4 in *Staphylococcus aureus* and *Pseudomonas aeruginosa*-inoculated control subgroups, there was a tendency for microbial load reduction. That is, the bacteriophage-treated animals showed significantly lower counts than the control animals in all three groups on t1 and t4.

[0244] Wound closure kinetics (planimetry)

[0245] FIG. 8 illustrates results of wound closure analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention. Wound area was assessed on t1 and t9, and the differences between the two timepoints calculated.

[0246] Planimetry analysis of wounds in the rat model showed a statistically significant difference between the negative control group and all the inoculated control subgroups wound areas, with a tendency for wound area reduction between control and test subgroups in all groups; and a statistically significant difference between control and test subgroups wound areas in the *S. aureus*-inoculated and *P. aeruginosa*-inoculated groups. That is, bacteriophage treatment reduced wound size in both *S. aureus*- and *P. aeruginosa*-infected wounds ($p < 0.05$).

[0247] Histological analysis

[0248] FIG. 9 illustrates results of histological analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a rat model of an exemplary phage cocktail composition in accordance with the instant invention.

[0249] There was a statistically significant difference between the negative control group and all the inoculated control subgroups in both the epidermal gap (EG) and the dermal gap (DG). There was a statistically significant difference between control and test subgroups EG in the *Staphylococcus aureus*-inoculated and *Pseudomonas aeruginosa*-inoculated groups ($p < 0.05$). In DG, the difference between test and control subgroups only obtained statistical significance in *Pseudomonas aeruginosa*-inoculated group. These results correlate with the fact that *Pseudomonas aeruginosa* enters more deeply into tissues than *Staphylococcus aureus*. *Acinetobacter baumannii* is a colonizer appearing later in the process in patients which is why it is important for the bacteriophage cocktail, in certain embodiments, to comprise this bacteriophage.

[0250] Results using the Pig Model

[0251] Similar results as those illustrated above were obtained using a pig model. To recap, a previously optimized pig wound infection model in chemically induced animals, as described by Hirsch et al. (Hirsch T, et al. 2008. BMC Surg 8:5), was modified to fit the needs of the instant study. Three animals (negative control, inoculated-control, and inoculated-test) with a total of 48 excisional wounds (12 negative control wounds, 12 *Pseudomonas aeruginosa*-inoculated wounds, 12 *Staphylococcus aureus*-inoculated wounds, and 12 *Acinetobacter baumannii*-inoculated wounds) were used. As noted above, FIG. 3 illustrates the study protocol. The same dosing and dosing schedule was used as in the rat model, described above.

[0252] Microbiology analysis

[0253] FIG. 10 illustrates results of microbial load analyses for control (C) and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0254] After induction therapy (t1), there was a statistically significant difference in colony count in selective media between control and test subgroups in the *S. aureus*-inoculated and *P.*

aeruginosa-inoculated groups, and a tendency for microbial load reduction in average colony count for the *A. baumannii*-inoculated test and control subgroups ($p < 0.05$). At day four after treatment initiation (t4), there was a statistically significant difference in colony count between control and test subgroups in *Staphylococcus aureus*-inoculated and *Pseudomonas aeruginosa*-inoculated groups. There was no statistically significant difference in average colony count in *Acinetobacter baumannii*-inoculated test and control subgroups, although there is a trend for a decrease in colony count.

[0255] Wound closure kinetics (planimetry)

[0256] FIG. 11 illustrates results of wound closure analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0257] Histological analysis

[0258] FIG. 12 illustrates results of histological analyses for negative, control (C), and test (T) groups for *Staphylococcus aureus*-inoculated, *Pseudomonas aeruginosa*-inoculated, and *Acinetobacter baumannii*-inoculated animals, demonstrating in vivo efficacy in a pig model of an exemplary phage cocktail composition in accordance with the instant invention.

[0259] There was a statistically significant difference between the negative control group and all the inoculated control subgroups in EG. There also was a statistically significant difference between control and test subgroups in the *Staphylococcus aureus*-inoculated and *Pseudomonas aeruginosa*-inoculated groups with respect to EG ($p < 0.05$).

[0260] Discussion of Results

[0261] Based on previous rodent studies (Mendes JJ, et al. 2012. Comp Med 62:1-12), it was known that the bacterial colony counts in tissue cultured from infected wounds at t4 are, on average, $7.54 \pm 0.19 \log(\text{CFU})$ per ulcer. The instant study used high bacteriophage doses (10^8 to 10^9 pfu per administration), which yields a multiplicity of infection of 10 to 100. It is believed that this initial dose is sufficiently in excess of the target bacterium population to cause reductions without the need for bacteriophages to replicate and complete their life cycle. This is in contrast with previous bacteriophage therapy studies that employed relatively low bacteriophage doses and mainly relied on active therapy, which involves phage infection/replication cycles to reduce the target bacterium (Loc Carrillo C, et al. , 2005, Appl

Environ Microbiol. 71(11):6554-6563). These processes of active and passive bacteriophage therapy have been described for in vitro and in vivo studies (Cairns BJ, et al., 2011, PLoS Pathog. 5(1):e1000253; and Hooton SP, et al., 2011, Int J Food Microbiol. 151(2): 157-163).

[0262] All three outcomes were improved by bacteriophage treatment in animals infected with *S. aureus* and *P. aeruginosa*, and bacterial reduction was observed in those infected with *A. baumannii*. Without being limited by theory, this can be explained by studies (e.g., Simoes LC, et al., 2008, Appl Environ Microbiol. 74(4): 1259-1263) in which the presence of *Acinetobacter* spp. in a biofilm community was found to facilitate surface colonization by other species, namely *Staphylococcus* spp. Results herein are in line with this finding, in that excess bacteria growing in non-selective media in *A. baumannii*-inoculated groups were determined to be primarily *Staphylococcus* spp.

[0263] In the instant work, bacterial counts were assessed at t4 (day 4 after treatment initiation), and colony counts were significantly different for *S. aureus* and *P. aeruginosa* test conditions compared to control. especially with respect to *P. aeruginosa*. These findings are in agreement with previous studies (e.g., Mendes JJ, et al. 2012. Comp Med 62:1-12; and Fazli M, et al., 2009, J Clin Microbiol. 47(12): 4084-4089). In particular, Fazli et al. used confocal laser scanning microscopy of clinical wound-biopsy specimens to demonstrate that the distance from *P. aeruginosa* aggregates to the wound surface was significantly greater than that of *S. aureus* aggregates, which led to an underestimation of the former in swab samples. This observation supports the possibility that factors intrinsic to each pathogenic bacterial strain can contribute to differences among studies that compare cultures grown from swabs and tissue samples.

[0264] Planimetric assessments revealed statistically significant differences between the control and test groups treated with *S. aureus* and *P. aeruginosa*, and the same trend was observed for *A. baumannii*. These results were similar to the EG and DG measurements in harvested histological specimens.

[0265] Importantly, the results obtained in the rodent model were largely corroborated by experiments in swine, as pigs are considered the ideal large animal model for studying cutaneous disease (Greenhalgh DG., 2005, J Burn Care Rehabil. 26(4): 293-305). In both models, there was a significant reduction of bacterial counts at both time points (t1 and t4) for *S. aureus* and *P. aeruginosa* infections.

[0266] Significant results were observed in the *S. aureus*-inoculated and *P. aeruginosa*-inoculated test animals with regard to EG measurements.

[0267] Accordingly, this study suggests that bacteriophage-containing TAT provides a viable treatment for DFIs, including infections caused by drug-resistant bacteria, offering an effective and novel therapeutic approach for addressing the serious problems associated with DFIs and other chronic skin and soft tissue infections. That is, bacteriophage treatment effectively decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps, in *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections, and thus topically administered bacteriophage treatment is effective in resolving chronic infections, especially when applied in conjunction with wound debridement.

6.1.9 TOXICOLOGY PROGRAM FOR FIRST HUMAN STUDY

[0268] A 4-week dermal irritation study in mini-pigs, as shown below, is proposed to support an initial clinical study. If necessary, a 4 week intravenous (iv) study in rats also can be carried out, although it is believed that the iv study will not lead to any conclusion as the bacteriophage strains do not replicate if they are not in the presence of specific bacteria; the iv study, however, can confirm this belief and also that the bacteriophage strains are safe when given in much higher quantities (as using the same dose in an iv study as topical application reflects a much higher dose when compared to maximum absorption possible).

[0269] 4-week Dermal Irritation Study in Minipigs, 4-week Recovery Period (GLP)

[0270] The study design involves 5 female minipigs, where the dose route is dermal (2 sites/site; wrapped/washed) with a frequency of once daily. The dose preparation is to use as received. Observations are made twice daily (mortality/morbidity), while a detailed clinical observation is made weekly, including measuring body weight. Physical examinations are conducted by a staff veterinarian on all animals prior to initiation of administering an exemplary phage cocktail composition in accordance with the instant invention, as the test article (TA).

[0271] To evaluate skin reaction, each animal is evaluated for erythema and edema daily prior to each dose beginning on Day 2. Any non-test site lesions also are noted and described. A Draize scale for scoring skin irritation is used, as follows. For erythema and eschar formation - 0 indicates no erythema; 1 indicates very slight erythema (barely perceptible); 2 indicates well-defined erythema; 3 indicates moderate to severe erythema; while 4 indicates severe erythema (beet redness) to slight eschar formation (injuries in depth). For edema formation - 0 indicates

no edema; 1 indicates very slight edema (barely perceptible); 2 indicates slight edema (edges of area well defined by definite raising); 3 indicates moderate edema (raised approximately 1 millimeter); while 4 indicates severe edema (raised more than 1 millimeter and extending beyond area of exposure). If there is no necropsy, the animal is returned to stock.

[0272] Punch biopsies are performed for histological analyses. Three samples (naïve, placebo, TA) from the left side are collected on day 29; 3 samples from the right side are collected on day 57; all samples are preserved, processed to slides, and microscopically evaluated. Formulation analysis involves a certificate of analysis, provided by a third party.

[0273] 4-week Dermal Toxicology Study in Rats, 4-week Recovery Period (GLP)

[0274] The study design involves a dosage schedule for administering an exemplary phage cocktail composition according to the invention, as presented in the table below.

	Main Study		Recovery	
	Males	Females	Males	Females
Vehicle Control	10	10	5	5
Mid Dose	10	10	5	5
High Dose	10	10	5	5

[0275] Additional animals/sex/treatment group are included as replacement animals.

[0276] The dose route is iv bolus, with a frequency of once daily. The dose preparation is to use as received. Observations are made twice daily (mortality/morbidity), while a detailed clinical observation is made weekly, including measuring body weight. Food consumption occurs weekly as well.

[0277] Ophthalmology tests are performed on all animals pre-test and on all surviving main study animals at termination and recovery. Clinical pathology tests, including hematology, coagulation, clinical chemistry, and urinalysis, are performed on all surviving main study animals once at the terminal or recovery necropsy. Necropsy tests are performed on all main study and recovery animals; TK animals are euthanized and discarded. Organ weights are

determined for adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, and uterus.

[0278] Slide preparation and microscopic pathology is performed for all animals in the vehicle control and high dose groups, as well as for all found-dead animals, and includes the preparation of a full set of standard tissues (approximately 65) and target organs in low and mid dose groups, and for all recovery animals. Slide preparation and microscopic pathology also is performed on gross lesions from all animals.

[0279] Standard statistical analyses are used. Standard parameters for toxicokinetic analysis also is used, such as AUC, $t_{1/2}$, t_{max} , and C_{max} .

6.1.10 CLINICAL STUDIES

[0280] Clinical Trial/Proof of Concept

[0281] FIG. 13 illustrates diabetic foot infection classifications and application of phage therapy thereto using exemplary phage cocktail compositions in accordance with the instant invention. Basically, a bacteriophage cocktail composition in accordance with the invention is applied in grade 2-3 ulcers based on the PEDIS classification. Administration involves use of a topically-applied liquid formulation after debridement of the wound.

[0282] FIG. 14 illustrates a clinical study design for exemplary phage cocktail compositions in accordance with the instant invention for use in therapy for diabetic foot ulcers. The study type is interventional, with the intervention model of parallel assignment; the study design involves randomized allocation with open label masking; endpoint classification includes safety and efficacy, while the primary purpose is for treatment. Primary outcome measures include microbial tissue burden (biopsy); wound fluid microbial burden (time frame: with every foam change); and tissue microbial burden (time frame: 24h/3rd day/5th day/clinical signs off/on). Secondary outcome measures include wound assessment in each follow up visit (time frame 4 weeks and 12 weeks).

[0283] After the screening visit and debridement of wounds, the eligible patient population randomly receive placebo or a bacteriophage cocktail composition in accordance with the invention at 10^9 phages/cm²/application for 4h/4h the first 24hours and then once a day for 5 days. Safety and efficacy of the drug is compared to the placebo group; however, if a patient is determined to be at risk of requiring an amputation, the patient also can be included in the therapy group.

[0284] Criteria involve the following inclusion and exclusion criteria. Inclusion criteria include a clinical bacterial infection at the ulcer site of grade 2 or 3 according to PEDIS classification and no contraindications to negative wound pressure therapy. Exclusion criteria include topical application on the wound of any agents for advanced wound care (e.g., use of a growth factor) within the prior 7 days.

[0285] Certain modifications and improvements will occur to those skilled in the art upon a reading of the foregoing description. It should be understood that all such modifications and improvements have been deleted herein for the sake of conciseness and readability but are properly within the scope of the following claims.

[0286] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition comprising a first and a second purified strain of bacteriophage, each of said strains having a genome which comprises at least 95% sequence identity to the nucleotide sequence selected from the group of consisting of SEQ ID NO:1 and SEQ ID NO:2, and showing antibacterial activity against *Staphylococcus aureus*;

a third purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:3 and showing antibacterial activity against *Pseudomonas aeruginosa*;

a fourth purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:4, and showing antibacterial activity against *Pseudomonas aeruginosa*; and

a fifth purified strain of bacteriophage having a genome which comprises at least 95% sequence identity to the nucleotide sequence SEQ ID NO:5 and showing antibacterial activity against *Acinetobacter baumannii*

2. The composition according to claim 1, wherein

said first and said second purified strain of bacteriophage each has a genome which comprises at least 99% sequence identity to the nucleotide sequence selected from the group of consisting of SEQ ID NO:1 and SEQ ID NO:2, and shows antibacterial activity against *Staphylococcus aureus*;

said third purified strain of bacteriophage has a genome which comprises at least 99% sequence identity to the nucleotide sequence SEQ ID NO:3 and shows antibacterial activity against *Pseudomonas aeruginosa*;

said fourth purified strain of bacteriophage has a genome which comprises at least 99% sequence identity to the nucleotide sequence SEQ ID NO:4, and shows antibacterial activity against *Pseudomonas aeruginosa*; and

said fifth purified strain of bacteriophage has a genome which comprises at least 99% sequence identity to the nucleotide sequence SEQ ID NO:5 and shows antibacterial activity against *Acinetobacter baumannii*.

3. The composition according to claim 1 or 2, wherein each of said first, second, fourth and fifth bacteriophage strains is present in said composition in an amount about 10 times that of said third bacteriophage strain.

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4. The composition according to claim 2 or 3, wherein said composition comprises said five bacteriophage strains comprising nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.
5. A pharmaceutical composition comprising the composition according to any one of claims 1-4 and a pharmaceutically acceptable carrier.
6. The pharmaceutical composition according to claim 5, wherein said composition is formulated for topical application.
7. The pharmaceutical composition according to claim 5 or 6, wherein said composition further comprises a sterile buffer comprising about 0.05 M Tris-HCl, about 0.1M NaCl, and about 10mM MgSO₄.7H₂O.
8. The pharmaceutical composition according to any one of claims 5-7, wherein said composition is contained in an ampoule.
9. The pharmaceutical composition according to any one of claims 5-8, further comprising an additional agent, said additional agent selected from the group consisting of an antibiotic agent, an anti-inflammatory agent, an antiviral agent, a local anesthetic agent, a growth factor, and a corticosteroid
10. The pharmaceutical composition according to claim 9, wherein said antibiotic agent has antibacterial activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and/or *Staphylococcus aureus*.
11. The pharmaceutical composition according to any one of claims 5-10, wherein said composition is for use in treating or reducing the incidence of a bacterial infection associated with an area of non-intact skin, wherein said third phage strain is present in said composition in an amount corresponding to 10^3 to 10^{13} phage particles/cm² of said area; and
 wherein each of said first, second, fourth, and fifth bacteriophage strains is present in said composition in an amount about 10 times that of said third bacteriophage strain.

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12. The pharmaceutical composition according to claim 11, wherein said third phage strain is present in said composition in an amount corresponding to 10^7 to 10^9 phage particles/cm² of said area.

13. The pharmaceutical composition according to claim 12, wherein said third phage strain is present in said composition in an amount corresponding to 10^8 phage particles/cm² of said area.

14. A pharmaceutical composition comprising the composition according to claim 2 and a pharmaceutically acceptable carrier,

wherein said composition is formulated for topical application; and

wherein said composition is for use in treating or reducing the incidence of a bacterial infection associated with an area of non-intact skin, and said third phage strain is present in said composition in an amount corresponding to 10^7 to 10^9 phage particles/cm² of said area; and

wherein each of said first, second, fourth, and fifth bacteriophage strains is present in said composition in an amount about 10 times that of said third bacteriophage strain.

15. A method of treating or reducing the incidence of a bacterial infection in a subject in need thereof comprising administering to said subject a therapeutically effective amount of the pharmaceutical composition according to any one of claims 5-14.

16. Use of a therapeutically effective amount of the pharmaceutical composition according to any one of claims 8-13 in the preparation of a medicament for treating or reducing the incidence of a bacterial infection in a subject in need thereof.

17. The method according to claim 15, or use according to claim 16, wherein said bacterial infection is an infection by one or more of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

18. The method or use according to claim 17, wherein the pharmaceutical composition is administered topically and wherein said subject is a human.

19. The method or use according to any of claims 15-18, wherein said bacterial infection is associated with area of non-intact skin selected from cellulitis sore, a chronic ulcer, a burn

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wound, a diabetic ulcer, a cutaneous ulcer, an erysipelas lesion, a decubitus ulcer, and a pressure sore.

20. The method or use according to claim 19, wherein said cutaneous ulcer is associated with a diabetic foot infection.

21. A method of treating or reducing the incidence of a bacterial infection in a subject in need thereof comprising administering to said subject a therapeutically effective amount of the pharmaceutical composition according to claim 14,
wherein said bacterial infection is diabetic foot infection.

22. Use of a therapeutically effective amount of the pharmaceutical composition according to claim 14 in the preparation of a medicament for treating or reducing the incidence of a bacterial infection in a subject in need thereof,
wherein said bacterial infection is diabetic foot infection.

23. The method according to claim 21, or use according to claim 22, wherein said treatment comprises topically administering said composition to a cutaneous ulcer associated with said diabetic foot infection

24. The method or use according to claim 22, wherein said administration follows mechanical debridement of said ulcer; and/or
wherein said administration comprises use of at least one of a dressing, an instillation device, and a negative pressure wound therapy device.

25. The method or use according to claim 23 or 24, wherein said pharmaceutical composition is administered every 4 hours or every 6 hours for an initial 24 hours.

26. The method or use according to claim 25, wherein, following said initial 24 hours, said pharmaceutical composition is administered every 12 hours or every 24 hours for at least 3 or 4 additional days.

27. The method or use according to any one of claims 21-26, wherein said treatment is used in combination with a standard therapy for diabetic foot infection,

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wherein said standard therapy is selected from the group consisting of extracellular matrix replacement therapy, moist wound therapy, negative pressure wound therapy, arterial re-vascularization therapy, hyperbaric oxygen therapy, administration of an antibiotic agent, and administration of a growth factor.

28. The method or use according to claim 27, wherein said moist wound therapy comprises use of an adhesive-backing film, a silicone-coated foam, a hydrogel, and/or a hydrocolloid;

wherein said extracellular matrix replacement therapy comprises use of bio-engineered tissue;

wherein said antibiotic agent has antibacterial activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and/or *Staphylococcus aureus*; and/or

wherein said growth factor is at least one selected from the group consisting of platelet-derived growth factor, granulocyte colony-stimulating factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, and vascular endothelial growth factor.

29. The method or use according to claim 27, wherein said antibiotic agent is administered via systemic administration and/or wherein said growth factor is administered via topical administration.

30. The method or use according to any one of claims 21-26, wherein said treatment is used in combination with a non-standard therapy for diabetic foot infection, wherein said diabetic foot infection is refractory to a standard therapy.

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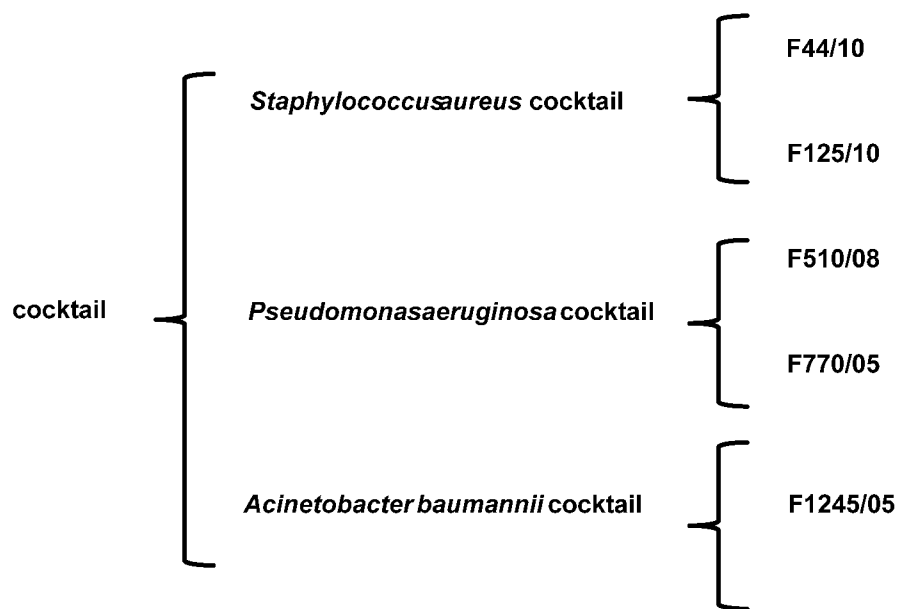


FIG. 1

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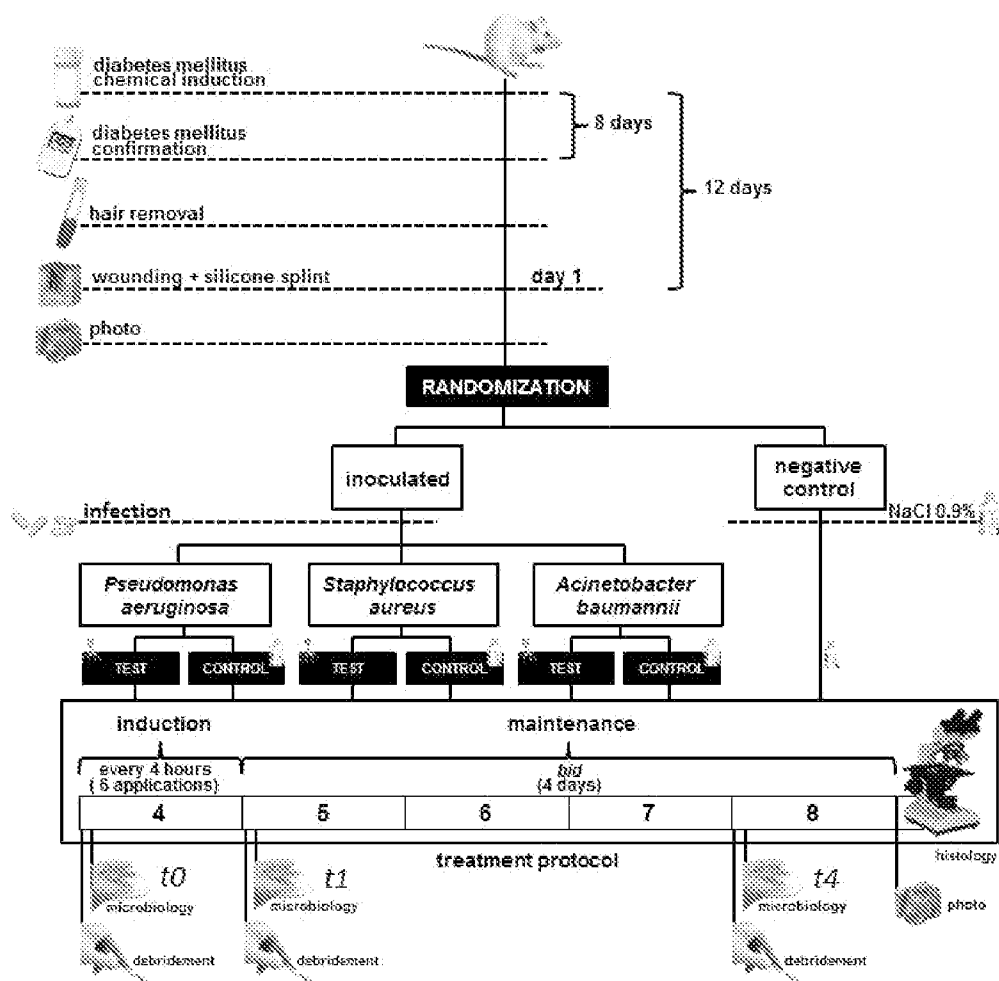


FIG. 2

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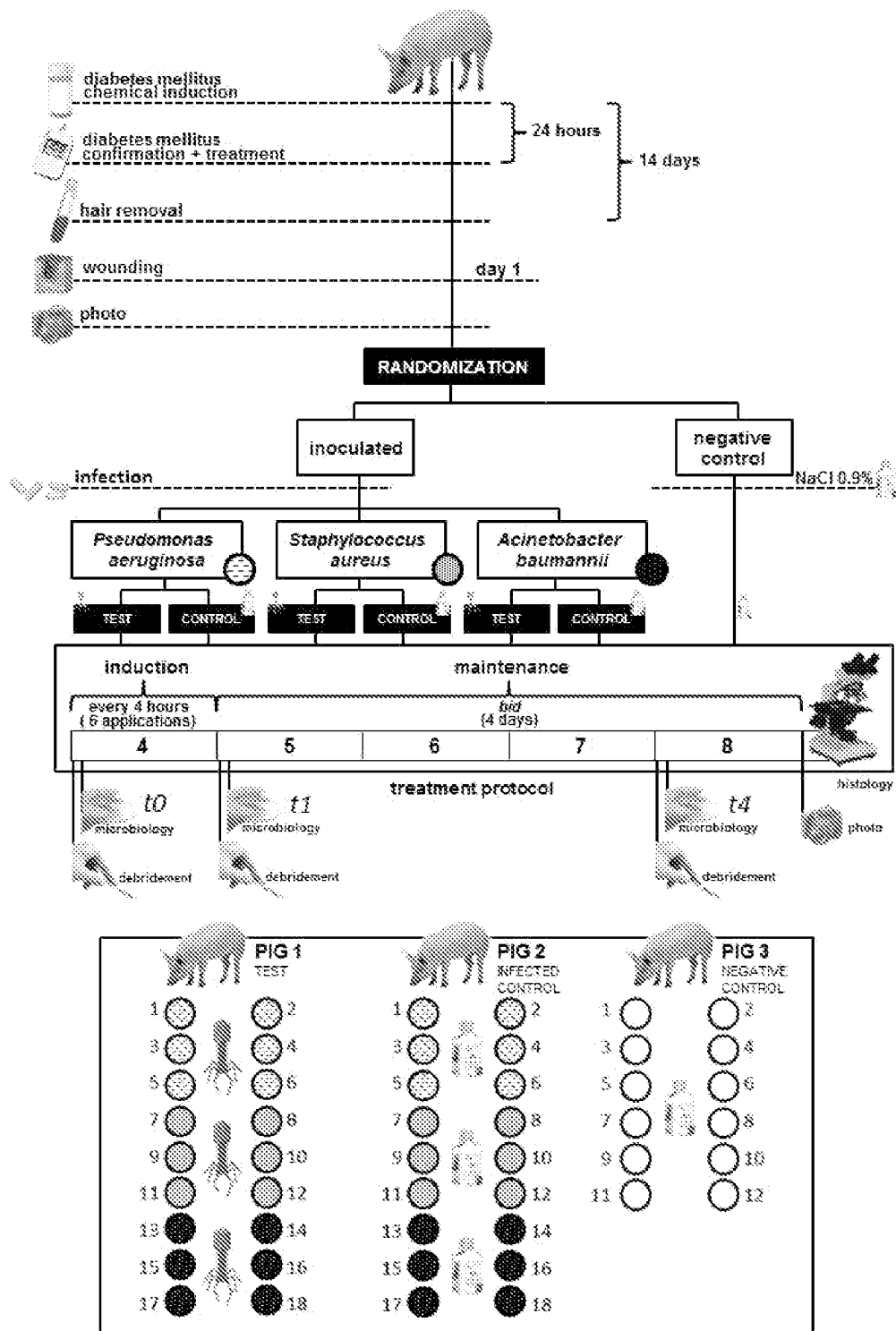


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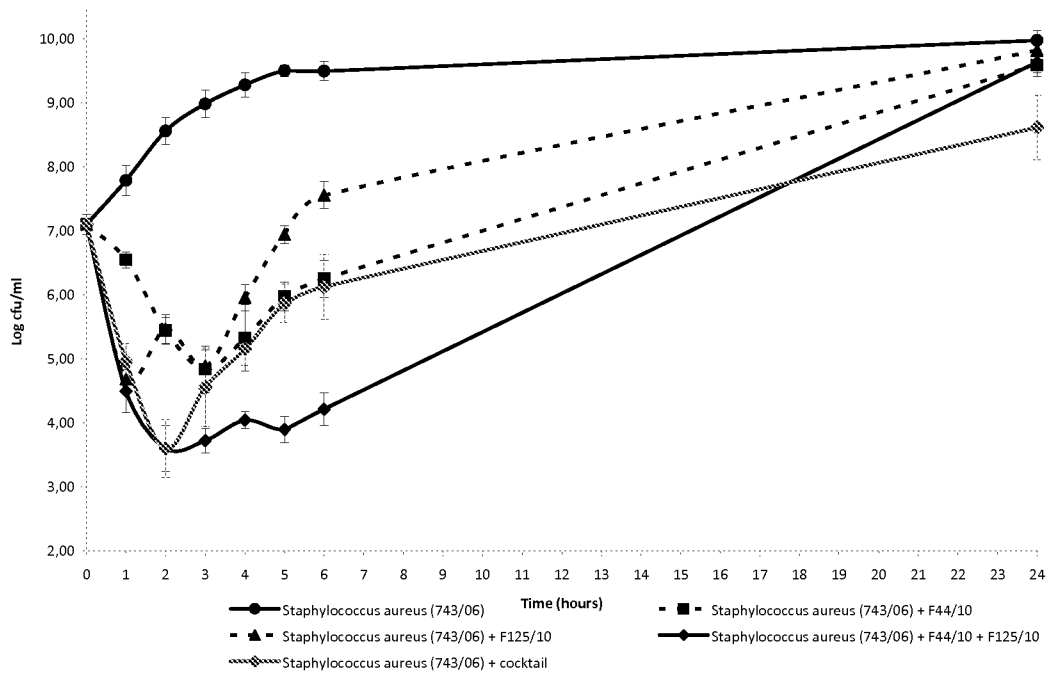


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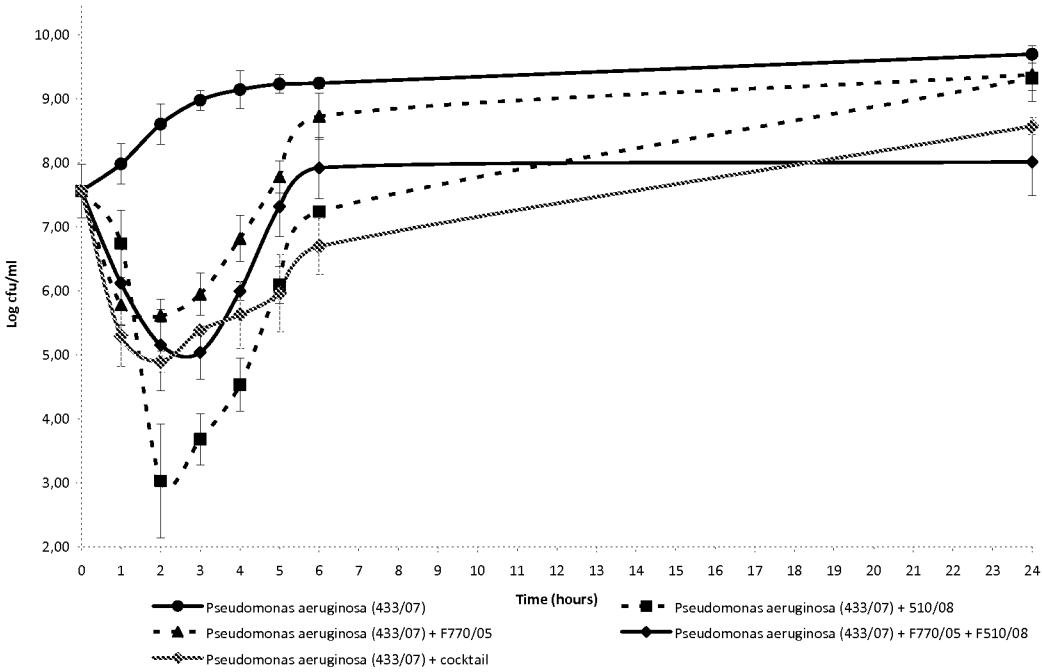


FIG. 5

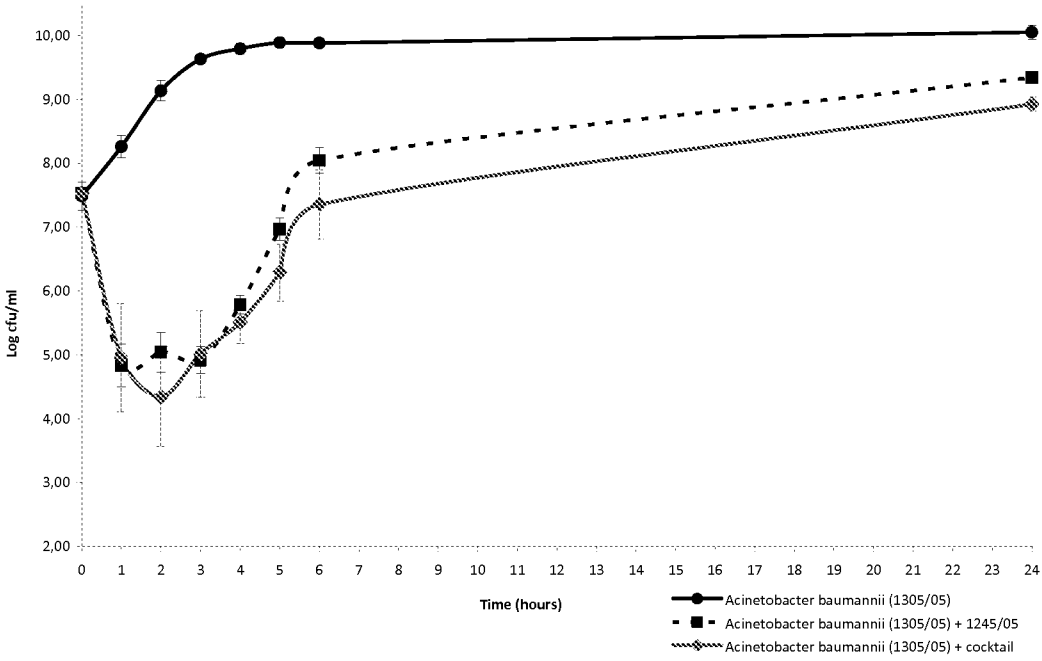


FIG. 6

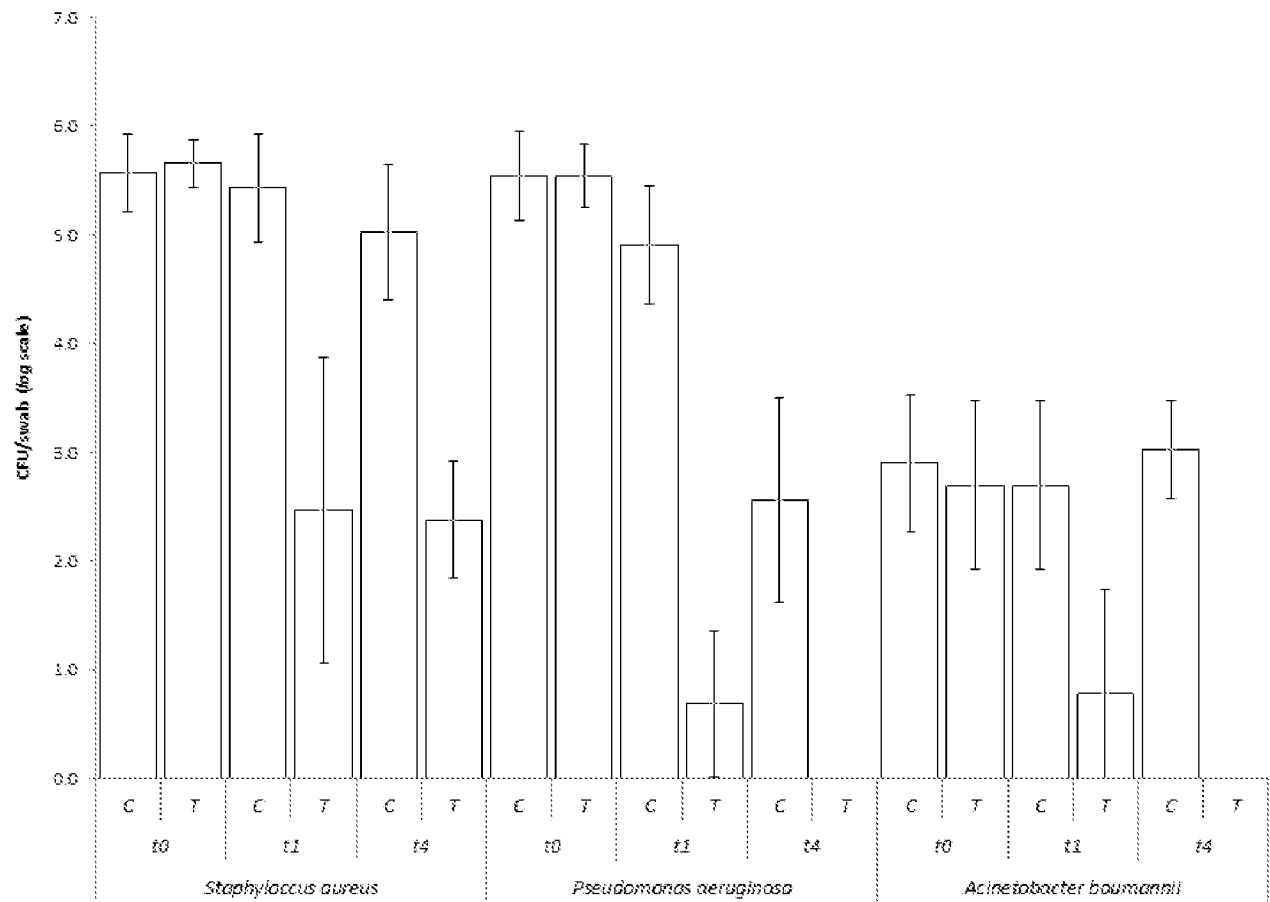


FIG. 7

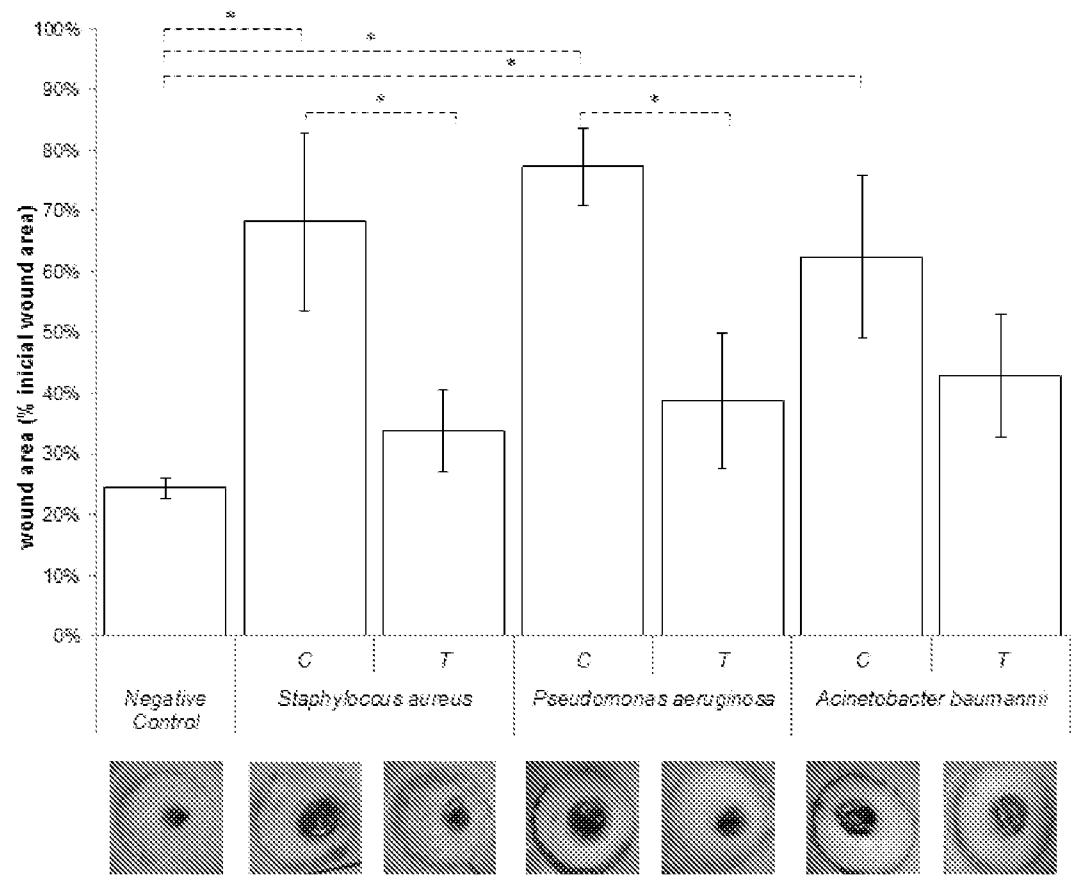


FIG. 8

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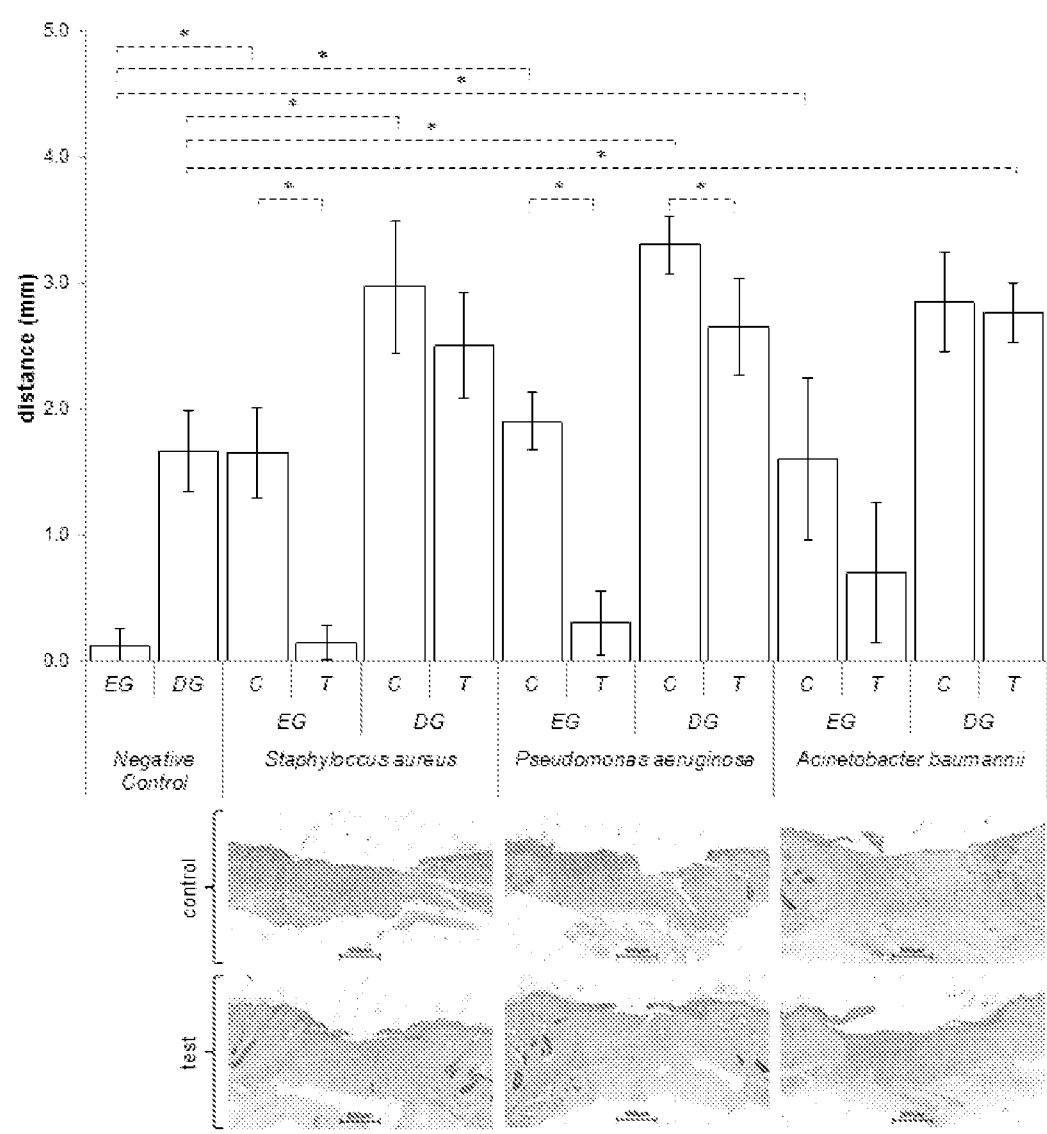


FIG. 9

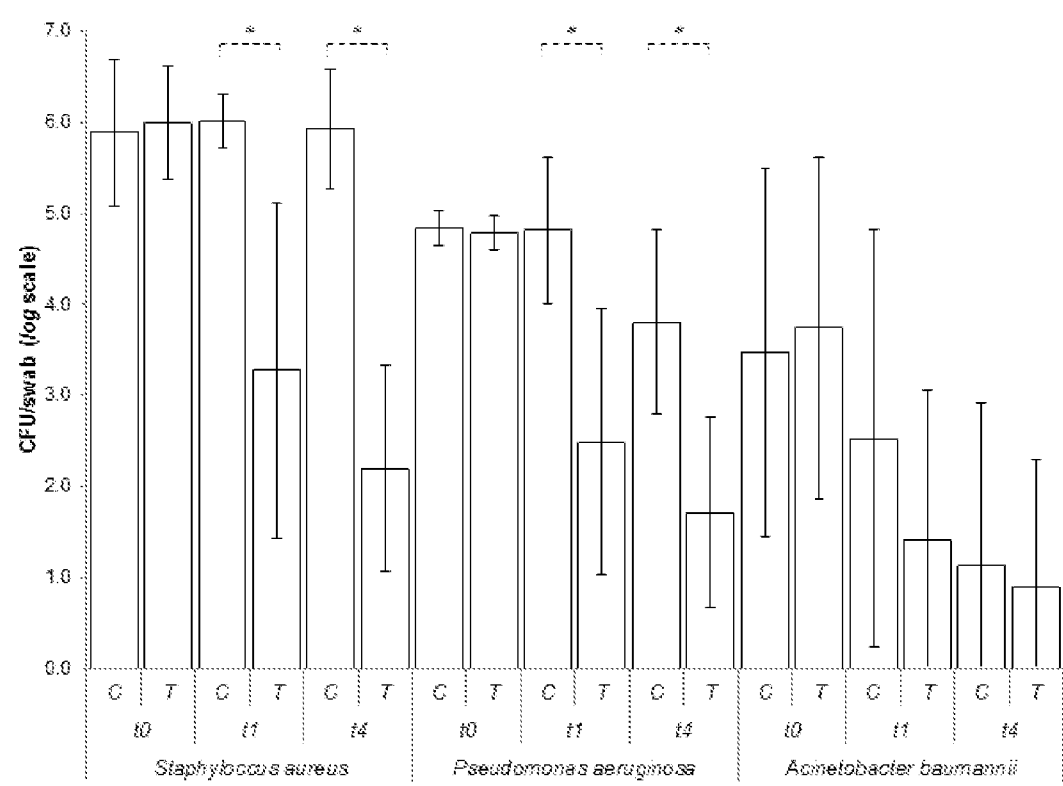


FIG. 10

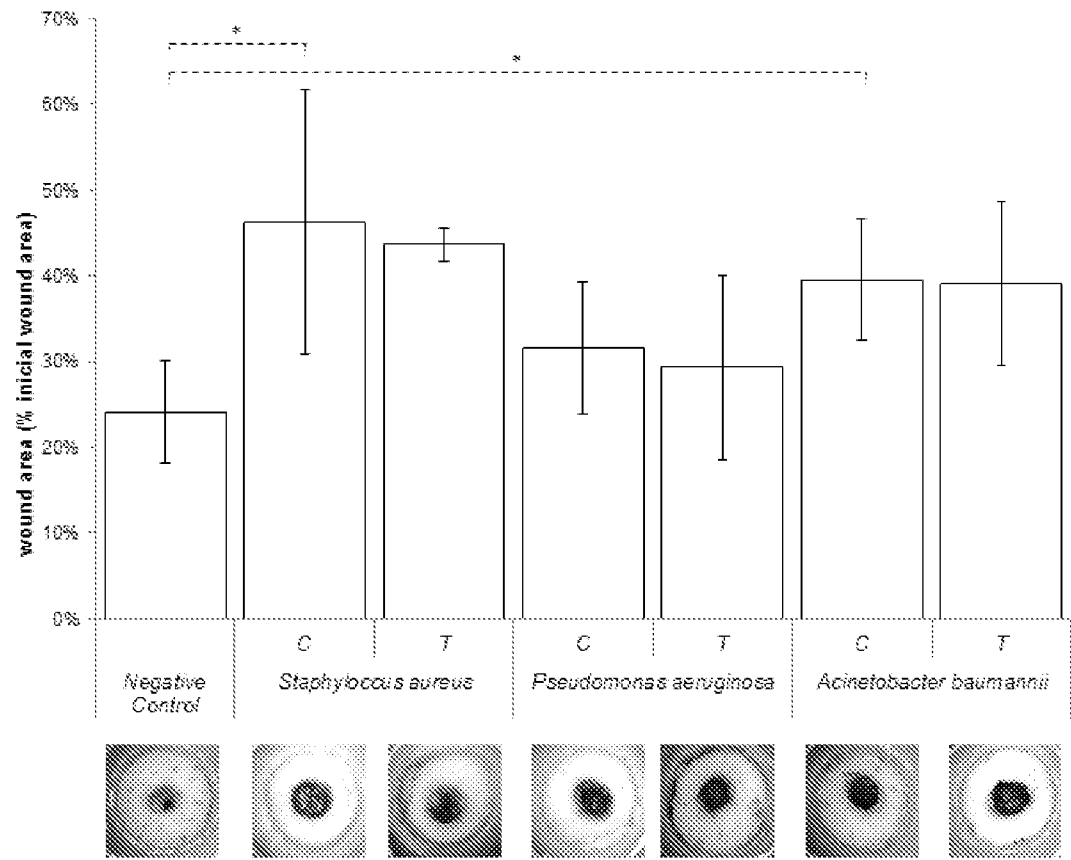


FIG. 11

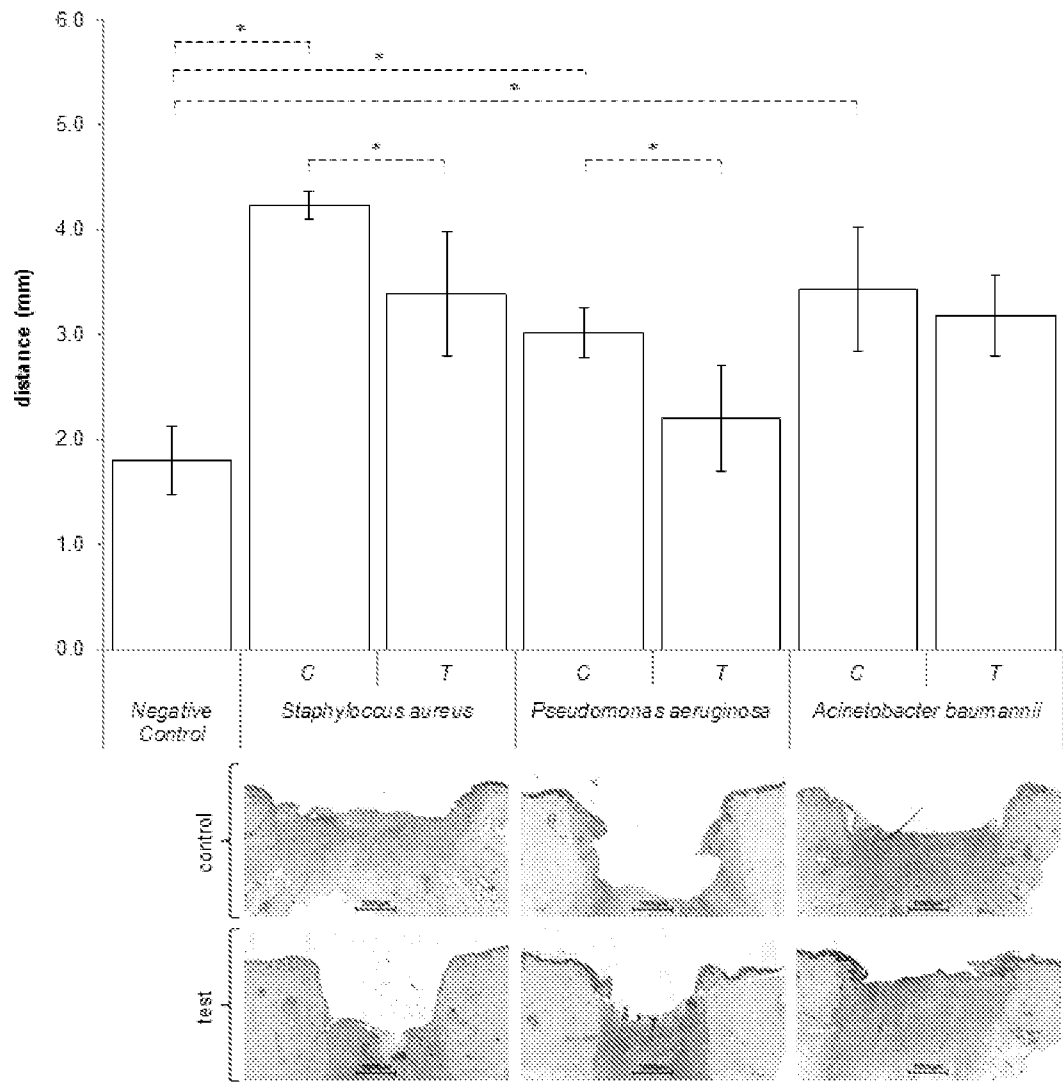


FIG. 12

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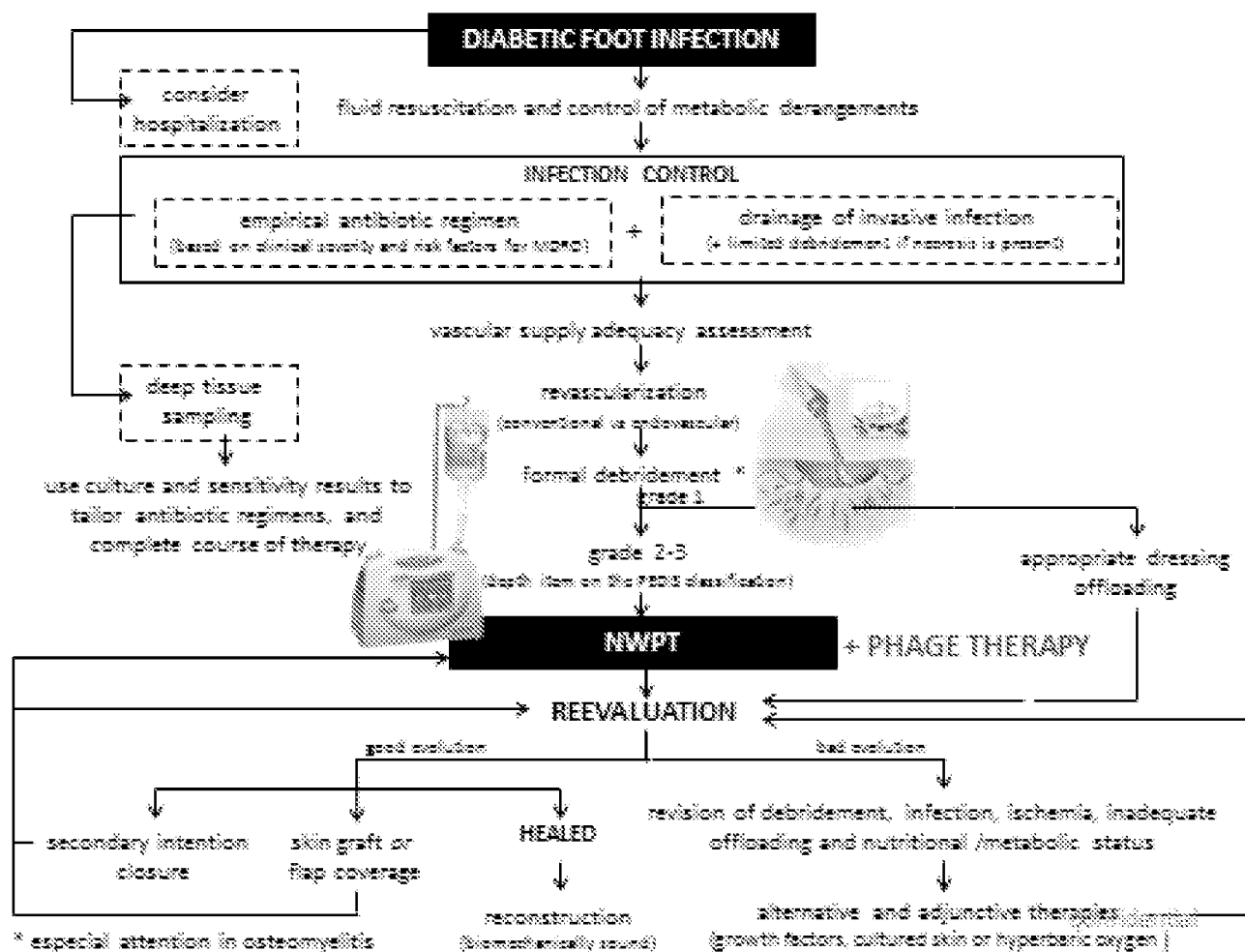
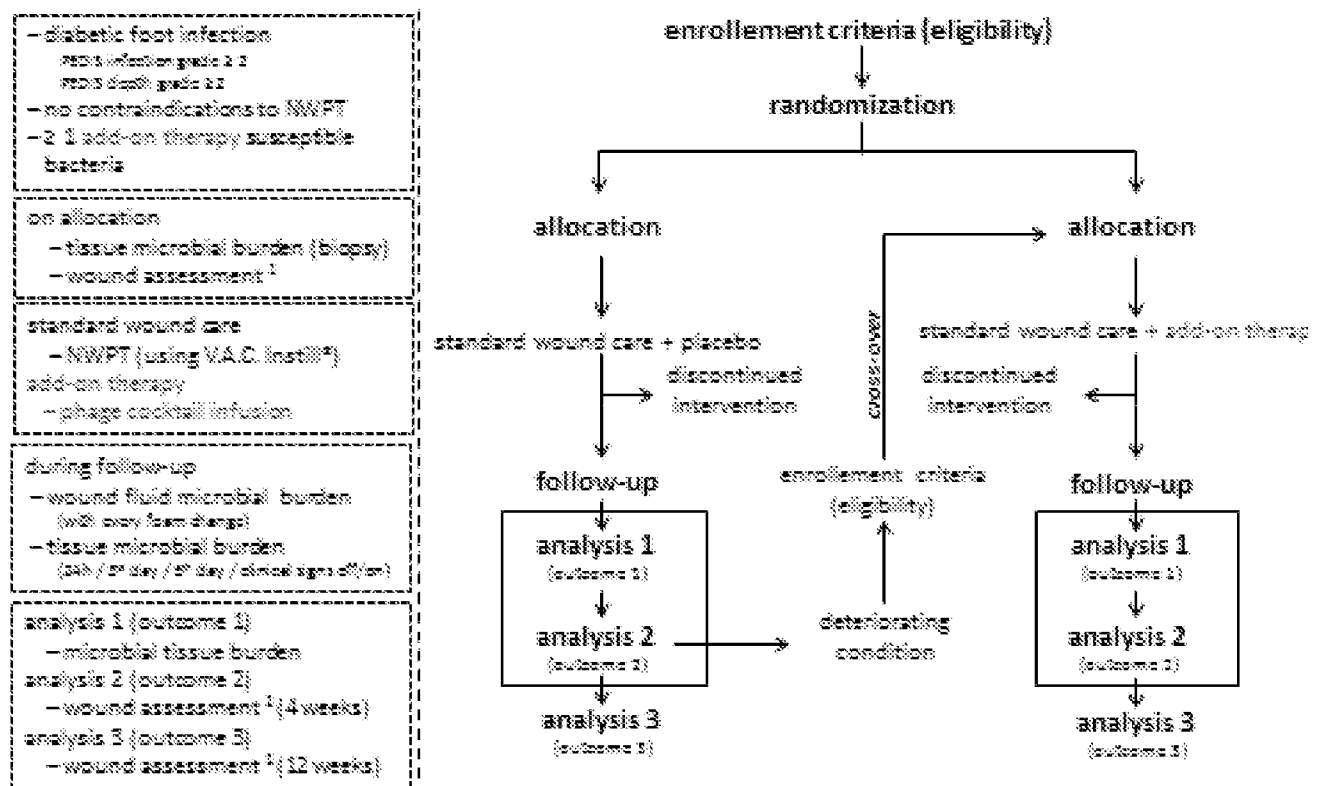


FIG. 13

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randomized placebo-controlled trial
(with possible end-of-therapy "rescue" crossover)



¹ wound assessment: diabetic foot ulcer PEDIS classification system including techniques to assess surface area (high resolution digital photography, and computerized planimetry software), type of exudate, and wound healing, infection and exudate containment/containment

FIG. 14

16395105
SEQUENCE LISTING

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LEANDRO, CLARA

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<140>

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t aat t at t ct t acagt act a at at at caca ggt ct t t t t c t aggt cgt t t t t aaat t t ct	2760
cct cat aaga act agcat aa gt t act t cat aacct at t ac ct t agt at aa t ct at gcaaa	2820
gt aat t t at a at t ggact t t at t t t aat at cct ct gat t g t t ct at t t t a t t gat aact t	2880
cat t t agct c at t cgaagag t aat gt t t at t at caact t t t at t gt t t t t cct t ggg t at	2940
agat at caat t t ct t gt at c at cat t t cat cct t t t gat t at t cat t at t t gat t at aag	3000
t ct ct aaat c at caat gt t a t ct gt at ct g aacct t t t ac t aacct t t ct cct ct ct t ct	3060
t aaggaggt c at caaat t t c t cat gt t ct t t aat t at ct t ct ct act t ca ct cggt at t a	3120
gaacagct ct agcgt aat t t at at gccaca tagacat at t at caat aaga t aat t aacca	3180
t t ct t at aag t t cct t ct ca t t t gccat at accaacct cc t t at at ct aa t act aat at a	3240
agagaaaagc agact t at t a aaagt ct gct t ct gt acct a at t ct aat ct t t t at t t t t c	3300

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at at gagga	t cat t t t t c t	at ct cct g t t	aat agagat a	at t ct ct agc	t t t t t c t t t a	3360
gat aat g t t a	at agt ccat t	at aat t at ct	act t t c t t at	t at at t ccat	aat t aagcgc	3420
t ct agct cat	at gat at at c	t t cgagt t ct	cct gat t t aa	ct ccaagt aa	ct t t ct at ac	3480
at at cat aat	ct t cagaaag	act t t ct act	t t at t t t t ag	at acagaat c	at aaact gct	3540
t gt aaat t ac	ct t ct t caat	aagt t t aaag	t t at g t t cac	ct at gat t aa	t t cct cct ca	3600
gaagaat caa	gcgt t act aa	t ccgct t gt a	t t acct g t aa	agt cacct t t	at aat ct aca	3660
acaat acct t	cagt t act t t	gt cacct aat	t caat agt cc	cat ct t cat t	t t c t t t aaat	3720
t t at gagcat	cat at act t c	t act t t gt ca	cct aat ct ca	aat ct t gagt	t aagt t at gt	3780
t t accaat aa	t t ct at ccat	t act caat ct	ct cct t t at t	aat aggg t ct	t gt g t t aaga	3840
acat t t ct ag	at t ct ct t t t	gt aat aggt a	acccaaaat a	t t t act t t cc	ggaat t gt aa	3900
t t gt at agaa	at cct cat ct	t t g t t aact t	t aat at t aac	at ct g t aaac	t cat cct gca	3960
t t aaccaat g	agt t acagt t	aagt t at at g	aacct cact	aacat acct	aaat caat at	4020
cat gt ct aaa	agccaaat ct	t ct aaat g t t	ct aat aaat c	gt t ct t t t ca	t t at g t t t t	4080
ct t ct t ct gt	at t at t t t t a	at t ggg t t aa	t t aact ct gt	gcaaacaat a	t cgt acaat t	4140
cacct ct gt	aacct cat ag	t t ct t t t caa	t t aat acat c	t t gt at t t t a	t t gat t gagt	4200
t t gt aact ac	t t t cccat at	t ct t ct t ct g	t aaact t aca	t t t at ct aaa	t caacat ct g	4260
t aat t aat t c	t gcaat ccat	t t at t t aaaa	t t gat act gc	cat t g t t ct a	gaaat aat ac	4320
t gt cgt at ac	cat at t t at t	t aat ct cct t	at t t aggt ga	at gt ggt ct t	ct aat gaaaa	4380
at caaaaggc	gct acaccat	t t ct t t t at t	at t t g t t t ct	t t t t t aagt a	t aacat aagt	4440
t agt gaaaaa	gt caagat ag	t t act acaac	cat t gat aaa	aat t t aat ca	gg t t t t t cat	4500
aat t act ct a	act cct t aag	t t t at t t t t t	act t t ct ct t	t at cgt act t	at aat ct t t a	4560
ct agagt t t t	cat t t t t t t c	t t t ct ct t ct	t cat t aagt t	ct ct at act g	agcct ct t ct	4620
acct ct t gt t	ct t t at t at c	at t acct t ct	t ct gct t t t t	gaat t t ct ac	at t t t t act a	4680
ct accaccat	t t acct t t t t	t ct aaaaaga	aaccaaagt a	t t aat aaaat	gat gagt aaa	4740
at aat aat gc	t t aat acaac	agcccaaat a	t t at t aacca	t t acaacct a	cct ccgaat a	4800
gt t t t t t t ac	ggct ct t aag	t t t t cagat g	aat cat t at t	t at at caat a	cct at gct ag	4860
aat caaaaaat	t acagcat t a	t caagt at at	gct ct gt caa	t t t at t accg	t aact act t t	4920
t act t accac	act accat aa	ccat gat t ag	t t aggt caac	cat at caggt	t caact t ct a	4980
gt act ct aaa	agat at t ct a	cgt aagaat g	aaggat t t ac	caagt aaaag	gaagat t t aa	5040
aaacat t t aa	t ct t t gat aa	gaat g t t t t a	t at t aacaac	aaaccct g t t	aact t at t t t	5100
cat at t ct ga	at t t gat aac	t t acct aggt	aaagg t t t at	act at at cct	t t t g t t t ct a	5160
at g t t t gaat	agcact t aac	at t at agccc	ct ct at aagc	aagg t t t t ca	gggt ct t cca	5220
t ccaact aat	act agaat t a	t aaaat acat	caat aact t t	ct t ct ct gct	t t aact ct t t	5280
gct gagacat	cat agaat t a	gg t aaccct t	t t at agcat t	aggt acgt ga	gg t t gat acc	5340

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cttccggagc	tacgacaggt	tttcttttta	ctgacttatc	cat t ct aaat	aat gcat ct g	5400
tcat t t t t t t	aag t t t aact	accat at cat	at gat t ct ct	at caccct t a	accat t aagt	5460
t at aggc t t c	t t gaaaact a	t gagt t cct g	t aaaat cat a	gct acct gt a	t ct gat gaat	5520
t t t ct ct acc	t gaaact ct a	t t c t t t t t a	aagcagaaaa	gaaat caggt	agaccat cat	5580
at t t aat t ac	at t t aat t ct	gagt t at ct a	t t aat cgt ct	acccat t gat	t t t cct cct a	5640
t t ct aat cct	aact t at cca	t aat t gt at c	aaagt ccatt	gaat c t t t t g	at gt act at t	5700
agat t t t ct a	gg t t cct gt t	t aggt t ct t g	t t gcat acct	aaaagct t t c	t t gt t gct t c	5760
t gt at at ct g	t t acct t cag	gt aaagagct	aat aaat t ga	t t aat ct cat	ct t t cggt ac	5820
agat t t aaag	at aat act t t	ct acaacaaa	ct cat ct t cc	at t act ccat	ct aat t t act	5880
accat t aat a	at t gcacgca	t t gagaat ac	at aaggt aat	cct t t t t cat	cat t ct cat g	5940
t ct t aat t gt	t gt acaaagt	t t act aagt c	t t cat t gct t	gat agt t gat	gt t ccacct t	6000
agt at cat ag	t caaat t caa	ct t gagcaaa	gcgggt ct aat	gt agct ccat	ct aat t gt t g	6060
t ct acct aca	t aaat at ggt	ct gct cct gt	t cccat agt a	t t acct gct g	acacaact ct	6120
gaaat ct t ca	t gagct gt t a	cacgt ccaat	agggaagt ca	aagt at t t at	t t gcaat agc	6180
t gaat t aaga	at t aat agt a	ct t caggaat	agat gcat cc	at t t cat ct a	agaagaat aa	6240
cccacct t t t	gt aaat gct t	t at agaat t g	agt t t cat ga	aact t accat	t t gcat caat	6300
aaat cct gt t	aat t t aaat t	ct t gagt aat	t gcat t act a	aaat agaaat	ct aaat ct ag	6360
ggct t ct gct	act t gt t cca	at acat ggt t	ct t acct gaa	cct gct ccgc	ct t t t aaaaa	6420
t act ggaat a	t t t t ggt t aa	ct aact t t ag	t at at ct t ga	t at ct gt aat	ggaagat t cc	6480
t gagat at ct	t t aat t gt t t	t t cct t ct t g	t t gt aat t ca	at t t t aact g	gt aaat t act	6540
aagt t gt t ct	t ct acat act	ct t caat t t g	t t t t t t aaca	t cagt aat aa	t aat t t ct ct	6600
act ct cagt t	cct gct t t ct	caacaat t gc	at ct acaat t	gct t gt t cat	acggat t aga	6660
gt t t t t ct ct	cct agt t t t t	t t gct aaat c	t gct gt t gt t	t ccat t t gt t	gct ct accaa	6720
t ct ct ct aat	ct t t caat ag	t at ct t gct t	t gccat at t t	at cat t ct cc	t t t gat t t gt	6780
t at acat t t a	t t at at t aca	agt at t t gaa	t t t gt caaca	act t t ct aaa	act t t t t t ag	6840
t t gct aat aa	aaaaat acct	t acacct at a	act t aacat a	gggt aaggt a	at t gt caaca	6900
ct t t t gt t aa	aaat acat t a	at t t aaaaaa	at cat caat a	t ct t t agt t c	cat gt gt at c	6960
cat at cat ac	at aaacat ac	aat t at at gt	at gact at t c	at t at t t ct a	acat gt t at g	7020
cat agaagt t	gcatt at t ga	at t cct ct aa	at caat agt t	accgt aaggt t	ct t gacct t c	7080
at aaagt at g	t t t gct at at	aat at t t cct	aacacct t cc	at t gt t ccat	gggaagt t t c	7140
at t at gat t a	agt act t ct a	cacct agt ga	agg t aaat at	t ct gaaaagt	aat at t t aca	7200
gaaat at at a	aaat t gt ct g	t t c t t t t aga	cacgagt act	at ct ccgt ac	t t t at at t t c	7260
t t t ct aat cg	t acat aat at	gt t t t aat t t	t t t gt act t c	t t t at ct act	gcat cct t t c	7320
t t cct aacct	t gt agt at at	t t t acaat at	t aaat at cat	agaat caaca	aagccat cat	7380

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aagaaaaat g t t c t t c t a g a a a g a a a t a a c a t c c t t a c t a c c t t t a t a a t g t t c a g g t a	7440
a a t g t g c a t c t a c t t g t a t a t t a t a a t a a t c t t c t a a a g a c c t a t a c t c t c a c c a a g a c	7500
t a g a t a a a g c g t a a c c t a a a t c a t t t t g a a t c a t t a g a c c a t t c t t t a g a t a c t g a t a g t g	7560
c a t c t t c t a t a a t t g t t a c t t t t a a t t t a t c t a a a t a a t c t t c t a c t t g a g c t t g t g t t t	7620
t c a t a a a t t c t t t t g c g t t c a t g t a a t a c c c t c c t a a a t t a t a t a a a a a a a c a c c c t g c	7680
t t g g a t a c a a g c a a g g t g a a a a g g a a a g a t a t t a t g g a a g t g t a c t a t c t a a g t a c a c c	7740
t c a t a a t a t a a c a g t t t t c c t t g c t a g t t a t t a c t t a t t t t t a a g g t c t t c t t c t t t g a	7800
c a a a c a c t c c g t t a a t a a g c t t a c c t t t c c t t t c t t t a t c t c a t c a t a a g c c a t a t c a a	7860
t a c a c t c t t c a a t a t c t a t a t c t a a c t g t a a a c a t a g t a c t g t t a a t a c t a c a a a a t a t	7920
c c c c a a c a c t a t c t c t t g t t a c a t g g t c a t t a c t t t t a g c a a t a c c t g a a g c t a a t t c t c	7980
c t g c t t c t t c t a a t a a t t t t a a c a t t t g a c c c t c g g g t t t a c c t g t t t g t a a g t t t c t a t	8040
c t t t t g c c c a t t g t t t a a t a a g t t c t a c t t t t c c a t t a t t c t a t a t c t c c t t t a a t t t c	8100
t g t a t c t t t g a t a a t t a g g t t a t c a g a g t c a c t t g t t a c a t t t a a g t t a t c t t c a a c t a a	8160
t t c a t g t a g a t t a t t a g t a a t a t c t t c t c a t a c c t a t a a c c t a c a c g a a c a t a a g c t t t	8220
a a c t c t g a t a t c t a t a t t a a c a t a a t c t t c t t g g a a t t t t c c a t t t c t a a c t t c c t t t a	8280
t t a t a t c a t a t t a t t a t a c t a t t g t c a a t t a a t c t g a g t a g t t t c c t t t a g c a a g t t g a t	8340
a c t t t t t g t g t a a t t c t t c a t a t a a t t c t c t c a t a c c t t c g t a g t t t c t c a t a t c a t c t t	8400
c c a a g a a a c t a a g a t a a t c t a a t a a t a c t t t t a c a t c c t c a g g t t c t a a a g t t a t a a c t g	8460
g t t t t a c c a t t a g g c a a c c t c c t t a a a t t c t t c t t t a t t t a t t t c t t a a t a t c t t t t c	8520
t a a t g c t t c t t t t a a t t c a t t a g g t a a t t t a t a g g c a t c a a t t g a t t g t t g t t g a c c t a a	8580
t a c a t a t c c a t t a t c t g t g a t a c g t a t t t c c a c t g t a a a c c a t g a a t t a t c t a a a t c t t c	8640
t t c t a a t c t t g c t a a t a a t a t t a a c a a c t a t t t t t t a a a t t c t a t t a g c a t a c c c g c c	8700
a a c a c a a t g a g a t a a c a t t t t a c c t t c a t c t t t a a g t t t a c t t a c a g t a t c t g c a g g a a g	8760
g a a t t t t a c t t t t c t a c c a t c t t t t a a t t t a t a a g t t t t a t c a a t t a t t t t t t c t a a t t t	8820
a t t a t c a t a t t t a g c t t t a a g t t c c g c a t c a t c t a a t t g t t g t t g t a t a g a t t g t t t c t c	8880
a t c t g t a a c t a t a t c a t g t t c t a g t t t t a a t g a g a a t g g t g t t a g g t t a a c a c t t t c t a a	8940
t g t t c t a t a a c c t t c t c t t a t t a a t a t t g a t a a a t c a t g t a a g t a a t c t a a g t a a t a g t t	9000
a t c t a g t g c a t a t c c t g t t a t a c g t t g t c t g t c t t g a g c a t c t a c a t c t a a a t a g t g a g t	9060
c a t c t t t t t g t a g t t a g c a a a a g a t a t a g a t a a t a t t t c a t t t a c a a t a g g t t t t a c t t t	9120
t a a a g c a t t t g t a a c a t c t c t t g a a t c t c t a a c c a t t a a a a a g g t a t c g t c a a a t a a t t g	9180
g t g t a a a t t a a c t t c a t t g t g t a a a t g a t t a t a g t t c t t a t a g a g a g t a t t a g c a a a t c t	9240
t a a g t a a t t a c c t t g c t c a a a t t a t t t a a a g t t a g t a g c t t t t t a a a g t c t g t t t t g t	9300
a a g a t t g a a g g c t t c a t g a a c t t t c c a t t t a g g g t t t t t a g g t a t a t g g a a t a g t a a t g c	9360
a t t t c t t t c a a a t a a t c c a a a t t c t t c t a a g t t a t t t a t t t a t c a a t a t t t t t a a c a a t	9420

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at ct gt t aaa	gt t at t aagt	aat t agaagt	t gaat t t t ct	cct at aaaaa	t cct at act t	9480
at cccct ct a	t aat t t at at	gacat aaac	at ct at at t a	t caggacacc	aact agaaaa	9540
at caaaat t a	t gat gct ct a	at g t t g t t c	t at t at ct t t	at t at aat t c	ct ct at t t aa	9600
gt t aggt t gt	gaat agt t t t	t t aaaat aac	at t t aat aaa	acagat aat g	t caat t cat t	9660
t t t gt at t ca	ct t t t act aa	t at cat ct t t	at at aggt t t	aaagct at t t	ct t t at t aac	9720
aagact at ct	gt t aagaaaa	cct t gact gc	t cct gt ct t a	acgt caaat g	aact t t t at t	9780
t t ct aaaacc	cat t t gt t ac	ccat at t at g	ct t at ccct g	at at gt ct aa	ct t t aagacc	9840
aaaagat gaa	t t at t ct cag	t act t gggg t g	cat gt accaa	acacgact at	acaat gaat c	9900
t gacat t t cc	t t at aat act	cact agaacc	t t t t t ct at a	t ct t cat t ca	t aacaat aat	9960
agat gaat t t	at aagaacat	at t t acct t g	gt ct agt aca	t ccat gat at	cat t at t t aa	10020
act at ct act	gct t cct t at	act cat ct aa	t t gt ct agct	t cat at cccc	at aaacggg t	10080
t t cat t t t ct	aat t ct t t aa	t t t t t ct t c	aacat accct	t t agat t gt a	t t t gt t t t ct	10140
acgt ct act a	ccat at aaag	gaaaat ct t t	t ct t t ct cct	ct at cagct t	caat at act c	10200
t t t gt aat t t	ct t cct t t at	t at t accaat	cacacct t ca	act aat t t t t	caact gt t t c	10260
at aggggt ca	cct t caaagt	t t gt t act t c	t t t at t acca	cat agggct a	aaaat aat g	10320
t at t t ct gt a	gct gt at caa	aact aaat at	at t at gaat a	t ct ct aaat a	at t ct t t aga	10380
acct aagt t a	at t at at t at	t t t t ct t t t t	ct t aagaaat	acat ct t ct t	ct cct at at a	10440
gat acat cct	t t at t aacct	t aggt aaat t	aat aat t t ct	t gt t ct gt t a	at cct t t t t g	10500
t t t at aagt t	at t gccat t t	aaaat cact c	ct t at t t gt t	at gt act aat	cat accat ag	10560
t aaat aat at	t t gt caacaa	aaaaagaaga	act t t t t aaa	gt t ct t ct aa	at gagt t t cg	10620
t at at aact t	t t t gaat t t t	at t t aat ggt	t ct aaat ct a	aat t cct aat	aagt t t t t ca	10680
t act t t ct t g	aat t t t t aaa	at t gat agt a	t t t ggcat ag	caagagct t c	at caacat ct	10740
t t agt at agc	t t acaacat c	t gaat agat a	t ct act t ct t	t t acat at ag	acct t gagt t	10800
aaact cct aa	at act acct c	at t at gt gct	at aat t t ct t	ct t t ct t t t c	t at gct cat t	10860
t at aaacct c	ct ggt ct act	ct acacaaac	aagt acgt at	t ct aaat t ag	t t aaagaaac	10920
t gat t t aat a	t t gt t t aat t	ct t gt aat t t	ct t aat t t cc	acat cat agt	t ct t act t at	10980
agt ccat aat	gt ct ct cct g	ct ct t act t t	gt gat aat at	t t at t t ccct	ct t t gat aag	11040
gt cat t caat	at t acct acc	t cct t gagt a	at aat t agct	t gt agat aac	at at aagt at	11100
aagaacaaaag	t t t acaaat t	cagt agct at	aat at gaaca	t aggt at gt g	t t aaaaccat	11160
act t acaat t	aat gaagct a	at cct aat cc	aat aat aaga	aat agaaat c	t at t t gt t cc	11220
t t ct gcact t	t t agt t t t at	aaaagg t t gt	t at ct gagt t	acat acgcaa	ggat aat agt	11280
aat agt t gct	acagt t t gt g	t t aaggct gt	aaagt cact t	aat aaaaat a	gt aacagt ga	11340
gaacacaat a	at aaaagg t a	t agagaaat a	gt cct t t t t t	ct at at gaag	ct act aat aa	11400
gcaaacaat a	cct agagt t a	aat t aagacc	aact gat act	act t gaaaca	t cgt agcat c	11460

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agt t agaagt	aaat t gt aaa	aact aat acc	t act gt agct	acaat t aaat	acccaaaaat a	11520
act act aaca	cct t t aacac	t at ct gact t	aact agggct	at t aaacct g	gt at at aacc	11580
t act gt aact	aat at agcat	at aat at act	t aagt aat gt	gat aaat t at	ccat ct t gt t	11640
cccct aat t t	ct ct aat ct a	t t agt aact t	ct t cccat ga	aat aaat cct	t ct ccgt t t g	11700
t t aat t ct aa	aacct atcca	t acacaaat t	gg t t t gt act	aaat t cagct	ct gt cagggt	11760
cat t gt at gg	t t t accat ga	ccct gt ct aa	t at cagagca	gt agat t aat	acgggt t t t t	11820
t t aaaat at a	t t cct gct ct	ccaat t t gt c	ct t gt aaaat	at cat at gt t	t ct gat agt t	11880
cat ct at ct c	at t gaact t a	aat aact cag	act gt t ct t t	t aat t gt t t t	at t gt t t ct t	11940
gt gct t gat g	t t t cat acct	aat aaaat ac	ct agt t ct gc	aat t gt t cct	aat cct t cat	12000
t aagaacat c	aaat acaaaa	at at ct gat t	ct t gcat agc	ct t aaagt ca	t t agt t aaaa	12060
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g t t t a t t t t t	t t c g t a a t t t	a a t t t t g a t a	a a t g t a a t a a	c t a t g a t a t a	c t a t a t g t a a	32820
t t g a t a t t a a	t a c a t a a a a a	a t a t t a a t a t	t t c a c t t a c a	a g t t a t t a t t	g t t a t a t t a t	32880
t a a c g t a a a a	g t a a a t a a a a	t a a c a a g t g g	a g g t g t a g a c	a c c t t t g g a a	g a a a t a a a a t	32940
t t a a t g c t t t	t g t a c c t a t g	g a t t t g a a g a	a a t c t g t a t c	a a c a g c t t c t	g a t a c t a a t g	33000
a g t a t t c t a t	c g t t t c a g g a	t g g g c t a g t a	c t c c a a g t a t	g g a t t t a c a g	a a t g a t a t a g	33060
t t a a t c c t a a	a g g a a t a g a t	a t a g a g t a t t	t t a a g t c a c a	a g g g t a c a t t	a a t t a t g a g c	33120
a t c a a a g t g a	t a a a g t t g t a	g g t a t a c c t a	c a g a g a a t t g	c t a t g t g g a t	a t a g a a a a a g	33180
g t t t a t t t a t	t g a a g c a a a g	c t a t g g a a g a	a t g a c g a a a a	t g t t g t t a a g	a t g c t t g a t t	33240
t a g c t g a g a a	a t t a g a a a a a	t c a g g t a g t g	g a a g a c g t t t	a g g t t t t t c t	a t t g a a g g t g	33300
c a g t t a a a a a	a c g t a a t a t a	a a t g a c a a t c	g a g t t a t t g a	t g a a g t t a t g	a t a a c c g g a g	33360
t t g c a t t a g t	t a a a a a c c c t	g c t a a t c c t g	a a g c a a c a t g	g g a a a g c t t t	a t g a a a t c a t	33420
t t t t a a c t g g	t c a t g g t a c a	t c a c c t g a c a	c t c a a g t t g a	t g c a g g a g c t	t t a a g a a a a g	33480
a a g a a a t a g c	a t c t a g c a t t	a c a a a t t t a g	c t t a c g t c a c	t a a g a t t a a a	g a t t t a a a a g	33540
a g t t t a a t g a	t g t a t g g a a t	g g c g t t g t t g	a a g a t t t g a g	t a a a t c t a a t	a g t a t g g g a t	33600
a t g a g g a a t c	a g t c c t t a c g	t t a c a a c t a g	c t a a a g g t t t	a t c t c g t a a a	g a t g c a g a a c	33660
t a g c a g t a a t	g g a t a t a a a c	a a a c a a a a a c	t a g a a t a g g t	a a g g a g a a t a	c a t t c t a t g a	33720
g t a a g a a a t	g c a a a a t a t t	t t a g a a g a g t	a t g a t a a g t t	a a a t g c t c a a	g a g g c a g t t t	33780
c g a a a t c t g t	a g a a g a t g a t	g a a a a g a a t a	c a g t a g a a t c	t a c c g a a g a g	c a a g t a g c a g	33840
a a a c a a c t g a	a g a a c c t g c t	a a a g a a c c t g	a a a a a g t a t c	t g a g g a a g a t	g c t a a a g a a g	33900

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t c t t a c c a t c	a t t a g a g a a g	t a t c t a g g t a	g g a a a t t a g a	t a t a t a a t a a	c a t t c a g a t a	40080
a g g a g a a t t a	a a t a t g g c a g	t a g a a c c a t t	c c c a a g a a g a	c c t a t t a c c c	g t c c t c a t g c	40140
a t c t a t t g a a	g t a g a t a c t t	c a g g t a t c g g	t g g c t c a g c a	g g t t c a a g t g	a a a a a g t a t t	40200
t t g c t t a a t c	g g t c a g g c t g	a a g g c g g a g a	a c c a a a t a c a	g t t t a t g a a t	t a c g t a a c t a	40260
t g c a c a a g c t	a a a c g t t t a t	t c c g t t c a g g	a g a a t t a c t t	g a t g c a a t a g	a a t t a g c a t g	40320
g g g t t c t a a c	c c t a a c t a t a	c a g c a g g t a a	g a t t t t a g c t	a t g c g t a t a g	a a g a t g c t a a	40380
a c c t g c t t c a	g c g g a a a t c g	g t g g a t t a a a	a g t a a c a t c t	a a a a t c t a t g	g t a a t g t t g c	40440
t a a c a a c a t t	c a a g t a g g a t	t a g a a a a g a a	t a c a t t a a g t	g a t t c a t t a c	g t t t a a g a g t	40500
a a t c t t c c a a	g a t g a c c g t t	t c a a t g a g g t	t t a t g a t a a t	a t c g g t a a t a	t c t t c a c a a t	40560
c a a g t a c a a a	g g a g a a g a a g	c t a a c g c a a c	t t t c t c t g t a	g a a c a t g a t g	a a g a a a c t c a	40620
a a a a g c a a g t	c g t t t a g t a t	t a a a a g t t g g	a g a c c a a g a a	g t t a a g t c a t	a t g a t t t a a c	40680
t g g t g g a g c t	t a t g a c t a c a	c t a a t g c t a t	t a t t a c a g a c	a t t a a t c a a t	t a c c t g a t t t	40740
c g a a g c t a a a	t t a t c a c c t t	t c g g a g a t a a	g a a c t t a g a a	t c t a g t a a a t	t a g a t a a a a t	40800
t g a a a a t g c a	a a t a t c a a a g	a t a a a g c t g t	a t a t g t a a a a	g c a g t t t t t g	g t g a c t t a g a	40860
a a a a c a a a c a	g c t t a c a a c g	g t a t c g t a t c	t t t c g a g c a a	c t t a a t g c a g	a a g g a g a a g t	40920
a c c a a g t a a t	g t a g a g g t t g	a a g c a g g a g a	a g a a t c a g c t	a c a g t a a c t g	c t a c t t c a c c	40980
t a t t a a a a c t	a t t g a g c c g t	t t g a g t t a a c	t a a g t t a a c g	g g c g g t a c t a	a t g g a g a a c c	41040
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c c c a t t a t c a	t c t a a a c a a t	c a g t t c a t g c	a g a g g t a g c t	t c t t t t g t t a	a a g a a c g t t c	41160
t g a t g c a g g g	g a a c c a a t g a	g a g c t a t t g t	t g g t g g a g g a	t t c a a t g a a t	c t a a a g a a c a	41220
a t t g t t c g g t	a g a c a a g c a t	c a t t a t c t a a	t c c a c g a g t a	t c a t t a g t a g	c t a a c t c a g g	41280
t a c t t t t g t t	a t g g a t g a t g	g a c g t a a a a a	c c a c g t a c c t	g c t t a c a t g g	t a g c c g t a g c	41340
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t g t a a g t t c a	t t a g a c c a a a	t c t a t g a g t c	a a t a g a c t t a	g a t g a a t t a a	a t g a a a a t g g	41460
t a t t a t t a g t	a t a g a g t t t g	t t c g t a a c c g	t a c t a a t a c a	t t c t t c a g a a	t c g t t g a t g a	41520
c g t a a c t a c a	t t c a a t g a t a	a a t c a g a c c c	a g t t a a g g c t	g a a a t g g c t g	t t g g g g a a g c	41580
t a a t g a c t t c	t t a g t a a g t g	a g c t t a a a g t	t c a a c t t g a a	g a c c a a t t t a	t t g g t a c t c g	41640
t a c t a t c a a t	a c a a g t g c t t	c a a t c a t t a a	a g a c t t t a t c	c a a t c t t a c t	t g g g t c g t a a	41700
g a a a c g t g a t	a a t g a a a t t c	a a g a c t t c c c	t g c t g a a g a c	g t a c a a g t t a	t t g t t g a a g g	41760
t a a c g a a g c a	a g a a t t t c a a	t g a c a g t t t a	c c c a a t c a g a	a g c t t c a a g a	a a a t t t c t g t	41820
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a a t a t t t c a a	a t g a t a g t a c	a g g t t c t a g t	g t a g a c t t t t	t t g a a g g g t a	t t g c a c t t t t	68700
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a a t a c a a c a a	a a g t a g c a t c	a t g g a a t g a a	a a g g a a c g t a	t a a a g g t a t a	c a a t a t t a t g	68820
g g a a t t a a t a	g a g g a t a a a g	a a a g g t g g a a	t a a a a a a a a c	t a t g g c t g t t	a a a t a t g a t a	68880
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c a g t a g g a g g	g t a t g a t a a g	g a g t t a g g a c	a a g t a a a a g t	t a a t a g a g a a	a t c t t a c c t g	69000
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a a a a t a c t g t	a c c t a a a g a a	c a a t a t g a t a	g t t t a a a a g a	a g a a c t a g a a	c t t a t g a g a a	69180
a a c a a c a a g a	a g c t a t g a t g	g a a a t g c t t c	a a a a a c t c t t	a g g t c a a a a g	g g g t a a t a a t	69240
a a a t g g c a t t	a a a t t t t a c t	a c a a t a a c g g	a a a a c a a t g t	t a t t a a a g a c	c t g a c t a c t c	69300
a g g t c a a t a a	c a t t g g g g a a	g a a t t a a c a a	a a g a a a g a a a	t a t a t t t g a c	a t t a c a g a t g	69360
a t t t a g t t t a	t a a t t t t a a t	a a a t c a c a g a	a g a t t a a a c t	a a c t g a t g a t	a a a g g a t t a a	69420
c t a a a t c g t a	t g g a a a c a t a	a c a g c t c t t a	g a g a t a t a a a	a g a a c c a g g t	t a c t a c t a t a	69480
t a g g c g c t a g	a a c a t t a g c a	a c a t t a t t a g	a t a g a c c t g a	t a t g g a g t c t	c t t g a t g t t g	69540
t t t t a c a t g t	a g t a c c t c t t	g a t a c t t c t a	g t a a g g t a g t	t c a a c a t t t a	t a t a c a c t a t	69600
c t a c t a a c a a	t a a c c a a a t t	a a a a t g t t a t	a t a g a t t t g t	c t c a g g a a a c	t c t a g t t c a g	69660
a a t g g c a a t t	t a t t c a a g g a	t t a c c g a g t a	a t a a a a t g c	t g t t a t a t c a	g g a a c t a a t a	69720
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g t g g t g t a g a	t t c a g g t t t t	t t a g a t t t a a	g t g t a g a t g c	t a a t g a c a a t	a g a t t a g c t a	69840
g a c t a a c t g a	t g c t g a a a c t	g g t a a a g a a t	a t a c t a g t a t	t a a g a a g c c t	a c a g a a g t a t	69900
a c a c a g c t t g	g a a a a a a g a a	t t t g a g c c a a	a a g a t a t g g a	g a a a t a t t t a	c t a a g t a g t a	69960
t c a g a g a c g a	t g g t a g t g c a	t c a t t c c c a c	t c c t a g t t t a	t a c t a g t g a t	a a t a a a a c g t	70020
t t c a a c a a g c	t a t t a t a g a c	c a t a t a g a t a	g a a c a g g t c a	a a c a a c c t t t	a c t t t c t a c g	70080
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a t g t a a c a g g	t t c a g t g g t a	g g a g g t a a t t	g g a c t t c a c c	a a a g a c a t c a	c c t t c c c a t a	70260
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c a g a g t t c t a	t a g a g t a g g t	g t a t c t t t c a	a a g g t t c a a c	a t g g a c g c t t	g t a g a t a g t g	70560
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a a c t g g a g g a	a a a a t a a t g a	a a t t a a a g a t	t t t a g a t a a a	g a t a a t g c a a	c a c t t a a t g t	78960
g t t t c a t c g t	a a t a a g g a g c	a t a a a a c g a t	a g a t a a t g t a	c c g a c t g c t a	a t t t a g t t g a	79020
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a t t t a a c a t g	a a a t c a g a g g	a a t c a c a a t t	g a a t g a a g c a	c t t a a a g a g a	g t g a t t t t t c	79440
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a a t t a g t a a a	t a g t a a g g a a	g g a t a t t a t g	a g g t t t g a a g	a c t t t t t a a c	c c a a g a a t t a	79560
g g a g a a c c a a	a a g a a a a t a c	t a t a g g t g a g	c t a a g a t a c t	g t t g t c c g t t	t t g t g g a g a a	79620
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a t t a a a t c t a	t t a a t g c t c c	t g c t a a a c a a	g a t g a a g t a g	g g a g a a a a g a	t g t c a t a t t t	80220
a a t t t g a a t a	t a g c a a g a a a	g a a a a a g t t c	t t a g t t a t a a	c t g a a g g t g t	a t t t g a t g c t	80280
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a a a a a a a t a a	t t g a t t a c g t	t a g t a t a g a t	a c a t c a a t a t	a t a t t a t g t t	a g a c a c t g a t	80400
g c a t t a g a t a	a t a a t a t a g a	c t t a g c t t a t	a a g t t a a a a a	c a c a t t t t a a	t a a a g t t t a c	80460
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a a a c t t a a a c	t t t a g g c t t g	a c c t t a g a g a	a g t t t t a t g t	t a t a c t a g t a	a t t a a g t a a t	80640
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aaagaaat ac	t t acagat aa	t aat ggt aat	t at t t ct at g	cact t acagg	agaaacaat g	104760
ct ct t t gat g	aaaat aaat t	aaat aaagt t	aaagat t agg	gt t gacagct	t ct at agt t t	104820
at gat at agt	at at gt at ac	t aaaaat aaa	ggagct aaca	at t at gt t t a	t t t cat t aaa	104880
t caagaagag	aaagaat t at	t aact aaaga	ggaaagt aaa	t acacaccac	t agaaacat c	104940
aagagagt t t	aacacacct a	aagaagaat t	cat t gt aaca	agt t at aacg	aaggt aaacc	105000
ct t agat t ac	at t gcaaaag	aagct aaggt	aagt at ggga	t t aat t t aca	cagt t ct aaa	105060
ct act at aaa	gt aggt aagc	gt aat aagaa	at cacct gt a	gaagaaagaa	t t gcacat at	105120
ct t aaaagat	aaaaact t ag	t caaagagat	t at t aaggat	t accaat at a	t gaat t t aca	105180
ggacat t t at	agt aaat at a	at ct t cat aa	gaat ggt t t a	t at t acat ct	t agat t t at a	105240
ccat gt agaa	agaaaaat ct g	aact t aagga	caaagcat t a	gaagaggat a	at at t gt cgt	105300

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t g a g t a a g t a	a a g a g g t t a t	a a t a t g a g a a	a t a a a a a a t c	a t t t c a a g a g	c a g t t a a a t g	105360
a c a t g c g t a a	t a a a g a g a a a	t g g g t a t c t g	a a g a g g a g t t	c a c t g a a g a a	g t g g c t c c t t	105420
c t g a a g a a c c	t g a a g t a g a a	g a a g a a a a a c	t a t a t a c t t t	a a a t g a g t t a	a a a g a g a a c t	105480
t a c t a g a t g c	t c a a g g a t t a	a a a g a t g t t g	t a g c t g a t t t	t c c t g c a t c t	a a a g a t t t a t	105540
a t g a a c c t a a	t a a a c t a t a t	a t t t g t a c a a	t a c c a a a a g g	a t a t c g t t c t	a c a g a a g t a c	105600
a a c c a g g t c a	a t a t a t t g g t	a t c a g t a c a g	g a t t a t t a t c	a g a a t c a g a a	g a t t t t a g t c	105660
a t t t a a g a g g	t c a a a t g c c t	a g a a a t c t t t	a t g a a a c t t c	t c a t g t t t t a	a a a c c t t t a g	105720
t a c g t a t t a a	t a a t a c a a a t	c t c g a a t a t c	a a c a g c a t g a	g t t a c t t g a a	g a t a t t a a a g	105780
a t g a c a a g a a	g a t a t a c g a t	g t t g a a t t a g	a a g a c c t g a g	a t t a g t a a c a	g g a g a a g a a a	105840
t a t c c c a t t t	a g a a a t t g t c	g a t a g t a a g t	t t t t t g a a a g	t c g t a t t a a t	g a a a t t c t a g	105900
a c c g c t a t a c	t g a a t t a a c g	g a t t c c g a t g	a t t t g c t t a t	a t a c t a t a g t	a a a t t a c g a g	105960
a a t t a g t t g g	t a g t g a c a a a	a t g a t t t a t t	g t t c a c t t t t	a g a t a a a t g t	g t t a a a a t t a	106020
t a g a t t a a t a	g t t a g t c t c c	t c t t a t a t t a	t a a c t g t a a g	a g g a g a c a t t	t t t g t a t a g a	106080
g g t g t t a a t t	a t g t c a a g a a	a a g c a a g t a t	a t t c t a t a t a	c t a g t g g t t a	t t g t t t t g g c	106140
t t t t t c t a t t	t c a t c t t a t t	a t a t a t c t t c	t t t c a t g t a t	c a c g a c a a a g	c a a a g a a t g a	106200
a g t c t c t a c t	g a g t t a t c a a	a c a c g g g a a a	g a t t a a a g a a	g a a a a g a a c g	t a g a a t t t g t	106260
c g g t g a c t a t	a c a c t t a a a a	a a g t g g a a a a	t a a t a a a g c t	t a t t t t a t g g	a a a c a t t a c c	106320
t a c t t a c c t a	c c c g g t a g a a	c a g g a g a t a a	c a g c a t a g a t	a t g a g g t a c t	a c a a a a c a a g	106380
t a g a t t t a a a	g a a g g g g t a a	a t t t c a a g c t	t a t t a g g g t a	t a t a c t g a a g	a t g g g g a a g a	106440
t a a t c c a a t t	c a t a a g t a t a	g g t t t g a a g c	a g t a c c a a c c	a a a a a g t a a t	a a g g a g g t g a	106500
c t t a a a t g a c	a a c a t t a a t t	g t c g t c a t c t	t t a t t g c t a t	c a t t t a t t a c	t t a t g g a a c a	106560
g t g a t t g a g t	c a a g t t a a t t	c t t g a c t c t c	t t t t t g t t t t	a t g g t a t a t t	a a t a t a t a g a	106620
a a g g a g a g a t	t a a t t a t g g a	a a t g g c a g a t	t t a g a a a g a t	t c g a t a c g t t	t g t a a g a t t a	106680
g t t t c a g a t g	a t g a g c t t t c	g g a g g a g a g a	g c a t t a g a a t	t a a g t g t a g a	c t t a t t a a a t	106740
c c g a t a c t a g	a a g g a g g t a c	a g c t t a c c a a	g c t a a a a a a c	g c a t t a g a a g	t a a g t t c g g t	106800
a a a a t a g a a g	c a a a a a a c t t	t a a a a g a a a t	t a t a a a t t c t	t a c t c a a g t c	g a t a g c t c a a	106860
a t a g a c c a a a	g g a g a t a g g a	c a a t g a t a g a	a a g g g a a a a g	t t a g t t a a a g	a a a t t g a a g a	106920
t g c t a a t a g a	g a c a t a c a a t	t g a g g t t a a a	a g a a g t a g a t	g a t t a t a a g g	a t a g t a t a c g	106980
t t c t a a a g g a	a c a a g a a a c t	a t g t a t c t a c	t a a g g t a t t a	g a t t c a g t t a	t g g t a g g g c t	107040
a a t t a t a a g t	t t c t t t a t t c	t t g t a a t g t t	a c g t g t a c t t	g a a t a t t t t g	t a a c a g g t a a	107100
t g c t g t t t a t	t c a c c t t t a g	c a c c c g c a g t	t a t t a t t a t g	t t t g t t t t a g	c c t t a g g t a c	107160
a t g g a a a g t a	a g t a a a a t g a	a t a a a a t a g t	a t c c t a t a g g	g g a a c t a t t a	a g a t g t a c t g	107220
g g a a t t a a g t	a a t g c t g a a c	a g a a c c a a g c	t a a g g t a t t t	a a g t a t c c t a	a t g a t g a a g t	107280
a g a t a t t g t a	t c a a a c a t a	a c t t a a g a c a	a a t a a c t t t t	a g t g a g a t t a	a t a t a c t t c a	107340

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t c t t a a a t a t	a t g a g a t a t a	a t a a g g c a g t	a g a a c a g c a t	a c t a a g t t a t	c t a a a g a a c t	107400
t t t t a a a a a a	g a t a a g a a a a	c t g t t g a c a a	g a a t a a a t a a	g t g t a g t a t a	g t a t t a c t a a	107460
a g g a g g a g a g	a t a t t a t g g t	t a t a c c t a g t	a t t a a a g c a c	a a a a c a a a t t	c a a g a a t g a a	107520
t t a g a g t a t t	a t a a g c a a g g	t c a c a t t a g t	g a a a g t a a a a	t g t t a g a a t t	a g c t t t t g a t	107580
t a c a t t c a a g	a a t t a g a a c a	a a a t a a t g a a	t a c g t t a c t a	a t t t g c t a g a	a g a g g a g a g a	107640
t a c g g t g a g t	a a a t t t a t t g	g a g t g t a c t t	a t t t a a t t t a	t t a g t a g t a g	c a c t a a t t t a	107700
c a c a g t a g g a	t t t t t a t t c t	t t t a t g g t g t	a g c t a g c t t a	g t t a t t a t t t	t a a c t c a t g c	107760
t a c t a t t g a c	c c g t t c g t a t	t a g c t a c t t t	c t t a g g a a t a	g g a t t c t t a g	t t a t t a g a a c	107820
t g c a c a c a g a	a t c a t g g c a c	g a g t a a t t a a	t g a t g c a g t a	g c t a a a g c t a	t t a a g g a t a a	107880
a g a a a a t g a a	t a a a g g g g a a	t t t a t t a t g g	a t a a a c a t t	a c c a a a g t t t	a g t g t a t a t g	107940
a a g t t a t t g t	a a a g a c t g t a	a t t a t g a c a c	c a a c a g a a g g	a a g t t c t g a c	c t a g a a t c a t	108000
t t t a c t t t t c	a a c t a g a g a g	t t a g c a g a a a	g a t t t g t t g a	a g a a a t a c a	g t g g a a a c a a	108060
a a a a c g g t a a	a c g t g t a t c t	t t t g c t g t t a	a a g a a c g t a a	a g t a a a t c a a	c c a g g c t a a c	108120
a t t a a t t t g t	t a g c t t t t t t	t a t t g a c a a a	t c a t t t t a t a	t a g t g t a t a g	t a a t a t t a t a	108180
c a g a a a a g g a	g g a a t t a t t a	t g a a a g t t t c	a g a a g a a g t a	a a a c a g a g t t	a t c t a g a g a a	108240
t a g a g c t a a t	a c t a a a a t g g	a t a a g a t a a g	t t g g t c t g a g	t t a a g g t c t a	g t c c t t t a g g	108300
t a t t a c c t t a	g g t g a t a t t a	t a t t t t a t a g	t g t g g t t a t t	a t a g a t a a c a	t t a t a g c t a t	108360
t a t t t t a a c t	t t a a c c t t a a	t a g g t a c t a t	t a c t g a c t c a	a t t g a g a g t a	c t t t a g c c c a	108420
a a t a a t c g t a	g g g a t g t t c a	t a a t c a t t a c	t a t a t a t g g a	a t c c t a t c a g	c g t t a a t a c c	108480
t a t t c t a g t t	c a t a a a g c t g	t a t c a c c g g g	a t g g a g c t a t	a c t g a a t g g a	a t g a a t c c t a	108540
t t a c a t c a g a	t t a c c t g g a g	a a g a g a a c t a	c a a g t a c t a t	a g t a a a t g g t	a t t t a g a t t t	108600
a t t a g g a g t t	a a a g a a t t t t	a c t a t a a g a g	a g a c a a t g g a	g a a g a a g t a a	a a g a a a a a a t	108660
a t a t c a t g g g	c t t t t c a a g c	t g a a g t a a a a	a g a c c t g a a g	a t g t t a a c c a	c t g g a a a a a c	108720
c a a t t g c t t a	c t a a t a g a c c	t t t a a c a a t t	t t a g a a t a t a	a a a a a t t a a a	g a a a t t a g a t	108780
a a g g a a a g t g	a a a t t a g g a a	a c a a g a a g a t	t t a g a a g a a t	a t a a a c a a t a	c a a t a g t a a t	108840
t a a a g a g g t g	g a a a g c a a t g	a t a a g c t c a t	t t g a t a g t a t	a c t a c t t g t c	a t a t a c a t t a	108900
t t a t a g c t t t	t g c a g t a g c t	a t g g c a a t t a	t c t a c t t a g t	a t t t a a a g g t	a t g a c t a t t c	108960
t a c t a g a t a a	g c t a a t g a t g	t t a t t a t t a a	g t a a a a c t a c	a t t a g a t g t a	g a a g c t t g c t	109020
c t a t g a t a a t	g g c a g t c a t c	a g t a c a a t t g	t g t t t g g a a t	t a t t g t a c t t	t t a a t a t g g c	109080
t a g c a g t a a a	t a a t a t t t t a	c t a t a a g g a g	a t t t a c t a t g	g a t t t t a a t g	a c t t t a t a a a	109140
c a g t g a a t c g	g a t a g g g t a g	g t a a g c c t a a	a c a a a a g a a g	a a g g t a g a g a	a t a a g c t a c c	109200
t t c t t c t a c t	c c t a t t g a a g	a t a a g g a a a a	g a a a t t a a a a	g a g a t a a g a a	a g a a a t c a t t	109260
a t a t a t t g a t	t t a a g g a g a a	a a a g a a a t g a	c t a a a g a a a c	a a a t g t a c t t	t a c a a a g a t a	109320
a g t a t a g a g a	t t a t a c t a t a	g t t g t a a g a t	t a g c a g g g a a	t a t t a t t g t t	a c t g a a g t a g	109380

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at aagaaaca	t aaaacagca	ttt acacct a	tt at at t t ga	caat ggt gt a	gaaggcgt ag	109440
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gct t gacaga	t gaagcat t t	t aat at at ac	t aaaagt at a	aat aaaat aa	agaaaagagg	109620
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at act t gt at	t aggaggt t g	t at agat gat	t gat at act a	gt t at t cact	at gaagaaac	109860
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t t t acct at t	aaaacaacag	aacgt at t gc	gct aggt aga	t t aacagat t	ct t t acagt t	110460
t gt t at ct ca	gaaat agaag	t agt t aaaga	ccaaat t at a	gat gaagaaa	at gaagct t a	110520
cat t gat t t t	gt gat ggaag	act ggaact g	ggat t aat gc	ct at ggact t	at t aact at t	110580
gct t ct gt t g	ct t t t at agc	t gt agt cat t	at t gat t t ga	t t aat gat ga	t at gagct at	110640
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gagt caat ac	t at at t ccag	t gct t cat t a	at at gt gcac	t aggaat gat	aat aaat ct t	110820
ct t t cat t t t	ct t aaaaat a	agt at t gaca	cct t t gt act	t t t gt at t at	act t agt at a	110880
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ggat agt agaa	act t aggaag	ct t t gt agt t	t t t acaaaca	t t gat t t t ga	t t at gat aaa	111120
ct agaaggaa	ct t caacaca	at at at gat t	aat t act t t g	ct aagaaat t	aact aaagat	111180
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agat t aat gg	t at acct at t	gaccacgt t a	acat t gaagt	t ggt at ct ct	t t ct at gaaa	111420

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aact aaagga	gct t caagga	aagagaat cc	aat t t gt agg	cact gt t t ac	aagt at gt t a	111480
aacat gct ag	agggcgcaaa	ggg agaat t a	aaggat t t t a	t aaagaggat	t at agcgt aa	111540
ct t t agat aa	gaagt t acaa	aaggaggaaa	aat aat gact	gaat ggt at g	ct t t at gct a	111600
t t at gat aaa	gt aggt aaaa	agaaaat acc	t aggcaagt t	agagcgcaca	gagat at t t c	111660
agt at t agaa	gaat t aaaag	aaagat t aga	agaaagaaat	cct aat acag	aat act ct at	111720
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t cat t aaaag	aacaacaaaa	gaaat t aagt	aaaaagaaga	aat aact gt t	gacaaat gag	111840
t gt gcat agg	t t at act t aa	gt t aacaaat	aaagaggagg	t at gacct at	gt t at t cat a	111900
at t t t t at t c	t agcagt at t	ct t t gt act a	ggat t t at t a	acggat ggaa	ct cagaagac	111960
t aaaaaagga	gt ggt t at ag	t gaagt t aga	agat aaagt a	t t agaaagaa	t t gat t ct ct	112020
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t at at at ggt	at t at agacc	t t at agt agg	t at t gt agt t	at agcat t aa	ct t t at t t t t	112140
at ggaaggt a	t t t at t aat c	aacat aagaa	ggg aaat gat	at ggat agag	at gat gat t a	112200
t agt t t act a	t t t gaagat t	gt gaagat t t	at caggcat a	ggg t t gt t t t	at gt aat agt	112260
t acat cat t a	at at cact at	t t gcat t t at	at act t aat c	t at ggaat ac	ct at ggat at	112320
t at aaagat a	t t aaaccct g	aagt at t t gc	agt aaaagac	t t aat agaac	aagct aaagg	112380
aggaaat t aa	t at gaaacaa	agagat t t cg	aat t t gaaga	ggat t t t gt a	t t aact t at g	112440
aat gt gagga	t t gcaaacat	t t cgaggat t	gggggt cat ga	t gaagagcct	gaagaat gca	112500
gt gaat gt gg	t agt agt gac	t t aat t aaca	at acaagt ca	t gaagacact	gagt gt gat a	112560
t gt gt aaagg	at acat t gat	at gt ggcaag	at ggt t at ag	at acat ggga	gat aat aaag	112620
cat acct t ga	aaaagaagat	t cagggt t t aa	t t t gt gaaga	t t gct at gag	aaat t agat a	112680
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aaggat t t t c	agccat ggt a	t ggcac caag	t at t agt agg	t ct aggggt t t	at t t t at t ag	112800
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16395105

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ct aaagagt t	acatt t t at ca	aaaact aaac	ct t cacagca	gt t att t t t	gt aaat aagg	4200
aaaat aaaga	t att t gat att	aaaacagt at	ttt t agat t a	aggat ggt t a	at aaat ggat	4260
acaatt t t t a	tt at aggt gt	agcgt t t at a	act t t t gcaa	cat t t aacat	agt ct t t aga	4320

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ct at t t g a t t	t a t g g a c t a c	c g a g a a a a a a	a t g g t a a g t c	a a g g a c a a c c	c c c g c t a a g t	4380
a a t t t t g a g t	a c t a t c a t g t	g a t a g t a c c t	t a c t t a g t a g	g t g t t a t t g t	t a t t a c a c t a	4440
a g t a t t a t t t	t t a g a g a t t c	c t t g t a t t c c	g c a c a a t c a g	g g t t c g g t a t	t a t t a t t a c a	4500
a g c t t t a t t t	a c a t g c t a g t	t t a t g t t a t a	a t t g g t c t t g	t a g g g t c a t t	t a t a c t t a c a	4560
a t a t t c c a a g	c t a g a a a a g c	t a g a c a g t a t	c a a a c a c a g g	a g g a t a a t a a	t g a a g t t c a a	4620
t g a t a t t t a t	g a g c a a t t a a	t t a a a a a t g a	t a c a g t a c a a	a a c a t t c a t g	a g t c t c a a g a	4680
t g a c a a a g g a	a a t a t t t a t a	c a a t c c a a t t	t g a t a a a g g t	a a t g a t a a g t	a t t t a t t t a a	4740
t g t t a t t a a t	g a t g g a t t c t	t g a a a g a a a t	g a c a a a c g g t	a t g g t a g a c c	a t c c t g a a g g	4800
t c a g c c a t a c	t c a g t a a g t t	t a a t c a a t a a	a g a a a c a c c t	a g t a t g t c a g	t g a a a c a a t a	4860
t t t a a c a g a t	g t a g a a g a t a	t t g t a c c t a c	t a t t a g a a a a	a t g g a a a a g g	a t t t c t t a t a	4920
g a g t c a a g t c	t t t a c t t g a c	t c t t t t t a c t	a t a t a t g g t a	t a t t a a t a t a	g a g g t g a c t t	4980
a a a a a t g g a t	t t t a a t t t t a	g t g c t t t t g a	t a a t a g c t c a	t t a g c a a t g a	g a a t t a g t g a	5040
g g g t g t a t a c	t a t t t c a a t g	a t a c t c c t t a	t t a c t t t a t t	g a g c a t g t a g	a a g a a g a a a t	5100
g t c t g a g t a t	g t t a t t g t g t	a t g a c a t a c a	t g a c a g a g a g	g a a a a a g a a a	a t c c t c a g a a	5160
g a a a t a t a g a	a t a g a a c c t t	a c c a a c g t a c	a a t a c c t g g g	g g a a c a c c t c	t t a g t a a c t t	5220
a a t t a a g a g t	a t g a t g c c t c	a a c g t a a g t a	t c c t a a g a a g	g t t a c a g a a g	a c c c t a t a t t	5280
t g t a g c t a a t	g t t a t t c c t t	t a g g a a c a g a	t a c a g t a a c a	g g t a a a a c c g	g t a a a g g a t t	5340
t t t t g a a a g a	g a t a a g g a t a	g a a c t a t c t a	t t c t c a a a a g	g a a c c a a c t a	a a g t c g t t c a	5400
t g g t c a a t a t	a c a g g t g t t t	t t a t a g g t c t	a a c a a g t g t t	a a g t g g a a t a	g a a c a t a t a c	5460
c c c t c t a g a a	a g t g t t g t t g	a g t a c t a c a a	a a g g g t t a a a	g g g g a t a g g t	t a a a t g t c t a	5520
a t g a t g t a g t	t a a g t t t t a t	g a a a a a g a t a	t t a a a g a c c t	t a t c a g a a c t	a a a a a c a c a	5580
t g t t c a a a g a	c g a t g a a a t a	a c t a g t g a t a	t a a a c g a t a t	a c g a a t c t t t	a a t g a g a a a g	5640
t c a t t t g t c a	a g g t a a g t g c	a g a a c a g a t t	g t t t a g t a c t	a g a c c g t a a t	g g t a c a g t a a	5700
t g g g t a t a g a	g a t a a a a a c a	g a a c g a g a c t	c t a c a c a g a g	a c t a a a t a a c	c a a t t a a a g t	5760
a t t a t a g t c t	a g t a t g t a a g	t a t g t a t a t g	t a a t g t g t c a	t g a t a a a c a t	g t a c c t a a a g	5820
t a g a a c a a a t	a c t t a a a a g g	t a t a a a c a t a	a t c a t g t a g g	t a t a a t g a g c	t a c a t t a g t t	5880
t t a a a g g c a a	a c c t g t t g t a	g g t a a a t a c a	a a g a t g c t a c	a c c a t c a c c a	c a t a g a a g c c	5940
c t t a t c a t a c	a a t g a a t a t a	t t a t g g a a g a	c a a a c t t a a t	g a c a a t a c t t	a g a t t g a t t a	6000
g a g a c c c t c a	t a c g t a t a g a	a c a g g g t a t a	g c t a t a a t g c	t a g t g g t a g a	t a t a g t g g c g	6060
g a g a a g g t a a	t t t c t c c c a a	a c a a c t c a a a	g t a a a a g a a t	g a a a a a a c c t	g c t a t t a t t a	6120
a t c a a a t a a t	t c a t t a t g t a	g g g g t a g a t a	a t a c t t a t a a	a c t c t t t a c a	a g a g g t g t t a	6180
t c t a t g g t t a	t a a t a a t a g g	t g g g a a g t t a	t a g a a g a a g a	t t t c t t t a a t	a c t a t g a a g a	6240
a t g g g g t a a g	a g t a a t c a a t	g a g c a a a g a c	a a a c c a a a t a	g a c g t a a a g a	g a t a c a a c a t	6300
c a g c c t g t t a	a c t t t g c c c c	t a t g a a t a c t	c t a a c a g g g g	c t a a t a a t a g	t t t c t t t g c t	6360

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t t c t g c a a t c	g t t c c t a g c c	c c t c a t t a a g	a a t g t c a a a t	a c a a a a a t a t	c t g a t t c t t g	57420
c a t a g c t t t a	a a a t c a t t g t	t t a a g a t a c g	t t c a g c t a a t	c c a g t t t g t t	c t g c a t t a g c	57480
t t t a t c g t t g	a t t g a t t t g t	c c t g a t g a g g	g c t a t a a g g a	g t t a c t c c t a	c a a t a c c c t c	57540
t a c t t g t t c g	t g t t g t t t a g	t t c t a t a t t c	t a c c a t a g c t	t g g t t a a g t a	g a t g a c c t c c	57600
c a t g t a a c a a	a c c t t a t c t t	t a g t a t a a t t	a a c c a t c t a t	a g t a t c t c c t	t t t t c t t c t a	57660
g a a t a c c t c t	t a a a a t g t g t	g g c a t c t t t t	t c t t a a t t t g	t t t t t c t a t a	a t t t t c a t c a	57720
t a t t t t c t t t	t t c t t c t t c c	a t g a t a t c a t	c a a c a a a a t t	t t g a c c t a c t	t g t t t c a t a a	57780
t t a a a c c a a a	a t t t t c c a a t	t c t a a a t c a t	c t t t a g a c a a	t c t g t t t t c t	t c t a t a g c t c	57840
t a a a a a t c a t	t t t t t c c a t t	c t t g a t t t t g	t g a t a g c a t a	a t c t g c t a c a	g a a t c a t t a c	57900
t t c t a a c t t c	t g a t t t c a t t	t t c t t a c g a c	t a a a c t c t t t	a a a t t c c t t a	g a t a c t a a t t	57960
t a a a a t a a t c	a t c a t g t t c t	g a t t t a c c a t	c t a a a t a t t t	a a t a a c a a t a	c c t t c t c c t g	58020
t a t t a g g t t t	a a c a g t c a t a	t c t g a t t t t c	c t a c t a a a t c	t t g a a t t t c t	t g a g g g t t t a	58080
a t t t a t t a a g	a t a a a a g a t	g g t t c t a t a a	t c a t t a a a g t	t t t t a c a g t t	t t c a a a c c t a	58140
a t g t t t c t g a	t a a a g a a a t g	a c t t c t g a a t	a a g g t a a a t a	g g t t t c a c t a	t c c t t a t c g t	58200
a t a c a t c g a a	t a c a t a a a a a	t t a t t a t a a c	a c t c t t c t t t	a t a a t t t a c c	t t a t g t t t a a	58260
c t a a c c a t t c	t c c a a a t a t a	a t a a t a c c t t	c t a a t a t a g a	t a a a t c t a a t	t t a t c t g t c a	58320
t g t t t t c a t g	t a c c c a a t t a	t a a a a a c c g t	t t a a t g t t t c	g t t t t c a t t t	a a t t t t t t t c	58380
t a c g a g a g a a	a c a t a c t a a c	t c a c c a t t c t	c t g t a g t a a a	a c t t g c g t t g	c t t c c a t c t a	58440
a t t t t t c t t g	a a c t a c a a a a	c c t c t a t c t t	t a a a t t t g t c	t a a a g a t a a t	c c t t t a t t t t	58500
t t a c t t t a g t	a t a a g a t t t c	a t t a a t t a g t	t a t c c t c c t t	t g a a t t a t g t	a c t a t t g a a a	58560
a t a a a a t a a g	a c t t a c a c t t	g c c a a a a a t g	c t a a t a c t a c	t a a a c c a g g t	a a a t t t a g a a	58620
c t g t t g a t a a	g a a t a a t g c t	a c t g c a c t t a	t a a c a t a a a c	t a g a c c g c c t	a a t a a t a a a g	58680
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a a t a a t t c a t	a a a a a a g c c t	c c t t a g t t a t	t a t a a t a t a a	g t a t a c c a t a	t c t a a g g a g g	58800
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a t t a c a g t a g	a a t t t t t t g t	a t g a a c c c t t	g t a a g a a c t t	t t t t a a g t t c	a c t a c g t t t a	59040
a a g t t c t g t g	c t t c a t t t a t	a a t t a t a g t a	g a a t c t c t t a	g a t t t c c a c c	t c t t a g g a a t	59100
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a t c a t t a a c a	t t t c a g t t a t	t t g t t g t t c a	g g a t t c a t a t	t a a g t t c a a t	a a g g g c a t c g	59220
t g t a a t c c c a	t g a a a t a a g c	c a t t t c t t t t	t c t g t c t g a t	t a c c t g g t c t	g c t t c c t a a a	59280
t c c t c t g a t a	c t g g t g a a a t	t a t a a a t a c t	a g c t t t c t a t	c t t t a t t a a g	a t a g t c t g c g	59340
t a a g c a c a g g	c t a c t g a g c a	c a t t g t t t t a	c c t g t a c c g g	c t t g a c t c t c	a t t c c a a a g t	59400

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g a t g t t a c t a	t t c a a t t c t a	c c a a c c c t t a	g a t a t g a g a a	t g t a t a t a c c	t g c a g g a a c a	107580
a c t t t t a c t t	c a a c a c g a c a	a g a a t a c c c t	c a g c a a t t t g	a a a c a t t a g t	t g a t t a t t a t	107640
g c a g a g c c t g	a t t c t a c t g a	g a t t g t t g t t	g a a g t t t a t t	g t a a a g a a a c	a g g g g t t g c a	107700
g g t a a t g t t c	c t g a a g g a a c	g a t t a a t a c t	a t a g c a t c a g	g t t c t a g t t t	g a t t a g a a g t	107760
g t t a a t a a t g	a g t a t t c t t t	t a a t a c a g g a	a c t a a a g a a g	a a a g t c a g g a	a g a c t t t a a a	107820
c g t a g a t t c c	a c t c t t t t g t	a g a a t c t a g a	g g t a g a g c a a	c t a a t a a a t c	a g t a a g a t a t	107880
g g t g c a c t g c	a g a t a c c t g a	t g t a g a a g g t	g t t t a t g t t t	a t g a a g a a a c	a g g g c a t a t t	107940
a c a g t a t t t g	c t c a t g a t a g	a a a c g g t a a t	t t a t c a g a t a	c c t t a a a a g a	a g a t a t a a t t	108000
g a t g c t t t a c	a a g a c t a t a g	a c c a a g t g g t	a t a a t g t t a g	a t g t t a c a g g	t g t a g a a a a a	108060
g a a g a a g t t a	a t g t t t c t g c	t a c a g t a a c t	a t a t c t a a t a	a a t c t a g a a t	t g g t g a t a c a	108120
t t a c a a a a a c	a t a t c g a a a g	t g t t a t t a g a	a g c t a t t t a a	a t a a t t t a a a	a a c t t c t g a t	108180
g a c c t a a t a a	t t a c a g a c c t	t a t t c a a g c t	a t a a t g a a t a	t t g a t g a c g t	a t t a a t a t a t	108240
g a t g t g t c a t	t t g a t a a c t t	a g a t g a g a a c	a t t a t a g t a c	c a c c a c a a g g	g a t t a t t a g a	108300
g c g g g a g a a a	t a a a a g t a g a	a t t a a a g t a a	a g a g a g g t g a	a a c t t a a g t c	g t g g c t a a t t	108360

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t t t t a a a g a a	t c t t c a t c c a	t t a t t a a g a a	g a g a t a g a a a	t a a a a a a g a t	a a t c a a g a c c	108420
c t a a c t t t g c	t c t g a t a g a t	g c a c t c a a t g	a a g a g a t g a a	t c a a g t g g a g	a a a g a t g c t a	108480
t a g a a a g t a a	a t t a c a a t c c	t c t c t a a a g a	c a t c t a c a a g	t g a a t a t t t a	g a t a a g t t t g	108540
g g g a t t g g t t	t g g a g t t t a t	c g t a a g a c c g	a t g a g a a c g a	t g a t g t t t a t	a g a g c a a g a a	108600
t t a t a a a a t a	t t t a c t c t t g	a a a a g a g g a a	c t a a t a a t g c	t a t a a t a g a t	g c t a t a a a a g	108660
a t t a t t t a g g	t a g a g a t g a t	a t t g a t g t a a	g t g t a t a t g a	g c c c t t t a c a	a a t a t t t t t t	108720
a t a c g a a c a a	a t c a c a t t t a	a a t g g t g a a g	a c c a t t t a a t	g g g a t a c t a t	t a t a g a t t t g	108780
c t g t t a t t a a	t g t a t c t a t a	g g t g a t t a t t	t t c c t g t a g a	g a t t a t a g a t	g t a a t t a a t g	108840
a a t t c a a a c c	t g c a g g t g t a	a c t c t a t a t g	t c a c t t a t g a	t g g a g c t t c t	a c t a t t a g a g	108900
g t g g a g c a a t	t a t t a a g t g g	t t a g a t g g g t	t a c c t a a a a t	a g a a a c a t a c	c a a g a g t t t g	108960
a t a g a t t t a c	a g g t t a t g a t	g a t a c a t t c t	a t g g t c a t a t	t a a t a t g a a t	c a a a g t a a a g	109020
a t a c t g a t a a	c a g t t c a t c a	g a t a t t t t t a	a a a c a a a t c a	t a g c t t a a t t	a a t a g t t t a g	109080
a t g t t t t a a c	a g g t t c a t c t	a g t g t a g g t a	g a c a g t a t a t	t a a c t a t g g a	t a t g t a a c a t	109140
c a t a t g t t t a	t a a t c c a g g t	a t g a c a t c c t	c t g t a a a c c a	a a t a a g t g c t	a g t a c a g a a g	109200
g t a g a g g t c a	a g a a g t a c c t	a c t g a t t a c t	a t a t g t a t a c	t a g t a c t a a g	a a t a a c a a t a	109260
c a g t a g a a c t	t a g t a t g c a a	a c t a c t t c c g	g t g t g t c t t a	t t t a t a t a a t	a a c t t t a a t t	109320
t t a g a g a t t a	t a t g a g t a a a	t a t a g a c c t c	a a g t a g a t t t	a c a a t c t g a t	g a g g c t a g a a	109380
g a a t t g t a t c	t g a t t a t a t a	a a a g a a t t a a	g t a t t g a t t a	c t a t c t t a g t	g c t g t g a t a c	109440
c t c c t g a t g a	a a g t a t a g a a	a t t a a a c t a c	a a g t t t a t g a	t t t t t c t a t t	a a t a g a t g g c	109500
t t a c a g t a t c	a a t t a a t a a c	t t a t c t t t c t	a t g a a a a a a a	t a t t g g g a g t	a a t a t a g g g t	109560
a t a t a a a a g a	c t a t c t a a a c	a g t g a a t t a a	a t a t g t t t a c	t a g g t t a g a g	a t a a a t g c a g	109620
g t a a a a g a g a	t t c a g t a g a t	a t t a a a g t t a	a t t a c t t a g a	t t t a a t g t t t	t a t t a c t a t g	109680
a a c g a g g t a t	t t a t a c a a t a	a a a c c t t a t a	a a g c a t t a a t	a g a a a a t t a t	t t a g a t a t a t	109740
c t a g a g a g a c	t t a t g t a g a a	g c a t t t a a a a	t a g c a t c a t t	a t c t a a t g g a	g a t a t t a t a a	109800
c t a a a a c a g g	t t t t c a g c c t	a t a g g g t a t t	t a a a a c t a g t	t g g t a a t t a t	g a a a a t a c a a	109860
g a c c t a g c a c	a a t a a a t a t a	g t a g c t a a a g	a t a c a g a t a a	t a a c c c t a t a	g a a t c t a a t g	109920
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a t a c g a t a g c	t a g a g a a a t a	a c t t c t a c a a	a a g a g t t t a c	t g t a t c a g g a	t g g g c t a a a g	110040
a g a t a t a c t c	a a c t a a t t a t	c t t t c t a a a g	t a t t a a a a c c	a g g t a a a g t g	t a t a c g t t a t	110100
c t t t t g a t a t	a g a a a t a a c a	g g t a a t g a c c	t a a c t c t t a a	a t c t t a t t c t	g a t a a t c a t g	110160
g t a t a t a t t t	a t a c a g t a a t	a c t a a g g g a a	t t g t t g t t a a	t g g t g t t a a a	t c t a t g g a a c	110220
g t a c t a t a g g	t a a c a a a g t a	t c c g t a a c t c	a a a c t t t t a c	a g c c c c t a c t	a t t a c t g a c c	110280
a t a g a t t a t t	a a t a t a t a c t	g g a a g a t a t a	c a t c t g a t g g	t a a a g c a t c a	a c t c c t c c a g	110340
t g t t c t t t a a	t a c a g t t a a a	a t t a c g g a a t	t a a a a t t g t c	t g a g g g t a c c	t c t a a t c t a g	110400

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ct t t at at t a	t t ct t act at	ggg gat aat t	gggt agaact	aaagact ct a	ggaaat at t a	110580
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16395105

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