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(54) Title: ENGINEERED LYSIN POLYPEPTIDE CONSTRUCTS ACTIVE AGAINST GRAM-NEGATIVE BACTERIA

(57) Abstract: The present disclosure is directed to an engineered lysin comprising: (a) a Gram-negative base lysin; and (b) one or more antimicrobial peptide (AMP) and/or cell penetrating peptide (CPP), and optionally an outer membrane binding motif and at least one linker, or an active fragment thereof, or a variant thereof having at least 80% sequence identity. Also disclosed herein are methods of treating a bacterial infection caused by at least one Gram-negative bacteria and methods of killing at least one Gram-negative bacteria, comprising administering the engineered lysin disclosed herein.



ENGINEERED LYSIN POLYPEPTIDE CONSTRUCTS ACTIVE AGAINST GRAM-NEGATIVE BACTERIA

CROSS-REFERENCE TO RELATED APPLICATION

5 [001] This application claims the benefit of, and relies on the filing date of, U.S. Provisional Application No. 63/508,506, filed on 15 June 2023, the contents of which are incorporated herein by reference in its entirety.

SEQUENCE LISTING

10 [002] This application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on 12 June 2024, is named 0497_0034-PCT_SL.xml and is 196,743 bytes in size.

FIELD OF THE DISCLOSURE

15 [003] The present disclosure relates to the field of antimicrobial agents and more specifically to engineered, phage-derived lysins that infect Gram-negative bacteria and the use of these agents in killing Gram-negative bacteria and combatting bacterial infection and contamination.

BACKGROUND OF THE DISCLOSURE

20 [004] Gram-negative bacteria are an important cause of serious and potentially life-threatening invasive infections. *Pseudomonas* infection, for example, presents a major problem in burn wounds, chronic wounds, chronic obstructive pulmonary disorder (COPD), cystic fibrosis, surface growth on implanted biomaterials, and within hospital surface and water supplies where it poses a host of threats to vulnerable patients.

25 [005] Likewise, *Burkholderia* species are pervasive throughout the environment and are known to cause lung infections, particularly in cystic fibrosis patients, diabetics, or patients who are otherwise immunocompromised. Melioidosis and glanders are two examples of Gram-negative bacterial infections caused by *Burkholderia* species (i.e., *Burkholderia pseudomallei* and *Burkholderia mallei*, respectively) that are of concern. Another Gram-negative bacteria
30 responsible for devastating human diseases is *Yersinia pestis*, a non-motile coccobacillus bacteria that may be found in small mammals and fleas. *Yersinia pestis* is the causative agent of bubonic,

septicemic, and pneumonic plagues. Both *Burkholderia* and *Yersinia* pathogens have a potential to be used as biowarfare agents.

5 [006] Bacteriophage lysins are molecules produced by bacterial viruses (bacteriophages) that are capable of lysing the bacterial cell wall, resulting in death of the target bacteria. These bacteriophage lysins, or “lysins,” may be highly efficient at killing pathogenic bacteria, including various pathogenic *Burkholderia* and/or *Yersinia* species.

[007] Accordingly, there is a need for therapies and agents effective in the diagnosis and treatment of bacterial contamination and infection, particularly with respect to *Burkholderia* and *Yersinia* species.

SUMMARY OF THE DISCLOSURE

[008] This application discloses novel lysins, which have been engineered to cross the outer membrane of target Gram-negative pathogens, such as *Burkholderia* and *Yersinia* species, as well as other Gram-negative bacteria species as disclosed herein. The peptidoglycan (PG) layer of Gram-negative bacteria is shielded by the outer membrane (OM) and is generally not readily cleaved by native (unmodified) lysins. For lysins to target Gram-negative bacteria, it has been discovered that the sequences generally must be engineered by the addition of specific functional domains to mediate translocation across the OM into the subjacent murein matrix to result in lysis. These functional domains may include, for example, one or more antimicrobial peptide (AMP) and/or cell penetrating peptide (CPP). In certain embodiments the AMP may be selected from BK3 (SEQ ID NO: 19), HIP_A (SEQ ID NO: 27), HIP_{ABC} (SEQ ID NO: 28), HIP_{AB} (SEQ ID NO: 181), BK5 (SEQ ID NO: 17), AM1 (SEQ ID NO: 123), AM13 (SEQ ID NO: 124), MMAV-2 (SEQ ID NO: 125), MFVI-3 (SEQ ID NO: 126), BCM1 (SEQ ID NO: 18), BK3h2 (SEQ ID NO: 178), BK3h3 (SEQ ID NO: 179), ATRA (SEQ ID NO: 21), ATRA-1A Δ aa2-9 (SEQ ID NO: 22), ATRA-1A (SEQ ID NO: 23), PMAP-36 (SEQ ID NO: 24), RI18 (SEQ ID NO: 25), H2A (SEQ ID NO: 26), HIP_{Anat} (SEQ ID NO: 180), BK9 (SEQ ID NO: 29), or active fragments or variants thereof having at least 80% sequence identity with the AMP. In certain embodiments, the CPP may be selected from Zf5.3 (SEQ ID NO: 38), PRR (SEQ ID NO: 39), PRR₅ (SEQ ID NO: 40), or PRR₃ (SEQ ID NO: 48), or active fragments or variants thereof having at least 80% sequence identity with the CPP.

[009] Such AMPs and CPPs may be added to native (unmodified) Gram-negative base lysins or modified Gram-negative base lysins. In certain embodiments, the native or modified Gram-negative base lysin may be selected from the protein family phage lysozyme pfam00959; peptidase_M15_4 pfam13539; muramidase pfam11860, or peptidase_M15_3 pfam08291, wherein the Gram-negative base lysin is modified, e.g., by making a non-naturally occurring modification to the native amino acid sequence or truncating the native lysin. The AMPs and CPPs may, in addition to those listed above, further be selected from other AMPs and CPPs known in the art, such as RI18, PRR, PRR₃ and PRR₅.

[0010] In some embodiments, ATRA comprises SEQ ID NO: 21 or is an active fragment thereof or a variant thereof, such as ATRA-1A Δ aa2-9, set forth as SEQ ID NO: 22 and/or variant ATRA-1A set forth as SEQ ID NO: 23. In some embodiments, BK3 is a full length BK3, e.g., as

set forth in SEQ ID NO: 19. In other embodiments, BK3 comprises a variant, such as the variant set forth in SEQ ID NO: 20.

[0011] Therefore, in one aspect, the present application discloses and claims novel engineered lysins and fragments and variants thereof comprising (a) a Gram-negative base lysin in addition
5 to (b) one or more antimicrobial peptides (AMP) and/or cell-penetrating peptides (CPPs) that can be used, for example, to treat bacterial infections, including infections caused by Gram-negative bacteria, including, but not limited to *Burkholderia* and *Yersinia* species.

[0012] As described herein, the engineered lysins may be included in compositions comprising an excipient or carrier, particularly in pharmaceutical compositions comprising a
10 pharmaceutically acceptable excipient or carrier that can be used, for example, to kill Gram-negative bacteria or treat bacterial infections. Also provided herein, *inter alia*, are methods for using the lysins and engineered lysins per se or in a composition or pharmaceutical composition disclosed herein, for treating bacterial infections, and, generally, inhibiting the growth, reducing the population, or killing Gram-negative bacteria. Polynucleotides encoding the engineered
15 lysins and variants thereof, recombinant vectors comprising the polynucleotide and host cells comprising the recombinant vector disclosed herein are also provided.

[0013] It has also surprisingly been discovered that certain native lysins without addition of any AMP or CPP are active against Gram-positive bacteria, such as *Burkholderia*. Therefore, in another aspect, pharmaceutical compositions comprising certain native lysins, such as GN569 or
20 GN568, and the use of these certain native lysins for inhibiting the growth, reducing the population, or killing Gram-negative bacteria and treating bacterial infection are also contemplated.

[0014] In a particular embodiment of one aspect of the disclosure, the present disclosure is directed to an engineered lysin comprising:

25 I) (a) a Gram-negative base lysin selected from the protein family phage lysozyme pfam00959; peptidase_M15_4 pfam13539; muramidase pfam11860; or peptidase_M15_3 pfam08291; and (b) one or more antimicrobial peptide (AMP) and/or cell penetrating peptide (CPP) selected from BK3 as defined herein, HIP_{ABC} set forth as SEQ ID NO: 28, HIP_A set forth as SEQ ID NO: 27, BK5 set forth as SEQ ID NO: 17, BCM1 set forth as SEQ ID NO: 18, Zf5.3
30 set forth as SEQ ID NO: 37, ATRA as disclosed herein, BK9 set forth as SEQ ID NO: 29,

AM13 set forth as SEQ ID NO: 124, MMAV-2 set forth as SEQ ID NO: 125, or MFVI-3 set forth as SEQ ID NO: 126; or

II) (a) a Gram-negative base lysin selected from GN3(R100Q,R116H) set forth as SEQ ID NO: 14, GN515(R100D,R116H) set forth as SEQ ID NO: 15, GN685 set forth as SEQ ID NO: 2, GN569(K87H) set forth as SEQ ID NO: 16, GN685(K78H) set forth as SEQ ID NO: 106; and optionally (b) one or more AMP and/or CPP; or

III) (a) a Gram-negative base lysin selected from the protein family phage lysozyme pfam00959; peptidase_M15_4 pfam13539; muramidase pfam11860; or peptidase_M15_3 pfam08291; and (b) RI18 set forth as SEQ ID NO: 25 and a linker, or PRR set forth as SEQ ID NO: 39 and an outer membrane binding motif, PRR₃ set forth as SEQ ID NO: 48 and an outer membrane binding motif, PRR₅ set forth as SEQ ID NO: 40 and an outer membrane binding motif; or

IV) (a) a modified Gram-negative base lysin selected from the protein family phage lysozyme pfam00959; peptidase_M15_4 pfam13539; muramidase pfam11860; or peptidase_M15_3 pfam08291; and (b) RI18 set forth as SEQ ID NO: 25, AM1 set forth as SEQ ID NO: 123, PRR set forth as SEQ ID NO: 39, or an active fragment thereof, or a variant thereof having at least 80% sequence identity, such as at least 85% sequence identity, such as at least 90% sequence identity, such as at least 95% sequence identity, such as at least 98% sequence identity, such as at least 99% sequence identity with said engineered lysin.

[0015] In some embodiments, the Gram-negative base lysin is from the phage lysozyme pfam00959 protein family, such as GN867 (SEQ ID NO: 13), GN3 (R100Q,R116H) set forth as SEQ ID NO: 14, GN515 (R100D,R116H) set forth as SEQ ID NO: 15, QPI14001 (SEQ ID NO: 9), or GN917 (SEQ ID NO: 10).

[0016] In some embodiments, the Gram-negative base lysin is from the peptidase_M15_4 pfam13539 protein family, such as GN685 (SEQ ID NO: 2), GN685 (K78H) set forth as SEQ ID NO: 106, GN37 (SEQ ID NO: 8), GN569 (SEQ ID NO: 7), GN568 (SEQ ID NO: 1), GN569 (K87H) set forth as SEQ ID NO: 16, and GN577 (SEQ ID NO: 12).

[0017] In some embodiments, the Gram-negative base lysin is from the muramidase pfam11860 protein family, such as GN428 (SEQ ID NO: 4) and CAH0532154 (SEQ ID NO: 11).

[0018] In some embodiments, the Gram-negative base lysin is from the peptidase_M15_3 pfam08291 protein family, such as GN588 (SEQ ID NO: 5).

[0019] In some embodiments, disclosed herein is an engineered lysin comprising: (a) a Gram-negative base lysin selected from GN515(R100D, R116H) (SEQ ID NO: 15), GN3 (SEQ ID NO: 3), GN3(R100Q, R116H) (SEQ ID NO: 14), GN3(R100Q, R116Q) (SEQ ID NO: 177), GN515 (SEQ ID NO: 6), GN568 (SEQ ID NO: 1), GN685 (SEQ ID NO: 2), GN428 (SEQ ID NO: 4),
 5 GN588 (SEQ ID NO: 5), GN569 (SEQ ID NO: 7), GN37 (SEQ ID NO: 8), QPI14001 (SEQ ID NO: 9), GN917 (SEQ ID NO: 10), CAH0532154 (SEQ ID NO: 11), GN577 (SEQ ID NO: 12), GN867 (SEQ ID NO: 13), GN4 (SEQ ID NO: 175), or GN4 (K100D, R116H) (SEQ ID NO: 176); and (b) (1) one or more antimicrobial peptide (AMP) selected from BK3 (SEQ ID NO: 19), HIP_A (SEQ ID NO: 27), HIP_{ABC} (SEQ ID NO: 28), HIP_{AB} (SEQ ID NO: 181), BK5 (SEQ ID
 10 NO: 17), AM1 (SEQ ID NO: 123), AM13 (SEQ ID NO: 124), MMAV-2 (SEQ ID NO: 125), MFVI-3 (SEQ ID NO: 126), BCM1 (SEQ ID NO: 18), BK3h2 (SEQ ID NO: 178), BK3h3 (SEQ ID NO: 179), ATRA (SEQ ID NO: 21), ATRA-1AΔaa2-9 (SEQ ID NO: 22), ATRA-1A (SEQ ID NO: 23), PMAP-36 (SEQ ID NO: 24), RI18 (SEQ ID NO: 25), H2A (SEQ ID NO: 26), HIP_{Anat} (SEQ ID NO: 180), BK9 (SEQ ID NO: 29); and/or (b) (2) a cell penetrating peptide
 15 (CPP) selected from Zf5.3 (SEQ ID NO: 38), PRR (SEQ ID NO: 39), PRR₅ (SEQ ID NO: 40), or PRR₃ (SEQ ID NO: 48); or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

[0020] In certain embodiments, the Gram-negative base lysin is selected from GN515(R100D,R116H) (SEQ ID NO: 15), GN3(R100Q,R116H) (SEQ ID NO: 14), or GN3
 20 (SEQ ID NO: 3). In certain embodiments, the Gram-negative base lysin is selected from GN917 (SEQ ID NO: 10), QP14001 (SEQ ID NO: 9), or GN867 (SEQ ID NO: 13), GN685 (SEQ ID NO: 2), GN569(K87H) (SEQ ID NO: 16), GN37 (SEQ ID NO: 8), GN685(K78H) (SEQ ID NO: 106), GN428 (SEQ ID NO: 4), or CAH0532154 (SEQ ID NO: 11), or GN588 (SEQ ID NO: 5).

[0021] In some embodiments, the engineered lysin of the disclosure further comprises one or
 25 more outer membrane binding motifs, and in some embodiments, the engineered lysin comprises at least one linker. In some embodiments, the outer membrane binding motif is selected from FIRL_{Bc} (SEQ ID NO: 143), FMRL_{Yp} (SEQ ID NO: 144), FMRL_{Nm} (SEQ ID NO: 147), FIRL (SEQ ID NO: 146), or or FIQL_{BC} (SEQ ID NO: 173), and in certain embodiments, the at least one linker is selected from TAGGTAGG (SEQ ID NO: 41), IGEM peptide linker
 30 GGS GSGSGSGSP (SEQ ID NO: 42), GGGGSGGGGSGGGGS (SEQ ID NO: 43), AGAGAGAGAGAGAGAS (SEQ ID NO: 44), LSKLGG (SEQ ID NO: 45),

LSKLGGLGGGPRRLGG (SEQ ID NO: 46), LGGLGG (SEQ ID NO: 47), GGGGGGGGGGGG (SEQ ID NO: 107), G, GT, TG, SKY, or LGGKSLG (SEQ ID NO: 174).

[0022] In some embodiments, the engineered lysin of the disclosure further comprises one or more amino acid extensions selected from MG or LKWI (SEQ ID NO: 127).

5 **[0023]** In some embodiments, the engineered lysin of the disclosure further comprises a cationic peptide, such as KFFKFFKFFK (SEQ ID NO: 36).

[0024] In some embodiments, the engineered lysin comprises: (a) a Gram-negative base lysin selected comprising GN515(R100D, R116H) (SEQ ID NO: 15), (b) an AMP comprising BK3 (SEQ ID NO: 19), and (c) an outer membrane binding motif comprising FMRL_{Nm} (SEQ ID NO: 147); or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin. In certain embodiments, the engineered lysin comprises GN1074 (SEQ ID NO: 62), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

15 **[0025]** In some embodiments, the engineered lysin comprises: (a) a Gram-negative base lysin selected comprising GN3(R100Q, R116H) (SEQ ID NO: 14), (b) an AMP comprising HIP_{ABC} (SEQ ID NO: 28), and (c) a linker comprising GT; or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin. In certain embodiments, the engineered lysin comprises GN1119 (SEQ ID NO: 63), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

20 **[0026]** In some embodiments, the engineered lysin comprises: (a) a Gram-negative base lysin selected comprising (a) a Gram-negative base lysin comprising GN3 (SEQ ID NO: 3), and (b) an AMP comprising HIP_{AB} (SEQ ID NO: 181); or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin. In certain embodiments, the engineered lysin comprises GN1120 (SEQ ID NO: 149), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

25 **[0027]** In some embodiments, the engineered lysin comprises: (a) a Gram-negative base lysin comprising GN3(R100Q, R116H) (SEQ ID NO: 14), (b) two AMPs comprising HIP_A (SEQ ID NO: 27) and BK3 (SEQ ID NO: 19), and (c) a linker comprising GT; or or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin. In certain embodiments, the engineered lysin comprises GN1125 (SEQ ID NO: 64).

[0028] In some embodiments, the engineered lysin is selected from GN1074 (SEQ ID NO: 62), GN1119 (SEQ ID NO: 63), GN1125 (SEQ ID NO: 64), or GN1120 (SEQ ID NO: 149). In some embodiments, the engineered lysins of the disclosure is selected from GN693 (SEQ ID NO: 55), GN694 (SEQ ID NO: 56), GN751 (SEQ ID NO: 57), GN757 (SEQ ID NO: 58), GN1040 (SEQ ID NO: 59), GN1041 (SEQ ID NO: 60), GN1072 (SEQ ID NO: 61), GN737 (SEQ ID NO: 65), GN808 (SEQ ID NO: 66), GN848 (SEQ ID NO: 67), GN887 (SEQ ID NO: 68), GN907 (SEQ ID NO: 69), GN918 (SEQ ID NO: 70), GN937 (SEQ ID NO: 71), GN585 (SEQ ID NO: 72), GN686 (SEQ ID NO: 73), GN727 (SEQ ID NO: 74), GN758 (SEQ ID NO: 75), GN764 (SEQ ID NO: 76), GN765 (SEQ ID NO: 77), GN873 (SEQ ID NO: 78), GN916 (SEQ ID NO: 79), GN687 (SEQ ID NO: 108), GN688 (SEQ ID NO: 109), GN689 (SEQ ID NO: 110), GN690 (SEQ ID NO: 111), GN692 (SEQ ID NO: 112), GN695 (SEQ ID NO: 113), GN696 (SEQ ID NO: 114), GN697 (SEQ ID NO: 115), GN869 (SEQ ID NO: 116), GN870 (SEQ ID NO: 117), GN871 (SEQ ID NO: 118), GN872 (SEQ ID NO: 119), GN874 (SEQ ID NO: 120), GN875 (SEQ ID NO: 121), GN905 (SEQ ID NO: 122), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

[0029] In some embodiments, the engineered lysins of the disclosure is selected from GN1127 (SEQ ID NO: 150), GN1071 (SEQ ID NO: 151), GN1077 (SEQ ID NO: 152), GN1078 (SEQ ID NO: 153), GN1117 (SEQ ID NO: 154), GN1118 (SEQ ID NO: 155), GN1236 (SEQ ID NO: 156), GN1238 (SEQ ID NO: 157), GN1239 (SEQ ID NO: 158), GN1250 (SEQ ID NO: 159), GN1251 (SEQ ID NO: 160), GN1252 (SEQ ID NO: 161), GN1253 (SEQ ID NO: 162), GN1283 (SEQ ID NO: 163), GN1287 (SEQ ID NO: 164), GN1313 (SEQ ID NO: 165), GN1316 (SEQ ID NO: 166), GN1328 (SEQ ID NO: 167), GN1336 (SEQ ID NO: 168), GN1339 (SEQ ID NO: 169), GN1344 (SEQ ID NO: 170), GN1108 (SEQ ID NO: 171), GN1140 (SEQ ID NO: 172), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin

[0030] In some embodiments, the engineered lysin comprises at least one activity selected from inhibiting Gram-negative bacterial growth, reducing a Gram-negative bacterial population and/or killing Gram-negative bacteria.

[0031] In some embodiments, the Gram-negative bacteria demonstrating inhibited growth, population reduction or which is killed by the engineered lysins of the invention is selected from a *Burkholderia* spp., a *Yersinia* spp., such as *Yersinia pestis*, a *Pseudomonas* spp., such as,

Pseudomonas aeruginosa, an *Acinetobacter* spp., such as *Acinetobacter baumannii*, an *Enterobacter* spp., such as *E. cloacae*, an *Escherichia* spp., such as *E. coli*, a *Serratia* spp., such as *S. marcescens* and/or a *Klebsiella* spp., such as *Klebsiella pneumoniae*.

[0032] In some embodiments, the *Burkholderia* spp. are selected from *Burkholderia cepacia* complex species and/or *Burkholderia pseudomallei* complex species, wherein the *Burkholderia cepacia* complex species comprise *Burkholderia cepacia*, *Burkholderia cenocepacia*, and/or *Burkholderia multivorans* and wherein the *Burkholderia pseudomallei* complex species comprise *Burkholderia thailandensis*, *B. pseudomallei* and/or *Burkholderia humptydooensis*.

[0033] In another aspect, the disclosure is directed to an isolated polynucleotide comprising a nucleic acid molecule encoding the engineered lysin of the disclosure. In some embodiments, the isolated polynucleotide comprises cDNA.

[0034] In yet another aspect, the disclosure is directed to a recombinant vector comprising the isolated polynucleotide sequence as described herein. In some embodiments, the isolated polynucleotide sequence is operatively linked to a heterologous promoter. In some embodiments, the recombinant vector is a recombinant expression vector.

[0035] The present disclosure is also directed to an isolated host cell comprising the recombinant vector as described herein.

[0036] In another aspect, the present disclosure is directed to a composition comprising a carrier, such as a pharmaceutically acceptable carrier, comprising the engineered lysin of the disclosure, or GN568 or GN569 as herein described.

[0037] The present disclosure is also directed to an AMP comprising AM13 set forth as SEQ ID NO: 124, MMAV-2 set forth as SEQ ID NO: 125, and/or MFVI-3 set forth as SEQ ID NO: 126.

[0038] In yet another aspect, the present disclosure is directed to a method of treating a bacterial infection caused by a Gram-negative bacteria, which method comprises administering to a subject in need thereof an effective amount of the engineered lysin as herein described. In some embodiments, the Gram-negative bacteria is a Gram-negative ESKAPE bacteria.

[0039] In addition, the present disclosure is directed to a method of killing a Gram-negative bacteria, which method comprises contacting an effective amount of the engineered lysin as herein described. In some embodiments, the bacteria is *Burkholderia* spp., *Yersinia* spp., *Acinetobacter* spp., *Klebsiella* spp., or *Pseudomonas* spp. In some embodiments, the

Burkholderia spp. are selected from *B. cepacia*, *B. cenocepacia*, *B. thailandensis*, *B. multivorans*, or *B. humptydooensis*.

[0040] In some embodiments, the *Yersinia* spp. is *Yersinia pestis*. In some embodiments, the *Acinetobacter* spp. is *A. baumannii*. In some embodiments, the *Klebsiella* spp. is *K. pneumoniae*.

5 [0041] In some embodiments, Gram-negative bacteria include *Serratia* spp., such as *Serratia marcescens*.

BRIEF DESCRIPTION OF THE DRAWING

10 [0042] **Figure 1** is a graph showing the $\log_{10}$ CFU/mL over time for GN751 against *B. humptydooensis* (BAA-2767), as described in Example 5.

[0043] **Figure 2** is a graph showing the $\log_{10}$ CFU/mL over time for GN757 against *B. humptydooensis* (BAA-2767), as described in Example 5.

[0044] **Figure 3** is a graph showing the $\log_{10}$ CFU/mL over time for GN751 against *B. thaillandensis* (NR-9909), as described in Example 5.

15 [0045] **Figure 4** is a graph showing the $\log_{10}$ CFU/mL over time for GN757 against *B. thaillandensis* (NR-9909), as described in Example 5.

[0046] **Figure 5** is a graph showing the relative fluorescence units (RFUs) for GN751 against *B. thailandensis* (NR-9909) over time for varying concentrations of GN751, as described in Example 5.

20 [0047] **Figure 6** is a graph showing the relative fluorescence units (RFUs) for GN808 against *Y. Pestis* (Yokohama D11) over time for varying concentrations of GN808, as described in Example 5.

[0048] **Figure 7** is a bar graph showing the percent of hemolysis for GN751 over a range of concentrations, as described in Example 5.

25 [0049] **Figure 8** is a bar graph showing the percent of hemolysis for the RR12Wpolar control lysin over a range of concentrations, as described in Example 5.

DETAILED DESCRIPTION

Definitions

30 [0050] As used herein, the following terms and cognates thereof shall have the following meanings unless the context clearly indicates otherwise:

[0051] “**Carrier**” refers to a solvent, additive, excipient, dispersion medium, solubilizing agent, coating, preservative, isotonic and absorption delaying agent, surfactant, propellant, diluent, vehicle and the like with which an active compound is administered. Such carriers can be sterile liquids, such as water, saline solutions, aqueous dextrose solutions, aqueous glycerol solutions, and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like.

[0052] “**Pharmaceutically acceptable carrier**” refers to any and all solvents, additives, excipients, dispersion media, solubilizing agents, coatings, preservatives, isotonic and absorption delaying agents, surfactants, propellants, diluents, vehicles and the like that are physiologically compatible. The carrier(s) must be “acceptable” in the sense of not being deleterious to the subject to be treated in amounts typically used in medicaments. Pharmaceutically acceptable carriers are compatible with the other ingredients of the composition without rendering the composition unsuitable for its intended purpose. Furthermore, pharmaceutically acceptable carriers are suitable for use with subjects as provided herein without undue adverse side effects (such as toxicity, irritation, and allergic response). Side effects are “undue” when their risk outweighs the benefit provided by the composition. Non-limiting examples of pharmaceutically acceptable carriers or excipients include any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, water, and emulsions such as oil/water emulsions and microemulsions. Suitable pharmaceutical carriers are described, for example, in Remington’s Pharmaceutical Sciences by E.W. Martin, 18th Edition. The pharmaceutically acceptable carrier may be a carrier that does not exist in nature.

[0053] “**Bactericidal**” or “**bactericidal activity**” refers to the property of causing the death of bacteria or capable of killing bacteria to an extent of at least a 3-log₁₀ (99.9%) or better reduction among an initial population of bacteria over an 18-24 hour period.

[0054] “**Bacteriostatic**” or “**bacteriostatic activity**” refers to the property of inhibiting bacterial growth, including inhibiting growing bacterial cells, thus causing a 2-log₁₀ (99%) or better and up to just under a 3-log reduction among an initial population of bacteria over an 18-24 hour period.

[0055] “**Antibacterial**” refers to both bacteriostatic and bactericidal agents.

[0056] “**Effective amount**” refers to an amount which, when applied or administered in an appropriate frequency or dosing regimen, is sufficient to prevent, reduce, inhibit, or eliminate

bacterial growth or bacterial burden or to prevent, reduce, or ameliorate the onset, severity, duration, or progression of the disorder being treated (for example, Gram-negative bacterial pathogen growth or infection), prevent the advancement of the disorder being treated, cause the regression of the disorder being treated, or enhance or improve the prophylactic or therapeutic effect(s) of another therapy, such as bacteriostatic therapy.

[0057] “**Co-administer**” refers to the administration of two agents, such as an a lysin or an engineered lysin and another antibacterial agent, in a sequential manner, as well as administration of these agents in a substantially simultaneous manner, such as in a single mixture/composition or in doses given separately, but nonetheless administered substantially simultaneously to the subject, for example at different times in the same day or 24-hour period. Such co-administration of lysins, or engineered lysins with one or more additional antibacterial agents can be provided as a continuous treatment lasting up to days, weeks, or months. Additionally, depending on the use, the co-administration need not be continuous or coextensive. For example, if the use were as a topical antibacterial agent to treat, *e.g.*, a bacterial ulcer or an infected diabetic ulcer, the lysin, or engineered lysin could be administered only initially within 24 hours of an additional antibacterial agent, and then the additional antibacterial agent use may continue without further administration of the lysin, or engineered lysin.

[0058] “**Subject**” refers to a mammal, a plant, a lower animal, a single cell organism, or a cell culture. For example, the term “subject” is intended to include organisms, *e.g.*, prokaryotes and eukaryotes, which are susceptible to or afflicted with bacterial infections, for example Gram-positive bacterial infections, Gram-negative bacterial infections, or acid-fast bacterial infections. Examples of subjects include mammals, *e.g.*, humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In certain embodiments, the subject is a human, *e.g.*, a human suffering from, at risk of suffering from, or susceptible to infection by Gram-negative bacteria, whether such infection be systemic, topical or otherwise concentrated or confined to a particular organ or tissue.

[0059] “**Polypeptide**” is used herein interchangeably with the term “**peptide**” and refers to a polymer made from amino acid residues and generally having at least about 30 amino acid residues. The term includes not only polypeptides in isolated form, but also active fragments and derivatives thereof, including modified variants. The term “polypeptide” also encompasses fusion proteins or fusion polypeptides comprising an antimicrobial peptide (AMP), a cell-

penetrating peptide (CPP), a lysin, and/or an engineered lysins as described herein and maintaining, for example a lytic function. Depending on context, a polypeptide can be a naturally occurring polypeptide or a recombinant, engineered, or synthetically produced polypeptide. A particular AMP, CPP, lysin or engineered lysin polypeptide construct can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (such as those disclosed in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)) or can be strategically truncated or segmented yielding active fragments, maintaining, e.g., lytic activity against the same or at least one common target bacterium, e.g., *Burkholderia* and/or *Yersinia* species.

[0060] “Fusion polypeptide” refers to an expression product resulting from the fusion of two or more nucleic acid segments, resulting in a fused expression product typically having two or more domains or segments, which typically have different properties or functionality. In a more particular sense, the term “fusion polypeptide” may also refer to a polypeptide or peptide comprising two or more heterologous polypeptides or peptides covalently linked, either directly or via an amino acid or peptide linker. The polypeptides forming the fusion polypeptide are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. The term “fusion polypeptide” can be used interchangeably with the term “fusion protein.” The open-ended expression “a polypeptide comprising” a certain structure includes larger molecules than the recited structure, such as fusion polypeptides.

[0061] “Heterologous” refers to nucleotide, peptide, or polypeptide sequences that are not naturally contiguous. For example, in the context of the present disclosure, the term “heterologous” can be used to describe a combination or fusion of two or more peptides and/or polypeptides wherein the fusion peptide or polypeptide is not normally found in nature, such as for a lysin or engineered lysin polypeptide construct fragment or active fragment thereof and a cationic and/or an AMP, a CPP, a polycationic peptide, an amphipathic peptide, a sushi peptide (Ding et al. Cell Mol Life Sci., 65(7-8):1202-19 (2008)), a defensin peptide (Ganz, T. Nature Reviews Immunology 3, 710-720 (2003)), and/or a hydrophobic peptide, which may have

enhanced lytic activity. Included in this definition are two or more lysin polypeptides or active fragments or variants thereof. These can be used to make a fusion polypeptide with lytic activity.

[0062] “**Active fragment**” refers to a portion of a polypeptide that retains one or more functions or biological activities of the isolated polypeptide from which the fragment was taken, for example bactericidal activity against one or more Gram-negative bacteria, including without limitation *Burkholderia* and/or *Yersinia* species.

[0063] “**Amphipathic peptide**” refers to a peptide having both hydrophilic and hydrophobic functional groups. In certain embodiments, secondary structure may place hydrophobic and hydrophilic amino acid residues at opposite sides (e.g., inner side vs outer side when the peptide is in a solvent, such as water) of an amphipathic peptide. These peptides may in certain embodiments adopt a helical secondary structure, such as an alpha-helical secondary structure.

[0064] “**Cationic peptide**” refers to a peptide having a high percentage of positively charged amino acid residues. In certain embodiments, a cationic peptide has a pKa-value of 8.0 or greater. The term “cationic peptide” in the context of the present disclosure also encompasses polycationic peptides that are synthetically produced peptides composed of mostly positively charged amino acid residues, such as lysine (Lys) and/or arginine (Arg) residues. The amino acid residues that are not positively charged can be neutrally charged amino acid residues, negatively charged amino acid residues, and/or hydrophobic amino acid residues.

[0065] “**Hydrophobic group**” refers to a chemical group such as an amino acid side chain that has low or no affinity for water molecules but higher affinity for oil molecules. Hydrophobic substances tend to have low or no solubility in water or aqueous phases and are typically apolar but tend to have higher solubility in oil phases. Examples of hydrophobic amino acids include glycine (Gly), alanine (Ala), valine (Val), Leucine (Leu), isoleucine (Ile), proline (Pro), phenylalanine (Phe), methionine (Met), and tryptophan (Trp).

[0066] “**Augmenting**” refers to a degree of activity of an agent, such as antimicrobial activity, that is higher than it would be otherwise. “Augmenting” encompasses additive as well as synergistic (superadditive) effects.

[0067] “**Treatment**” refers to any process, action, application, therapy, or the like, wherein a subject, such as a human being, is subjected to medical aid with the object of curing a disorder, eradicating a pathogen, or improving the subject’s condition, directly or indirectly. Treatment also refers to reducing incidence, alleviating symptoms, eliminating recurrence, preventing

recurrence, preventing incidence, reducing the risk of incidence, improving symptoms, improving prognosis, or combinations thereof. "Treatment" may further encompass reducing the population, growth rate, or virulence of a bacteria in the subject and thereby controlling or reducing a bacterial infection in a subject or bacterial contamination of an organ, tissue, or environment. Thus "treatment" that reduces incidence may, for example, be effective to inhibit growth of at least one Gram-negative bacteria in a particular milieu, whether it be a subject or an environment. On the other hand, "treatment" of an already established infection refers to inhibiting the growth, reducing the population, killing and/or eradicating, a Gram-negative bacteria responsible for an infection or contamination.

[0068] "Preventing" refers to the prevention of the incidence, recurrence, spread, onset or establishment of a disorder such as a bacterial infection. It is not intended that the present disclosure be limited to complete prevention or to prevention of establishment of an infection. In some embodiments, the onset is delayed, or the severity of a subsequently contracted disease or the chance of contracting the disease is reduced, and such constitute examples of prevention.

[0069] "Contracted diseases" refers to diseases manifesting with clinical or subclinical symptoms, such as the detection of fever, sepsis, or bacteremia, as well as diseases that may be detected by growth of a bacterial pathogen (*e.g.*, in culture) when symptoms associated with such pathology are not yet manifest.

[0070] The term "derivative" in the context of a peptide or polypeptide or active fragments thereof is intended to encompass, for example, a polypeptide modified to contain one or more chemical moieties other than an amino acid that do not substantially adversely impact or destroy the lytic activity. The chemical moiety can be linked covalently to the peptide, *e.g.*, via an amino terminal amino acid residue, a carboxy terminal amino acid residue, or at an internal amino acid residue. Such modifications may be natural or non-natural. In certain embodiments, a non-natural modification may include the addition of a protective or capping group on a reactive moiety, addition of a detectable label, such as antibody and/or fluorescent label, addition or modification of glycosylation, or addition of a bulking group such as PEG (pegylation) and other changes known to those skilled in the art. In certain embodiments, the non-natural modification may be a capping modification, such as N-terminal acetylations and C-terminal amidations. Exemplary protective groups that may be added to peptides include, but are not limited to, t-Boc and Fmoc. Commonly used fluorescent label proteins such as, but not limited to, green

fluorescent protein (GFP), red fluorescent protein (RFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and mCherry, are compact proteins that can be bound covalently or noncovalently to a peptide or fused to a peptide without interfering with normal functions of cellular proteins. In certain embodiments, a polynucleotide encoding a fluorescent protein may be inserted upstream or downstream of the polynucleotide sequence. This will produce a fusion protein (e.g., lysin::GFP) that does not interfere with cellular function or function of a peptide to which it is attached. Polyethylene glycol (PEG) conjugation to proteins has been used as a method for extending the circulating half-life of many pharmaceutical proteins. Thus, in the context of peptide derivatives, the term “derivative” encompasses peptides chemically modified by covalent attachment of one or more PEG molecules. It is anticipated that pegylated peptides will exhibit prolonged circulation half-life compared to the unpegylated peptides, while retaining biological and therapeutic activity.

[0071] “**Modified variant**” refers to a lysin or engineered lysin polypeptide construct wherein a non-naturally occurring modification has been made to the amino acid sequence that either enhances the lytic activity or does not substantially adversely impact or destroy the lytic activity of the lysin or engineered lysin polypeptide construct. Exemplary modifications that may be made to modified variants include modifying an amino acid of the lysin peptide, such as a positively charged amino acid, from an L-form to a D-form; adding or deleting one or more amino acid residue or residues to the C-terminus and/or the N-terminus, forming fusion polypeptides, and forming charge array variants, wherein amino acid charges have been reordered. In certain embodiments, an N-terminal methionine residue is deleted from the lysin or engineered lysin polypeptide construct. Cleavage of an initial methionine residue, e.g., by a methionine aminopeptidase enzyme, after protein synthesis is a known mechanism recognized in the art. Wingfield, P., *N-Terminal Methionine Processing*, Curr. Protoc. Protein Sci. 2017, 88:6.14.1-6.14.3. Accordingly, in the embodiments disclosed herein a modified variant of a lysin or engineered lysin as disclosed herein may include the lysin or engineered lysin with or without an initial methionine residue. It is also understood that for lysin, AMP, and/or CPP sequences identified herein, the sequence may include the initial methionine residue or the sequence may exclude the initial methionine residue. By way of non-limiting example, the HIP_{ABC} AMP identified herein as SEQ ID NO: 28 may contain an initial methionine residue such that the sequence is

MKGRGKTGGKARAKWKTRSSRAGLQWPVGRVHRLLRKGNYAHRVGAGAPVWL

(SEQ ID NO: 28) or it may exclude the initial methionine residue such that the HIP_{ABC} AMP sequence is

KGRGKTGGKARAKWKTRSSRAGLQWPVGRVHRLLRKGNYAHRVGAGAPVWL (amino

5 acid residues 2-52 of SEQ ID NO: 28). Thus, as used herein HIP_{ABC} (SEQ ID NO: 28) may indicate either a 52 amino acid sequence containing an initial methionine residue or a 51 amino acid sequence containing lysine (K) as the initial residue.

[0072] “Percent amino acid sequence identity” refers to the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, such as a specific lysin or engineered construct polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for example, using publicly available software such as BLAST or software available commercially, for example from DNASTAR. Two or more polypeptide sequences can be anywhere from 0-100% identical, or any integer value there between. In the context of the present disclosure, two polypeptides may have at least 80% of the amino acid residues (such as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92.5%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5%) that are identical. The term “percent (%) amino acid sequence identity” as described herein applies to peptides as well. Two polypeptides having certain percent amino acid sequence identity as disclosed herein will encompass mutated, truncated, fused, or otherwise sequence-modified forms of isolated polypeptides and peptides described herein, and active fragments thereof (*e.g.*, at least about 80%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92.5%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% identity) as measured for example by one or more methods referenced above) as compared to the reference (wild type or other intact) polypeptide.

[0073] As used herein, a “**conservative amino acid substitution**” is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine),
5 acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

10 [0074] “**Inhalable composition**” refers to pharmaceutical compositions of the present disclosure that are formulated for direct delivery to the respiratory tract during or in conjunction with routine or assisted respiration (e.g., by intratracheobronchial, pulmonary, and/or nasal administration), including, but not limited to, atomized, nebulized, dry powder, and/or aerosolized formulations.

15 [0075] “**Outer Membrane**” or “**OM**” refers to a feature of Gram-negative bacteria. The outer membrane is comprised of a lipid bilayer with an internal leaflet of phospholipids and an external amphiphilic leaflet largely consisting of lipopolysaccharide (LPS). The LPS has three main sections: a hexa-acylated glucosamine-based phospholipid called lipid A, a polysaccharide core and an extended, external polysaccharide chain called O-antigen. The OM presents a non-
20 fluid continuum stabilized by three major interactions, including: i) the avid binding of LPS molecules to each other, especially if cations are present to neutralize phosphate groups; ii) the tight packing of largely saturated acyl chains; and iii) hydrophobic stacking of the lipid A moiety. The resulting structure is a barrier for both hydrophobic and hydrophilic molecules. Below the OM, the peptidoglycan forms a thin layer that is very sensitive to hydrolytic cleavage
25 - unlike the peptidoglycan of Gram-negative bacteria which is 30-100 nanometers (nm) thick and consists of up to 40 layers, the peptidoglycan of Gram-negative bacteria is only 2-3 nm thick and consists of only 1-3 layers.

[0076] As used herein, “**ESKAPE bacteria**” refers to a group of pathogens that are known for their ability to escape the effects of antimicrobial drugs. The acronym “ESKAPE” stands for the
30 following bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. Among these

pathogens, the group of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species are referred to herein as Gram-negative ESKAPE bacteria.

POLYPEPTIDES

5 *Native and Modified Lysins*

[0077] The present disclosure is directed to isolated polypeptides comprising base lysins and modified base lysins, as well as active fragments and variants thereof as herein described. In certain embodiments, the N-terminal methionine residue is deleted from the native or modified base lysin or an engineered lysin, e.g., a base lysin, which includes a variant base lysin, as herein
10 described, which is modified to include linker polypeptides, AMPs, CPPs and/or other features as herein described. Cleavage of an initial methionine residue, e.g., by a methionine aminopeptidase enzyme, after protein synthesis is a known mechanism recognized in the art. Wingfield, P., *N-Terminal Methionine Processing*, Curr. Protoc. Protein Sci. 2017, 88:6.14.1-6.14.3. Accordingly, in the embodiments disclosed herein a base lysin or engineered lysin may
15 include an initial methionine residue or may not include an initial methionine residue.

[0078] In some embodiments, the isolated polypeptides comprise a lysin, variant lysin, active fragments thereof or derivatives thereof, wherein the lysin, variant, fragment, or derivative thereof is combined with at least one antimicrobial peptide (“AMP”) and/or at least one cell-penetrating peptides (“CPP”) to form an engineered lysin, wherein the engineered lysin has lytic
20 activity. As used herein “lytic activity” encompasses the ability of an engineered lysin polypeptide construct to kill bacteria, such as a Gram-negative bacteria (e.g., *P. aeruginosa*, *B. cepacia*, *Burkholderia spp.*, *Yersinia spp.*), reduce the population of bacteria or inhibit bacterial growth (e.g., by penetrating the outer membrane of a Gram-negative bacteria), optionally in the presence of human serum.

[0079] In some embodiments, the present isolated lysins and engineered lysins, fragments, or variants thereof are capable of penetrating the outer membrane of Gram-negative bacteria. Without being limited by theory, after penetration of the outer membrane, the present isolated lysins and engineered lysins, fragments, or variants thereof can degrade peptidoglycan, a major structural component of the bacterial cell wall, resulting in e.g., cell lysis or non-lethal damage
30 that inhibits bacterial growth. In some embodiments, the present isolated lysins and engineered lysins, fragments, or variants thereof disclosed herein contain positively charged (and

amphipathic) N- and/or C-terminal α -helical domains that facilitate binding to the anionic outer membrane of a Gram-negative bacteria to effect translocation into the sub-adjacent peptidoglycan.

[0080] The ability of a lysin to penetrate an outer membrane of a Gram-negative bacteria may be assessed by any method known in the art, such as described in WO 2017/049233, which is herein incorporated by reference in its entirety. For example, the lysin may be incubated with Gram-negative bacteria and a hydrophobic compound. Most Gram-negative bacteria are strongly resistant to hydrophobic compounds, due to the presence of the outer membrane and, thus, do not allow the uptake of hydrophobic agents such as 1-N-phenylnaphthylamine (NPN), crystal violet, or 8-anilino-1-naphthalenesulfonic acid (ANS). NPN, for example, fluoresces strongly under hydrophobic conditions and weakly under aqueous conditions. Accordingly, NPN fluorescence can be used as a measurement of the outer membrane permeability.

[0081] More particularly, the ability of a lysin to penetrate an outer wall may be assessed by incubating, e.g., NPN with a Gram-negative bacteria, e.g., *Burkholderia spp.* or *Yersinia spp.*, in the presence of the lysin to be tested for activity. A higher induction of fluorescence in comparison to the fluorescence emitted in the absence of a lysin (negative control) indicates outer membrane penetration. In addition, fluorescence induction can be compared to that of established permeabilizing agents, such as EDTA (ethylene diamine tetraacetate) or an antibiotic such as an antibiotic of last resort used in the treatment of Gram-negative bacterial infections, *i.e.*, Polymyxin B (PMB), to assess the level of outer membrane permeability.

[0082] In some embodiments, the present isolated lysins and engineered lysins, fragments, or variants thereof exhibit lytic activity in the presence and/or absence of human serum. Suitable methods for assessing the activity of a lysin in human serum are known in the art and described herein. Briefly, a minimum inhibitory concentration ("MIC") value (*i.e.*, the minimum concentration of peptide sufficient to suppress at least 80% of the bacterial growth compared to control) may be determined for a lysin and compared to, *e.g.*, a parent lysin or compound inactive in human serum, *e.g.*, T4 phage lysozyme or artilysin GN126. T4 phage lysozyme is commercially available, *e.g.* from Sigma-Aldrich, Inc. GN126 corresponds to Art-175, which is described in the literature and may be obtained by fusing AMP SMAP-29 to GN lysin KZ144. *See, e.g.*, Briers et al., *Antimicrob. Agents Chemother.* 2014, 58:3774-3784, which is herein

incorporated by reference in its entirety. MIC values may be expressed, e.g., in $\mu\text{g/mL}$, and MIC may be determined by any method known in the art.

[0083] More particularly, MIC values for a lysin may be determined against a Gram-negative bacterial strain, e.g., a *Burkholderia* spp in e.g., AST medium containing % CAA (casamino acid media) with 0.02% TWEEN80®, and 0.5micromolar MgSO_4 , or 25% CAMHB with 0.02% TWEEN80® when the Gram-negative bacterial strain are e.g., *Yersinia* spp.

[0084] In some embodiments, the present isolated lysins and engineered lysins, fragments, and variants thereof show low toxicity against erythrocytes. Any methodology known in the art may be used to assess the potential for hemolytic activity of the present isolated lysins and engineered construct, fragments, or variants thereof. Examples of suitable lysins of the present disclosure, particularly for use in preparing the engineered lysins described herein, include Gram-negative base lysins.

[0085] As used herein, a “Gram-negative base lysin” is an isolated lysin, which may be modified, e.g., by fusing the Gram-negative base lysin with an AMP and/or CPP or other element as herein described, e.g, at the N-terminus or C-terminus. This term includes native lysins, as well as native lysins modified to contain one or more insertions, deletions and/or amino acid substitutions in comparison to a reference lysin polypeptide, e.g., a naturally occurring lysin or a parent lysin, which itself may be a variant lysin. Such variant base lysins may be further modified to include AMPs, CPPs or other features as disclosed, e.g, at the N-terminus or C-terminus.

[0086] Examples of suitable Gram-negative base lysins include those from the phage lysozyme pfam00959 protein family, such as GN867 set forth as SEQ ID NO: 13, GN3 set forth as SEQ ID NO: 3, QPI14001 set forth as SEQ ID NO: 9 and GN917 set forth as SEQ ID NO: 10. Other suitable base lysins without e.g., substitutions include peptidase_M15_4 pfam13539 protein family, such as GN685 set forth as SEQ ID NO: 2, GN37 set forth as SEQ ID NO: 8, GN569 set forth as SEQ ID NO: 7, GN568 set forth as SEQ ID NO: 1 and GN577 set forth as SEQ ID NO: 12. In some embodiments, the Gram-negative base lysin is from the phage lysozyme pfam00959 protein family, such as GN867 set forth as SEQ ID NO: 13, GN3 set forth as SEQ ID NO: 3, GN515 set forth as SEQ ID NO: 6, QPI14001 set forth as SEQ ID NO: 9 and GN917 set forth as SEQ ID NO: 10. In some embodiments, the Gram-negative base lysin is from the peptidase_M15_4 pfam13539 protein family, such as GN685 set forth as SEQ ID NO: 2, GN568

set forth as SEQ ID NO: 1, GN37 set forth as SEQ ID NO: 8, GN569 set forth as SEQ ID NO: 7, and GN577 set forth as SEQ ID NO: 12. Other suitable Gram-negative base lysins are from the muramidase pfam11860 protein family, such as GN428 set forth as SEQ ID NO: 4, and CAH0532154 set forth as SEQ ID NO: 11. GN588 as set forth in SEQ ID NO: 5 from the
 5 peptidase_M15_3 pfam08291 protein family is also suitable for use as a Gram-negative base lysin. The foregoing Gram-negative base lysins are detailed below.

[0087] Table 1 – Exemplary Base Lysins

Lysin	Source of lysin (protein family)	NCBI Accession number	Amino acid sequence
GN568	<i>Yersinia</i> phage PY100 (Peptidase_M15_4; pfam13539)	CAJ28446	MEVQPTIEEVSMGFKLGSRSLQRLQG VHPDLVKVVKRAIEISPVDFTVTEGL RTLERQKELFAKGASKTMRSRHLTG HAVDISPLVDGKVSWDWKYYYPMA DAMKQAAKELNIPVEWGGDWKTFK DGPHEFQLPYGVYK (SEQ ID NO: 1)
GN685	<i>Yersinia</i> phage PY100 (Peptidase_M15_4; pfam13539)	CAJ28446	MGFKLGSRSLQRLQGVHPDLVKVVK RAIEISPVDFTVTEGLRTLERQKELFA KGASKTMRSRHLTGHAVDISPLVDG KVSWDWKYYYPMADAMKQAAKEL NIPVEWGGDWKTFKDGPHEFQLPYGV YK (SEQ ID NO: 2)
GN3	<i>Pseudomonas</i> (Phage lysozyme; pfam00959)	ABY99279; WP_012273008	MRTSQRGLSLIKSFEGRLRLQAYQDSV GVWTIGYGTTRGVKAGMKISKDQAE RMLLNDVQRFEPERLIKVPLNQD QWDALMSFTYNLGAANLESSTLRRL LNAGNYAAAAEQFPRWNKAGGQVL AGLTRRRRAERELFLGAA (SEQ ID NO: 3)
GN428	<i>Escherichia</i> phage (Muramidase; pfam 11860)	YP_04957781.1	MAILKLGNRGSEVKALQQSLNKIGFS LTADGIFGKATENAVKSVQAGAGLV IDGIAGPKTFYAIRNAGDAHQEHLTE ADLVDAARELGVELASMKAVNQVE SRGTGFTKTGKIKTLFERHIMYKKVT AKFGQARANALYQLYPTLVNPNSSG YIGGDAELERLQGAIALDEDCAYESA SYGLFQIMGFNCQICGYSNAKEMFTD FLTGERAHLALFVKFIKADANMWKA LKNKNWAEFARRYNGPAYAKNQYD TKLAAAYKSFC (SEQ ID NO: 4)

GN588	<i>Burkholderia stagnalis</i> (Peptidase_M15_3; pfam08291)	WP_060150081	MQLTPHFTLEEFTRSDTARARQIDNS PSPAAAANLRRLAQTLERARVLLGG KPMQITSGYRSPALNRAVGGVPMAS HAQGLAADFVCSGYGPPLDVCRVLA ASDLFPDQVINEFGRWVHIGLAADG VTPRRQVL TARKVGGRTTYVAGLQA (SEQ ID NO: 5)
GN515	<i>Pseudomonas aeruginosa</i> (Phage_lysozyme; pfam00959)	WP_058157505.1	MRTSQRGIDLIKSFEGRLRLSAYQDSV GVWTIGYGTTRGVTRYMTITVEQAE RMLSNDLQRFEPDLRLVKVPLNQ QWDALMSFVYNLGAANLESSTLRL LNAGDYAGAAEQFLRWKAGGKVL PGLVRRRASERELFLGAA (SEQ ID NO: 6)
GN569	<i>Burkholderia ambifaria</i> (Peptidase_M15_4; pfam13539)	WP_175712571.1	MPVSGFKFSRRSEVNIGVKPSLVSL ARRALELSTVDFGVTEGLRTKARQR ELVAAKKSQTMNSRHLTGDAVDVIA YVRGAGTWEWKYYEDIARAFKQAA AELGIPIVWGGDWKTLKDGVFHFLA R (SEQ ID NO: 7)
GN37	<i>Micavibrio aeruginosavorus</i> (Peptidase_M15_4; pfam13539)	AEP08879	MTYTLSKRSLDNLKGVHPDLVAVVH RAIQLTPVDFAVIEGLRSVSRQKELV AAGASKTMNSRHLTGHAVDLAAYV NGIRWDWPLYDAIAVAVKAAAKEL GVAIVWGGDWTTFKDGPHELDRLSK YR (SEQ ID NO: 8)
QPI14001	<i>Serratia</i> phage 4S (Phage_lysozyme; pfam00959)	QPI14001.1	MTIFDMLRIDEGLKLTVYKDTTEGF TVGIGHLLTKDSSKAVAIKELDRLTG RSTDGSI SVGEAENIFKLDVSKAEN KGNSVLAPVYESLDSVRKMAMVNM VFQLGVAGVAGFKNSLALLKAKDW DKAAVNLAQSKWYNQTPNRAKRVIS VFKTGTMDAYK (SEQ ID NO: 9)
GN917	<i>Yersinia</i> phage phiR1-RT Phage_lysozyme; pfam00959)	YP_007235955.1	MNLFEMRLRYDEGLKLEVYFDTEGYP TVGVGHLLTKVHNLMSGISILDKELG RSTQGRITTEDAEYLFSKDVEKAKAG IKSNTVLNDLYLSLDPIRQMALVNM VFQMGVAGVAGFNNSIKLLKAKQW DQAALNLAQSKWFKQTPNRAKRVIN VFKTGTMDAYK (SEQ ID NO: 10)
CAH0532154	<i>Ralstonia</i> phage UAM5 (Muramidase; pfam11860)	CAH0532154.2	MPQSTKQITPDDYARAAAALGVPVA AVKAVTDVESNGHGFLSDGRPLILYE RHIMRRQLIAAGRAADADSHEASEP NIVNRAPGGYKGGAAEWDRLDAV HIDRACALESASWGLFQIMGFHWKS LGYESVQAFVNAMYASEGAQLDAF

			VAFVKANPNLLRALRAKDWPGFAR GYNGPGYAANKYDTKLAAAYARHI KDAA (SEQ ID NO: 11)
GN577	<i>Burkholderia cenocepacia</i>	WP_200884908 .1	MKNFRFSRRSERNLVGVKPDILKLAR RALELSEVDFGVTEGLRTKDRQRQL VAEGKSRTANSRHLTGDAIDVVAYL GAAVTWEWKYYEQIAVAFKRASAE LGIPVEWGGDWKSLKDGPHFQLPW (SEQ ID NO: 12)
GN867	<i>Yersinia</i> phage vB_YenM_TG1	YP_009200388. 1	MNLFEMRLRYDEGLKLEVYFDTEGY TVGVGHLLTKVHNLMSGISILDKELG LSTQGRITTEDAEYLFSKDVEKAKAG IKSNTVLNDLYLSLDPIRQMALVNM VFQMGVAGVAGFNNSIKLLKAKQW DQAALNLAQSKWFKQTPNRAKRVIN VFKTGTMGAYK (SEQ ID NO: 13)
GN4	<i>Pseudomonas</i> phage PAJU2	YP 002284361.1	MRTSQRGIDLIKSFEGLRLSAYQDSV GVWTIGYGTTRGVTRYMTITVEQAE RMLSNDIQRFEPELDRLAKVPLNQNQ WDALMSFVYNLGAANLASSTLLKLL NKGDYQGAADQFPRWVNAGGKRLD GLVKRRAAERALEPLS (SEQ ID NO: 175)

[0088] In some embodiments, the present engineered lysins may comprise a base lysin variant that contains, e.g, substitutions, insertions or deletions relative to a native base lysin, and further includes AMPs, CPPs and the additional features disclosed herein, e.g., linkers. In other

[0089] In some embodiments, the engineered lysin, active fragment thereof or derivative thereof has at least about 80%, such as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity with a reference lysin and/or active fragment thereof described herein. In certain embodiments, the engineered lysin, active fragment thereof or derivative thereof has at least two amino acid substitution relative to a reference lysin and/or active fragment thereof described herein, and in certain embodiments, the engineered lysin, active fragment thereof or derivative

thereof has at least two amino acid substitutions relative to a reference lysin and/or active fragment thereof described herein. In certain embodiments, the engineered lysin, active fragment thereof or derivative thereof has two amino acid substitution relative to a reference lysin and/or active fragment thereof described herein.

5 [0090] In some embodiments, such as when an engineered lysin includes a base lysin which base lysin includes, e.g., substitutions, deletions or insertions and further includes the AMPs, CPPs and optionally other elements, such as linkers, as described herein or in embodiments, such as those when an engineered lysin includes substitutions, deletions or insertions, but does not include the AMPs, CPPs or other features described herein, function the same as a reference
10 lysin. In other embodiments, such engineered lysins retain one or more functional or biological activities of a reference lysin. In some embodiments, the modification improves the antibacterial activity in comparison to the reference lysin. Typically, the lysin variant has improved *in vitro* antibacterial activity (e.g., in buffer and/or media) in comparison to the reference lysin. In other embodiments, the lysin variant has improved *in vivo* antibacterial activity (e.g., in an animal
15 infection model). In some embodiments, the modification improves the antibacterial activity of the lysin in the presence of human serum, and in some embodiments, the modification improves the antibacterial activity of the lysin in the absence of human serum.

[0091] Suitable variant lysins, particularly those which may be used as a Gram-negative base lysin, include variants of GN685, GN3, GN4, GN515, and GN569. In certain embodiments, the
20 variant of GN685 comprises an amino acid substitution of K78H relative to GN685 (in embodiments wherein GN685 includes an initial methionine residue) or amino acid substitutions of K77H relative to GN685 (in embodiments wherein GN685 does not include an initial methionine residue). In certain embodiments, the variant of GN3 comprises amino acid substitutions of R100Q and R116H relative to GN3 (in embodiments wherein GN3 includes an
25 initial methionine residue) or amino acid substitutions of R99Q and R115H relative to GN3 (in embodiments wherein GN3 does not include an initial methionine residue). In certain embodiments, the variant of GN3 comprises amino acid substitutions of R100Q and R116Q relative to GN3 (in embodiments wherein GN3 includes an initial methionine residue) or amino acid substitutions of R99Q and R115Q relative to GN3 (in embodiments wherein GN3 does not
30 include an initial methionine residue). In certain embodiments, the variant of GN4 comprises amino acid substitutions of K100D and R116H relative to GN4 (in embodiments wherein GN4

includes an initial methionine residue) or amino acid substitutions of K99D and R115H relative to GN4 (in embodiments wherein GN4 does not include an initial methionine residue). The GN4 lysin may be obtained from *Pseudomonas* phage PAJU2 (NCBI Reference Sequence YP 002284361.1, and is described in PCT Publication No. 2019/191633, which reference is incorporated by reference herein in its entirety. In certain embodiments, the variant of GN515 comprises amino acid substitutions of R100D and R116H relative to GN515 (in embodiments wherein GN515 includes an initial methionine residue) or amino acid substitutions of R99D and R115H relative to GN515 (in embodiments wherein GN515 does not include an initial methionine residue). In certain embodiments, the variant of GN569 comprises an amino acid substitution of K87H relative to GN569 (in embodiments wherein GN569 includes an initial methionine residue) or amino acid substitutions of K86H relative to GN569 (in embodiments wherein GN569 does not include an initial methionine residue). Exemplary variant lysins are detailed below in Table 2.

[0092] Table 2 – Exemplary Variant Lysins

Parent Lysin	Modifications (with initial methionine residue)	Amino acid sequence of variant lysin
GN685	K78H	MGFKLGSRSLQRLQGVHPDLVKVVKRAIEISPVDFTVT EGLRTLERQKELFAKGASKTMRSRHLTGHAVDISPLVD GHVSWDWKYYYPADAMKQAAKELNIPVEWGGDW KTFKDGPHFQLPYGVYK (SEQ ID NO: 106)
GN3	R100Q; R116H	MRTSQRGLSLIKSFEGRLRLQAYQDSVGVWTIGYGTTTR GVKAGMKISKDQAERMLLNDVQRFEP EVERLIKVPLN QDQWDALMSFTYNLGAANLESSTLRQLLNAGNYAAA AEQFPHWNKAGGQVLGLTRRRRAERELFLGAA (SEQ ID NO: 14)
GN515	R100D; R116H	MRTSQRGIDLIKSFEGLRLSAYQDSVGVWTIGYGTTTRG VTRYMTITVEQAERMLSNDLQRFEP ELDRLVKVPLNQ NQWDALMSFVYNLGAANLESSTLRDLLNAGDYAGAA EQFLHWNKAGGKVLPLVRRRASERELFLGAA (SEQ ID NO: 15)
GN569	K87H	MPVSGFKFSRRSEVNIGVKPSLVSLARRALELSTVDF GVTEGLRTKARQRELVA AKKSQTMNSRHLTGDAVDVI AYVRGAGTWEWHYYEDIAFAFKQAAAEELGIPIVWGG DWKTLKDG VHFELAR (SEQ ID NO: 16)
GN3	R99Q, R115Q	RTSQRGLSLIKSFEGRLRLQAYQDSVGVWTIGYGTTTRGV KAGMKISKDQAERMLLNDVQRFEP EVERLIKVPLNQD

		QWDALMSFTYNLGAANLESSTLRQLLNAGNYAAAAE QFPQWNKAGGQVLAGLTRRRAAERELFLGAA (SEQ ID NO: 177)
GN4	K99D, R115H	RTSQRGIDLIKSFEGRLRSAYQDSVGVWTIGYGTTRGV TRYMTITVEQAERMLSNDIQRFEPELDRLAKVPLNQNQ WDALMSFVYNLGAANLASSTLLDLLNKGDYQGAADQ FPHWVNAGGKRLDGLVKRRAAERALFLEPLS (SEQ ID NO: 176)

[0093] In some embodiments, the lysins disclosed herein have a predicted overall isoelectric point (pI) of greater than 8, in some embodiments, 8-12, in some embodiments 8-11.5, in some embodiment 9-11.5, in still another embodiment, 9.5-11.5, in still another embodiment, 10.5-11.5. In other embodiments, the variant lysins are obtained by modifying a reference lysin to include a modification resulting in a change in the overall isoelectric point (pI) of the lysin, *i.e.*, the pH at which a molecule has a net neutral charge by, for example, incorporating a single pI-increasing mutation, such as a single point mutation, into a reference lysin.

[0094] In some embodiments, the lysin variants of the present disclosure are typically designed to retain an α -helix domain, the presence or absence of which can be readily determined using various software programs, such as Jpred4 (compio.dundee.ac.uk/jpred), Helical Wheel (hael.net/helical.htm), HeliQuest (zhanglab.ccmb.med.umich.edu/I-TASSER/) and PEP-FOLD 3 (bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3). In some embodiments, the α -helix domain is located at the C-terminus of a lysin. In other embodiments, the α -helix domain is located at the N-terminus of a lysin. More typically, the α -helix domain is located at the C terminus. The α -helix domain of the lysins of the present disclosure varies in size between about 20 and 40 amino acids, more typically between about 15 and 33 amino acid residues.

[0095] Lysin variants may be formed by any method known in the art and as described in WO 2017/049233, which is herein incorporated by reference in its entirety, *e.g.*, by modifying any of the lysins, active fragments thereof and derivatives described herein through site-directed mutagenesis or via mutations in hosts that produce the present lysins which retain one or more of the biological functions as described herein. The present lysin variants may be truncated, chimeric, shuffled or “natural,” and may be in combination as described, for example, in U. S. Patent No. 5,604,109, which is incorporated herein in its entirety by reference.

[0096] For example, one of skill in the art can reasonably make and test substitutions or replacements to, e.g., the α -helix domain or regions outside of the α -helix domain. Sequence comparisons to the Genbank database can be made with e.g., a full amino acid sequence as described herein, for instance, to identify amino acids for substitution.

5 [0097] Mutations can be made in the amino acid sequences, or in the nucleic acid sequences encoding the lysins and engineered lysin polypeptide constructs, active fragments or variants thereof, such that a particular codon is changed to a codon which codes for a different amino acid, an amino acid is substituted for another amino acid, or one or more amino acids are deleted.

[0098] Such a mutation is generally made by making the fewest nucleotide changes possible. A

10 substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (for example, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (for example, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an

15 amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present disclosure should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein. Thus, one of skill

20 in the art, based on a review of the sequence of lysins provided herein and on their knowledge and the public information available for other lysin polypeptides, can make amino acid changes or substitutions in the lysin polypeptide sequence. Amino acid changes can be made to replace or substitute one or more, one or a few, one or several, one to five, one to ten, or such other number of amino acids in the sequence of the lysin(s) provided herein to generate mutants or variants

25 thereof. Such mutants or variants thereof may be predicted for function or tested for function or capability for anti-bacterial activity as described herein against, e.g., *Burkholderia spp.* or *Yersinia spp.*, and/or for having comparable activity to the lysin(s) as described and particularly provided herein. Thus, changes made to the sequence of lysin, and mutants or variants described herein can be tested using the assays and methods known in the art and described herein. One of

30 skill in the art, on the basis of the domain structure of the lysin(s) hereof can predict one or more, one or several amino acids suitable for substitution or replacement and/or one or more amino

acids which are not suitable for substitution or replacement, including reasonable conservative or non-conservative substitutions.

[0099] In some embodiments, the present isolated lysins and engineered lysin polypeptide constructs comprise active fragments of lysins or derivatives. The term “active fragment” refers to a portion of a full-length lysin, which retains one or more biological activities of the reference lysin. Thus, as used herein, an active fragment of a lysin or variant lysin inhibits the growth, or reduces the population, or kills a Gram-negative bacteria, such as a *Burkholderia spp.* and/or *Yersinia spp.* and optionally at least one additional species of Gram-negative bacteria as described herein in the absence or presence of human serum. The active lysin fragments may retain an α -helix domain.

[00100] In some embodiments, the isolated lysin or engineered lysin polypeptide construct comprises a lysin selected from GN568, GN685, GN3, GN4, GN428, GN588, GN515, GN37, GN569, QPI14001.1, GN917, CAH0532154, GN577, and GN867 or an active fragment thereof, wherein the lysin or active fragment thereof inhibits the growth, or reduces the population, or kills at least one Gram-negative bacteria, such as *Burkholderia spp.* or at least one *Yersinia spp.* In some embodiments, the lysin or active fragment thereof contains at least one substitution, deletion, or insertion modification relative to GN568, GN685, GN3, GN4, GN428, GN588, GN515, Peptidase_M15_4, GN37, GN569, QPI14001.1, GN917, CAH0532154, GN577, and GN867. In certain embodiments, the at least one amino acid substitution is a conservative amino acid substitution. In some embodiments, the at least one amino acid substitution is chosen from R100D, R100Q, R116H, R116Q, K100D, K78H, or K87H, when the base lysin polypeptide comprises an initial methionine residue. In some embodiments, the at least one amino acid substitution is chosen from R99D, R99Q, R115H, K99D, or K77H, K86H, when the base lysin polypeptide does not comprise an initial methionine residue. In certain embodiments, when the base lysin comprises an initial methionine residue and the base lysin is GN3, the lysin or active fragment thereof contains two amino acid substitutions of R100Q and R116H or two amino acid substitutions of R100Q and R116Q. In certain embodiments, when the base lysin does not comprise an initial methionine residue and the base lysin is GN3, the lysin or active fragment thereof contains two amino acid substitutions of R99Q and R115H or two amino acid substitutions of R99Q and R115Q. In certain embodiments, when the base lysin comprises an initial methionine residue and the base lysin is GN4, the lysin or active fragment thereof contains

two amino acid substitutions of K100D and R116H. In certain embodiments, when the base lysin does not comprise an initial methionine residue and the base lysin is GN4, the lysin or active fragment thereof contains two amino acid substitutions of K99D and R115H.

Antimicrobial Peptides (AMPs)

5 **[00101]** In various embodiments of the engineered lysin polypeptide constructs, fragments thereof, and variants thereof disclosed herein, the construct comprises an AMP in addition to the lysin polypeptide, e.g., an engineered lysin, which is a lysin-AMP polypeptide construct, optionally further comprising a linker and/or an outer membrane binding motif, as disclosed herein.

10 **[00102]** The term “antimicrobial peptide” (AMP) as used herein refers to a member of a wide range of short (generally 3 to 50 amino acid residues in length) gene-encoded peptides, that can help penetrate the outer membrane of a Gram-negative bacteria or kill bacteria, reduce the population of bacteria or inhibit bacterial growth. The term encompasses helical peptides, β -sheet peptides and those that display largely disordered random coil structures. AMPs include
15 defensins, cathelicidins, sushi peptides, cationic peptides, polycationic peptides, amphipathic peptides, hydrophobic peptides and/or AMP-like peptides, e.g., amurin peptides as described herein. Fragments of AMPs, AMP variants and derivatives of AMPs are also encompassed by this term.

20 **[00103]** The term “AMP activity” as used herein encompasses the ability of an AMP or fragment or variant thereof to kill bacteria, reduce the population of bacteria or inhibit bacterial growth e.g., by penetrating the outer membrane of a Gram-negative bacteria in the presence and/or absence of human serum. Typically, translocation of the AMPs is driven by a primary electrostatic interaction with the lipopolysaccharide portion of the outer membrane followed by cation displacement, membrane disorganization and transient openings, and in some cases,
25 internalization of the AMP.

30 **[00104]** In some embodiments, the present AMPs are variant AMPs having at least 80%, such as at least about 85%, such as at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about
98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity with any of the AMPs described herein, wherein the variant AMP thereof retains an AMP activity.

[00105] In some embodiments, the present AMPs comprise a helical domain, such as an α -helical domain. In some embodiments, the α -helical domain spans most of the molecule. In other embodiments, the α -helix domain is either interrupted or truncated. The α -helix domain of the present AMPs may vary in size from between about 3 to 32 amino acids, more typically between about 10 and 25 amino acid residues. Generally, the helical domains are required for activity and typically must be retained when fused to a C- or N-terminus of a lysin.

[00106] Typically, helical peptides display amphipathic characteristics and contain a substantial proportion (e.g. 50%) of hydrophobic residues, frequently appearing in repeated patterns. Upon formation of an α -helical structure, the hydrophilic residues typically end up on the same side of the helix, thereby resulting in a conformation-dependent amphiphilicity. Frequently, these peptides are unstructured in an aqueous environment, but adopt a helical conformation upon encountering lipid membranes. Peptides belonging to this group typically display an overall positive charge ranging from +2 to +11 and usually kill microbes, such as Gram-negative bacteria, by creating membrane defects, leading to a loss of gradients in electrolytes, signal substances and other factors.

[00107] Suitable AMPs for use in the present engineered lysin-AMP constructs include, for example BK5, AM1, AM13, MMAV-2, MFV1-3, BCM1, BK3, BK3h2, BK3h3, ATRA, RI18, HIP_A, HIP_{AB}, or HIP_{ABC}, HIP_{Anat}, BK9 as detailed in Table 3 below.

[00108] Table 3 – Exemplary AMPs

AMP	NCBI Accession number	Amino acid sequence
BK5	WP_087740524	MSKGKGARPGRPQPLRGTKGKRKGARLWYVGGQQF (SEQ ID NO: 17)
AM1		MRLKMARRRYRLPRRRSRRLFSRTALRMHPRNRLRRIM RGGIRF (SEQ ID NO: 123)
AM13		MRRRVRRTRRHQWRLTHSARSIRRARIMPSRPRGGRRF (SEQ ID NO: 124)
MMAV-2		MLAKNKAEFAYKNIKTFARKFKNLLTKSIRRQFTKNF (SEQ ID NO: 125)
MFV1-3		MFSLLKIKKRSFPFYCKRTTANLLYIKK (SEQ ID NO: 126)
BCM1	YP_007236750.1	MPKTHSEFRHRPFLRALTPEQRRRWREMRRARGMKPLY NSRIRGR (SEQ ID NO: 18)
BK3	QJB18883	MHSKHRKHVNKRKSARTFRKHSARTKSANMQKAPQRG GWRL (SEQ ID NO: 19)

		Or a variant thereof represented by MYRKPVNKRRSARTFRRNAKRTKSANLQKAPQRRGGWRL (SEQ ID NO: 20) Or a variant BK3h2 represented by: MYRKPVNKRRSARSFRKNARRTKSANMQKSTTTWRL (SEQ ID NO: 178) Or a variant BK3h3 represented by: MKILHRKYMSKKAHSKTFRKHGRKTKSPNTHSSPQRRGGWRL (SEQ ID NO: 179)
ATRA	B6S2X0.1	MEGFFWKTLLVVGALTISGTSSFPHKPLTYEEAVDLAVSVYNSKSGEDSLYRLLEAVPALKWDALSESNOELNFSVKETVCQMAEERSLEECDQFEAGAVMGCTGYFFGESPPVLVLTCKSVGNEEEEQKQEEGNEEEEKEVEKEEKEEDQKDQPKRVKRFKKFFKKLKNVKKRAKKFFKKPKVIGVTFPF (SEQ ID NO: 21) Or a fragment thereof from amino acid residues 166-168 with an initial methionine residue, as represented by MKLK (SEQ ID NO: 22) Or a modified fragment thereof from amino acid residues 172-183 with an optional initial methionine residue, as represented by MKRAKKFFKKLK (SEQ ID NO: 23)
PMAP-36, RI18	NP_001123437.1	METQRASLCLGRWSLWLLLLGLVVPSASQAQALSYREAVLRAVDRLNEQSSEANLYRLLELDQPPKADEDPGTPKPVSFTVKETVCPRPTWRPPELCDFKENGVRVKQCVGTVTNLNSNDPLDINCDEIQSVGRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG (SEQ ID NO: 24) Or a fragment thereof from amino acid residues 137-154, as presented by RKKTRKRLKKIGKVLKWI (SEQ ID NO: 25)
Histone H2A, HIP _{Anat} , HIP _{AB} , HIP _A and HIP _{ABC}	XP_034433694.1	MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYLAHVLEYLTAEILELAGNAARDNKKSRIPRHLQLAVRNDEELNKLGGVTIAQGGVLPNIQAVLLPKKTEKAAKSK (SEQ ID NO: 26) Or HIP_{Anat}, a fragment thereof represented by: MSGRGKTGGKARAKAK (SEQ ID NO: 180) Or a modified fragment thereof HIP_A derived from amino acid residues 3-14, as represented by MKGRGKTGGKARAKWK (SEQ ID NO: 27) Or a modified fragment thereof HIP_{ABC} derived from amino

		acid residues 3-52, as represented by MKGRGKTGGKARAKWKTRSSRAGLQWPVGRVHLLR KGNYAHRVGAGAPVWL (SEQ ID NO: 28) Or a fragment thereof HIP _{AB} represented by: MKGRGKTGGKARAKWKTRSSRAGLQWPVGRVHLLR K (SEQ ID NO: 181)
BK9		RRRLRSRRRFGRRRRVGLRIGYRM (SEQ ID NO: 29)

[00109] Various AMPs are disclosed, for example, in Blower, R. et al. *Snake Cathelicidin NA-CATH and Smaller Helical Antimicrobial Peptides are Effective Against Burkholderia thailandensis*, PLoS Negl Trop Dis. 2015, 9(7): e0003862; Lyu, Y. et al, *Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and Candida*, Sci Rep. 2016, 6:27258; and Bustillo, M. et al., *Modular analysis of hipposin, a histone-derived antimicrobial peptide consisting of membrane translocating and membrane permeabilizing fragments*, Biochim Biophys Acta 2014, 1838(9):2228-2233, all of which are incorporated herein by reference in their entireties.

[00110] In some embodiments, the AMPs are modified to produce variant AMPs. As described herein, AMPs typically comprise a helical domain such as an α -helical domain. Typically, the variant AMPs retain the α -helical domain. The retention of the α -helical domain in any variant AMP is typically accurately identified using various software programs, such as Jpred4 (compio.dundee.ac.uk/jpred), Helical Wheel (hael.net/helical.htm), HeliQuest (zhanglab.ccmb.med.umich.edu/I-TASSER/) and PEP-FOLD 3 (bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3). In some embodiments, the AMP variants are modified by converting (=) charged residues, such as arginine and lysine, within the AMP to a "D" amino acid form. The utility of conversions to the D form is described in the literature, e.g., Manabe *et al.*, *Sci. Rep.*, 2017, pages 1-10, which is herein incorporated by reference in its entirety. Variant AMPs may be prepared according to any method known in the art including as described herein above for the lysins, active fragments thereof and variants thereof.

[00111] In some embodiments, the AMPs for use in the engineered lysins of the present disclosure include a fragment of a larger AMP that retains antibacterial activity. For example, in certain embodiments, the AMP portion of the lysin-AMP polypeptide construct may include a fragment of porcine myeloid antimicrobial peptide-36 ("PMAP-36") (SEQ ID NO: 25) that

retains antibacterial activity. PMAP-36 is a cathelicidin-related AMP deduced from porcine myeloid cDNA with an amphipathic α -helical conformation at the N-terminus. Accordingly, suitable PMAP-36 fragments are typically selected from the N-terminus to obtain fragments retaining antibacterial activity. In some embodiments, the PMAP-36 fragment of the present disclosure includes the hydrophobic amino acid (Trp) at position 23. In other embodiments, the random coil C-terminal is omitted from the PMAP-36 fragment to reduce or eliminate hemolysis that may be caused by PMAP-36. Further features of PMAP-36 fragments are described, for example, in Lyu *et al.*, *Scientific Reports*, 2016, 6, pages 1-12, which is herein incorporated by reference in its entirety.

[00112] Particularly desirable PMAP-36 fragments include RI12 having the amino acid sequence RLKKIGKVLKWK (SEQ ID NO: 30), RI18 having the amino acid sequence RKKTRKRLKKIGKVLKWI (SEQ ID NO: 25), and TI15 having the amino acid sequence TRKRLKKIGKVLKWI (SEQ ID NO: 31).

[00113] In some embodiments, the AMPs of the engineered lysins of the present disclosure include an outer membrane binding motif. A particularly desirable outer membrane binding motif for use with the engineered lysins of the present disclosure includes FIRL (SEQ ID NO: 146). Without being limited by theory, FIRL (SEQ ID NO: 146), which is related to a sequence of a protein involved in outer membrane protein biogenesis, BamD, appears to increase the permeability of the outer membrane to antibiotics.

[00114] In certain embodiments, the outer membrane binding motif is FIQL_{Bc} set forth as SEQ ID NO: 33, FMRL_{Yp} set forth as SEQ ID NO: 34, or FMRL_{Nm} (FRRL) set forth as SEQ ID NO: 35. Additional outer membrane binding motifs that are disclosed herein include FIRL_{Bc}, (SEQ ID NO: 143), derived from the outer membrane protein assembly factor BamD of *Burkholderia cenocepacia* (NCBI Accession No.: WP_226210925.1).

[00115] MIDRFMRL_{Yp}NPTH (SEQ ID NO: 144) and MIERFRRLHPQH (FMRL_{Nm}) (SEQ ID NO: 147) are outer membrane binding motifs derived from the outer membrane protein assembly factor BamD of *Yersinia pestis* or *Neisseria meningitidis* (NCBI Accession No: WP_046596486.1 and NCBI Accession No: WP_148082483.1, respectively). Further information regarding the proposed mechanisms can be found, for example, in Mori, N. et al., *A peptide based on homologous sequence of the β -barrel assembly machinery component BamD*

potentiates antibiotic susceptibility of Pseudomonas aeruginosa, J. Antimicrob. Chemother., 2012, 67: 2173-2181, which is herein incorporated by reference in its entirety.

[00116] Other peptides, which may be incorporated into the engineered lysin polypeptide constructs of the present disclosure includes the cationic peptide KFFKFFKFFK (SEQ ID NO: 36) described in Vaara and Porro, *Group of peptides that act synergistically with hydrophobic antibiotics against gram-negative enteric bacteria*, Antimicrobial Agents Chemother. 1996, 40(8):1801-1805, which is herein incorporated by reference in its entirety.

[00117] In some embodiments, the foregoing additional peptides are resistant to salts and serum inactivation as described, for example, in Monhanram et al., *Salt-resistant short antimicrobial peptides*, Peptide Science 2016, 106(3):345-356, which is herein incorporated by reference in its entirety.

Cell-Penetrating Peptides (CPPs)

[00118] In various embodiments of the engineered lysin polypeptide constructs, fragments thereof, and variants thereof disclosed herein, the construct comprises a cell-penetrating peptide (CPP) in addition to the lysin polypeptide, *e.g.*, an engineered lysin-CPP polypeptide construct, optionally further comprising a linker and/or an outer membrane binding motif, as disclosed herein.

[00119] As used herein, “Cell-penetrating peptides” or “CPPs” refers to small peptides that have the ability to translocate across biological membranes and may be used, for example, to facilitate intracellular protein delivery. Like AMPs, CPPs are also typical short, cationic peptide sequences that have a high affinity for cell membranes and may be either unstructured or alpha-helical.

[00120] In some embodiments, the present CPPs are variant CPPs having at least about 80%, such as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity with any of the CPPs described herein, wherein the variant CPP thereof retains a CPP activity.

[00121] In certain embodiments, the engineered lysin polypeptide constructs, fragments thereof, and variants thereof may comprise both a CPP and an AMP in addition to the lysin polypeptide.

[00122] Suitable CPPs for use in the present engineered lysin-CPP constructs include, for example ZF5.3 and PRR, PRR₃, and PRR₅, as detailed in Table 4 below.

[00123] Table 4 – Exemplary CPPs

CPP	NCBI Accession number or journal article	Amino acid sequence
ZF5.3	KAI4044133.1	MDFTLGDWEQLGLEQGDTFWDTALDNCQDLFLLDPPRP NLTSHPDGSEDLEPLAGGSPEATSPDVTETKNSPLMEDFF EEGFSQEIIEMLSKDGFWNSNFGEACIEDTWLDSLLGDPE SLLRSDIATNGESPTECKSHELKRGLSPVSTVSTGEDSMV HNVSEKTLTPAKSKEYRGEFFSYSDHSQQDSVQEGEKPY QCSECGKSFSGSYRLTQHWITHTREKPTVHQECEQGFR NASLSVYPKTHGTGYKFYVCNEYGTTFSQSTYLWHQKTH TGEKPCKSQSDSHPPSHDTQPGEHQKTHDTSKSYNCNE CGKAFTRIFHLTRHQKIHTRKRYECSKCQATFNLRKHLI QHOKTHAAKTTSCECGKIFRHSSLLIEHQALHAGEEP YKCNERGKSFRHNSTLKIHQRVHSGEKPYKCSECGKAF HRHHTLNEHRIHTGYRPHKCQECVRSFSRPSHLMRHQ AIHTAEKPYSCAECKETFSDNNRLVQHOKMHTVKTPYE CQECGERFICGSTLKCHESVHAREKQGFFVSGKILDQNP EQKEKCFKCNKCEKTFSCSKYLTQHERIHTRGMKPFEC QCGKAFGQSTRLIHHQRIHSRVRLYKWGEQGKAISSASL IKLQSFHTKEHPFKCNECGKTFSSAHLKHLIHA GENP FKCSKCDRVFTQRNYLVQHERTHARKKPLVCNECGKTF RQSSCLSKHQRIHSGEKPYVCDYCGKAFGLSAELVRHQR IHTGEKPYVCQECGKAFTQSSCLSIHRRVHTGEKPYRCG ECGKAFAQKANLTQHQRHTGEKPYSCNVCGKAFVLSA HLNQHLRVHTQETLYQCQRCQKAFRCHSSLSRHQRVHN KQQYCL (SEQ ID NO: 37) Or a modified fragment thereof derived from amino acid residues at about 802-828, as represented by the amino acid sequence MYSCNVCGKAFVLSRHLNRHLRVHRRAT (SEQ ID NO: 38)
PRR	Daniels et al. <i>J. Am. Chem. Soc.</i> 2007, 129, 47, 14578–14579	GPRRPRRPGRAPV (SEQ ID NO: 39)
PRR ₅	DAS01177.1	PRRPRRGRRRPHRGRL (SEQ ID NO: 40)
PRR ₃	MBI0245460.1	RKPRQSPRPQQVRPPRPQVEENQPRPVPV (SEQ ID NO: 48)

[00124] ZF5.3 is described, for example, in Wissner, R. et al., *Fluorescence Correlation Spectroscopy Reveals Efficient Cytosolic Delivery of Protein Cargo by Cell-Permanent Miniature Proteins*, ACS Cent. Sci. 2018 4(10):1379-1393, incorporated by reference herein. The PPR residue represented by GPRRPRRPGRRAPV (SEQ ID NO: 39) is described, for example, in Daniels and Scepartz, *Intrinsically cell-permeable miniature proteins based on a minimal cationic PPII motif*, J. Am. Chem. Soc. 2007, 129:14578-14579, which is incorporated by reference herein.

Linkers and Structure Stabilizing Components

[00125] In some embodiments, the engineered lysin polypeptide construct disclosed herein further comprise at least one linker. In some embodiments, the engineered lysin polypeptide constructs of the present disclosure further comprise at least one structure stabilizing component to maintain at least a portion of the structure of the first and/or second component in the construct, e.g., the lysin and/or AMP and/or CPP, substantially the same as in the unconjugated lysin and/or AMP or CPP. In some embodiments, the stabilizing structure is also a linker. Typically, the at least one structure stabilizing component, such as a linker, enables the lysin and AMP or CPP to substantially preserve the three-dimensional structure of the first and/or second protein moieties, such that at least one biological activity of the lysin and/or AMP or CPP is retained.

[00126] Suitable linkers and structure stabilizing components are disclosed, for example, in PCT Publication Nos. WO 2019/191633 and 2020/046747, both of which are incorporated by referenced herein.

[00127] Suitable linkers for connecting two polypeptides are known in the art. In certain embodiments, the linker is a peptide, such as a peptide comprising glycine and serine residues. Specific suitable linkers include, but are not limited to, a TAGGTAGG linker (SEQ ID NO: 41), an IGEM linker GGSGSGSGSGSP (BBa_K1485002) (SEQ ID NO: 42). GGGSGGGGSGGGS (BBA_K1486037) (SEQ ID NO: 43), or a linker as described in Briers *et al.*, *mBio*, 2014, 5:e01379-14, which is herein incorporated by reference in its entirety, *i.e.*, AGAGAGAGAGAGAGAS (SEQ ID NO: 44).

[00128] In certain embodiments disclosed herein, the linker is a glycine (G) amino acid, and in certain embodiments, the linker is a glycine optionally with a threonine residue having the sequence GT or TG. In certain embodiments, the linker is GT. In certain embodiments, the linker

has the amino acid sequence LSKLGG (SEQ ID NO: 45). In certain embodiments, the linker has the amino acid sequence LSKLGGLGGGPRRLGG (SEQ ID NO: 46). In certain embodiments, the linker has the amino acid sequence LGGLGG (SEQ ID NO: 47). In certain embodiments, the linker has the amino acid sequence SKY. In certain embodiments, the linker has the amino acid sequence LGGKSLG (SEQ ID NO: 174).

[00129] In some embodiments, the structure stabilizing component is a peptide moiety, e.g., an RPP or PP moiety. Such peptide moieties may be included in the present engineered lysin polypeptide constructs to assist in maintaining the structure of the lysin and/or AMP and/or the CPP protein moieties. For example, the structure stabilizing component may be inserted at the C-terminus or N-terminus of a linker.

[00130] In other embodiments, the engineered lysins of the disclosure may further include additional amino acid extensions, such as MG or LKWI (SEQ ID NO: 127).

Examples of Engineered Lysin Polypeptide Constructs

[00131] In some embodiments, the engineered lysin polypeptide construct comprises a base lysin polypeptide or fragment or variant thereof and at least one AMP or CPP or fragment or variant thereof. In certain embodiments the engineered lysin polypeptide construct comprises a lysin polypeptide or fragment or variant thereof and at least one AMP or CMP or fragment or variant thereof and further comprises at least one linker and optionally at least one outer membrane binding motif structure and at least one structure stabilizing component, such as at least two structure stabilizing components. It is understood that the at least one AMP or CPP or fragment or variant thereof can be at the N-terminus or the C-terminus of the lysin polypeptide or fragment or variant thereof. It is further understood that the optional structure stabilizing component and outer membrane binding motif can be at the N-terminus or the C-terminus of the engineered lysin polypeptide construct.

[00132] Table 5, below, depicts specific examples of the engineered lysin polypeptide constructs described herein. For each of the constructs, double underlines correspond to a lysin. Structure stabilizing components, outer membrane binding motifs, Other Amino Acid Extensions, and linkers are italicized. AMPs and CPPs are indicated in bold.

[00133] Table 5 – Exemplary Engineered Lysins

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN693	GN685+BK5	SEQ ID NO: 80	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>HFQLPYGVYKMSKGKGARPGRPQPLRGT</u> KGKRKGARLWYVGGQQF (SEQ ID NO: 55)
GN694	GN685+BCM1	SEQ ID NO: 81	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>HFQLPYGVYKMPKTHSEFRHRPFLRALTP</u> EQRRRWREMRRARGMKPLYNSRIRGR (SEQ ID NO: 56)
GN751	FIRL _{BC} (SEQ ID NO: 143)+BK3+GN3 (R100Q, R116H)	SEQ ID NO: 82	<u>MVDRFIQLHPDHMYRKPVNKRRSARTFRR</u> <u>NAKRTKSANLQKAPQRGGWRLRTSQRGL</u> <u>SLIKSFEGLRLQAYQDSVGVWTIGYGTTRG</u> <u>VKAGMKISKDQAERMMLNDVORFEPEVER</u> <u>LIKVPLNQDQWDALMSFTYNLGAANLESST</u> <u>LROLLNAGNYAAAAEOFPHWNKAGGOVL</u> <u>AGLTRRRAAERELFLGAA</u> (SEQ ID NO: 57)
GN757	FIQL _{BC} (SEQ ID NO: 173)+G+BK3+GN428	SEQ ID NO: 83	<u>MVDRFIQLHPDHGMYRKPVNKRRSARTFR</u> <u>RNAKRTKSANLQKAPQRGGWRLAILKLG</u> <u>NRGSEVKALQOSLNKIGFSLTADGIFGKATE</u> <u>NAVKSVOAGAGLVIDGIAGPKTFYAIRNAG</u> <u>DAHQEHLTEADLVDAARELGVELASMKAV</u> <u>NQVESRGTGFTKTGKIKTFLFERHIMYKKVT</u> <u>AKFGQARANALYQLYPTLVNPNSSGGYIGG</u> <u>DAELERLQGAIALDEDCAYESASYGLFQIM</u> <u>GFNCQICGYSNAKEMFTDFLTGERAHL</u> <u>LAFVKFIKADANMWKALKKNWAEFARRYNG</u> <u>PAYAKNOYDTKLAAAYKSEC</u> (SEQ ID NO: 58)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1040	ZF5.3+LSKLG G (SEQ ID NO: 45)+PRR+GN4 28	SEQ ID NO: 84	MYSCNVC GKAFVLSRHLNRHLRVHRRAT <u>LSKLGGGPRRPRRPGRRAPVAILKLGNRGS</u> <u>EVKALQOQSLNKIGFSLTADGIFGKATENAV</u> <u>KSVQAGAGLVIDGIAGPKTFYAIRNAGDAH</u> <u>QEHLTEADLVDAARELGVELASMKAVNOV</u> <u>ESRGTGFTKTGKIKTLFERHIMYKKVTAKE</u> <u>GOARANALYQLYPTLVNPNSSGGYIGGDAEL</u> <u>ERLOGAIALDEDCAYESASYGLFOIMGFNC</u> <u>QICGYSNAKEMFTDELTGERAHLALAFVKFIK</u> <u>ADANMWKALKKNKNWAEFARRYNGPAYA</u> <u>KNQYDTKLAAAYKSEC</u> (SEQ ID NO: 59)
GN1041	ZF5.3+LSKLG G (SEQ ID NO: 45)+PRR+GN5 88	SEQ ID NO: 85	MYSCNVC GKAFVLSRHLNRHLRVHRRAT <u>LSKLGGGPRRPRRPGRRAPVQLTPHFTLEE</u> <u>FTRSDTARARQIDNSPSPAAAANLRLAQTL</u> <u>ERARVLLGGKPMQITSGYRSPALNRAVGGV</u> <u>PMSAHAQGLAADFVCSGYGPPLDVCRVLA</u> <u>ASDLPFDQVINEFGRWVHIGLAADGVTPRR</u> <u>QVLTARKVGGRTTYVAGLQA</u> (SEQ ID NO: 60)
GN1072	FMRL _{Yp} (SEQ ID NO: 144) +BK3+GN515 (R100D, R116H)	SEQ ID NO: 86	MIDRFMRLNPTHMYRK PVNKRRSARTFRR NAKRTKSANLQKAPQ RGGWRLRTSQRGI <u>DLIKSFEGLRLSAYQDSVGVWTIGYGTTTG</u> <u>VTRYMTITVEQAERMLSNLQRFEPELDRL</u> <u>VKVPLNQNOWDALMSFVYNLGAANLESST</u> <u>LRDLLNAGDYAGAAEQFLHWNKAGGKVL</u> <u>PGLVRRRASERELFLGAA</u> (SEQ ID NO: 61)
GN1074	FMRL _{Nm} (SEQ ID NO: 147)+BK3+GN 515 (R100D, R116H)	SEQ ID NO: 87	MIERFRLHPQHMYRK PVNKRRSARTFRR NAKRTKSANLQKAPQ RGGWRLRTSQRGI <u>DLIKSFEGLRLSAYQDSVGVWTIGYGTTTG</u> <u>VTRYMTITVEQAERMLSNLQRFEPELDRL</u> <u>VKVPLNQNOWDALMSFVYNLGAANLESST</u> <u>LRDLLNAGDYAGAAEQFLHWNKAGGKVL</u> <u>PGLVRRRASERELFLGAA</u> (SEQ ID NO: 62)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1119	HIP _{ABC} +GT(Kpn1)+GN3 (R100Q, R116H)	SEQ ID NO: 88	MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNIAHRVGAGAPVWLGT <u>RTSQRGLSLIKSFEGLRLQAYODSVGVWTI</u> <u>GYGTTRGVKAGMKISKDQAERMMLNDVQR</u> <u>FEPEVERLIKVPLNQDQWDALMSFTYNLGA</u> <u>ANLESSTLRQLLNAGNYAAAAEQFPHWNK</u> <u>AGGOVLAGLTRRRAAEREFLGAA</u> (SEQ ID NO: 63)
GN1125	HIP _A +BK3+GT (Kpn1)+GN3 (R100Q, R116H)	SEQ ID NO: 89	MKGRGKTGGKARAKWKMYRKPVNKRR SARTFRRNAKRTKSANLQKAPQRRGWR <u>LGTRTSQRGLSLIKSFEGLRLQAYODSVGV</u> <u>WTIGYGTTRGVKAGMKISKDQAERMMLND</u> <u>VORFEPEVERLIKVPLNQDQWDALMSFTYN</u> <u>LGAANLESSTLRQLLNAGNYAAAAEQFPH</u> <u>WNKAGGOVLAGLTRRRAAEREFLGAA</u> (SEQ ID NO: 64)
GN737	ATRA+1AΔaa2-9+GN37	SEQ ID NO: 90	MKLKTYTLSKRSLDNLKGVHPDLVAVVHR <u>AIOLTPVDFAVIEGLRSVSRQKELVAAGASK</u> <u>TMNSRHLTGHAVDLAAYVNGIRWDWPLY</u> <u>DAIAVAVKAAAKELGVAIVWGGDWTTFKD</u> <u>GPHELDRSKYR</u> (SEQ ID NO: 65)
GN808	GN685+LSKL GG (SEQ ID NO: 45)+RI18	SEQ ID NO: 91	MGFKLGSRSLQRLQGVHPDLVKVVKRAIEI <u>SPVDFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYP</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>HFQLPYGVYKLSKLGGRRKKTRKRLKKIGK</u> VLKWI (SEQ ID NO: 66)
GN848	GN569+LSKL GGLGGGPRR LGG (SEQ ID NO: 46)+RI18	SEQ ID NO: 92	MPVSGFKFSRRSEVNLIGVKPSLVSLARRAL <u>ELSTVDFGVTEGLRTKARQRELVAAKKSQT</u> <u>MNSRHLTGDAVDVIAAYVRGAGTWEWHYY</u> <u>EDIARAFKQAAAELGIPIVWGGDWKTLKDG</u> <u>VHFELARLSKLGGGLGGGPRRLGGRRKKTRKR</u> LKKIGKVLKWI (SEQ ID NO: 67)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN887	FMRL _{Yp} (SEQ ID NO: 146)+PRR+QPI 14001	SEQ ID NO: 93	<u>MIDRFMRLNP</u> <u>THGPRRPRRP</u> <u>GRRAPVTIFD</u> <u>MLRIDEGLKLTVYKDTEGFWTVGIGHLLTK</u> <u>DSSKAVA</u> <u>IKELDRLTGRSTDGSISVGEAENIF</u> <u>KLDVSKAENG</u> <u>IKGNSVLAPVYESLDSVRKM</u> <u>AMVNMVFQ</u> <u>LG</u> <u>VAGVAGFKNSLALLKAKD</u> <u>WDKAAVNLAQSKWYNQTPNRAKRVISVFK</u> <u>TGTMDAYK</u> (SEQ ID NO: 68)
GN907	GN685+LGGL GG (SEQ ID NO: 47)+ZF5.3	SEQ ID NO: 94	<u>MGFKLGS</u> <u>RS</u> <u>LORLOGVHPDLVKVVKRAIEI</u> <u>SPVDEFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHA</u> <u>VDISPLVDGKVS</u> <u>WDWKYYYP</u> <u>ADAMKOA</u> <u>AKELNIPVEWGGDWKTEKDG</u> <u>PFQ</u> <u>LPYGVYK</u> <u>LGGLGGM</u> <u>YSCNVC</u> <u>GKAFV</u> <u>LSRHLNRHLRVHRRAT</u> (SEQ ID NO: 69)
GN918	FMRL _{Yp} (SEQ ID NO: 146)+PRR+GN 917	SEQ ID NO: 95	<u>MIDRFMRLNP</u> <u>THGPRRPRRP</u> <u>GRRAPVN</u> <u>LFE</u> <u>MLRYDEGLKLE</u> <u>VYFDTEGYPTVG</u> <u>VGHLLT</u> <u>KVHNLM</u> <u>SGISILDKELGRSTQGRITTEDAEY</u> <u>LFSKDVEKAKAGIKSNTVLNDLYLSLDPIRQ</u> <u>MALVNMVFQ</u> <u>MGVAGVAGFNNSIKLLKAK</u> <u>QWDQAALNLAQSKWFKQTPNRAKRVINVE</u> <u>KTGTMDAYK</u> (SEQ ID NO: 70)
GN937	FIRL (SEQ ID NO: 148)+PRR+CA H0532154	SEQ ID NO: 96	<u>MIDRFIRLNP</u> <u>THGPRRPRRP</u> <u>GRRAPV</u> <u>POST</u> <u>KQITPDDYARAAAALGVPVAAVKAVTDVE</u> <u>SNGHGFLSDGRPLILYERHIMRRQLIAAGRA</u> <u>ADADSHEA</u> <u>SEPNI</u> <u>VNRAPGGYKGGAAEWD</u> <u>RLDA</u> <u>AVHIDRACALESASWGLFQIMGFHW</u> <u>KSLGYESVQAFVNAMYASEGAQLDAFVAE</u> <u>VKANPNLLRALRAKDWPGEARGYNGPGYA</u> <u>ANKYDTKLAAAYARHIKDAA</u> (SEQ ID NO: 71)
GN585	GN577+SKY+ RI18	SEQ ID NO: 97	<u>MKNFRFSRRSERNL</u> <u>VG</u> <u>VKPD</u> <u>LIKLARRALE</u> <u>LSEVDFGVTEGLRTKDRQRQLVAEGKSRTA</u> <u>NSRHLTGDAIDVVAYLGAAVTWEWKYYE</u> <u>QIAVAFKRASAE</u> <u>LGPVEWGGDWKSLKDGP</u> <u>HFQLPW</u> <u>SKYRKKTRKRLKKIGKVLKWI</u> (SEQ ID NO: 72)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN686	GN685+RI18	SEQ ID NO: 98	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYP</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>HFQLPYGVYKRKKTRKRLKKIGKVLKWI</u> (SEQ ID NO: 73)
GN727	ATRA-1A+GN37+A ₋ ATRA-A1	SEQ ID NO: 99	<u>MKRAKKFFKKLKYTL</u> <u>SKRSLDNLKGVH</u> <u>PDLVAVVHRAIQLTPVDFAVIEGLRSVSROK</u> <u>ELVAAGASKTMNSRHLTGHAVDLAAYVNG</u> <u>IRWDWPLYDAIAVAVKAAAKELGVAIVWG</u> <u>GDWTFKDGPHFELDRSKYRAKRAKKFFK</u> <u>KLK</u> (SEQ ID NO: 74)
GN758	FIRL _{Bc} (SEQ ID NO: 143)+G+BK9+GN428	SEQ ID NO: 100	<u>MVDRFIQLHPDHGRFRRLRSRRRFGRRR</u> <u>RVGLRIGYRMAILKLGNRGSEVKALOQSL</u> <u>NKIGFSLTADGIFGKATENAVKSVQAGAGL</u> <u>VIDGIAGPKTFYAIRNAGDAHQEHLTEADL</u> <u>VDAARELGVELASMKAVNQVESRGTGFTK</u> <u>TGKIKTLEFERHIMYKKVTAKFGOARANALY</u> <u>QLYPTLVNPNSSGGYIGGDAELERLQGAIAL</u> <u>DEDCAYESASYGLFOIMGFNCOICGYSNAK</u> <u>EMFTDFLTGERAHL LAFVKFIKADANMWK</u> <u>ALKNKNWAEFARRYNGPAYAKNOYDTKL</u> <u>AAAYKSFC</u> (SEQ ID NO: 75)
GN764	GN569+RI18	SEQ ID NO: 101	<u>MPVSGFKFSRRSEVN</u> <u>LIGVKPSLVSLARRAL</u> <u>ELSTVDFGVTEGLRTKARQRELVAACKSQT</u> <u>MNSRHLTGDAVDVIAYYVRGAGTWEWHYY</u> <u>EDIARAFKQAAAELGIPIVWGGDWKTLKDG</u> <u>VHFELARRKKTRKRLKKIGKVLKWI</u> (SEQ ID NO: 76)
GN765	GN685+RI18	SEQ ID NO: 102	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGHVSWDWKYYYP</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>HFQLPYGVYKRKKTRKRLKKIGKVLKWI</u> (SEQ ID NO: 77)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN873	FMRL _{Yp} (SEQ ID NO: 146)+LSKLGG (SEQ ID NO: 45)+PRR ₅ +GN 867	SEQ ID NO: 103	<u>MIDRFMRLNPTHLSKLGGPRRPRRGRRRP</u> <u>HRGRLNLFEMRLRYDEGLKLEVYFDTEGYPTVGVGHLLTKVHNLMSGISILDKELGLSTQ</u> <u>GRITTEDAEYLFSKDVEKAKAGIKSNTVLN</u> <u>DLYLSLDPIROMALVNMVFQMGVAGVAGF</u> <u>NNSIKLLKAKQWDQAALNLAQSKWFKQTP</u> <u>NRAKRVINVEKTGTMGAYK</u> (SEQ ID NO: 78)
GN916	ZF5.3+LSKLGG (SEQ ID NO: 45)+PRR+GN8 67	SEQ ID NO: 104	<u>MYSCNVC GKAFVLSRHLNRHLRVHRRAT</u> <u>LSKLGGGPRRPRRPGRRAPVNLFEMRLRYD</u> <u>EGLKLEVYFDTEGYPTVGVGHLLTKVHNL</u> <u>MSGISILDKELGLSTQGRITTEDAEYLFSKD</u> <u>VEKAKAGIKSNTVLNDLYLSLDPIROMALV</u> <u>NMVFQMGVAGVAGFNNSIKLLKAKQWDQ</u> <u>AALNLAQSKWFKQTPNRAKRVINVEKTGT</u> <u>MGAYK</u> (SEQ ID NO: 79)
GN687	GN685 + (GGGGS) ₃ (SEQ ID NO: 43)+PRR+LKWI (SEQ ID NO: 127)	SEQ ID NO: 128	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLRLERQKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>PFQLPYGVYKGGGGSGGGGSGGGGSGPRR</u> <u>PRRPGRRAPVLKWI</u> (SEQ ID NO: 108)
GN688	FIRL _{Bc} (SEQ ID NO: 143)+PRR + GN685	SEQ ID NO: 129	<u>MVDRFIQLHPDHGPRRPRRPGRRAPVGFK</u> <u>LGSRSLQRLOGVHPDLVKVVKRAIEISPVDF</u> <u>TVTEGLRTLRLERQKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>PFQLPYGVYK</u> (SEQ ID NO: 109)
GN689	GN685 + AM1	SEQ ID NO: 130	<u>MGFKLGSRSLQRLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLRLERQKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>PFQLPYGVYKMRLKMARRRYRLPRRRSR</u> <u>RLFSRTALRMHPRNRLRRIMRGGIRF</u> (SEQ ID NO: 110)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN690	[MG] + AM1 + GN685	SEQ ID NO: 131	<u>MGMRLKMARRRYRLPRRRSRRLFSRTAL</u> <u>RMHPRNRLRRIMRGGIRFGFKLGSRSLOR</u> <u>LOGVHPDLVKVVKRAIEISPVDFTVTEGLRT</u> <u>LEROKELFAKGASKTMRSRHLTGHAVDISP</u> <u>LVDGKVSWDWKYYYPMADAMKQAAKEL</u> <u>NIPVEWGGDWKTFKDGPHFQLPYGVYK</u> (SEQ ID NO: 111)
GN692	GN685 + AM13	SEQ ID NO: 132	<u>MGFKLGSRSLORLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLRLEROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDGPHFQLPYGVYK</u> <u>MRRRVRRTRRRHQWRLTH</u> <u>SARSIRRARIMPSRPRGGRRF</u> (SEQ ID NO: 112)
GN695	GN685 + MMAV-2 (for this construct, MMAV codon optional)	SEQ ID NO: 133	<u>MGFKLGSRSLORLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLRLEROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDGPHFQLPYGVYK</u> <u>MRRRVRRTRRRHQWRLTH</u> <u>SARSIRRARIMPSRPRGGRRF</u> (SEQ ID NO: 113)
GN696	GN685 + MFV1-3	SEQ ID NO: 134	<u>MGFKLGSRSLORLOGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLRLEROKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDGPHFQLPYGVYK</u> <u>MFSLLKIKKRSFPFYCKRT</u> <u>TANLLYIKK</u> (SEQ ID NO: 114)
GN697	[MG] + MFV1-3 + GN685	SEQ ID NO: 135	<u>MGMFSLKIKKRSFPFYCKRTTANLLYIK</u> <u>KMGFKLGSRSLORLOGVHPDLVKVVKRAI</u> <u>EISPVDFTVTEGLRTLRLEROKELFAKGASKTM</u> <u>RSRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>MADAMKQAAKELNIPVEWGGDWKTFKDGPHFQLPYGVYK</u> (SEQ ID NO: 115)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN869	FMRL _{Yp} (SEQ ID NO: 144)+ PRR ₃ + GN867	SEQ ID NO: 136	<u>MIDRFMRLNP</u> THRKPRQSPRPQQVRPPRP QVEENQPRPVPV <u>NLFEM</u> <u>RLRYDEGLKLEVY</u> <u>FDTEGYPTVG</u> <u>VGHLLTKVHNLMSGISILDK</u> <u>ELGLSTQGRITTEDAEYLFSKDVEKAKAGIK</u> <u>SNTVLNDLYLSLDPIRQ</u> <u>MALVNMVFQMGV</u> <u>AGVAGFNNSIKLLKAKQWDQAALNLAQSK</u> <u>WFKQTPNRAKRVINVFKTGTMGAYK</u> (SEQ ID NO: 116)
GN870	FMRL _{Yp} (SEQ ID NO: 144)+ PRR ₃ + LGGKSLG (SEQ ID NO: 174)+ GN867	SEQ ID NO: 137	<u>MIDRFMRLNP</u> THRKPRQSPRPQQVRPPRP QVEENQPRPVPV <u>LGGKSLGNLFEM</u> <u>RLRYDE</u> <u>GLKLEVYFDTEGYPTVG</u> <u>VGHLLTKVHNLMS</u> <u>SGISILDKELGLSTQGRITTEDAEYLFSKDVE</u> <u>KAKAGIKSNTVLNDLYLSLDPIRQ</u> <u>MALVNM</u> <u>VFQMGVAGVAGFNNSIKLLKAKQWDQAA</u> <u>LNLAQSKWFKQTPNRAKRVINVFKTGTMG</u> <u>AYK</u> (SEQ ID NO: 117)
GN871	FMRL _{Yp} (SEQ ID NO: 144)+ LGGKSLG (SEQ ID NO: 174)+ PRR ₃ + GN867	SEQ ID NO: 138	<u>MIDRFMRLNP</u> THLGGKSLGRKPRQSPRPQQ VRPPRPQVEENQPRPVPV <u>NLFEM</u> <u>RLRYDEG</u> <u>LKLEVYFDTEGYPTVG</u> <u>VGHLLTKVHNLMS</u> <u>GISILDKELGLSTQGRITTEDAEYLFSKDVEK</u> <u>AKAGIKSNTVLNDLYLSLDPIRQ</u> <u>MALVNMV</u> <u>FQMGVAGVAGFNNSIKLLKAKQWDQAAL</u> <u>NLAQSKWFKQTPNRAKRVINVFKTGTMG</u> <u>AYK</u> (SEQ ID NO: 118)
GN872	FIRL _{Bc} (SEQ ID NO: 143)+ LSKLGG (SEQ ID NO: 45)+ PRR ₃ + GN867	SEQ ID NO: 139	<u>MVDRFIQLHPDHLSKLGG</u> RKPRQSPRPQQV RPPRPQVEENQPRPVPV <u>NLFEM</u> <u>RLRYDEGL</u> <u>KLEVYFDTEGYPTVG</u> <u>VGHLLTKVHNLMSGI</u> <u>SILDKELGLSTQGRITTEDAEYLFSKDVEKA</u> <u>KAGIKSNTVLNDLYLSLDPIRQ</u> <u>MALVNMVF</u> <u>QMGVAGVAGFNNSIKLLKAKQWDQAALN</u> <u>LAQSKWFKQTPNRAKRVINVFKTGTMGAY</u> <u>K</u> (SEQ ID NO: 119)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN874	FMRL _{Yp} (SEQ ID NO: 144)+ PRR ₅ + GN867	SEQ ID NO: 140	<u>MIDRFMRLNP</u> TH <u>PRRPRRGRRRRPHRGRL</u> <u>NLFEMRLRYDEGLKLEVYFDTEGYPTVGVG</u> <u>HLLTKVHNLM</u> SGISILDKELGLSTQGRITTE <u>DAEYLFSKDVEKAKAGIKSNTVLNDLYLSL</u> <u>DPIRQMALVNMVFQMGVAGVAGFNNSIKL</u> <u>LKAKQWDQAALNLAQSKWFKQTPNRAKR</u> <u>VINVFKTGTMGAYK</u> (SEQ ID NO: 120)
GN875	FIRL _{Bc} (SEQ ID NO: 143)+ LSKLGG (SEQ ID NO: 45)+ PRR ₅ + GN867	SEQ ID NO: 141	<u>MVDRFIQLHPDHLSKLGG</u> PRRPRRGRRRRP HRGRL <u>NLFEMRLRYDEGLKLEVYFDTEGYPT</u> <u>TVGVGHLLTKVHNLM</u> SGISILDKELGLSTQ <u>GRITTEDAEYLFSKDVEKAKAGIKSNTVLN</u> <u>DLYLSLDPIRQMALVNMVFQMGVAGVAGF</u> <u>NNSIKLLKAKQWDQAALNLAQSKWFKQTP</u> <u>NRAKRVINVFKTGTMGAYK</u> (SEQ ID NO: 121)
GN905	FIRL _{Bc} (SEQ ID NO: 143)+ LSKLGG (SEQ ID NO: 45)+ Zf5.3 + GN867	SEQ ID NO: 142	<u>MVDRFIQLHPDHLSKLGG</u> MYSCNVC GKAF VLSRHLNRHLRVHRRAT <u>NLFEMRLRYDEG</u> <u>LKLEVYFDTEGYPTVGVG</u> HLLTKVHNLM S <u>GISILDKELGLSTQGRITTEDAEYLFSKDVEK</u> <u>AKAGIKSNTVLNDLYLSLDPIRQMALVNMV</u> <u>FQMGVAGVAGFNNSIKLLKAKQWDQAAL</u> <u>NLAQSKWFKQTPNRAKRVINVFKTGTMG</u> <u>AYK</u> (SEQ ID NO: 122)
GN1120	HIP _{AB} + GT + GN3 (R100Q, R116H)		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLRLRK <u>G7RTSORGLSLIKSFEGLR</u> <u>LQAYQDSVGVWTIGYGTTRGVKAGMKISK</u> <u>DQAERMLLNDVQRFEP</u> EVERLIKVPLNQDQ <u>WDALMSFTYNLGAANLESSTLRQLLNAGN</u> <u>YAAAAEQPHWNKAGGOVL</u> AGLTRRRAA <u>ERELFLGAA</u> (SEQ ID NO: 149)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1127	HIP _{ABC} + GT + GN428		<u>MKGRGKTGGKARAKWKTRSSRAGLQW</u> <u>PVGRVHRLLRKGNIAHRVGAGAPVWL</u> <u>GTAILKLGNRGSEVKALQOSLNKIGFSLTADG</u> <u>IFGKATENAVKSVOAGAGLVIDGIAGPKTF</u> <u>YAIRNAGDAHQEHLEADLVDAARELGVE</u> <u>LASMKA VNQVESRG TGFTKTGKI KTLFERH</u> <u>IMYKKVTAKFGQARANALYQLYPTLVNPN</u> <u>SGGYIGGDAELERLQGAIALDEDCAYESAS</u> <u>YGLFQIMGFNCQICGYSNAKEMFTDFLTGE</u> <u>RAHLLAFVKFIKADANMWKALKNKNWAE</u> <u>FARRYNGPAYAKNOYDTKLAAAYKSFC</u> (SEQ ID NO: 150)
GN1071	FIQL(Bc) (SEQ ID NO: 173)+ BK3 + GN515(R100D_R116H)		<u>MVDRFIQLHPDHMYRKPVNKRRSARTFRR</u> <u>NAKRTKSANLQKAPQRGGWRLRTSQRGI</u> <u>DLIKSFEGLRLSAYQDSVGVWTIGYGTTRG</u> <u>VTRYMTITVEQAERML SNDLQRFEPELDRL</u> <u>VKVPLNQNDALMSFVYNLGAANLESST</u> <u>LRDLLNAGDYAGAAEQFLHWNKAGGKVL</u> <u>PGLVRRRASERELFLGAA</u> (SEQ ID NO: 151)
GN1077	FIQL(Bc) (SEQ ID NO: 173)+ BK3 _{H2} + GN3(R100Q_R116H)		<u>MVDRFIQLHPDHMYRKPVNKRRSARSFRK</u> <u>NARRTKSANMQKSTTTWRLRTSQRGLSLI</u> <u>KSFEGLRLQAYQDSVGVWTIGYGTTRGVK</u> <u>AGMKISKDQAERM L LNDVQRFEP EVERLIK</u> <u>VPLNQDQWDALMSFTYNLGAANLESSTLR</u> <u>QLLNAGNYAAAAEQPHWNKAGGQVLAG</u> <u>LTRRRAAERELFLGAA</u> (SEQ ID NO: 152)
GN1078	FIQL(Bc) (SEQ ID NO: 173)+ BK3 _{H3} + GN3(R100Q_R116H)		<u>MVDRFIQLHPDHMKILHRKYMSKKAHSK</u> <u>TFRKHGRKTKSPNTHSSPQRGGWRLRTS</u> <u>QRGLSLIKSFEGLRLQAYQDSVGVWTIGYG</u> <u>TTRGVKAGMKISKDQAERM L LNDVQRFEP</u> <u>EVERLIKVPLNQDQWDALMSFTYNLGAAN</u> <u>LESSTLRQLLNAGNYAAAAEQPHWNKAG</u> <u>GQVLAGLTRRRAAERELFLGAA</u> (SEQ ID NO: 153)
GN1117	GN685 + GT(KpnI) + HIP _{ABC}		<u>MGFKLGSRSLQRLQGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLERQKELFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYPM</u> <u>ADAMKQAAKELNIPVEWGGDWKTEKDG</u> <u>HFOLPYGVYKGT</u> <u>KGRGKTGGKARAKWK</u> <u>TRSSRAGLQWPVGRVHRLLRKGNIAHR</u> <u>VGAGAPVWL</u> (SEQ ID NO: 154)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1118	GN685 + GT(KpnI) + LSKLGG (SEQ ID NO: 45)+ HIP _{ABC}		<u>MGFKLGSRSLQRLQGVHPDLVKVVKRAIEI</u> <u>SPVDFTVTEGLRTLEROKELEFAKGASKTMR</u> <u>SRHLTGHAVDISPLVDGKVSWDWKYYYP</u> <u>ADAMKQAAKELNIPVEWGGDWKTFKDG</u> <u>PFQLPYGVYK</u> <u>GTLSKLGGKGRGKTGGKAR</u> <u>AKWKTRSSRAGLQWPVGRVHRLLRKGN</u> <u>YAHRVGAGAPVWL</u> (SEQ ID NO: 155)
GN1236	HipAnat + BK3 + GT(KpnI) + GN3(R100Q_R 116H)		<u>MSGRGKTGGKARAKAKMYRKPVNKR</u> <u>ARTFRNAKRTKSANLQKAPQRGGWRL</u> <u>GRTSQRGLSLIKSFEGLRLQAYQDSVG</u> <u>WTIGYTTRGVKAGMKISKDQAERM</u> <u>LLNDVORFEPEVERLIKVPLNQDQWDAL</u> <u>MSFTYNLGAANLESSTLRQLLNAGNY</u> <u>AAAAEQPHWNKAGGOVL</u> <u>AGLTRRRRAAERELFLGAA</u> (SEQ ID NO: 156)
GN1238	HipA + BK3h2 + GT(KpnI) + GN3(R100Q_R 116H)		<u>MKGRGKTGGKARAKWKMYRKPVNKR</u> <u>RARSFRKNARRTKSANMQKSTTTWRL</u> <u>GTRTSQRGLSLIKSFEGLRLQAYQDS</u> <u>VGWWTIGYTTRGVKAGMKISKDQAER</u> <u>MLLNDVORFEPEVERLIKVPLNQDQWDAL</u> <u>MSFTYNLGAANLESSTLRQLLNAGNY</u> <u>AAAAEQPHWNKAGGOVL</u> <u>AGLTRRRRAAERELFLGAA</u> (SEQ ID NO: 157)
GN1239	HipA + BK3h3 + GT(KpnI) + GN3(R100Q_R 116H)		<u>MKGRGKTGGKARAKWKMKILHRKYMS</u> <u>KKAHSKTFRKHGRKTKSPNTHSSPQR</u> <u>GGWRLGTRTSQRGLSLIKSFEGLRLQAYQDS</u> <u>VGWWTIGYTTRGVKAGMKISKDQAER</u> <u>MLLNDVORFEPEVERLIKVPLNQDQWDAL</u> <u>MSFTYNLGAANLESSTLRQLLNAGNY</u> <u>AAAAEQPHWNKAGGOVL</u> <u>AGLTRRRRAAERELFLGAA</u> (SEQ ID NO: 158)
GN1250	FIQL(Bc) (SEQ ID NO: 173)+ BK3 + GN3(R100Q_R 116Q)		<u>MVDRFIQLHPDHMYRKPVNKR</u> <u>RSARTFRNAKRTKSANLQKAPQRGGWRL</u> <u>RTSQRGLSLIKSFEGLRLQAYQDSVG</u> <u>WWTIGYTTRGVKAGMKISKDQAER</u> <u>MLLNDVORFEPEVERLIKVPLNQDQWDAL</u> <u>MSFTYNLGAANLESSTLRQLLNAGNY</u> <u>AAAAEQPHWNKAGGOVL</u> <u>AGLTRRRRAAERELFLGAA</u> (SEQ ID NO: 159)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1251	HIP _{ABC} + GT(KpnI) + GN3(R100Q_R 116Q)		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNYAHRVGAGAPVWLG <u>7RTSQRGLSLIKSFEGLRLQAYQDSVGVWTI</u> <u>GYGTTRGVKAGMKISKDQAERMMLNDVQR</u> <u>FEPEVERLIKVPLNQDQWDALMSFTYNLGA</u> <u>ANLESSTLROLLNAGNYAAAAEQFPOWNK</u> <u>AGGOVLAGLTRRRAAEREFLGAA</u> (SEQ ID NO: 160)
GN1252	HIP _{AB} + GT(KpnI) + GN3(R100Q_R 116Q)		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRK <u>7RTSQRGLSLIKSFEGLR</u> <u>LOAYQDSVGVWTIGYGTTRGVKAGMKISK</u> <u>DQAERMMLNDVQRFEPEVERLIKVPLNQDQ</u> <u>WDALMSFTYNLGAANLESSTLROLLNAGN</u> <u>YAAAAEQFPOWNKAGGOVLAGLTRRRAA</u> <u>EREFLGAA</u> (SEQ ID NO: 161)
GN1253	HIP _A + BK3 + GT(KpnI) + GN3(R100Q_R 116H)		MKGRGKTGGKARAKWKMYRKPVNKRR SARTFRRNAKRTKSANLQKAPQRRGWR <u>LG7RTSQRGLSLIKSFEGLRLQAYQDSVGV</u> <u>WTIGYGTTRGVKAGMKISKDQAERMMLND</u> <u>VQRFEPEVERLIKVPLNQDQWDALMSFTYN</u> <u>LGAANLESSTLROLLNAGNYAAAAEQFPQ</u> <u>WNKAGGOVLAGLTRRRAAEREFLGAA</u> (SEQ ID NO: 162)
GN1283	HIP _A + BK3 + GT(KpnI) + GN4(K100D_R 116H)		MKGRGKTGGKARAKWKMYRKPVNKRR SARTFRRNAKRTKSANLQKAPQRRGWR <u>LG7RTSQRGIDLIKFEGLRLSAYQDSVGVW</u> <u>TIGYGTTRGVTRYMTITVEQAERMLSNDIQ</u> <u>RFEPELDRLAKVPLNQDQWDALMSFVYNL</u> <u>GAANLASSTLLDLLNKGDYQGAADQPHW</u> <u>VNAGGKRLDGLVKRRAAERALEPLS</u> (SEQ ID NO: 163)
GN1287	HIP _{ABC} + GT(KpnI) + GN4(K100D_R 116H)		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNYAHRVGAGAPVWLG <u>7RTSQRGIDLIKFEGLRLSAYQDSVGVWTI</u> <u>GYGTTRGVTRYMTITVEQAERMLSNDIQRE</u> <u>EPELDRLAKVPLNQDQWDALMSFVYNLGA</u> <u>ANLASSTLLDLLNKGDYQGAADQPHWVN</u> <u>AGGKRLDGLVKRRAAERALEPLS</u> (SEQ ID NO: 164)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1313	HIP _A + BK3 + GT(KpnI) + GN588		MKGRGKTGGKARAKWKMYRKPVNKRR SARTFRRNAKRTKSANLQKAPQRGGWR <u>LGTQLTPHFTLEEFTRSDTARARQIDNSPSP</u> <u>AAAANLRRLAOTLERARVLLGGKPMQITSG</u> <u>YRSPALNRAVGGVPMSAHAQGLAADFVCS</u> <u>GYGPPLDVCRVLAASDLPFDQVINEFGRWV</u> <u>HIGLAADGVTPRRQVLTARKVGGRTTYVA</u> <u>GLQA</u> (SEQ ID NO: 165)
GN1316	HIP _{ABC} + GT(KpnI) + GN588		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNIAHVRGAGAPVWL <u>G7QLTPHFTLEEFTRSDTARARQIDNSPSPA</u> <u>AANLRRLAOTLERARVLLGGKPMQITSGYR</u> <u>SPALNRAVGGVPMSAHAQGLAADFVCSGY</u> <u>GPPLDVCRVLAASDLPFDQVINEFGRWVHI</u> <u>GLAADGVTPRRQVLTARKVGGRTTYVAGL</u> <u>QA</u> (SEQ ID NO: 166)
GN1328	HIP _{ABC} + GT(KpnI) + QPI14001		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNIAHVRGAGAPVWL <u>G7TIFDMLRIDEGLKLTIVYKDTEGFWTVGIGH</u> <u>LLTKDSSKAVAIKELDRLTGRSTDGSISVGE</u> <u>AENIEKLDVSKAENGIGNSVLAPVYESLDS</u> <u>VRKMAMVMVMVFOLGVAGVAGFKNSLALL</u> <u>KAKDWDKAAVNLAQSKWYNQTPNRAKRV</u> <u>ISVEKTGTMDAYK</u> (SEQ ID NO: 167)
GN1336	HIP _A + BK3 + GT(KpnI) + GN917		MKGRGKTGGKARAKWKMYRKPVNKRR SARTFRRNAKRTKSANLQKAPQRGGWR <u>LGTNLFEMLRYDEGLKLEVYFDTEGYPTVG</u> <u>VGHLLTKVHNLMSGISILDKELGRSTQGRIT</u> <u>TEDAEYLFSKDVEKAKAGIKSNTVLNDLYL</u> <u>SLDPIRQMALVNMVFQMGVAGVAGFNNSI</u> <u>KLLKAKQWDQAALNLAQSKWFKQTPNRA</u> <u>KRVINVFKTGTMDAYK</u> (SEQ ID NO: 168)
GN1339	HIP _{ABC} + GT(KpnI) + GN917		MKGRGKTGGKARAKWKTRSSRAGLQW PVGRVHRLLRKGNIAHVRGAGAPVWL <u>G7NLFEMLRYDEGLKLEVYFDTEGYPTVGVG</u> <u>HLLTKVHNLMSGISILDKELGRSTQGRITTE</u> <u>DAEYLFSKDVEKAKAGIKSNTVLNDLYLSL</u> <u>DPIRQMALVNMVFQMGVAGVAGFNNSIKL</u> <u>LKAKQWDQAALNLAQSKWFKQTPNRAKR</u> <u>VINVFKTGTMDAYK</u> (SEQ ID NO: 169)

Engineered Lysin Polypeptide Construct	Components	DNA	Amino Acid Sequence
GN1344	HIP _A + BK3 + GT(KpnI) + CAH0532154		<u>MKGRGKTGGKARAKWKMYRKPVNKRR</u> <u>SARTFRRNAKRTKSANLQKAPQRRGGWR</u> <u>LGTPQSTKOITPDDYARAAAALGVPVAAVK</u> <u>AVTDVESNGHGF LSDGRPLILYERHIMRRO</u> <u>LIAAGRAADADSHEASEPNIVNRAPGGYKG</u> <u>GAAEWDRLDAAVHIDRACALESASWGLFQ</u> <u>IMGFHWKSLGYESVQAFVNAMYASEGAQL</u> <u>DAFVAFVKANPNLLRALRAKDWPGEARGY</u> <u>NGPGYAANKYDTKLAAAYARHIKDAA*</u> (SEQ ID NO: 170)
GN1108	Zf5.3 + LSKLGG (SEQ ID NO: 45)+ PRR + GN588		<u>MYSCNVC GKAFVLSRHLNRHLRVHRRAT</u> <u>LSKLGGGPRRRPRRPGRRAPVOLTPHFTLEEF</u> <u>RSDTARARQIDNSPSPAAAANLRLAQTLE</u> <u>RARVLLGGKPMQITSGYRSPALNRAVGGVP</u> <u>MSAHAQGLAADFVCSGYGPPLDVCRVLAA</u> <u>SDLPFDQVINEFGRWVHIGLAADGVTPRRO</u> <u>VTARKVGGRTTYVAGLQA*</u> (SEQ ID NO: 171)
GN1140	FMRL _{Nm} (SEQ ID NO: 147)+ BK3 + GN685		<u>MIERFRR LHPQHMYRKPVNKRRSARTFRR</u> <u>NAKRTKSANLQKAPQRRGGWR L GFKLGSR</u> <u>SLORLOGVHPDLVKVVKRAIEISPVDFTVTE</u> <u>GLRTLROKELFAKGASKTMR SRHLTGHA</u> <u>VDISPLVDGKVSWDWKYYYPMADAMKQA</u> <u>AKELNIPVEWGGDWKTEKDGPHFQLPYGV</u> <u>YK*</u> (SEQ ID NO: 172)

[00134] In certain embodiment, disclosed herein is an engineered lysin selected from SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO:

151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, SEQ ID NO: 172, or fragments or variants of any of SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, or SEQ ID NO: 172. In certain embodiments, a variant of SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, or SEQ ID NO: 172 has at least about 80% sequence identity to the engineered lysin polypeptide construct, such as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least

about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity to the engineered lysin polypeptide construct.

5 [00135] In some embodiments, the engineered lysins of the present disclosure are chemically modified. A chemical modification includes but is not limited to, adding chemical moieties, creating new bonds, and removing chemical moieties. Chemical modifications can occur anywhere in a engineered lysin polypeptide construct, including the amino acid side chains, as well as the amino or carboxyl termini. For example, in certain embodiments,
10 engineered lysin polypeptide constructs comprise an N-terminal acetylation modification. In certain embodiments, the engineered lysin polypeptide constructs comprise a C-terminal amidation modification. Such modification can be present at more than one site in an engineered lysin polypeptide construct.

[00136] Furthermore, one or more side groups, or terminal groups of an engineered lysin
15 polypeptide construct may be protected by protective groups known to the person ordinarily-skilled in the art.

[00137] In some embodiments, the engineered lysins are conjugated to a duration enhancing moiety. In some embodiment, the duration enhancing moiety is polyethylene glycol. Polyethylene glycol ("PEG") has been used to obtain therapeutic polypeptides of enhanced
20 duration (Zalipsky, S., *Bioconjugate Chemistry*, 6:150-165 (1995); Mehvar, R., *J. Pharm. Pharmaceut. Sci.*, 3:125-136 (2000), which is herein incorporated by reference in its entirety). The PEG backbone, $(\text{CH}_2\text{CH}_2\text{-O})_n$, wherein n is a number of repeating monomers, is flexible and amphiphilic. When attached to another chemical entity, such as an engineered lysin polypeptide construct, PEG polymer chains can protect such polypeptides from immune response
25 and other clearance mechanisms. As a result, pegylation can lead to improved efficacy and safety by optimizing pharmacokinetics, increasing bioavailability, and decreasing immunogenicity and dosing amount and/or frequency.

POLYNUCLEOTIDES

30 *Engineered lysin polypeptide constructs, active fragments thereof or derivatives*

[00138] In one aspect, the present disclosure is directed an isolated polynucleotide comprising a nucleic acid molecule encoding an engineered lysin, or a variant or active fragment thereof as described herein. In some embodiments, the isolated polynucleotide sequence is a DNA sequence. In other embodiments, the isolated polynucleotide is a cDNA sequence.

5 **[00139]** In some embodiments, the polynucleotide is selected from SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, 10 SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 138, SEQ ID NO: 139, SEQ ID NO: 140, SEQ ID NO: 141, SEQ ID NO: 142 or a fragment or a variant thereof having at least about 80% sequence identity to the nucleic acid encoding the engineered lysin polypeptide construct, such 15 as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity to the nucleic acid encoding the engineered lysin polypeptide construct. In some embodiments, the 20 polynucleotide encodes an engineered polypeptide lysin construct selected from SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, 25 SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 30 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ

ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, or SEQ ID NO: 172, or a fragment or a variant thereof having at least about 80% sequence identity to the nucleic acid encoding the engineered lysin polypeptide construct, such as at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99%, or at least about 99.5% sequence identity to the nucleic acid encoding the engineered lysin polypeptide construct.

10 Vectors and Host Cells

[00140] In another aspect, the present disclosure is directed to a vector comprising an isolated polynucleotide comprising a nucleic acid molecule encoding any of the engineered lysin polypeptide constructs or variants or active fragments thereof disclosed herein or a complementary sequence of the present isolated polynucleotides. In some embodiments, the vector is a plasmid or cosmid. In other embodiments, the vector is a viral vector, wherein additional DNA segments can be ligated into the viral vector. In some embodiments, the vector can autonomously replicate in a host cell into which it is introduced. In some embodiments, the vector can be integrated into the genome of a host cell upon introduction into the host cell and thereby be replicated along with the host genome.

[00141] In some embodiments, particular vectors, referred to herein as “recombinant expression vectors” or “expression vectors”, can direct the expression of genes to which they are operatively linked. A polynucleotide sequence is “operatively linked” when it is placed into a functional relationship with another nucleotide sequence. For example, a promoter or regulatory DNA sequence is said to be “operatively linked” to a DNA sequence that codes for an RNA and/or a protein if the two sequences are operatively linked, or situated such that the promoter or regulatory DNA sequence affects the expression level of the coding or structural DNA sequence. Operatively linked DNA sequences are typically, but not necessarily, contiguous.

[00142] In some embodiments, the present disclosure is directed to a vector comprising a nucleic acid molecule that encodes an engineered lysin polypeptide construct chosen from SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ

ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, or SEQ ID NO: 172, or active fragments or variants thereof.

[00143] Generally, any system or vector suitable to maintain, propagate or express a polypeptide in a host may be used for expression of the engineered lysin polypeptide constructs disclosed herein or active fragments or variants thereof. The appropriate DNA/polynucleotide sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, eds., *Molecular Cloning: A Laboratory Manual* (3rd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory (2001). Additionally, tags can also be added to the engineered lysin polypeptide constructs or active fragments or variants thereof to provide convenient methods of isolation, *e.g.*, c-myc, biotin, poly-His, etc. Kits for such expression systems are commercially available.

[00144] A wide variety of host/expression vector combinations may be employed in expressing the polynucleotide sequences encoding the engineered lysin polypeptide constructs disclosed herein or active fragments or variants thereof. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Examples of suitable vectors are provided, *e.g.*, in Sambrook *et al.*, eds., *Molecular Cloning: A Laboratory Manual* (3rd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory (2001). Such vectors include, among others, chromosomal, episomal and virus derived vectors, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and

vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids.

[00145] Furthermore, the vectors may provide for the constitutive or inducible expression of the engineered lysin polypeptide constructs, or active fragments or variants thereof of the present disclosure. Suitable vectors include but are not limited to derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids colEI, pCRI, pBR322, pMB9 and their derivatives, plasmids such as RP4, pBAD24 and pBAD-TOPO; phage DNAs, *e.g.*, the numerous derivatives of phage A, *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 D plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like. Many of the vectors mentioned above are commercially available from vendors such as New England Biolabs Inc., Addgene, Takara Bio Inc., ThermoFisher Scientific Inc., etc.

[00146] Additionally, vectors may comprise various regulatory elements (including promoter, ribosome binding site, terminator, enhancer, various cis-elements for controlling the expression level) wherein the vector is constructed in accordance with the host cell. Any of a wide variety of expression control sequences (sequences that control the expression of a polynucleotide sequence operatively linked to it) may be used in these vectors to express the polynucleotide sequences encoding the engineered lysin polypeptide constructs or active fragments or variants thereof of the present disclosure. Useful control sequences include, but are not limited to: the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the lac system, the trp system, the TAC system, the TRC system, the LTR system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (*e.g.*, Pho5), the promoters of the yeast-mating factors, *E. coli* promoter for expression in bacteria, and other promoter sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. Typically, the polynucleotide sequences encoding the engineered lysin polypeptide constructs or active fragments or variants thereof are operatively linked to a heterologous promoter or regulatory element.

[00147] In another aspect, the present disclosure is directed to a host cell comprising any of the vectors disclosed herein including the expression vectors comprising the polynucleotide sequences encoding the engineered lysin polypeptide constructs or active fragments or variants thereof of the present disclosure. A wide variety of host cells are useful in expressing the present polypeptides. Non-limiting examples of host cells suitable for expression of the present polypeptides include well-known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture. While the expression host may be any known expression host cell, in a typical embodiment the expression host is one of the strains of *E. coli*. These include, but are not limited to, commercially available *E. coli* strains such as Top10 (ThermoFisher Scientific, Inc.), DH5a (Thermo Fisher Scientific, Inc.), XLI-Blue (Agilent Technologies, Inc.), SCS110 (Agilent Technologies, Inc.), JM109 (Promega, Inc.), LMG194 (ATCC), and BL21 (Thermo Fisher Scientific, Inc.).

[00148] There are several advantages of using *E. coli* as a host system including: fast growth kinetics, where under the optimal environmental conditions, its doubling time is about 20 min (Sezonov et al., *J. Bacterial.* 189 8746-8749 (2007)), easily achieved high density cultures, easy and fast transformation with exogenous DNA, etc. Details regarding protein expression in *E. coli*, including plasmid selection as well as strain selection are discussed in detail by Rosano, G. and Ceccarelli, E., *Front Microbial.*, 5: 172 (2014).

[00149] Efficient expression of the present engineered lysin polypeptide constructs or active fragments or variants thereof depends on a variety of factors such as optimal expression signals (both at the level of transcription and translation), correct protein folding, and cell growth characteristics. Regarding methods for constructing the vector and methods for transducing the constructed recombinant vector into the host cell, conventional methods known in the art can be utilized. While it is understood that not all vectors, expression control sequences, and hosts will function equally well to express the polynucleotide sequences encoding engineered lysin polypeptide constructs or active fragments or variants thereof of the present disclosure, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this disclosure.

[00150] The engineered lysin polypeptide constructs or active fragments or variants thereof of the present disclosure can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. High performance liquid chromatography can also be employed for peptide purification, including purification of the engineered lysin polypeptide constructs or active fragments and variants thereof, as well as their component parts, *e.g.*, isolated lysin polypeptides, linkers, stabilizing components, AMPs, and/or CPPs.

[00151] Alternatively, the vector system used for the production of engineered lysin polypeptide constructs or active fragments or variants of the present disclosure may be a cell-free expression system. Various cell-free expression systems are commercially available, including, but are not limited to those available from Promega, LifeTechnologies, Clontech, etc.

[00152] As indicated above, there is an array of choices when it comes to protein production and purification. Examples of suitable methods and strategies to be considered in protein production and purification are provided in WO 2017/049233, which is herein incorporated by reference in its entirety and further provided in Structural Genomics Consortium *et al.*, *Nat. Methods.*, 5(2): 135-146 (2008).

Pharmaceutical Compositions

[00153] The compositions of the present disclosure can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, tampon applications emulsions, aerosols, sprays, suspensions, lozenges, troches, candies, injectants, chewing gums, ointments, smears, time-release patches, liquid absorbed wipes, and combinations thereof.

[00154] Administration of the compositions of the present disclosure or pharmaceutically acceptable forms thereof may be topical, *i.e.*, the pharmaceutical composition may be applied directly where its action is desired (for example directly to a wound), or systemic. In turn, systemic administration can be enteral or oral, *i.e.*, the composition may be given via the digestive tract, parenteral, *i.e.*, the composition may be given by other routes than the digestive tract such as by injection or inhalation. Thus, the engineered lysin polypeptide constructs of the

present disclosure or active fragments or variants thereof and compositions comprising them can be administered to a subject orally, parenterally, by inhalation, topically, rectally, nasally, buccally, via an implanted reservoir, or by any other known method. The engineered lysin polypeptide constructs of the present disclosure or active fragments or variants thereof can also
5 be administered by means of sustained release dosage forms.

[00155] For oral administration, the engineered lysin polypeptide constructs of the present disclosure or active fragments or variants thereof can be formulated into solid or liquid preparations, for example tablets, capsules, powders, solutions, suspensions, and dispersions. The composition can be formulated with excipients such as, *e.g.*, lactose, sucrose, corn starch,
10 gelatin, potato starch, alginic acid, and/or magnesium stearate.

[00156] For preparing solid compositions such as tablets and pills, an engineered lysin polypeptide construct of the present disclosure or active fragments or variants thereof may be mixed with a pharmaceutical excipient to form a solid pre-formulation composition. If desired, tablets may be sugar coated or enteric coated by standard techniques. The tablets or pills may be
15 coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can include an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer, which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of
20 materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

[00157] The topical compositions of the present disclosure may further comprise a pharmaceutically or physiologically acceptable carrier, such as a dermatologically or an otically
25 acceptable carrier. Such carriers, in the case of dermatologically acceptable carriers, may be compatible with skin, nails, mucous membranes, tissues, and/or hair, and can include any conventionally-used dermatological carrier meeting these requirements. In the case of otically acceptable carriers, the carrier may be compatible with all parts of the ear. Such carriers can be readily selected by one of ordinary skill in the art. Carriers for topical administration of the
30 compositions of the present disclosure include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene and/or polyoxypropylene

compounds, emulsifying wax, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol, and water. In formulating skin ointments, the active components of the present disclosure may be formulated, for example, in an oleaginous hydrocarbon base, an anhydrous absorption base, a water-in-oil absorption base, an oil-in-water water-removable base, and/or a water-soluble base. In formulating otic compositions, the active components of the present disclosure may be formulated, for example, in an aqueous polymeric suspension including such carriers as dextrans, polyethylene glycols, polyvinylpyrrolidone, polysaccharide gels, gellan gums such as Gelrite®, cellulosic polymers such as hydroxypropyl methylcellulose, and carboxy-containing polymers such as polymers or copolymers of acrylic acid, as well as other polymeric demulcents. The topical compositions according to the present disclosure may be in any form suitable for topical application, including aqueous, aqueous-alcoholic or oily solutions; lotion or serum dispersions; aqueous, anhydrous or oily gels; emulsions obtained by dispersion of a fatty phase in an aqueous phase (O/W or oil-in-water) or, conversely, (W/O or water-in-oil); microemulsions or alternatively microcapsules, microparticles or lipid vesicle dispersions of ionic and/or nonionic type; creams; lotions; gels; foams (which may use a pressurized canister, a suitable applicator, an emulsifier, and an inert propellant); essences; milks; suspensions; and patches. Topical compositions of the present disclosure may also contain adjuvants such as hydrophilic or lipophilic gelling agents, hydrophilic or lipophilic active agents, preserving agents, antioxidants, solvents, fragrances, fillers, sunscreens, odor-absorbers, and dyestuffs. In a further aspect, the topical compositions disclosed herein may be administered in conjunction with devices such as transdermal patches, dressings, pads, wraps, matrices, and bandages capable of being adhered to or otherwise associated with the skin or other tissue of a subject, being capable of delivering a therapeutically effective amount of one or more engineered lysin polypeptide construct or active fragment or variant thereof as disclosed herein.

[00158] In one embodiment, the topical compositions of the present disclosure additionally comprise one or more components used to treat topical burns. Such components may include, but are not limited to, a propylene glycol hydrogel; a combination of a glycol, a cellulose derivative, and a water soluble aluminum salt; an antiseptic; and a corticosteroid. Humectants such as solid or liquid wax esters; absorption promoters such as hydrophilic clays or starches; viscosity building agents; and skin-protecting agents may also be added. Topical formulations may be in the form of rinses such as mouthwash. See, *e.g.*, WO 2004/004650.

[00159] The compositions of the present disclosure may also be administered by injection of a therapeutic agent comprising the appropriate amount of an engineered lysin polypeptide construct or active fragment or variant thereof and a carrier. For example, the engineered lysin polypeptide construct or active fragment or variant thereof can be administered intramuscularly, intrathecally, subdermally, subcutaneously, or intravenously to treat infections by Gram-negative bacteria, such as those caused by *Burkholderia* and/or *Yersinia spp.* The carrier may be comprised of distilled water, a saline solution, albumin, a serum, or any combinations thereof. Additionally, pharmaceutical compositions of parenteral injections can comprise pharmaceutically acceptable aqueous or nonaqueous solutions of engineered lysin polypeptide constructs as disclosed herein or active fragments or variants thereof in addition to one or more of the following: pH buffered solutions, adjuvants (*e.g.*, preservatives, wetting agents, emulsifying agents, and dispersing agents), liposomal formulations, nanoparticles, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

[00160] In cases where parenteral injection is the chosen mode of administration, an isotonic formulation may be used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol, and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers can include gelatin and albumin. A vasoconstriction agent can be added to the formulation. The pharmaceutical preparations according to this type of application may be provided sterile and pyrogen free.

[00161] The diluent may further comprise one or more other excipient such as ethanol, propylene glycol, an oil, or a pharmaceutically acceptable emulsifier or surfactant.

[00162] In another embodiment, the compositions of the present disclosure are inhalable compositions. The inhalable compositions of the present disclosure can further comprise a pharmaceutically acceptable carrier. In one embodiment, the engineered lysin polypeptide constructs of the present disclosure or active fragments or variants thereof may be formulated as a dry, inhalable powder. In specific embodiments, an inhalation solution comprising engineered lysin polypeptide constructs or active fragments or variants thereof may further be formulated with a propellant for aerosol delivery. In certain embodiments, solutions may be nebulized.

[00163] A surfactant can be added to an inhalable pharmaceutical composition of the present disclosure in order to lower the surface and interfacial tension between the medicaments

and the propellant. Where the medicaments, propellant and excipient are to form a suspension, a surfactant may or may not be used. Where the medicaments, propellant and excipient are to form a solution, a surfactant may or may not be used, depending, for example, on the solubility of the particular medicament and excipient. The surfactant may be any suitable, non-toxic compound which is non-reactive with the medicament and which reduces the surface tension between the medicament, the excipient and the propellant and/or acts as a valve lubricant.

[00164] Examples of surfactants useful for the invention include, but are not limited to: oleic acid; sorbitan trioleate; cetyl pyridinium chloride; soya lecithin; polyoxyethylene (20) sorbitan monolaurate; polyoxyethylene (10) stearyl ether; polyoxyethylene (2) oleyl ether; polyoxypropylene-polyoxyethylene ethylene diamine block copolymers; polyoxyethylene (20) sorbitan monostearate; polyoxyethylene(20) sorbitan monooleate; polyoxypropylene-polyoxyethylene block copolymers; castor oil ethoxylate; and combinations thereof.

[00165] Examples of propellants useful for the invention include, but are not limited to: dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, and carbon dioxide.

[00166] Examples of excipients for use in inhalable compositions include, but are not limited to: lactose, starch, propylene glycol diesters of medium chain fatty acids; triglyceride esters of medium chain fatty acids, short chains, or long chains, or any combination thereof; perfluorodimethylcyclobutane; perfluorocyclobutane; polyethylene glycol; menthol; lauroglycol; diethylene glycol monoethylether; polyglycolized glycerides of medium chain fatty acids; alcohols; eucalyptus oil; short chain fatty acids; and combinations thereof.

[00167] In some embodiments, the compositions of the present disclosure comprise nasal applications. Nasal applications include applications for direct use, such as nasal sprays, nasal drops, nasal ointments, nasal washes, nasal injections, nasal packings, bronchial sprays and inhalers, as well as applications for indirect use, such as throat lozenges and mouthwashes or gargles, or through the use of ointments applied to the nasal nares or the face, and any combination of these and similar methods of application.

[00168] In another embodiment, the pharmaceutical compositions of the present disclosure comprise a complementary agent, including one or more antimicrobial agents. In order to accelerate the treatment of the infection, or augment the antibacterial effect, the therapeutic agent containing an engineered lysin polypeptide construct of the present disclosure or active fragment

or variant thereof may further include at least one complementary agent that can also potentiate the bactericidal activity of the construct. In one embodiment, the complementary agent is an antimicrobial agent used for the treatment of infections caused by *Burkholderia spp.*

[00169] The compositions of the present disclosure may be presented in unit dosage form and may be prepared by any methods well known in the art. The amount of active ingredients that can be combined with a carrier material to produce a single dosage form will vary depending, for example, upon the host being treated, the duration of exposure of the recipient to the infectious bacteria, the size and weight of the subject, and the particular mode of administration. The amount of active ingredients that can be combined with a carrier material to produce a single dosage form may, for example, be that amount of each compound which produces a therapeutic effect. In certain embodiments, out of one hundred percent, the total amount may range from about 1 percent to about ninety-nine percent of active ingredients, such as from about 5 percent to about 70 percent, or from about 10 percent to about 30 percent.

Dosage and Administration

[00170] Dosages administered may depend on a number of factors such as the activity of infection being treated; the age, health and general physical condition of the subject to be treated; the activity of a particular engineered lysin polypeptide construct or active fragment or variant thereof; in certain embodiments, effective amounts of the engineered lysin polypeptide construct or active fragment or variant thereof to be administered may fall within the range of about 1-50 mg/kg (or 1 to 50 mcg/ml). In certain embodiments, effective amounts of the engineered lysin polypeptide construct or active fragment or variant thereof to be administered may fall within the range of about 1-50 µg/mL, such as within the range of about 1-10 µg/mL, about 1 µg/mL, or about 10 µg/mL. In certain embodiments, the engineered lysin polypeptide construct or active fragment or variant thereof may be administered 1-4 times daily for a period ranging from 1 to 14 days. All such dosages and regimens, however, (whether of the engineered lysin polypeptide construct or active fragment or variant thereof) are subject to optimization. Optimal dosages can be determined by performing *in vitro* and *in vivo* pilot efficacy experiments as is within the skill of the art but taking the present disclosure into account.

[00171] It is contemplated that the engineered lysin polypeptide constructs disclosed herein or active fragments or variants thereof may provide a rapid bactericidal and, when used in

sub-MIC amounts, may provide a bacteriostatic effect. It is further contemplated that the engineered lysin polypeptide constructs disclosed herein or active fragments or variants thereof may not be associated with evolving resistance. It is believed that existing resistance mechanisms for Gram-negative bacteria do not affect sensitivity to the lytic activity of the present engineered lysin polypeptide constructs or active fragments or variants thereof.

[00172] In some embodiments, time exposure to the lysins and engineered lysin polypeptide constructs disclosed herein or active fragments or variants thereof may influence the desired concentration of active peptide units per ml. Carriers that are classified as “long” or “slow” release carriers (such as, for example, certain nasal sprays or lozenges) may possess or provide a lower concentration of peptide units per ml but over a longer period of time, whereas a “short” or “fast” release carrier (such as, for example, a gargle) may possess or provide a high concentration peptide units (mcg) per ml but over a shorter period of time. There are circumstances where it may be desirable to have a higher unit/ml dosage or a lower unit/ml dosage.

[00173] For the engineered lysin polypeptide constructs or active fragments or variants thereof of the present disclosure, the therapeutically effective dose may be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model can also be used to achieve a desirable concentration range and route of administration. Obtained information can then be used to determine the effective doses, as well as routes of administration, in humans. Dosage and administration can be further adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Additional factors that may be taken into account include the severity of the disease state; age, weight and gender of the patient; diet; desired duration of treatment; method of administration; time and frequency of administration; drug combinations; reaction sensitivities; tolerance/response to therapy; and the judgment of a treating physician.

[00174] A treatment regimen can entail administration daily (*e.g.*, once, twice, thrice, etc. daily), every other day (*e.g.*, once, twice, thrice, etc. every other day), semi-weekly, weekly, once every two weeks, once a month, etc. In one embodiment, treatment can be given as a continuous infusion. Unit doses can be administered on multiple occasions. Intervals can also be irregular as indicated by monitoring clinical symptoms. Alternatively, the unit dose can be administered as a sustained release formulation, in which case less frequent administration may

be used. Dosage and frequency may vary depending on the patient. It will be understood by one of skill in the art that such guidelines will be adjusted for localized administration, *e.g.*, intranasal, inhalation, rectal, etc., or for systemic administration, *e.g.*, oral, rectal (*e.g.*, via enema), intramuscular (i.m.), intraperitoneal (i.p.), intravenous (i.v.), subcutaneous (s.c.), transurethral, and the like.

Methods

[00175] The engineered lysins and active fragments or variants thereof of the present disclosure can be used *in vivo*, for example, to treat bacterial infections due to Gram-negative bacteria, such as *P. aeruginosa*, *Burkholderia spp.*, and/or *Yersinia pestis*, in a subject, as well as *in vitro*, for example to reduce the level of bacterial contamination on, for example, a surface, *e.g.*, of a medical device or *ex vivo*. The engineered lysins and active fragments or variants thereof of the present disclosure can also be used prophylactically, for example, to prevent or ameliorate a bacterial infection due to Gram-negative bacteria, such as *P. aeruginosa*, *Burkholderia spp.*, and/or *Yersinia pestis*, in a subject at risk of acquiring such an infection.

[00176] In one aspect, the present disclosure is directed to a method of treating a bacterial infection caused by Gram-negative bacteria as described herein, comprising administering to a subject in need thereof an effective amount of an engineered lysin of the disclosure. In some embodiments, the subject is diagnosed with, at risk for, or exhibiting symptoms of a bacterial infection. In one aspect, the present disclosure is directed to a method of treating a bacterial infection caused by one or more Gram-negative bacteria, *e.g.*, one or more *Burkholderia spp.* and/or one or more *Yersinia spp.*, as described herein, comprising administering to a subject in need thereof, *e.g.*, diagnosed with, at risk for, or exhibiting symptoms of a bacterial infection, a lysin or an engineered lysin of the disclosure optionally in a pharmaceutical composition as herein described. In one aspect, the present disclosure is directed to a method of treating a bacterial infection caused by a Gram-negative bacteria that is used or has the potential to be used as a biowarfare agent, as described herein, comprising administering to a subject in need thereof, *e.g.*, diagnosed with, at risk for, or exhibiting symptoms of a bacterial infection, an engineered lysin of the disclosure optionally in a pharmaceutical composition as described herein described.

[00177] The terms “infection” and “bacterial infection” are meant to include respiratory tract infections (RTIs), such as respiratory tract infections in patients having cystic fibrosis (CF),

including pulmonary exacerbations in CF patients and/or patients with decline in lung function and mortality; lower respiratory tract infections, such as acute exacerbation of chronic bronchitis (ACEB); acute sinusitis; community-acquired pneumonia (CAP); hospital-acquired pneumonia (HAP); nosocomial respiratory tract infections; non-cystic fibrosis lung diseases such as bronchiectasis and/or acute pneumonias; sexually transmitted diseases, such as gonococcal cervicitis and gonococcal urethritis; urinary tract infections; acute otitis media; sepsis including neonatal septicemia and catheter-related sepsis; osteomyelitis; tuberculosis, and non-tuberculosis mycobacteria infections. Infections caused by drug-resistant bacteria and multidrug-resistant bacteria are also contemplated.

[00178] Non-limiting examples of infections caused by Gram-negative bacteria include: A) Nosocomial infections: 1. Respiratory tract infections, especially in cystic fibrosis patients and mechanically-ventilated patients, including pulmonary exacerbations of cystic fibrosis patients and bronchiectasis in non-cystic fibrosis patients; 2. Bacteremia and sepsis; 3. Wound infections, particularly those of burn victims; 4. Urinary tract infections; 5. Post-surgery infections on invasive devices; 6. Endocarditis by intravenous administration of contaminated drug solutions; 7. Infections in patients with acquired immunodeficiency syndrome, cancer chemotherapy, steroid therapy, hematological malignancies, organ transplantation, renal replacement therapy, and other conditions with severe neutropenia. B) Community-acquired infections: 1. Community-acquired respiratory tract infections such as tuberculosis; 2. Meningitis; 3. Folliculitis and infections of the ear canal caused by contaminated water; 4. Malignant otitis externa in the elderly and diabetics; 5. Osteomyelitis of the calcaneus in children; 6. Eye infections commonly associated with contaminated contact lens; 7. Skin infections such as nail infections in people whose hands are frequently exposed to water; 8. Gastrointestinal tract infections; and 9. Musculoskeletal system infections.

[00179] The one or more species of Gram-negative bacteria of the present methods may include any of the species of Gram-negative bacteria as described herein. For example, in some embodiments the bacteria are *Burkholderia* spp., *Yersinia* spp., *Acinetobacter* spp., *Klebsiella* spp., or *Pseudomonas* spp. In some embodiments, the bacteria are *B. cepacia*, *B. cenocepacia*, *B. thailandensis*, *B. multivorans*, or *B. humptydooensis*. In some embodiments, the bacteria are *Burkholderia* spp., e.g., *B. cepacia*, *B. cenocepacia*, *B. thailandensis*, *B. multivorans*, and/or *B. humptydooensis*. Typically, the *Yersinia* spp. is *Yersinia pestis*. In some embodiments, the

Acinetobacter spp. is *A. baumannii*. In some embodiments, the *Klebsiella* spp. is *K. pneumoniae*. In some embodiments, Gram-negative bacteria include *Serratia* spp., such as *Serratia marcescens*.

[00180] In some embodiments, infection with Gram-negative bacteria results in a localized infection, such as a topical bacterial infection, e.g., a skin wound. In other embodiments, the bacterial infection is a systemic pathogenic bacterial infection. Common Gram-negative pathogens and associated infections are listed in Table A of the present disclosure. These are meant to serve as examples of the bacterial infections that may be treated, mitigated or prevented with the present engineered lysin polypeptide constructs and active fragments and variants thereof and are not intended to be limiting.

Table A - Medically relevant Gram-negative bacteria and associated diseases

<i>Acinetobacter baumannii</i>	Wound infections
<i>Pseudomonas aeruginosa</i>	bloodstream infections and pneumonia
<i>Klebsiella pneumoniae</i>	UTIs, and bloodstream infections
<i>Serratia</i> spp.	Catheter contaminations, UTIs, and pneumonia
<i>Yersinia pestis</i>	Bubonic and pneumonic plague
<i>Burkholderia</i> spp.	Lung infections, e.g., pneumonia, bacteremia, skin infections

[00181] In some embodiments, the engineered lysins and active fragments and variants thereof of the present disclosure are used to treat a subject at risk for acquiring an infection due to a Gram-negative bacterium. Subjects at risk for acquiring a Gram-negative bacterial infection include, for example, cystic fibrosis patients, neutropenic patients, patients with necrotising enterocolitis, burn victims, patients with wound infections, and, more generally, patients in a hospital setting, in particular surgical patients and patients being treated using an implantable medical device such as a catheter, for example a central venous catheter, a Hickman device, or electrophysiologic cardiac devices, for example pacemakers and implantable defibrillators. Other patient groups at risk for infection with Gram-negative bacteria include without limitation patients with implanted prostheses such a total joint replacement (for example total knee or hip replacement).

[00182] The engineered lysins or active fragments or variants thereof can be further combined with additional permeabilizing agents of the outer membrane of the Gram-negative bacteria, including, but not limited to metal chelators, such as e.g. EDTA, TRIS, lactic acid, lactoferrin, polymyxins, citric acid (Vaara M. *Microbial Rev.* 56(3):395-441 (1992), which is
5 herein incorporated by reference in its entirety).

[00183] In yet another aspect, the present disclosure is directed to a method of inhibiting the growth, or reducing the population, or killing of at least one species of Gram-negative bacteria, the method comprising contacting the bacteria with the lysin or engineered lysin of the invention, or a composition containing an effective amount of the lysins or engineered lysin of
10 the invention or active fragment or variants thereof as described herein, wherein the lysin or engineered lysin polypeptide construct or active fragment or variant thereof inhibits the growth, or reduces the population, or kills at least one species of Gram-negative bacteria.

[00184] In some embodiments, inhibiting the growth, or reducing the population, or killing at least one species of Gram-negative bacteria comprises contacting bacteria with the
15 lysin or engineered lysins or active fragments or variants as described herein, wherein the bacteria are present on a surface of e.g., medical devices, floors, stairs, walls and countertops in hospitals and other health related or public use buildings and surfaces of equipment in operating rooms, emergency rooms, hospital rooms, clinics, and bathrooms and the like.

[00185] Examples of medical devices that can be protected using the engineered lysin
20 polypeptide constructs or active fragments or variants thereof described herein include but are not limited to tubing and other surface medical devices, such as urinary catheters, mucous extraction catheters, suction catheters, umbilical cannulae, contact lenses, intrauterine devices, intravaginal and intrainestinal devices, endotracheal tubes, bronchoscopes, dental prostheses and orthodontic devices, surgical instruments, dental instruments, tubings, dental water lines, fabrics,
25 paper, indicator strips (e.g., paper indicator strips or plastic indicator strips), adhesives (e.g., hydrogel adhesives, hot-melt adhesives, or solvent-based adhesives), bandages, tissue dressings or healing devices and occlusive patches, and any other surface devices used in the medical field. The devices may include electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types. Medical devices can also include any device which can be placed
30 at the insertion or implantation site such as the skin near the insertion or implantation site, and which can include at least one surface which is susceptible to colonization by Gram-negative.

EXAMPLES

Example 1: Identification of *Burkholderia* lysins – Screening Native and Engineered Derivatives

5 [00186] Native (n=104) and engineered (n=107) lysins, amurins (n=18), and amurin-like compounds (n=23) were synthesized and purified from bacteriophages and prophages of *Burkholderia* and related organisms, including mollicutes and cyanobacteria. An initial test panel included isolates of *B. cenocepacia*, *B. cepacia*, *B. multivorans*, and *B. thailandensis*. The majority of compounds tested were not active (MIC >256 µg/mL). Native (n=21) and engineered
10 (n=11) lysins having a MIC ≤256 µg/mL and amurins and amurin-like compounds (n=6) having a MIC ≤8 µg/mL were selected for further study.

[00187] Of the 21 selected native lysins, engineered lysins (n=52) were designed to incorporate amurins and amurin-like compounds previously identified. Additional engineered lysins (n=50) were designed to optimize domain placement, modify charge, and/or incorporate
15 amurins and amurin-like compounds previously identified. Additionally, the 6 amurins and amurin-like compounds were modified to optimize sequences and incorporate native and engineered lysins previously identified (n=12). Modifications may include optimization of an original sequence (*i.e.*, charge modification, codon optimization), fusion with cell penetrating peptides and outer membrane binding motifs, incorporation of spacer domains, and/or
20 improvement in induction condition and purification.

[00188] A total of 220 engineered and native lysins were identified and tested against the following four *Burkholderia* species: *B. cenocepacia*, *B. multivorans*, *B. thailandensis*, and *B. cepacia*, as shown below in Table 6.

[00189] Table 6 – Number of native and engineered GN lysins tested against *Burkholderia*
25 *ssp.*

	<i>B. cenocepacia</i> (AU24619)	<i>B. multivorans</i> (NR-707)	<i>B. thailandensis</i> (ATCC BAA-247)	<i>B. cepacia</i> (NR-9909)
Engineered GN lysins	167	38	58	100
Native peptidases	21	2	21	3
Native lysozymes	13	5	15	5
Native	14	5	15	5

muridamidases				
Others (including CHAP and amidases)	5	0	6	2
Total:	220	50	115	115

[00190] The native lysins had MICs >256 µg/mL. However, with modifications, activity was improved to MICs as low as ≤4 µg/mL. MICs of engineered derivatives were then compared to MICs of their parental compounds, leading to multiple iterative rounds of modifications.

5 Simultaneous analysis of novel amurin peptides against *Burkholderia* species allowed for identification of new compounds for fusion to lysin polypeptide sequences.

[00191] By way of example, GN568 (*Yersinia* phase PY100) and derivatives thereof were identified. GN568 (15.72 kDa) is a 138 amino acid cell wall peptidase (peptidase_M15_4) from *Yersinia* phage. GN568 was re-engineered by removing an alternate short start codon to create
 10 GN685, which allows for high-level production with >95% purity. GN685 was fused with a series of 8 peptides, including, for example, RI-18, having the amino acid sequence RKKTRKRLKKIGKVLKWI (SEQ ID NO: 25). GN685 was also fused to various linker variants, including FIRL_{BCC} (SEQ ID NO: 32), PRR peptide, and the last four amino acids from RI-18 (to stabilize helix). The activity of GN568 and the engineered constructs was measured
 15 against *B. cenocepacia*, *B. thailandensis*, and *B. multivorans*, and the results are shown below in Table 7.

[00192] Table 7 – MIC (µg/mL) activity of GN568 and Derivatives

Lysin	Description	<i>B. cenocepacia</i> (CFS# 1945)	<i>B. cenocepacia</i> (CFS# 2131)	<i>B. thailandensis</i> (CFS# 2639)	<i>B. multivorans</i> (CFS# 1946)
GN568 (SEQ ID NO: 1)	15.72 kDa cell wall peptidase	16	>256	>256	16
GN685 (SEQ ID NO: 2)	N-terminus, truncated version of GN568	16	>256	>256	16
GN688 (SEQ ID NO: 109)	FIQL _{Bc} (SEQ ID NO: 173)+PRR+GN685	4	32	32	4
GN686 (SEQ ID NO: 73)	GN685+RI18	1	2	2	1

GN687 (SEQ ID NO: 107)	GN685+(GGGGS) ₃ (SEQ ID NO: 43)+PRR+LKWI (SEQ ID NO: 127)	4	16	16	4
GN689 (SEQ ID NO: 110)	GN685+AM1	16	>256	>256	16
GN690 (SEQ ID NO: 111)	MG+AM1+GN685	16	>256	>256	16
GN695 (SEQ ID NO: 113)	GN685+MMAV-2	16	>256	>256	16
GN696 (SEQ ID NO: 114)	GN685+MFVI-3	16	>256	>256	16
GN697 (SEQ ID NO: 115)	MG+MFVI-3+GN685	16	>256	>256	16
GN692 (SEQ ID NO: 112)	GN685+AM13	16	>64	>64	16
GN693 (SEQ ID NO: 55)	GN685+BK5	1	2	2	1
GN694 (SEQ ID NO: 56)	GN685+BCM1	0.5	1	1	0.5

[00193] As shown in Table 7, all of GN688, GN686, GN687, GN693, and GN694 demonstrate improved MIC activity (e.g., ≤ 4 $\mu\text{g/mL}$) compared to GN568 and GN685 against a range of *Burkholderia* species, including *B. cenocepacia* and *B. multivorans*.

5

Example 2: Lysins having activity against *Burkholderia* spp.

[00194] Various derivatives of GN577, GN685, GN569, GN428, and GN867 were constructed as set forth in Table 8 below.

[00195] Table 8 – Engineered derivatives of GN577, GN685, GN569, GN428, and GN867

Lysin	Parent phage	Description
GN585 (SEQ ID NO: 72)	<i>B. cenocepacia</i>	GN577+SKY+RI18
GN686 (SEQ ID NO: 73)	<i>Yersinia</i> phage PY100	GN685+RI18
GN693 (SEQ ID NO: 55)	<i>Yersinia</i> phage PY100	GN685+BK5
GN694 (SEQ ID NO: 56)	<i>Yersinia</i> phage PY100	GN685+BCM1
GN727 (SEQ ID NO: 74)	<i>M. aeruginosavorus</i>	ATRA-1A+GN37+A+ATRA-1A

GN758 (SEQ ID NO: 75)	<i>Escherichia</i> phage	FIRL _{BC} (SEQ ID NO: 143)+G+BK9+GN428
GN764 (SEQ ID NO: 76)	<i>Burkholderia ambifaria</i>	GN569(K87H)+RI-18
GN765 (SEQ ID NO: 77)	<i>Yersinia</i> phage PY100	GN685(K78H)+RI-18
GN873 (SEQ ID NO: 78)	<i>Yersinia enterocolitica</i>	FMRL _{YP} (SEQ ID NO: 144)+LSKLGG (SEQ ID NO: 45)+PRR ₅ +GN867
GN916 (SEQ ID NO: 79)	<i>Yersinia enterocolitica</i>	ZF5.3+ LSKLGG (SEQ ID NO: 45)+PRR+GN867

[00196] Several of these lysins and derivative lysins demonstrate activity against *Burkholderia* spp. and *Y. pestis*. Accordingly, activity of various lysins was then evaluated for *B. thailandensis*, *B. humptydooensis*, and *Y. pestis*, and the results are shown in Table 9 below. As shown in Table 9, several engineered Gram-negative lysins, including GN873 and GN916 demonstrate activity against *B. thailandensis*, *B. humptydooensis*, and *Y. pestis*. Both *B. thailandensis* and *B. humptydooensis* are phylogenetically similar to *B. pseudomallei/mallei*, as they are part of the *B. pseudomallei* complex. Based on this phylogenetic similarity, *B. thailandensis* and *B. humptydooensis* may be used as surrogates for laboratory testing in lieu of the biohazardous *B. pseudomallei*.

[00197] Table 9 –MIC (μg/mL) activity against *B. pseudomallei* complex surrogates and *Y. pestis*

Lysin	<i>B. thailandensis</i>					<i>B. humptydooensis</i>	<i>Y. pestis</i>
	1950	1951	2638	2639	2736	2701	636
GN585 (SEQ ID NO: 72)	4	1	16	16	1	0.5	4
GN686 (SEQ ID NO: 73)	2	1	8	1	2	1	2
GN693 (SEQ ID NO: 55)	2	1	8	0.5	0.5	0.5	2
GN694 (SEQ ID NO: 56)	0.5	1	4	0.5	1	0.5	2
GN727 (SEQ ID NO: 74)	4	1	64	32	2	32	4
GN758 (SEQ ID NO: 75)	1	1	2	4	2	1	8
GN764 (SEQ ID NO: 76)	4	1	32	8	2	1	2
GN765	2	1	8	0.5	1	0.5	4

(SEQ ID NO: 77)							
GN873 (SEQ ID NO: 78)	1	1	4	1	1	1	2
GN916 (SEQ ID NO: 79)	1	1	4	1	1	2	2

[00198] Additionally, multiple variants of GN867 were constructed and to evaluate Gram-negative antibacterial activity, and the results shown in Table 10 below.

[00199] Table 10 – Engineered derivatives of GN867

Lysin	Description
GN867 (SEQ ID NO: 13)	<i>Yersinia</i> (lysozyme)
GN869 (SEQ ID NO: 116)	FMRL _{Yp} (SEQ ID NO: 144)+PRR ₃ + GN867
GN870 (SEQ ID NO: 117)	FMRL _{Yp} (SEQ ID NO: 144)+PRR ₃ +LGGKSLG (SEQ ID NO: 174)+GN867
GN871 (SEQ ID NO: 118)	FMRL _{Yp} (SEQ ID NO: 144)+ LGGKSLG (SEQ ID NO: 174)+PRR ₃ +GN867
GN872 (SEQ ID NO: 119)	FIQL _{Bc} (SEQ ID NO: 173)+LSKLGG (SEQ ID NO: 45)+PRR ₃ +GN867
GN873 (SEQ ID NO: 78)	FMRL _{Yp} (SEQ ID NO: 144)+ LSKLGG (SEQ ID NO: 45)+ PRR ₅ + GN867
GN874 (SEQ ID NO: 120)	FMRL _{Yp} (SEQ ID NO: 144)+PRR ₅ +GN867
GN875 (SEQ ID NO: 121)	FIQL _{Bc} (SEQ ID NO: 173)+LSKLGG (SEQ ID NO: 45)+PRR ₅ +GN867
GN905 (SEQ ID NO: 122)	FIQL _{Bc} (SEQ ID NO: 173)+LSKLGG (SEQ ID NO: 45)+ZF5.3+GN867
GN916 (SEQ ID NO: 79)	ZF5.3+LSKLGG (SEQ ID NO: 45)+PRR+GN867

5

[00200] In Table 10 above, PRR₃ is a PRR homolog from *Y. pestis* RNA chaperone ProQ and indicates (PRR₃) RKPRQSPRPQQVRPPRPQVEENQPRPVPV (SEQ ID NO: 48). PRR₅ is a PRR homolog from DAS01177.1 TPA: hypothetical protein [*Siphoviridae* sp.] and indicates (PRR₅) PRRPRRGRRRPHRGRL (SEQ ID NO: 40).

10 **[00201]** Activity of various lysins was evaluated for *B. cepacian*, *B. cenocepacia*, *B. thailandensis*, *B. multivorans*, and *B. humptydooensis*, and the results are shown in Table 11 below.

[00202] Table 11 – MIC ($\mu\text{g/mL}$) activity for GN867 and derivatives against various *Burkholderia* species.

Lysin	<i>B. cepacia</i> (CFS-2635)	<i>B. ceno- cepacia</i> (CFS-2131)	<i>B. thailand- ensis</i> (CFS-2639)	<i>B. multivorans</i> (CFS-1946)	<i>B. humpty- dooensis</i> (CFS-2701)
GN867 (SEQ ID NO: 13)	>256	>256	>256	256	>256
GN869 (SEQ ID NO: 116)	>64	>64	>64	64	>64
GN870 (SEQ ID NO: 117)	16	64	256	2	>256
GN871 (SEQ ID NO: 118)	32	128	256	8	>256
GN872 (SEQ ID NO: 119)	>256	>256	>256	16	>256
GN873 (SEQ ID NO: 78)	2	1	1	1	1
GN874 (SEQ ID NO: 120)	4	16	32	4	>32
GN875 (SEQ ID NO: 121)	n/a	n/a	n/a	n/a	n/a
GN905 (SEQ ID NO: 122)	16	32	>32	8	>32
GN916 (SEQ ID NO: 79)	1	4	1	1	2
GN1040 (SEQ ID NO: 59)	1	2	4	1	1
GN1041 (SEQ ID NO: 60)	2	1	2	0.25	0.25
GN1072 (SEQ ID NO: 61)	1	1	1	1	1
GN1108 (SEQ ID NO: 171)	1	1	1	<0.5	<0.5
GN1117 (SEQ ID NO: 154)	2	2	2	1	0.5
GN1118 (SEQ ID NO: 155)	2	2	2	1	0.5
GN1119 (SEQ ID NO: 63)	2	1	2	4	1
GN1120 (SEQ ID NO: 149)	2	8	2	2	1
GN1125 (SEQ ID NO: 64)	<0.5	<0.5	<0.5	<0.5	<0.5
GN1140 (SEQ ID NO: 172)	1	1	1	0.5	0.5
GN1236 (SEQ ID NO: 156)	2	2	1	1	2
GN1238 (SEQ ID NO: 157)	2	2	2	2	1
GN1239 (SEQ ID NO: 158)	0.5	0.5	1	0.25	0.5
GN1250 (SEQ ID NO: 159)	2	1	2	<0.5	1
GN1251 (SEQ ID NO: 160)	2	2	4	<0.5	2
GN1252 (SEQ ID NO: 161)	2	2	2	1	1
GN1253 (SEQ ID NO: 162)	0.5	0.5	0.5	0.25	0.25
GN1283 (SEQ ID NO: 163)	0.5	0.5	0.5	0.25	0.125
GN1287 (SEQ ID NO: 164)	2	1	1	0.25	0.25
GN1313 (SEQ ID NO: 165)	0.25	1	0.5	0.25	0.125
GN1316 (SEQ ID NO: 166)	0.5	1	1	0.5	0.25
GN1328 (SEQ ID NO: 167)	0.5	0.5	0.5	0.25	0.25
GN1336 (SEQ ID NO: 168)	0.5	1	0.5	0.25	0.25
GN1339 (SEQ ID NO: 169)	1	2	1	0.25	0.25
GN1344 (SEQ ID NO: 170)	0.5	0.5	0.5	0.25	0.5

[00203] As shown above in Table 11, GN873 and GN916 demonstrate lytic activity against multiple *Burkholderia* species.

Example 3 – Direct Lytic Agents’ activity against *B. cenocepacia* complex

5 [00204] Various engineered Gram-negative lysins were evaluated for activity against *B. cenocepacia* and *B. multivorans*, and the results are shown in Table 12 below. All of *B. cenocepacia*, *B. cepacia*, and *B. multivorans*, as well as *B. contaminans*, *B. ambifaria*, and *B. ubonensis* are phylogenetically similar as part of the *B. cenocepacia* complex.

10 [00205] Table 12 – MIC ($\mu\text{g/mL}$) activity for various lysins of the invention against *B. cenocepacia* complex bacteria

Lysin	<i>B. cenocepacia</i> (CFS-2131)	<i>B. multivorans</i> (CFS-1946)
GN585 (SEQ ID NO: 72)	4	2
GN686 (SEQ ID NO: 73)	2	1
GN693 (SEQ ID NO: 55)	2	1
GN694 (SEQ ID NO: 56)	1	0.5
GN727 (SEQ ID NO: 74)	16	1
GN758 (SEQ ID NO: 75)	4	1
GN764 (SEQ ID NO: 76)	2	1
GN765 (SEQ ID NO: 77)	2	1
GN873 (SEQ ID NO: 78)	1	1
GN916 (SEQ ID NO: 79)	4	1

[00206] As shown above in Table 12 above, both GN873 and GN916 demonstrate strong antibacterial activity against *B. cenocepacia* complex bacteria.

15 **Example 4 – Direct Lytic Agents’ activity against ESKAPE pathogens, *Stenotrophomonas melophilis*, *Escherichia coli* and *Serratia marcescens***

[00207] Various engineered Gram-negative lysins were evaluated for activity against *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *E. cloacae* and the results are shown in Tables 13-15 below. Likewise, the Gram-negative lysins were evaluated for activity against *Serratia marcescens*, and the results shown in Table 16 below, and various Gram-negative lysins were
20 evaluated for activity against *S. maltophilia* and *E. coli*, and the results shown in Table 17 below.
Table 13 – MIC ($\mu\text{g/mL}$) activity for engineered lysin polypeptide constructs against ESKAPE pathogens

Lysin	<i>P. aeruginosa</i>					<i>A. baumannii</i>		<i>K. pneumoniae</i>	
	1766	1595	2465	38	1558	30	1770	1139	1593
GN585 (SEQ ID NO: 72)	0.5	0.5	--	--	--	0.25	0.25	0.5	0.5
GN686 (SEQ ID NO: 73)	0.25	0.5	--	--	--	0.25	0.5	0.5	2
GN693 (SEQ ID NO: 55)	0.5	0.5	--	--	--	0.25	0.25	0.5	0.5
GN694 (SEQ ID NO: 56)	0.5	0.5	--	--	--	0.25	0.5	1	0.5
GN1074 (SEQ ID NO: 62)	1	--	1	1	1	0.5			
GN1119 (SEQ ID NO: 63)	1	--	1	1	1	1			
GN1120 (SEQ ID NO: 149)	1	--	1	2	1	0.5			
GN1125 (SEQ ID NO: 64)	2	--	1	1	0.5	0.5			

Table 14 – MIC (μg/mL) activity for engineered lysin polypeptide constructs against ESKAPE pathogens

Lysin	<i>P. aeruginosa</i>				<i>A. baumannii</i>			<i>K. pneumoniae</i>		
	PA01	PA20	AR769	ATCC 27853	ATCC BAA 747	CCUG 890	CCUG 61012	ATCC 700603	AR 115	AR 117
GN693 (SEQ ID NO: 55)	1	0.5	1	0.5	0.25	0.5	1	0.5	0.5	1
GN694 (SEQ ID NO: 56)	<0.5	<0.5	<0.5	1	1	1	1	<0.5	<0.5	<0.5
GN751	1	2	2	1	<0.5	<0.5	1	1	1	1

(SEQ ID NO: 57)										
GN757 (SEQ ID NO: 58)	1	1	1	2	0.25	0.5	1	1	1	1
GN1040 (SEQ ID NO: 59)	1	1	2	2	0.25	0.5	1	1	1	1
GN1041 (SEQ ID NO: 60)	1	1	2	1	0.16	1	2	1	1	1
GN1072 (SEQ ID NO: 61)	1	<0.5	1	1	<0.5	1	4	1	1	<0.5
GN569 (SEQ ID NO: 7)	8	16	32	4	<0.5	<0.5	<0.5	16	4	16
GN737 (SEQ ID NO: 65)	8	16	16	8	<0.5	1	<0.5	16	8	16
GN808 (SEQ ID NO: 66)	1	0.5	1	1	0.25	0.5	1	1	1	1
GN848 (SEQ ID NO: 67)	1	0.5	1	1	0.5	0.5	1	1	1	1
GN887 (SEQ ID NO: 68)	1	<0.5	1	2	<0.5	<0.5	<0.5	4	8	4
GN907 (SEQ ID NO: 69)	0.12	0.25	0.5	2	0.25	0.5	1	2	1	1
GN918 (SEQ ID NO:	1	1	<0.5	2	<0.06	0.5	0.16	2	2	2

70)										
GN937 (SEQ ID NO: 71)	4	4	4	4	0.5	2	2	8	16	8

Table 15 – MIC ($\mu\text{g/mL}$) activity for engineered lysin polypeptide constructs against ESKAPE pathogens

Lysin	<i>A. baumannii</i>				<i>K. pneumoniae</i>					<i>E. cloacae</i>		
	1771	1960	1570	2488	2365	2367	807	1589	1592	1985	1240	2386
GN1074 (SEQ ID NO: 62)	2	1	1	2	1	1	1	1	0.5	1	1	1
GN1119 (SEQ ID NO: 63)	1	0.5	1	1	1	2	1	0.5	1	2	2	1
GN1120 (SEQ ID NO: 149)	2	0.5	1	1	1	1	1	0.5	0.5	2	1	1
GN1125 (SEQ ID NO: 64)	1	1	1	1	1	1	1	0.5	0.5	1	1	1

5 Table 16 – MIC ($\mu\text{g/mL}$) activity for engineered lysin polypeptide constructs against *Serratia marcescens*

Lysin	<i>Serratia marcescens</i>							
	1871	1872	2371	2372	2373	2374	2380	2381
GN585 (SEQ ID NO: 72)	<0.5	0.5	2	2	2	2	2	2
GN686 (SEQ ID NO: 73)	<0.5	0.25	4	2	2	2	2	4
GN693 (SEQ ID NO: 55)	<0.5	0.125	1	1	1	1	2	1
GN694 (SEQ ID NO: 56)	<0.5	0.25	1	0.5	1	1	1	1

Table 17 – MIC ($\mu\text{g/mL}$) activity for engineered lysin polypeptide constructs against *S. maltophilia*

Lysin	<i>Stenotrophomonas maltophilia</i>			<i>Escherichia coli</i>		
	1527	2115	1612	2369	2378	813
GN1074 (SEQ ID NO: 62)	<0.5	2	2	0.5	0.5	0.5
GN1119 (SEQ ID NO: 63)	1	2	2	0.125	0.5	0.5
GN1120 (SEQ ID NO: 149)	1	2	2	0.125	0.25	0.5
GN1125 (SEQ ID NO: 64)	1	2	1	0.125	0.25	0.25

[00208] As shown in Tables 13-17, all of GN585, GN686, GN693, and GN694 demonstrate strong activity against a range of ESKAPE pathogens, as well as *S. marcescens*, and all of GN1074, GN1119, GN1120, and GN1125 further demonstrate strong activity against a range of ESKAPE pathogens, as well as *E. coli*, and *S. maltophilia*.

Example 5 – Identification of *Yersinia* lysins and additional *Burkholderia* lysins – Screening Native and Engineered Derivatives and Determining Characteristics

[00209] A structure and function guided screening approach was used to engineer and identify lysin polypeptides active versus attenuated (BSL2) strains of *Y. pestis* (N=5) and representative isolates of the *B. cepacia* complex, including *B. cepacia*, *B. cenocepacia*, and *B. multivorans*, as well as the *B. pseudomallei* complex, including *B. thailandensis* and *B. humptydooensis*. Compounds were purified by ion-exchange chromatography, and MICs were determined by broth microdilution $\pm$ human serum. Time-kill assays were performed in the same media over 24 hours. The fluorometric uptake assay for N-Phenyl-1-naphthylamine (NPN) was performed as described by Helander and Mattila-Sandholm, 2000 (PMID: 10735988). Hemolysis of human red blood cells (hRBCs) was examined according to the method of Lv et al., 2014 (PMID: 24466055).

[00210] Over 200 compounds were tested against *Y. pestis*, and 8 lead lysins were selected based on low MIC values determined $\pm$ human serum, compared to non-engineered controls. Over 300 compounds were tested against five *Burkholderia* species, and 10 lead lysins were likewise selected. Three leads were active against both *Y. pestis* and *Burkholderia* spp. Time-kill

assays conducted against *Y. pestis*, *B. thailandensis*, and *B. humptydooensis* demonstrated significant bactericidal activity (reductions of $\geq 3\text{-log}_{10}$ CFU/mL) $\pm$ human serum within 24 hours. Bactericidal activity was consistent with rapid outer membrane (OM) permeabilization observed in the NPN assay. No hemolysis of hRBCs was observed with all lead compounds (highest concentration tested was 128 $\mu\text{g/mL}$).

[00211] Table 18 below shows the MICs ($\mu\text{g/mL}$) against *Burkholderia cepacia* complex organisms, and Table 19 below shows the MICs ($\mu\text{g/mL}$) against *Burkholderia pseudomallei* complex organisms. For comparison, MICs typical for native (unmodified) lysins are shown for GN685 (an non-engineered progenitor of GN693). MICs are also indicated for GN370, as disclosed, for example, in in PCT Publication No. WO 2020/046747, incorporated by reference herein.

[00212] Table 18 – MICs ($\mu\text{g/mL}$) against *Burkholderia cepacia* complex organisms

Lysin	<i>B. cepacia</i>			<i>B. cenocepacia</i>				<i>B. multivorans</i>			
	NR-707	CCUG 59169	ATCC 39277	AU 39527	CCUG 61185	NR-701	AU 24619	ATC C BAA-247	NR-20534	AU 28638	AU 34527
GN693 (SEQ ID NO: 55)	1	4	1	16	0.5	1	1	0.5	1	1	1
GN694 (SEQ ID NO: 56)	1	4	2	4	<0.5	1	2	0.5	1	1	1
GN751 (SEQ ID NO: 57)	1	4	2	4	1	1	0.5	0.12	1	1	<0.5
GN757 (SEQ ID NO: 58)	1	4	1	8	0.25	1	1	0.25	2	2	1
GN1040 (SEQ ID NO: 59)	2	16	2	0.25	0.5	1	2	1	2	2	2
GN1041 (SEQ ID NO: 60)	2	4	1	>32	0.5	1	2	2	1	1	1
GN1072 (SEQ ID NO: 61)	2	16	1	4	0.5	1	2	0.5	1	1	<0.5
GN1074 (SEQ ID NO: 62)	<0.5	2	2	4	0.5	1	1	<0.5	1	1	1

NO: 62)											
GN1119 (SEQ ID NO: 63)	1	4	2	8	0.5	1	1	1	1	0.5	2
GN1125 (SEQ ID NO: 64)	0.5	0.5	1	1	0.25	0.5	0.5	0.25	1	0.25	0.5
GN685 (SEQ ID NO: 2)	>256	>256	>256	>256	128	8	>256	128	>256	>256	>256
GN370	32	16	8	128	2	2	8	4	4	8	4

[00213] Table 19 – MICs (μg/mL) against *Burkholderia pseudomallei* complex organisms

Lysin	<i>B. thailandensis</i>				<i>B. humptydooensis</i>
	NR-704	NR-9908	NR-9909	ATCC 700388	ATCC BAA-2767
GN693 (SEQ ID NO: 55)	1	2	2	1	0.5
GN694 (SEQ ID NO: 56)	1	2	2	1	1
GN751 (SEQ ID NO: 57)	1	1	0.5	1	0.5
GN757 (SEQ ID NO: 58)	2	1	1	2	1
GN1040 (SEQ ID NO: 59)	2	4	2	2	1
GN1041 (SEQ ID NO: 60)	2	4	2	1	1
GN1072 (SEQ ID NO: 61)	1	1	1	1	0.5
GN1074 (SEQ ID NO: 62)	2	1	<0.5	1	1
GN1119 (SEQ ID NO: 63)	1	1	1	1	0.5
GN1125 (SEQ ID NO: 64)	0.5	0.5	0.5	1	0.25
GN685 (SEQ ID NO: 2)	>256	>256	>256	16	>256
GN370	32	16	64	16	32

[00214] Table 20 below shows the MICs (μg/mL) against *Yersinia pestis*. MICs were also
5 determined in the absence and presence of human serum (“-” indicates no serum, and “+”
indicates human serum) for various *Y. pestis* strains, as shown in Table 21 below. For all strains
tested and shown in Table 21 (with examples shown for A1122 and KIM10+), serum does not
inhibit the engineered lysin polypeptide construct activity. A similar effect has been observed for
GN370 and exebacase (Schuch et al., 2022, PMID: 35308352; Indiani et al., 2019, PMID:
10 30670427). The results are shown below in Table 20.

[00215] Table 20 – MICs (μg/mL) against *Yersinia pestis* organisms

Lysin	NR636	NG636 (+10% Human serum)	NR-639	NR4693
GN693 (SEQ ID NO: 55)	8	2	<0.5	1

GN694 (SEQ ID NO: 56)	8	1	<0.5	<0.5
GN751 (SEQ ID NO: 57)	16	>256	0.5	2
GN757 (SEQ ID NO: 58)	32	8	<0.5	<0.5
GN1040 (SEQ ID NO: 59)	16	4	0.12	0.25
GN1041 (SEQ ID NO: 60)	32	32	2	4
GN1072 (SEQ ID NO: 61)	32	32	1	4
GN569 (SEQ ID NO: 7)	16	<0.5	<0.5	<0.5
GN737 (SEQ ID NO: 65)	2	<0.5	<0.5	<0.5
GN808 (SEQ ID NO: 66)	4	2	0.5	0.25
GN848 (SEQ ID NO: 67)	4	1	0.5	0.25
GN887 (SEQ ID NO: 68)	4	<0.5	<0.5	<0.5
GN907 (SEQ ID NO: 69)	8	<0.5	<0.5	<0.5
GN918 (SEQ ID NO: 70)	2	<0.5	<0.5	<0.5
GN937 (SEQ ID NO: 71)	2	<0.5	<0.5	<0.5

[00216] Table 21 – MICs (µg/mL) against *Y. pestis*

Lysin	A1122		KIM10+		Harbin 35	A12 (D5)	Yokohama (D11)	Kimberley (D12)
	-	+	-	+	-	-	-	-
GN569 (SEQ ID NO: 7)	16	<0.5	32	2	0.06	0.03	0.015	0.015
GN737 (SEQ ID NO: 65)	2	<0.5	8	2	0.5	0.12	0.5	0.03
GN808 (SEQ ID NO: 66)	4	2	8	8	1	0.12	0.5	0.5
GN848 (SEQ ID NO: 67)	4	1	8	8	1	0.06	0.25	0.5
GN887 (SEQ ID NO: 68)	4	<0.5	8	<0.5	0.007	0.03	0.003	0.0017
GN907 (SEQ ID NO: 69)	8	<0.5	8	1	1	0.0035	0.25	0.5
GN918 (SEQ ID NO: 70)	2	<0.5	1	<0.06	0.03	0.0035	0.003	0.06
GN937 (SEQ ID NO: 71)	2	<0.5	2	2	0.5	0.03	0.12	0.5

[00217] A kinetic analysis of killing was performed for several engineered lysin polypeptide constructs by time-kill assay. Rapid bactericidal activity (decreases of $\geq 3\text{-log}_{10}$ CFU/mL compared to starting concentrations) was observed in each case. Representative examples are shown for GN751 (SEQ ID NO: 57) and GN757 (SEQ ID NO: 58) against *B. humptydooensis* (as shown in Figures 1 and 2, respectively) and *B. thailandensis* (as shown in the Figures 3 and 4, respectively), which were bactericidal by 1 hour. Regrowth was not associated with changes in MIC or colony morphology (*i.e.*, not small colony variants).

[00218] The ability of various engineered lysin polypeptide constructs to disrupt the OM of *Burkholderia* spp. and *Y. pestis* was examined using the NPN uptake assay. If the OM is damaged or functionally compromised by pore formation, NPN partitions into the OM, exhibiting strong fluorescence. Rapidly induced fluorescence was observed for each construct examined, consistent with pore formation. Examples for GN751 (SEQ ID NO: 57) and GN808 (SEQ ID NO: 66) are shown for *B. thailandensis* and *Y. pestis* in Figure 5 and Figure 6, respectively.

[00219] Engineered lysins were tested for hemolytic activity. No significant hemolytic activity was observed for any of the engineered lysins polypeptide constructs tested, with a highest concentration tested of 128 $\mu\text{g/mL}$. A representative example is shown for GN751 (SEQ ID NO: 57) in Figure 7. RR12Wpolar, a positive control peptide, is hemolytic, as described (Mohanram and Bhattacharjya; PMID: 26849911), and as shown in Figure 8. Table 22 below shows the percent hemolysis observed for various engineered lysin constructs tested.

[00220] Table 22 – Percent Hemolysis of Engineered Lysin Polypeptide Constructs

Lysin	Percent hemolysis
GN569 (SEQ ID NO: 7)	-0.09
GN693 (SEQ ID NO: 55)	-0.21
GN694 (SEQ ID NO: 56)	-0.92
GN737 (SEQ ID NO: 65)	-0.53
GN751 (SEQ ID NO: 57)	-0.52
GN757 (SEQ ID NO: 58)	-0.37
GN808 (SEQ ID NO: 66)	-0.60
GN848 (SEQ ID NO: 67)	-0.10
GN887 (SEQ ID NO: 68)	-0.47
GN907 (SEQ ID NO: 69)	-0.60
GN918 (SEQ ID NO: 70)	-1.42
GN937 (SEQ ID NO: 71)	-1.28
GN1040 (SEQ ID NO: 59)	-2.25

GN1041 (SEQ ID NO: 60)	-2.11
GN1072 (SEQ ID NO: 61)	-1.24
GN1074 (SEQ ID NO: 62)	-0.09
GN1119 (SEQ ID NO: 63)	-0.14
GN1120 (SEQ ID NO: 149)	-0.16
GN1125 (SEQ ID NO: 64)	0.14

Example 6 – Further Characterization of Engineered Lysin Polypeptide Constructs

[00221] Exemplary engineered lysin polypeptide constructs as disclosed herein are further described in terms of their predicted pI (isoelectric point) and predicted molecular weight, in Daltons, as shown below in Table 23.

[00222] Table 23 – Predicted pI and Molecular Weight of Engineered Lysin Polypeptide Constructs

Lysin	Predicted pI (Expasy)	Predicted molecular weight in Daltons (Expasy)
GN693 (SEQ ID NO: 55)	10.21	36440
GN694 (SEQ ID NO: 56)	10.54	36440
GN751 (SEQ ID NO: 57)	10.92	21843
GN757 (SEQ ID NO: 58)	9.75	34798
GN1040 (SEQ ID NO: 59)	9.73	34157
GN1041 (SEQ ID NO: 60)	11.5	21496
GN1072 (SEQ ID NO: 61)	10.89	21990
GN1074 (SEQ ID NO: 62)	11.01	22079
GN1119 (SEQ ID NO: 63)	10.96	21692
GN1125 (SEQ ID NO: 64)	11.32	22254
GN569 (SEQ ID NO: 7)	9.84	14051
GN737 (SEQ ID NO: 65)	9.6	14268
GN808 (SEQ ID NO: 66)	10.19	17295
GN848 (SEQ ID NO: 67)	10.75	17799
GN887 (SEQ ID NO: 68)	10.03	20759
GN907 (SEQ ID NO: 69)	9.92	18240
GN918 (SEQ ID NO: 70)	9.95	21219
GN937 (SEQ ID NO: 71)	9.71	24623
GN568 (SEQ ID NO: 1)	9.1	15721
GN685 (SEQ ID NO: 2)	9.58	14477
GN686 (SEQ ID NO: 73)	10.17	16740
GN687 (SEQ ID NO: 108)	10.21	17574
GN688 (SEQ ID NO: 109)	10.08	17446
GN689 (SEQ ID NO: 110)	11.38	20169
GN690 (SEQ ID NO: 111)	11.38	20226
GN692 (SEQ ID NO: 112)	11.3	19327

GN695 (SEQ ID NO: 113)	10.09	19069
GN696 (SEQ ID NO: 114)	9.94	17899
GN697 (SEQ ID NO: 115)	9.94	18087
GN867 (SEQ ID NO: 13)	9.22	18126
GN869 (SEQ ID NO: 116)	9.83	22944
GN870 (SEQ ID NO: 117)	9.88	23556
GN871 (SEQ ID NO: 118)	9.88	23556
GN872 (SEQ ID NO: 108)	9.72	23476
GN873 (SEQ ID NO: 78)	10.22	22182
GN874 (SEQ ID NO: 120)	10.19	21626
GN875 (SEQ ID NO: 121)	10.07	22159
GN905 (SEQ ID NO: 122)	9.64	23348
GN916 (SEQ ID NO: 79)	10.14	23468
GN1108 (SEQ ID NO: 171)	11.5	21496
GN1117 (SEQ ID NO: 154)	10.46	20252
GN1118 (SEQ ID NO: 155)	10.48	20808
GN1120 (SEQ ID NO: 149)	10.96	20142
GN1140 (SEQ ID NO: 172)	10.71	20516
GN1236 (SEQ ID NO: 156)	11.31	22097
GN1238 (SEQ ID NO: 157)	11.23	22081
GN1239 (SEQ ID NO: 158)	10.93	22556
GN1250 (SEQ ID NO: 159)	10.92	21834
GN1251 (SEQ ID NO: 160)	10.96	21683
GN1252 (SEQ ID NO: 161)	10.96	20133
GN1253 (SEQ ID NO: 162)	11.32	22245
GN1283 (SEQ ID NO: 163)	10.98	22504
GN1287 (SEQ ID NO: 164)	10.56	21942
GN1313 (SEQ ID NO: 165)	11.67	22490
GN1316 (SEQ ID NO: 166)	11.49	21928
GN1328 (SEQ ID NO: 168)	10.24	23542
GN1336 (SEQ ID NO: 169)	10.44	24564
GN1339 (SEQ ID NO: 170)	10.18	24002
GN1344 (SEQ ID NO: 171)	10.32	27986

Example 6 – Lysins having activity against *B. pseudomallei*

[00223] Various engineered Gram-negative lysins were evaluated for activity against attenuated (BSL3) strains of *B. pseudomallei*, and the results are shown in Table 24 (24 hours) and Table 25 (48 hours) below.

[00224] Table 24 – MICs (μg/mL) against BSL3 strains of *B. pseudomallei* after 24 hours

Lysin	BSL3 strain								
	423	465a	503	644	730	E24	E40	E215	E27 7

GN1120 (SEQ ID NO: 149)	1	1	1	1	1	4	8	2	2
GN693 (SEQ ID NO: 55)	8	8	8	16	32	8	64	8	16
GN694 (SEQ ID NO: 56)	2	2	4	2	8	8	16	4	2
GN751 (SEQ ID NO: 57)	1	1	1	1	4	4	32	2	2
GN757 (SEQ ID NO: 58)	1	2	32	4	>64	16	64	2	4
GN1040 (SEQ ID NO: 59)	16	8	64	>64	>64	16	>64	4	16
GN1108 (SEQ ID NO: 171)	8	2	32	>64	>64	8	>64	2	64
GN1072 (SEQ ID NO: 61)	8	1	1	1	2	4	8	2	2
GN1074 (SEQ ID NO: 62)	1	1	1	1	2	4	8	1	2
GN1119 (SEQ ID NO: 63)	1	1	1	1	1	8	2	1	2
GN1125 (SEQ ID NO: 64)	0.5	1	1	1	1	2	4	1	1
GN370 (negative control)	>64	>64	>64	>64	>64	64	>64	>64	32
CF-296 (negative control)	>64	>64	>64	>64	>64	>64	>64	32	16
MMAV-1 (amurin)	>64	16	>64	>64	>64	>64	>64	1	>64
MMAV-2 (amurin)	1	2	8	4	64	2	16	1	1
BK9 (amurin)	1	1	2	2	32	8	16	1	1
Lysin	BSL3 strain								

	E280	E283	E300	E302	E304	576a	MIC50	MIC90	
GN1120 (SEQ ID NO: 149)	1	1	1	2	8	2	1	8	
GN693 (SEQ ID NO: 55)	2	16	16	16	32	16	16	32	
GN694 (SEQ ID NO: 56)	1	1	4	8	8	8	4	8	
GN751 (SEQ ID NO: 57)	2	1	1	2	16	2	2	16	
GN757 (SEQ ID NO: 58)	2	2	2	*	16	8	4	>64	
GN1040 (SEQ ID NO: 59)	4	4	4	32	32	32	16	>64	
GN1108 (SEQ ID NO: 171)	1	1	2	64	>64	64	32	>64	
GN1072 (SEQ ID NO: 61)	1	1	1	2	4	2	2	4	
GN1074 (SEQ ID NO: 62)	2	1	1	2	4	2	2	4	
GN1119 (SEQ ID NO: 63)	1	1	1	2	2	2	1	2	
GN1125 (SEQ ID NO: 64)	1	1	1	1	2	1	1	2	
GN370 (negative control)	>64	>64	>64	>64	64	>64	>64	>64	
CF-296 (negative control)	64	32	>64	>64	64	>64	>64	>64	
MMAV-1 (amurin)	2	4	8	32	>64	>64	>64	>64	
MMAV-2 (amurin)	1	1	1	4	32	4	2	32	
BK9	4	1	2	8	8	>64	2	32	

(amurin)									
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[00225] Table 25 – MICs (µg/mL) against BSL3 strains of *B. pseudomallei* after 48 hours

Lysin	423	465a	503	644	730	E24	E40	E215	E277
GN1120 (SEQ ID NO: 149)	0.5	1	1	1	2	4	4	2	2
GN693 (SEQ ID NO: 55)	8	8	8	16	32	8	64	8	16
GN694 (SEQ ID NO: 56)	4	4	4	8	16	8	16	2	4
GN751 (SEQ ID NO: 57)	1	2	2	2	16	4	32	2	2
GN757 (SEQ ID NO: 58)	16	2	>64	>64	>64	64	>64	2	4
GN1040 (SEQ ID NO: 59)	>64	>64	>64	>64	>64	32	>64	4	>64
GN1108 (SEQ ID NO: 171)	16	32	64	>64	>64	64	>64	2	>64
GN1072 (SEQ ID NO: 61)	0.5	1	4	4	8	4	>64	2	4
GN1074 (SEQ ID NO: 62)	1	2	2	2	4	8	>64	2	4
GN1119 (SEQ ID NO: 63)	1	1	1	1	2	8	4	1	2
GN1125 (SEQ ID NO: 64)	0.5	0.5	0.5	0.5	1	2	4	1	1
GN370 (negative control)	>64	>64	>64	>64	>64	>64	>64	>64	>64
CF-296 (negative control)	>64	>64	>64	>64	>64	>64	>64	>64	>64

MMAV-1 (amurin)	>64	>64	>64	>64	>64	>64	>64	4	>64
MMAV-2 (amurin)	4	4	16	32	>64	32	64	2	32
BK9 (amurin)	4	8	4	32	>64	16	>64	2	8
Lysin	E280	E283	E300	E302	E304	576a	MIC50	MIC 90	
GN1120 (SEQ ID NO: 149)	2	2	2	2	8	2	2	4	
GN693 (SEQ ID NO: 55)	8	16	16	16	32	16	16	32	
GN694 (SEQ ID NO: 56)	2	1	4	8	8	8	4	16	
GN751 (SEQ ID NO: 57)	2	2	2	8	32	4	2	32	
GN757 (SEQ ID NO: 58)	2	2	4	*	32	8	16	>64	
GN1040 (SEQ ID NO: 59)	4	8	>64	64	64	>64	>64	>64	
GN1108 (SEQ ID NO: 171)	2	2	>64	>64	>64	>64	>64	>64	
GN1072 (SEQ ID NO: 61)	2	1	2	2	8	2	2	8	
GN1074 (SEQ ID NO: 62)	2	2	2	2	8	4	2	8	
GN1119 (SEQ ID NO: 63)	2	1	1	2	2	4	2	4	
GN1125 (SEQ ID NO: 64)	1	1	1	1	16	1	1	4	
GN370	>64	>64	>64	>64	>64	>64	>64	>64	

(negative control)									
CF-296 (negative control)	>64	32	>64	>64	>64	>64	>64	>64	
MMAV-1 (amurin)	16	32	>64	32	>64	>64	>64	>64	
MMAV-2 (amurin)	2	2	2	4	32	16	16	64	
BK9 (amurin)	8	8	8	16	32	32	8	>64	

What is claimed is:

1. An engineered lysin comprising:

(a) a Gram-negative base lysin selected from GN685 (SEQ ID NO: 2), GN3(R100Q, R116Q) (SEQ ID NO: 177), GN515(R100D, R116H) (SEQ ID NO: 15), GN3 (SEQ ID NO: 3), GN3(R100Q, R116H) (SEQ ID NO: 14), GN515 (SEQ ID NO: 6), GN568 (SEQ ID NO: 1), GN428 (SEQ ID NO: 4), GN588 (SEQ ID NO: 5), GN569 (SEQ ID NO: 7), GN37 (SEQ ID NO: 8), QPI14001 (SEQ ID NO: 9), GN917 (SEQ ID NO: 10), CAH0532154 (SEQ ID NO: 11), GN577 (SEQ ID NO: 12), GN867 (SEQ ID NO: 13), GN4 (SEQ ID NO: 175), or GN4 (K100D, R116H) (SEQ ID NO: 176); and

(b) (1) one or more antimicrobial peptide (AMP) selected from BCM1 (SEQ ID NO: 18), BK3 (SEQ ID NO: 19), HIP_A (SEQ ID NO: 27), HIP_{ABC} (SEQ ID NO: 28), HIP_{AB} (SEQ ID NO: 181), BK5 (SEQ ID NO: 17), AM1 (SEQ ID NO: 123), AM13 (SEQ ID NO: 124), MMAV-2 (SEQ ID NO: 125), MFVI-3 (SEQ ID NO: 126), BK3h2 (SEQ ID NO: 178), BK3h3 (SEQ ID NO: 179), ATRA (SEQ ID NO: 21), ATRA-1A Δ aa2-9 (SEQ ID NO: 22), ATRA-1A (SEQ ID NO: 23), PMAP-36 (SEQ ID NO: 24), RI18 (SEQ ID NO: 25), H2A (SEQ ID NO: 26), HIP_{Anat} (SEQ ID NO: 180), BK9 (SEQ ID NO: 29); and/or

(b) (2) a cell penetrating peptide (CPP) selected from Zf5.3 (SEQ ID NO: 38), PRR (SEQ ID NO: 39), PRR₅ (SEQ ID NO: 40), or PRR₃ (SEQ ID NO: 48); or

an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

2. The engineered lysin of claim 1, further comprising one or more outer membrane binding motif.

3. The engineered lysin of claim 1 or 2, wherein the outer membrane binding motif is selected from FMRL_{Nm} (SEQ ID NO: 147), FIRL_{Bc} (SEQ ID NO: 143), FMRL_{Yp} (SEQ ID NO: 144), FIRL (SEQ ID NO: 146), or FIQL_{Bc} (SEQ ID NO: 173).

4. The engineered lysin of any one of claims 1-3, further comprising at least one linker.

5. The engineered lysin of claim 4, wherein the at least one linker is selected from GT, GGGGSGGGGSGGGGS (SEQ ID NO: 43), LSKLGG (SEQ ID NO: 45),
5 LSKLGGLGGPRLGG (SEQ ID NO: 46), LGGLGG (SEQ ID NO: 47), G, or SKY.

6. The engineered lysin of any one of claims 1-5, wherein the Gram-negative base lysin is selected from GN515(R100D,R116H) (SEQ ID NO: 15), GN3(R100Q,R116H) (SEQ ID NO: 14), or GN3 (SEQ ID NO: 3).

10

7. The engineered lysin of any one of claims 1-5, wherein the Gram-negative base lysin is selected from GN685 (SEQ ID NO: 2), GN917 (SEQ ID NO: 10), QP14001 (SEQ ID NO: 9), or GN867 (SEQ ID NO: 13), GN569(K87H) (SEQ ID NO: 16), GN37 (SEQ ID NO: 8), GN685(K78H) (SEQ ID NO: 106), GN428 (SEQ ID NO: 4), or CAH0532154 (SEQ ID NO: 11),
15 or GN588 (SEQ ID NO: 5).

8. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN685 (SEQ ID NO: 2), and

(b) an AMP comprising BCM1 (SEQ ID NO: 18); or

20 an active fragment thereof, or a variant thereof having at least 80% identity with said engineered lysin.

9. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN3(R100Q, R116H) (SEQ ID NO: 14),

25 (b) an AMP comprising BK3 (SEQ ID NO: 19), and

(c) an outer membrane binding motif comprising FIQL_{BC} (SEQ ID NO: 173); or

an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

30 10. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN428 (SEQ ID NO: 4),

(b) an AMP comprising BK3 (SEQ ID NO: 19), and

(c) an outer membrane binding motif comprising FIQL_{BC} (SEQ ID NO: 173); or
an active fragment thereof, or a variant thereof having at least 80% sequence identity
with said engineered lysin.

5

11. The engineered lysin of claim 10, further comprising a linker comprising G.

12. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN515(R100D, R116H) (SEQ ID NO: 15),

10 (b) an AMP comprising BK3 (SEQ ID NO: 19), and

(c) an outer membrane binding motif comprising FMRL_{Nm} (SEQ ID NO: 147); or

an active fragment thereof, or a variant thereof having at least 80% sequence identity
with said engineered lysin.

15 13. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN3(R100Q, R116H) (SEQ ID NO: 14), and

(b) an AMP comprising HIP_{ABC} (SEQ ID NO: 28), or

an active fragment thereof, or a variant thereof having at least 80% sequence identity
with said engineered lysin.

20

14. The engineered lysin of claim 13, further comprising a linker comprising GT.

15. The engineered lysin of claim 1, wherein the engineered lysin comprises:

(a) a Gram-negative base lysin comprising GN3(R100Q, R116H) (SEQ ID NO: 14), and

25 (b) an AMP comprising HIP_{AB} (SEQ ID NO: 181); or

an active fragment thereof, or a variant thereof having at least 80% sequence identity
with said engineered lysin.

16. The engineered lysin of claim 1, wherein the engineered lysin comprises:

30 (a) a Gram-negative base lysin comprising GN3(R100Q, R116H) (SEQ ID NO: 14), and

(b) two AMPs comprising HIP_A (SEQ ID NO: 27) and BK3 (SEQ ID NO: 19), or

an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

17. The engineered lysin of claim 16, further comprising a linker comprising GT.

18. The engineered lysin of claim 1, wherein the engineered lysin is selected from GN1074 (SEQ ID NO: 62), GN1119 (SEQ ID NO: 63), GN1125 (SEQ ID NO: 64), or GN1120 (SEQ ID NO: 149), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

19. The engineered lysin of claim 1, wherein the engineered lysin is selected from GN694 (SEQ ID NO: 56), GN751 (SEQ ID NO: 57), GN757 (SEQ ID NO: 58), GN693 (SEQ ID NO: 55), GN1040 (SEQ ID NO: 59), GN1041 (SEQ ID NO: 60), GN1072 (SEQ ID NO: 61), GN737 (SEQ ID NO: 65), GN808 (SEQ ID NO: 66), GN848 (SEQ ID NO: 67), GN887 (SEQ ID NO: 68), GN907 (SEQ ID NO: 69), GN918 (SEQ ID NO: 70), GN937 (SEQ ID NO: 71), GN585 (SEQ ID NO: 72), GN686 (SEQ ID NO: 73), GN727 (SEQ ID NO: 74), GN758 (SEQ ID NO: 75), GN764 (SEQ ID NO: 76), GN765 (SEQ ID NO: 77), GN873 (SEQ ID NO: 78), GN916 (SEQ ID NO: 79), GN687 (SEQ ID NO: 108), GN688 (SEQ ID NO: 109), GN689 (SEQ ID NO: 110), GN690 (SEQ ID NO: 111), GN692 (SEQ ID NO: 112), GN695 (SEQ ID NO: 113), GN696 (SEQ ID NO: 114), GN697 (SEQ ID NO: 115), GN869 (SEQ ID NO: 116), GN870 (SEQ ID NO: 117), GN871 (SEQ ID NO: 118), GN872 (SEQ ID NO: 119), GN874 (SEQ ID NO: 120), GN875 (SEQ ID NO: 121), GN905 (SEQ ID NO: 122), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

20. The engineered lysin of claim 1, wherein the engineered lysin is selected from GN1127 (SEQ ID NO: 150), GN1071 (SEQ ID NO: 151), GN1077 (SEQ ID NO: 152), GN1078 (SEQ ID NO: 153), GN1117 (SEQ ID NO: 154), GN1118 (SEQ ID NO: 155), GN1236 (SEQ ID NO: 156), GN1238 (SEQ ID NO: 157), GN1239 (SEQ ID NO: 158), GN1250 (SEQ ID NO: 159), GN1251 (SEQ ID NO: 160), GN1252 (SEQ ID NO: 161), GN1253 (SEQ ID NO: 162), GN1283 (SEQ ID NO: 163), GN1287 (SEQ ID NO: 164), GN1313 (SEQ ID NO: 165), GN1316 (SEQ ID NO: 166), GN1328 (SEQ ID NO: 167), GN1336 (SEQ ID NO: 168), GN1339 (SEQ ID NO:

169), GN1344 (SEQ ID NO: 170), GN1108 (SEQ ID NO: 171), GN1140 (SEQ ID NO: 172), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

21. The engineered lysin of claim 1, wherein the engineered lysin is selected from GN694 (SEQ ID NO: 56), GN751 (SEQ ID NO: 57), GN1074 (SEQ ID NO: 62), GN1119 (SEQ ID NO: 63), GN1125 (SEQ ID NO: 64), and GN1120 (SEQ ID NO: 149), or an active fragment thereof, or a variant thereof having at least 80% sequence identity with said engineered lysin.

22. An isolated polynucleotide comprising a nucleic acid molecule encoding the engineered lysin according to any of claims 1-21.

23. The isolated polynucleotide according to claim 22, wherein the isolated polynucleotide comprises cDNA.

24. A recombinant vector comprising the isolated polynucleotide according to claim 22 or 23.

25. The recombinant vector according to claim 24, wherein the isolated polynucleotide sequence is operatively linked to a promoter.

26. The recombinant vector according to claim 25, wherein the promoter is a heterologous promoter.

27. The recombinant vector according to any one of claims 24 to 26, wherein the recombinant vector is a recombinant expression vector.

28. An isolated host cell comprising the recombinant vector of claim 27.

29. A composition comprising a carrier and the engineered lysin according to any of claims 1-21, GN569 or GN568.

30. The composition according to claim 29, wherein the carrier is a pharmaceutically acceptable carrier.

31. The engineered lysin according to any one of claims 1-21, for use in a method of treating a bacterial infection caused by at least one Gram-negative bacteria, which method comprises administering to a subject in need thereof an effective amount of said engineered lysin.

5

32. The engineered lysin according to any one of claims 1-21, for use in a method of killing at least one Gram-negative bacteria, which method comprises administering to a subject in need thereof an effective amount of said engineered lysin.

10 33. A non-therapeutic method of killing at least one Gram-negative bacteria, which method comprises contacting an effective amount of the engineered lysin according to any one of claims 1 to 21 to the Gram-negative bacteria.

15 34. The engineered lysin for use according to any one of claim 31 or 32, or the method according to claim 33, wherein the at least one Gram-negative bacteria is a Gram-negative ESKAPE bacteria.

20 35. The engineered lysin for use according to any one of claim 31 or 32, or the method according to claim 33, wherein the at least one Gram-negative bacteria is selected from *Burkholderia* spp., *Yersinia* spp., *Acinetobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Stenotrophomonas* spp., *Escherichia* spp., *Serratia* spp., or *Pseudomonas* spp.

25 36. The engineered lysin for use according to claim 35, or the method of claim 35, wherein the *Burkholderia* spp. are selected from *B. cepacia*, *B. cenocepacia*, *B. thailandensis*, *B. multivorans*, *B. pseudomallei*, or *B. humptydooensis*.

37. The engineered lysin for use according to claim 35, or the method of claim 35, wherein the *Yersinia* spp. is *Yersinia pestis*.

30 38. The engineered lysin for use according to claim 35, or the method of claim 35, wherein the *Acinetobacter* spp. is *A. baumannii*.

39. The engineered lysin for use according to claim 35, or the method of claim 35, wherein the *Klebsiella* spp. is *K. pneumoniae*.

40. The engineered lysin for use according to claim 35, or the method of claim 35, wherein
5 the *Pseudomonas* spp. is *P. aeruginosa*.

41. The engineered lysin for use according to any one of claims 31 to 40, wherein the subject is a human.

10 42. The engineered lysin for use according to any one of claims 31 to 40, wherein the bacterial infection is selected from respiratory tract infections, such as respiratory tract infection in a patient having cystic fibrosis (CF), pulmonary exacerbations in CF patients and/or patients with decline in lung function and mortality; lower respiratory tract infections, such as acute exacerbation of chronic bronchitis (ACEB); acute sinusitis; community-acquired pneumonia
15 (CAP); hospital-acquired pneumonia (HAP); nosocomial respiratory tract infections; non-cystic fibrosis lung diseases such as bronchiectasis and/or acute pneumonias; sexually transmitted diseases, such as gonococcal cervicitis and gonococcal urethritis; urinary tract infections; acute otitis media; sepsis including neonatal septicemia and catheter-related sepsis; osteomyelitis; tuberculosis; and non-tuberculosis mycobacteria infections.

20

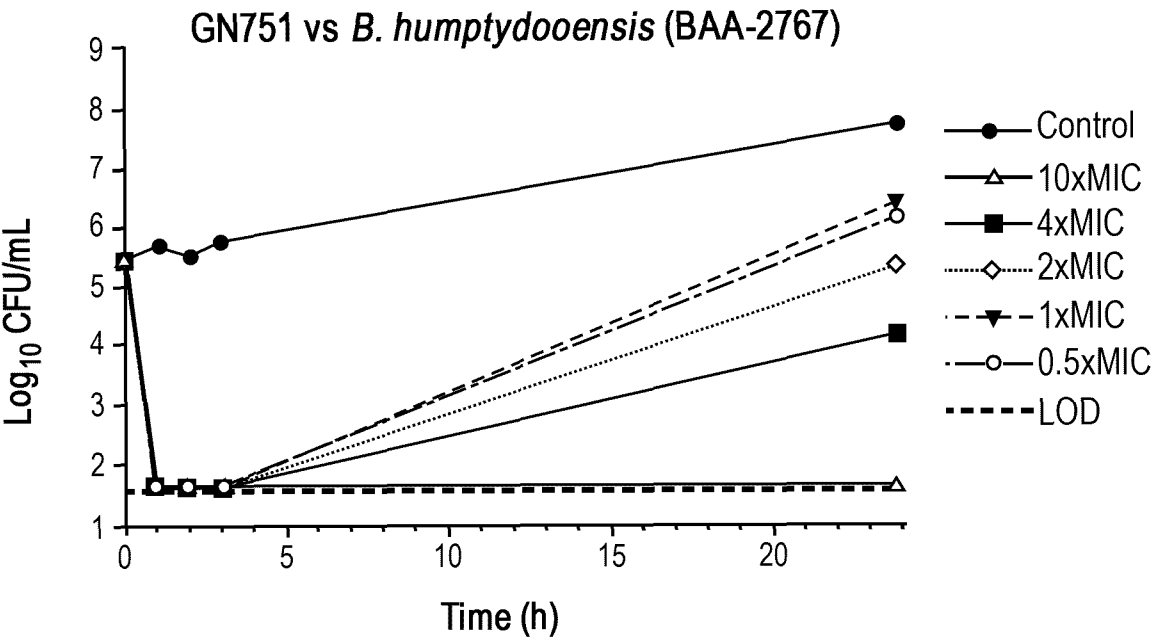


FIG. 1

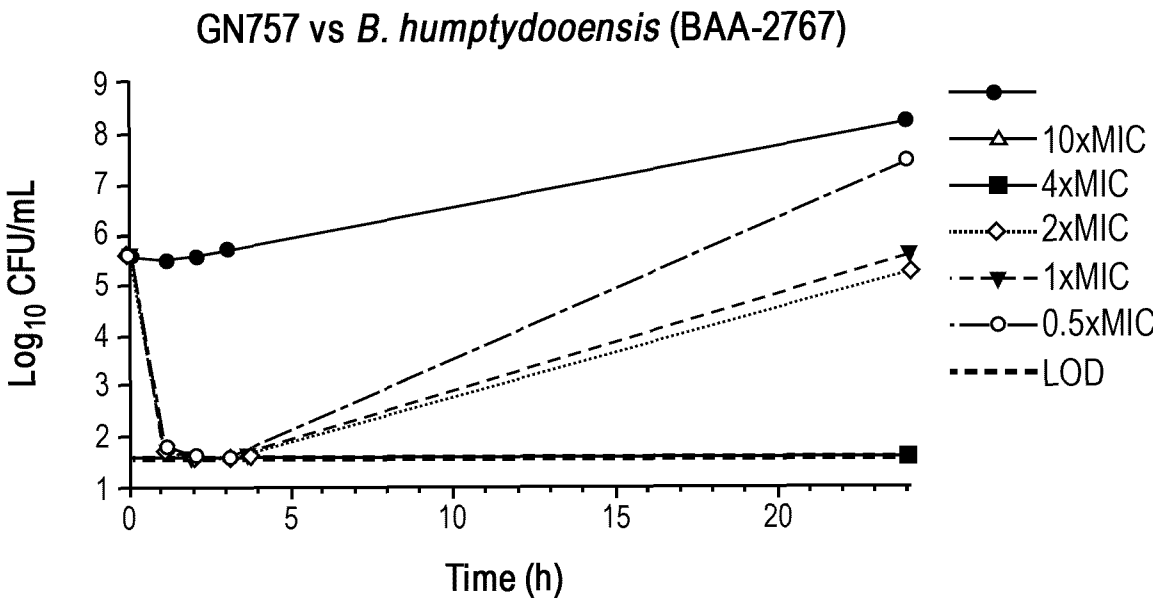


FIG. 2

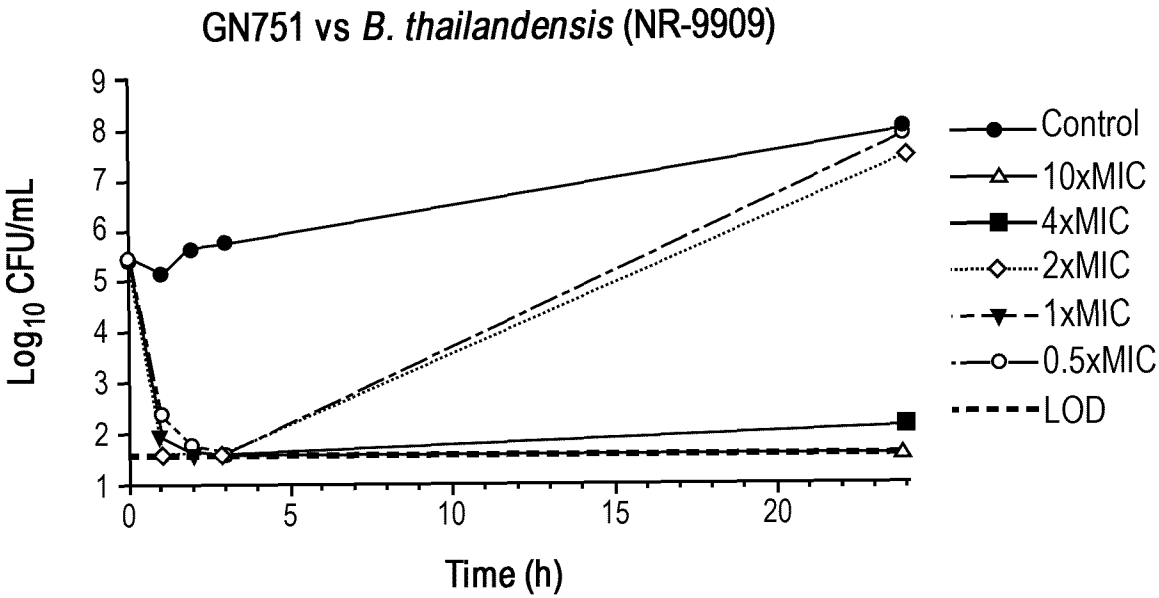


FIG. 3

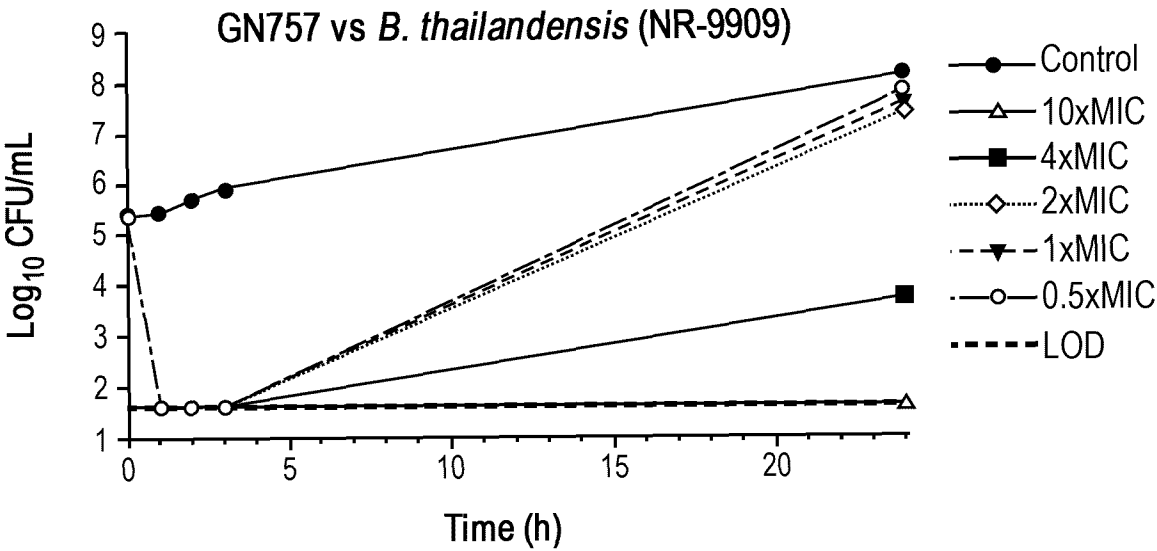


FIG. 4

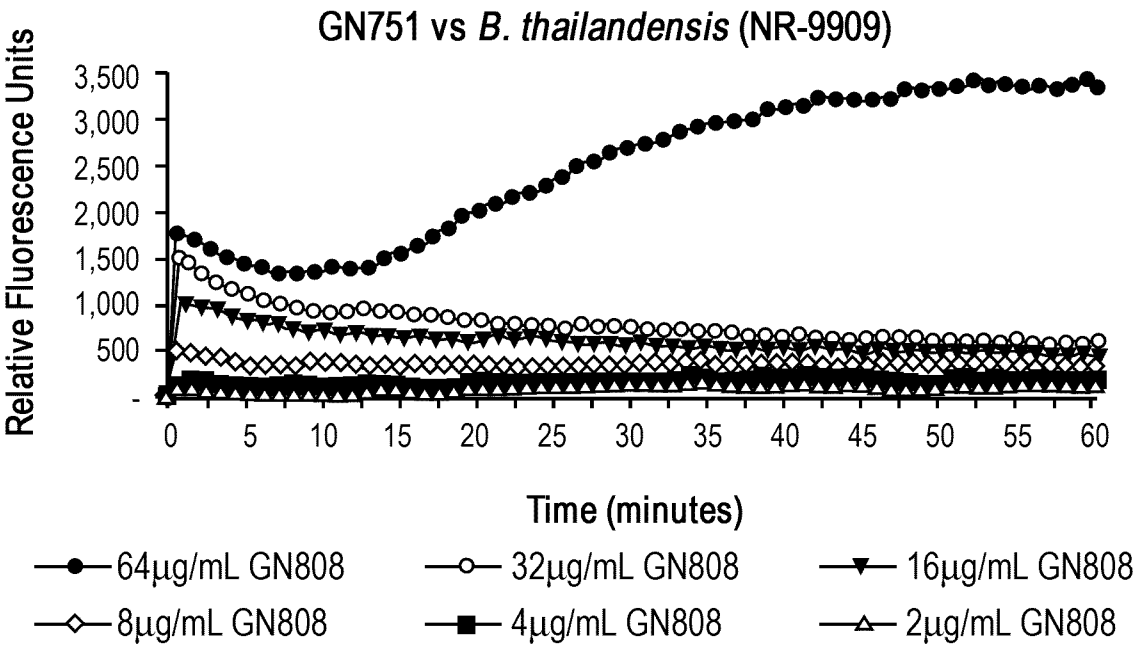


FIG. 5

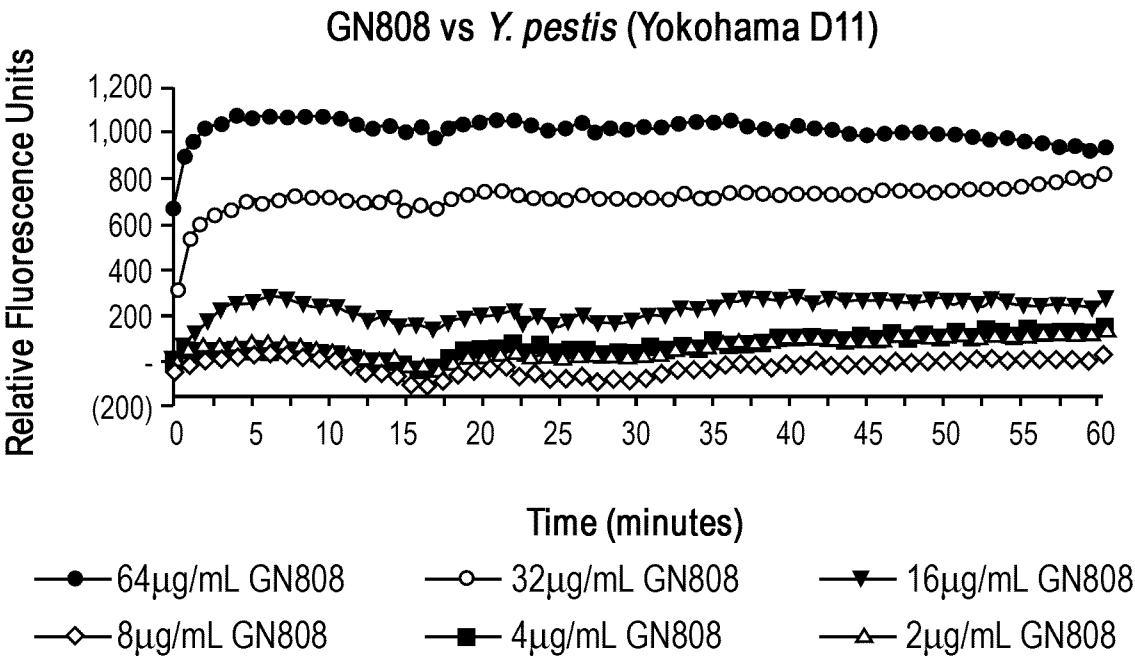


FIG. 6

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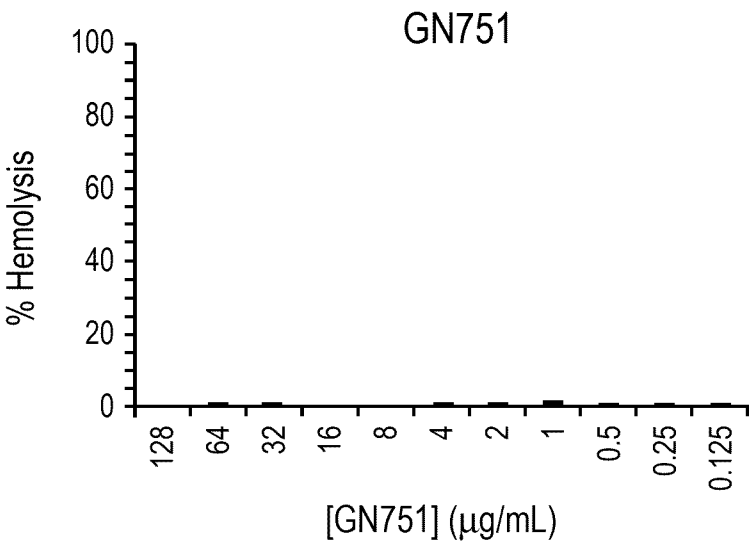


FIG. 7

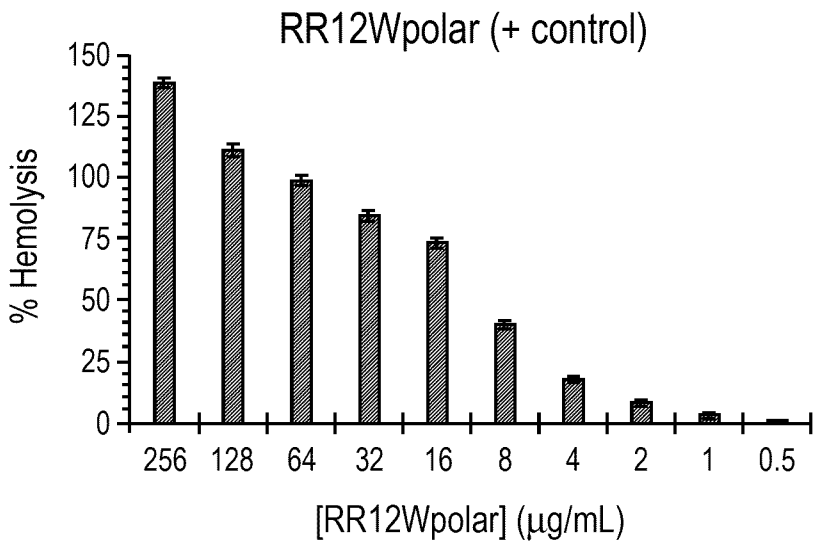


FIG. 8