

Anti-Bacterial Protein Complex

This application claims priority from GB 2301639.7, filed 06 February 2023, the contents and elements of which are herein incorporated by reference for all purposes.

5 **Field of the Invention**

The present invention relates to bacteriocins as therapeutic agents, and in particular to protein complexes comprising two or more bacteriocin molecules associated with a protein scaffold which comprises cognate immunity protein domains for the effector portions of the respective bacteriocins. The complexes of the invention may provide various advantages including increased cell-killing activity, reduced
10 incidence of resistance, enhanced strain coverage, and increased efficiency of production and purification.

Background

For Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and
15 *Escherichia coli*, therapeutic options are often limited due to the horizontal acquisition of antibiotic resistance determinants and the presence of a highly impermeable outer-membrane that severely limits the efficacy of many classes of antibiotics.

Consequently, there is an urgent need to consider alternative strategies for antibiotic development, to
20 bolster a developmental pipeline that in recent decades has yielded few antibiotics active against these difficult to treat bacteria.

An alternative strategy for the discovery of effective antibiotics is to exploit the potent narrow-spectrum antibiotics produced by many bacteria for intraspecies competition. The protein bacteriocins (PBs) are a
25 family of multi-domain protein antibiotics which includes S-type pyocins, klebicins colicins, cloacins, salmocins and pesticins, produced by *Pseudomonas aeruginosa*, *Klebsiella* (e.g. *K. pneumoniae*), *Escherichia coli*, *Enterobacter cloacae*, *Salmonella* (e.g. *Salmonella enterica*) and *Yersinia pestis*, respectively. PBs have evolved to efficiently cross the Gram-negative outer membrane by parasitising existing nutrient uptake pathways. The cellular targets of PBs are highly conserved, with cytotoxic activity
30 most commonly taking the form of an enzymatic activity (typically a nuclease or an enzyme that degrades peptidoglycan precursor molecules thus inhibiting cell wall synthesis), or a pore-forming activity targeting the cytoplasmic membrane.

However, whilst PBs are active against clinically relevant strains, each bacteriocin tends to target a
35 limited number of strains of a given species. In order to achieve therapeutically relevant strain coverage, it may therefore be desirable to use two or three different bacteriocins together. Producing cocktails of pharmaceutical grade protein bacteriocins presents a number of challenges, including high purification costs.

Summary of the Invention

The invention provides an anti-bacterial protein complex comprising:

- 5 (a) a first protein bacteriocin (PB) molecule and a second PB molecule;
and
(b) an immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain;
- 10 wherein the first and second immunity protein domains are non-covalently bound to the respective first and second PB molecules.

Although diverse in 3D-structure, PBs share a characteristic multi-domain architecture, comprising an effector (or cytotoxic) portion, typically at the C-terminus, and cell-targeting portions typically N-terminal to the effector domain, as discussed in more detail below. They are produced by Gram-negative bacteria, often of the same species against which the bacteriocins act. Organisms that express PBs also express cognate immunity proteins, which bind to the bacteriocin (typically to the effector portion of the bacteriocin) with ultra-high affinity (~ fM) and thus prevent the bacteriocin from killing the host. Examples of species-specific PBs and their targets include colicins (*Escherichia coli*), S-type pyocins (*Pseudomonas aeruginosa*), klebicins (*Klebsiella*, e.g. *K. pneumoniae*), cloacins (*Enterobacter cloacae*), salmocins (*Salmonella*, e.g. *Salmonella enterica*) and pesticins (*Yersinia pestis*). Thus, each bacteriocin typically comprises a cell targeting portion and an effector portion, and binds via its effector portion to the corresponding immunity protein domain of the immunity protein scaffold.

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25 The complexes of the present invention utilise an immunity protein scaffold containing a plurality of immunity protein domains. This configuration permits a single complex to contain two or more bacteriocin molecules, which are delivered to the target cell surface in close physical proximity to one another. Interaction between a bacteriocin molecule and its cognate receptor on the surface of a target bacterium typically leads to dissociation of the bacteriocin from the immunity protein scaffold. As described in more detail below, the inventors have found that such polyvalent complexes (i.e. complexes containing multiple bacteriocin molecules) provide a number of advantages over the corresponding single bacteriocins, including ease of preparation and purification, increased microbial killing activity, and reduced development of resistance.

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35 The immunity protein scaffold comprises a plurality of immunity protein domains coupled to one another. The immunity protein scaffold may contain any number of immunity protein domains, e.g. two, three, four, five immunity protein domains, or even more. At full occupancy, the anti-bacterial protein complex therefore contains the same number of bacteriocin molecules, each bacteriocin being non-covalently associated via its effector portion with a corresponding immunity protein domain of the immunity protein scaffold. (However, it will be understood that any population of such complexes may inevitably contain

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some complexes having less than full occupancy.) Two or three immunity protein domains may be preferred, such that the anti-bacterial protein complex contains two or three bacteriocin molecules, respectively. Thus the immunity protein scaffold may comprise a third immunity protein domain and the anti-bacterial protein complex may comprise a third bacteriocin molecule non-covalently bound to the
5 third immunity protein domain.

For the avoidance of doubt, it will be understood that the immunity protein scaffold and the respective bacteriocin molecules are separate molecules, associated via the non-covalent interactions between the immunity protein domains and the effector portions of the bacteriocin molecules. Those may be
10 substantially the only interactions between the various components of the complex.

The scaffold may comprise multiple (two or more) repeats of the same immunity protein domain. For example, all of the immunity protein domains may be the same. The complex will therefore contain multiple copies of the same bacteriocin. (Or at least, multiple bacteriocins each having the same effector
15 portion.)

Typically, though, the scaffold contains two or more different immunity protein domains. Thus the complex contains bacteriocin molecules having two or more different effector portions. The scaffold may contain two, three, four or five different immunity protein domains, or even more. In some embodiments,
20 each of the component immunity protein domains in a given scaffold molecule is different to each of the other component immunity protein domains. Two or three different immunity protein domains may be preferred, such that the anti-bacterial protein complex contains bacteriocin molecules having two or three different effector portions respectively.

25 The immunity protein scaffold is typically a fusion protein, i.e. a single peptide chain comprising the relevant immunity protein domains, optionally separated by linker peptides.

The immunity protein scaffold may further comprise a heterologous moiety, i.e. a moiety which is neither an immunity protein domain or a linker peptide. In some embodiments, the heterologous moiety may
30 comprise a cytotoxic domain, such that the immunity protein scaffold itself becomes a further toxin, in addition to its associated PBs. The heterologous moiety may comprise a cytotoxic domain and a portion capable of mediating translocation across the target cell outer membrane, such as a protein bacteriocin targeting portion. In preferred embodiments, the heterologous moiety is an M-type bacteriocin, such as colicin M (ColM), KpneM, KpneM2, KvarM, PaeM1 or PaeM4, or a functional variant thereof. In such
35 embodiments, the M-type bacteriocin is typically located at the N-terminus of the scaffold molecule, with the immunity protein domains located C-terminal of the M-type bacteriocin. A suitable linker will typically be present between the M-type bacteriocin and the first immunity protein domain.

An effector portion is typically an enzymatic effector portion. The enzymatic effector portion typically has
40 nuclease activity. The nuclease may, for example, be DNase (capable of degrading DNA) or RNase

(capable of degrading RNA). An RNase may, for example, be a rRNase (having activity against ribosomal RNA) or tRNase (having activity against transfer RNA).

Thus, within a given complex, each bacteriocin typically has an enzymatic effector portion with nuclease activity. The effector portions of the bacteriocins may be the same or different. Thus, a complex may comprise two or more bacteriocins having the same effector portion. For example, all of the bacteriocins within the complex may have the same effector portion. Alternatively, a complex may comprise two or more bacteriocins with different enzymatic effector portions. For example, all of the bacteriocins within the complex may have different effector portions. When the effector portions are different, they may nevertheless have the same enzymatic activity, e.g. DNase or RNase (which may be rRNase or tRNase). Thus, a complex may comprise two bacteriocins with different effector portions but wherein those effector portions have the same activity. Alternatively, some or all of the effector portions may have different enzymatic activities. In some embodiments, each of the effector portions in a given complex is an enzymatic effector portion, and each of those effector portions may have a different enzymatic activity.

Examples of suitable combinations include:

- an effector portion having DNase activity and an effector portion having RNase activity (e.g. rRNase or tRNase);
- an effector portion having rRNase activity and an effector portion having tRNase activity;
- an effector portion having DNase activity, an effector portion having rRNase activity, and an effector portion having tRNase activity.

Independently, a complex may comprise bacteriocins all having the same targeting portions, or having two or more different targeting portions. In some embodiments, each of the bacteriocins in a complex has a different targeting portion.

It may be desirable that all of the bacteriocins within the complex have targeting portions specific for the same species or strain of bacterium. They may nevertheless bind to two or more different receptors (e.g. each targeting portion binds to a different receptor) or use two or more different translocation portals (e.g. each targeting portion uses a different translocation portal).

However, it may also be desirable that two or more bacteriocins in a given complex each have targeting portions specific for a different species or strain, such that a single complex has activity against two or more species or strains of bacterium. It is thus possible to modulate the activity spectrum of a given complex by appropriate selection of targeting portions for the bacteriocins present in the complex.

A bacteriocin molecule comprises an effector (or cytotoxic) portion and a targeting portion. Within a given bacteriocin molecule, the effector and targeting domains may be derived from the same wild type bacteriocin. Alternatively, the bacteriocin may be chimeric, comprising an effector portion and a targeting portion from different wild type bacteriocins. It will be understood that the invention is not restricted to use of wild type bacteriocin sequences. Modified or engineered bacteriocin sequences may also be used, whether in their effector portions, targeting portions, or both.

An illustrative example of a complex of the invention is one which comprises:

a first PB comprising a colicin E9 effector portion;

a second PB comprising a colicin D effector portion; and

- 5 an immunity protein scaffold comprising a colicin E9 immunity protein domain ("Im9") and a colicin D immunity protein domain ("ImD") (together designated "Im9-ImD").

- The first PB may be colicin E9 (i.e. it also comprises a colicin E9 targeting portion). The second PB may be colicin D (i.e. it also comprises a colicin D targeting portion). Alternatively either or both may be
10 chimeric PBs, comprising targeting portions from PBs different to those of their effector portions.

A further illustrative example of a complex of the invention is one which comprises:

a first PB comprising a colicin E9 effector portion;

a second PB comprising a colicin E3 effector portion;

- 15 a third PB comprising a colicin D effector portion; and

an immunity protein scaffold comprising a colicin E9 immunity protein domain ("Im9"), a colicin E3 immunity protein domain ("Im3"), and a colicin D immunity protein domain ("ImD") (together designated "Im9-Im3-ImD").

- 20 The first PB may be colicin E9 (i.e. it also comprises a colicin E9 targeting portion). The second PB may be colicin E3 (i.e. it also comprises a colicin E3 targeting portion). The third PB may be colicin D (i.e. it also comprises a colicin D targeting portion).

- Alternatively one, two or all three of the PBs may be chimeric PBs, comprising targeting portions from
25 PBs different to those of their effector portions.

For example, independently:

the first PB may be a chimeric PB comprising KlebC targeting portion and a colicin E9 effector portion ("KlebC-E9");

- 30 the second PB may be a second chimeric PB comprising a CloDF13 targeting portion and a colicin E3 effector portion ("CloDF13-E3"); and

the third PB may be a chimeric PB comprising KlebG targeting portion and a colicin D effector portion (designated "KlebG-D").

The invention further provides an anti-bacterial method, comprising contacting a bacterium or population of bacteria with an anti-bacterial complex as described herein.

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The invention further provides an anti-bacterial complex as described herein for use in a method of medical treatment, e.g. for use in the prophylaxis or treatment of a bacterial infection or a condition caused by or associated with a bacterial infection.

10 The invention further provides the use of an anti-bacterial complex as described herein in the preparation of a medicament for use in the prophylaxis or treatment of a bacterial infection or a condition caused by or associated with a bacterial infection.

The invention further provides a method of prophylaxis or treatment of a bacterial infection or a condition
15 caused by or associated with a bacterial infection, comprising administering an anti-bacterial complex as described herein to a subject in need thereof.

The bacterial infection is typically an infection with a Gram-negative bacterium.

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The invention further provides a host cell comprising:

- (i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain;
 - (ii) nucleic acid encoding at least one bacteriocin, the or each bacteriocin having an effector domain
- 25 capable of binding to at least one of said immunity protein domains;
wherein the host cell is capable of expressing said immunity protein scaffold and said at least one bacteriocin.

On expression of the scaffold and bacteriocin components, an anti-bacterial complex of the invention may
30 be formed. Where the anti-bacterial complex contains an immunity protein scaffold having only one type of immunity protein domain, and the complex contains only one type of bacteriocin, the cell may encode and express just that single cognate bacteriocin.

Where the anti-bacterial complex contains at least two different types of bacteriocin (e.g. the immunity
35 protein scaffold contains at least two different immunity protein domains), the cell comprises nucleic acids encoding at least two bacteriocins, each having an effector capable of binding to at least one of said immunity protein domains, and is capable of expressing said bacteriocins.

Preferably the host cell comprises nucleic acids encoding bacteriocins having effector domains capable of
40 binding to each of the immunity protein domains of the immunity protein scaffold, and is capable of expressing said bacteriocins.

Thus, for example, the cell may comprise:

- (i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain;
- 5 (ii) first and second nucleic acids encoding respective first and second bacteriocins, each bacteriocin having an effector domain capable of binding to a respective one of said immunity protein domains; wherein the host cell is capable of expressing said immunity protein scaffold and said bacteriocins.

Further, the cell may comprise:

- 10 (i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising first, second and third immunity protein domains;
- (ii) first, second and third nucleic acids encoding respective first, second and third bacteriocins, each bacteriocin having an effector domain capable of binding to a respective one of said immunity protein domains;
- 15 wherein the host cell is capable of expressing said immunity protein scaffold and said bacteriocins.

As discussed above, it will be clear that immunity protein scaffolds may comprise more immunity protein domains if required, in which case the host cell may encode (and express) the corresponding number of bacteriocins as required.

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The invention further provides a method of producing an anti-bacterial complex comprising, providing a host cell as described and culturing said cell under conditions suitable for expression of the immunity protein scaffold and bacteriocin molecule(s). The method may further comprise a step of isolating the anti-bacterial complex, and optionally further steps of purification.

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- Alternatively, it may be desirable not to express all of the components of the anti-bacterial complex in the same host cell. Instead, components may be expressed in two or more different host cells, each comprising nucleic acid encoding one or more of the individual components, and being capable of expressing those components. For example, the immunity protein scaffold may be expressed in one host cell and one or more bacteriocin molecules in one or more other host cells.

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- Thus the invention further provides a method of generating an anti-bacterial complex, the method comprising contacting an immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain, with first and second bacteriocin molecules each having an effector portion capable of binding to a respective one of said immunity protein domains, to form a complex of the invention.

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In such cases, if a bacteriocin molecule is expressed in a host cell which is sensitive to that bacteriocin and does not also express the immunity protein scaffold, the host cell will typically also express a cognate

immunity protein for the relevant bacteriocin. In such cases, the bacteriocin may be dissociated from the immunity protein and isolated from it, before contacting the bacteriocin with the immunity protein scaffold.

The invention includes the combination of the aspects and preferred features described except where
5 such a combination is clearly impermissible or expressly avoided.

Summary of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with
reference to the accompanying figures in which:

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Figure 1. Schematic of (a) heterotrimeric complex (2 bacteriocin molecules) and (b) heterotetrameric complex (3 bacteriocin molecules). Immunity proteins are indicated "Im" (Im9, Im3 and ImD). The cognate cytotoxic domains are indicated as E9, E3 and D. The remainder of the bacteriocin molecule is shown simply as "bacteriocin". (c) Cartoon structures of Im9, Im3 and ImD, joined together with flexible
15 linkers represented by dotted line. (d) Cartoon structure of the Im9-Im3-ImD fusion protein associated with its cognate bacteriocins.

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Figure 2. Translocation pathways of Colicin E9 (Francis et al., 2021) and Colicin D across the cell envelope. Colicin E9 binds to BtuB and OmpF in the outer membrane, before interacting with TolB in the periplasm resulting in FtsH-dependent translocation of the C-terminal DNase domain to the cytoplasm. Colicin D crosses the outer membrane through interaction with FepA, interacts with TonB in the periplasm resulting in FtsH-dependent translocation of the C-terminal tRNase to the cytoplasm (Chauleau et al., 2011).

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Figure 3. Cloacin DF13 binds the ferric aerobactin receptor, IutA, before threading its unstructured N-terminus through trimeric porins to bind TolB within the periplasm (unpublished). Klebicin G binds the trimeric porin OmpK35 allowing its N-terminus to cross the outer membrane and bind TolA. KlebC binds the efflux pump TolC, with the N-terminus of the klebicin passing through the TolC lumen to interact with TonB in the periplasm.

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Figure 4. Superdex 200 increase 10/300 GL elution profile for (i) Im9-ImD + ColE9 + ColD, (ii) Im9-ImD + ColD, (iii) Im9-ImD + ColE9, and (iv) Im9-ImD. The central 0.5 ml of the elution peak for each complex was taken for analysis in killing assays.

Figure 5. A-C: Killing activity of ColD, ColE9 and [ColE9:Im9-ImD:ColD] against BL21 (DE3) (*btuB*-), *fepA*- BW25113, *tolA*- BW25113 and *tonB*- BW25113 *E. coli* cells. D: Killing activity of ColE9, ColD, ColE9 + ColD, and [ColE9:Im9-ImD:ColD] against *tolA*- BW25113 *E. coli* cells.

5 **Figure 6.** Growth inhibition of *E. coli* MG1655 by [ColE9:Im9-ImD:ColD], [Im9-ImD:ColD] and [ColE9:Im9-ImD]. Liquid cultures of *E. coli* MG1655 were grown in the presence of [ColE9:Im9-ImD:ColD], [Im9-ImD:ColD] or [ColE9:Im9-ImD] after 6 hours (top) and 24 hours (bottom) monitored via OD_{630nm}. Complexes were used at a concentration of 5.6 pM to 1 µM.

10 **Figure 7.** Nickel affinity purification of [ColE9:Im9-ImD:ColD] expressed in BL21 (DE3) cells from colicin E9, Im9-ImD, colicin D cloned into pET21a with an additional copy of colicin D cloned into pACYCDuet1. Protein was eluted from the column with a 0 to 500 mM imidazole gradient over 10 column volumes and fractions were analysed on a 12 % SDS-PAGE gel (fractions 18-23 shown).

15 **Figure 8.** 26/60 S200 purification of [ColE9:Im9-ImD:ColD] complex. [ColE9:Im9-ImD:ColD] was purified on a 26/60 S200 column equilibrated in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl and eluted fractions were analysed on a 12 % SDS-PAGE gel.

Figure 9. Activity of *in vivo* assembled trimeric [ColE9:Im9-ImD:ColD] complex against soft-agar lawns inoculated with *E. coli*. Threefold serial dilutions of [ColE9:Im9-ImD:ColD] were prepared over the concentration range of 100 nM to 137 pM. 5 µl of each dilution was spotted onto soft-agar lawns inoculated with against *E. coli* BW25113, *btuB*- BL21 (DE3), *fepA*- BW25113, *tolA*- BW25113 or *tonB*- BW25113. After overnight incubation at 37 °C, bacteriocin activity was seen a zones of clearance in the bacterial lawn.

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Figure 10. The assembled tetrameric complex [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D] was purified on a Superdex 200 increase 10/300 GL analytical gel filtration column (Cytiva). 12% SDS-PAGE analysis of eluted fractions from the peak is shown.

30 **Figure 11.** Killing activity of tetrameric complex [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D] against SG62, SR3 and SR6 *Klebsiella pneumoniae* cells, compared to previously observed activity of KlebC-E9, CloDF13-E3 and KlebG-D against the same strains.

Figure 12. Illustration of KvarM-immunity protein scaffolds and demonstration of cytotoxic activity. [A] Schematic illustration of KvarM-Im9-ImD protein scaffold. [B] Schematic illustration of KvarM-Im9-Im7-

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ImD protein scaffold. [C] Cytotoxic activity of wild type KvarM and KvarM-immunity protein scaffolds against *K. quasipneumoniae* SG96 cells in nutrient broth.

Figure 13. Schematic illustration of KvarM-immunity protein scaffold fusions, complexed with cognate protein bacteriocins.

Figure 14. Assembly and activity of trimeric complex [S5E9:Im9-Im7:S5E7] and activity against *P. aeruginosa*. (A) Schematic diagram of the composition of the three-component [S5E9:Im9-Im7:S5E7] complex. The chimeric pyocins S5E9 and S5E7 consist of the outer membrane transport (T_{om}) and receptor binding (R) domain of pyocin S5 followed by the inner membrane transport domain (T_{im}) of pyocin G and the DNase domains from colicin E9 and E7, respectively. These chimeric pyocins form a complex with the immunity protein scaffold via interactions between the E7 and E9 DNase domains and the cognate E7 and E9 immunity protein domains of the scaffold. (B) SDS PAGE of purified proteins. The free and immunity protein complex forms of S5E9 and S5E7 and the [S5E9:Im9-Im7:S5E7] complex were loaded onto a 16% SDS PAGE gel. In addition to the monomeric pyocin samples of the refolded uncomplexed S5E9 and S5E7 show bands indicating the presence of multimeric pyocin. (C) Activity of the [S5E9:Im9-Im7:S5E7] complex and component proteins. Three-fold dilutions from a starting concentration 0.14 mg/ml, of each protein or protein complex were spotted onto a growing lawn of *Pseudomonas aeruginosa* P8. Clear zones indicate killing of *P. aeruginosa*.

Detailed Description of the Invention

Aspects and embodiments of the present invention will now be discussed with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Whilst protein bacteriocins are active against clinically relevant strains, each bacteriocin tends to target a limited number of strains of a given species. In order to achieve therapeutically relevant strain coverage, it may therefore be desirable to use two or three different bacteriocins together. Producing cocktails of pharmaceutical grade protein bacteriocins presents a number of challenges, including high purification costs.

The complexes of the present invention provide a number of advantages, including the possibility of expressing all components (bacteriocin proteins and immunity protein scaffold) in a single culture, thus enabling the purification of a single multifunctional protein complex. Even if the individual components are expressed in two or more cultures, the affinity of the scaffold for the bacteriocin molecules may

facilitate purification of a single stoichiometrically defined complex from a relatively crude mixture of the separate cultures, without the need to purify each component individually.

- 5 The complexes described may also provide functional advantages, e.g. in terms of cell killing and reduced development of resistance. The complexes of the invention are believed to induce higher levels of cell killing than preparations of the same individual bacteriocin(s), likely due to the increased avidity effects provided by the physical association of multiple receptor-binding domains. This phenomenon exploits the fact that bacterial outer membrane proteins tend to associate in clusters.
- 10 Complexes containing different bacteriocins associated with a single immunity protein scaffold ("heterogeneous" complexes) also appear to demonstrate increased cell killing as compared to the corresponding individual bacteriocins, even in bacteria which are deficient in a component usually required for uptake of one of the bacteriocins, such as one of more of the outer or inner membrane proteins involved in membrane translocation (referred to herein as "translocation portals"). Without
- 15 wishing to be bound by theory, it is believed that the close spatial localisation of the different bacteriocin components may enable them to share components of each others' uptake systems, in a manner that would not be possible for individual bacteriocins. This is likely facilitated by the clustering of diverse bacterial outer membrane proteins in mega-clusters ("*Lipids Mediate Supramolecular Outer Membrane Protein Assembly in Bacteria*". Webby M.N. et al., Sci. Adv. 8, eadc9566 (2022); doi:
- 20 [10.1126/sciadv.adc9566](https://doi.org/10.1126/sciadv.adc9566)).

- The complexes of the invention can also be readily adapted for targeting different species or strains of bacteria. Typically, the cytotoxic domains are effective in many types of bacterium and can be readily exchanged between bacteriocin molecules. Strain specificity is determined primarily by the receptor
- 25 binding and/or translocation domains. Thus, it may be possible to tailor the strain specificity of the complex simply by exchanging one set of receptor binding and/or translocation domains of the bacteriocin molecules for another, thus enabling the same set of cytotoxic domains (and hence the same immunity protein scaffold) to be used against a wide range of bacterial types.
- 30 The use of complexes comprising two or more different bacteriocin molecules may also reduce the likelihood that resistance will develop to the relevant bacteriocins. For example, a single complex may contain two or more bacteriocin molecules which bind to different receptors, which use different translocation pathways, and/or which have different cytotoxic activities, all of which reduce the chance of resistance developing. (It will also be understood that similar effects may be achieved by using a
- 35 population or "cocktail" comprising a plurality of different complexes, wherein each given complex carries multiple copies of the same bacteriocin molecule, and the population comprises at least two different bacteriocin molecules, e.g. three, four, five or even more different bacteriocin molecules.)

Protein bacteriocins

The bacteriocins applicable in the context of the present invention are proteinaceous anti-microbial toxins produced by and effective against Gram-negative bacterial species, designated "protein bacteriocins" (PB).

Other structurally distinct types of bacteriocins are known, but are not included within the definition of "protein bacteriocins".

Thus, particulate bacteriocins, such as R-type (rod-like) pyocins and F-type (flexible and non-contractile) pyocins, are both related to phage tail proteins (from P2 phage and lambda phage respectively), and are also sometimes referred to as "tailocins" or "high molecular mass bacteriocins".

Peptide-like bacteriocins, sometimes referred to as microcins, are antibiotic peptides typically less than 10kDa in size, secreted by bacteria, primarily *Enterobacteriaceae*. They can be divided into Class I (less than 5 kDa) and Class II (5-10 kDa) microcins, and display various mechanisms of action including pore formation in the bacterial membrane (MccV, MccE492, and MccL), inhibition of aspartyl-tRNA synthetase (MccC), inhibition of DNA gyrase GyrB, resulting in double stranded DNA breaks (MccB17), inhibition of transcription, and inhibition of cellular respiration via cytochromes (MccJ25), the cellular proton channel (MccH47 and probably MccM and MccI) or the ATP synthase (MccH47). For a review, see Baquero et al., Front. Microbiol., October 2019, Vol. 10, 2019 (doi: [10.3389/fmicb.2019.02261](https://doi.org/10.3389/fmicb.2019.02261)).

PBs are believed to be evolutionarily related and share a characteristic multi-domain structure comprising a targeting portion and an effector (or cytotoxic) portion. Typically the targeting portion is at the N-terminal end of the molecule and the effector portion at the C-terminal end, especially for those having nuclease activity.

PBs include colicins (active against *Escherichia coli*), S-type pyocins (active against *Pseudomonas aeruginosa*), klebicins (active against *Klebsiella* species, e.g. *Klebsiella pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* and *K. aerogenes*), cloacins (active against *Enterobacter cloacae*), salmocins (active against *Salmonella enterica*) and pesticins (active against *Yersinia pestis*). They may also be referred to as "colicin-like bacteriocins". For a review, see Behrens et al., Emerging Topics in Life Sciences (2017) 1: 65–74 (doi: [10.1042/ETLS20160016](https://doi.org/10.1042/ETLS20160016)).

The effector portion may constitute a single independently folded domain. The targeting portion may also constitute a single independently folded domain or may be sub-divided into two or more independently folded domains.

The targeting portion binds to a receptor at the surface of the target organism (i.e. at the Gram negative outer membrane) and mediates translocation of the bacteriocin across the outer membrane. For the

avoidance of doubt, the term “receptor” is used simply to designate the molecule on the target organism to which the targeting portion binds, and should not be taken to imply a cooperative receptor-ligand interaction in the sense usually intended for a pair of molecules expressed by a single organism. The receptor is typically an outer membrane protein but may be any suitable molecule in the outer membrane, such as a lipopolysaccharide (e.g. the common polysaccharide antigen of *P. aeruginosa*, which is believed to serve as a receptor for a number of pyocins including PyoL1, PyoS2, PyoS3, PyoS5, PyoSD2 and PyoSD3).

In general, the targeting portion determines the species and strain specificity (or tropism) of the bacteriocin. In this specification, when a given targeting portion (or bacteriocin) is described as being “specific for” a particular target organism (such as a bacterial strain or species), it is simply meant that the targeting portion is capable of delivering the associated effector portion to the relevant species or strain, typically by binding to the receptor at the surface of the target organism and mediating translocation of the effector portion such that the effector portion can exert its activity against the target organism. It should be noted that a particular bacteriocin may have activity against more than one species of bacterium, e.g. if the receptor and translocation pathway used by the targeting portion of that bacteriocin are sufficiently similar between those different species. For example, the cloacin DF13 is capable of targeting *Klebsiella pneumoniae* strains (e.g. strain SG62) despite being a cloacin.

The targeting portions of most naturally occurring PBs have a characteristic modular structure containing up to four identifiable sub-regions, each of which may represent a separately folded domain or may lack recognisable secondary structure and thus form a flexible region of the molecule. Sub-region I, located at the N-terminus, is relatively unstructured and contains Tol or Ton binding epitopes. Sub-region II is a translocator-binding domain. Subregion III is a receptor-binding domain. Subregion IV is an inner membrane translocation domain. In some PBs, the translocation portal also serves as the receptor, in which case sub-regions II and III form a single domain. In others, the receptor and the translocation portal are different molecules, in which case sub-regions II and III are usually separate domains. These sub-regions typically (although not exclusively) occur in that order in an N- to C-terminal direction, although this is not always necessary (with the proviso that sub-region I is always at the N-terminus).

Without wishing to be bound by any particular theory, it is believed that sub-regions I, II, III and IV may be interchangeable between molecules, at least to some extent.

Within a given complex, the targeting portions of the bacteriocin molecules may be the same or different. Thus, a complex may comprise bacteriocins all having the same targeting portions, or having two or more different targeting portions. For example, each of the bacteriocins in a complex may have a different targeting portion.

It may be desirable that all of the bacteriocins within a complex have targeting portions specific for the same species or strain of bacterium. They may nevertheless bind to two or more different receptors on that strain or species. For example, each targeting portion may bind to a different receptor. Additionally

or alternatively, the targeting portions may use two or more different translocation portals, e.g. TolC, a trimeric porin, or a TonB-dependent translocator.

For example, a complex may comprise two or more bacteriocins having targeting portions specific for *E. coli*, e.g. two or more bacteriocins having different targeting portions specific for *E. coli*, e.g. derived from colicins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *E. coli*, e.g. derived from colicins. Colicins include colicin A, E1, E2, E3, E4, E6, E7, E8, E9, K, M, N, U, B, Ia, Ib, 5, 10, S4 and Y.

- 5 *coli*, e.g. two or more bacteriocins having different targeting portions specific for *E. coli*, e.g. derived from colicins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *E. coli*, e.g. derived from colicins. Colicins include colicin A, E1, E2, E3, E4, E6, E7, E8, E9, K, M, N, U, B, Ia, Ib, 5, 10, S4 and Y.
- 10 A complex may comprise two or more bacteriocins having targeting portions specific for *Pseudomonas*, e.g. *P. aeruginosa*, e.g. two or more bacteriocins having different targeting portions specific for *Pseudomonas*, e.g. *P. aeruginosa*, e.g. derived from S-type pyocins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Pseudomonas*, e.g. *P. aeruginosa*, e.g. derived from S-type pyocins. S-type pyocins include pyocin G, L1, L2, L3, M1, M2, M4, S1, S2, S3, S4,
- 15 S5, S6, S8, SD1, SD2, SD3, AP41, Sn, SX1 and SX2.

A complex may comprise two or more bacteriocins having targeting portions specific for *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*), e.g. two or more bacteriocins having different targeting portions specific for *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*), e.g. derived from klebicins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*), e.g. derived from klebicins. Klebicins include klebicin C, D, G, KpneA, KaerA, KpneA, Kvarla, KoxyY, KpneM, KpneM2 and KvarM.

- 20 *quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*), e.g. derived from klebicins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*), e.g. derived from klebicins. Klebicins include klebicin C, D, G, KpneA, KaerA, KpneA, Kvarla, KoxyY, KpneM, KpneM2 and KvarM.
- 25 A complex may comprise two or more bacteriocins having targeting portions specific for *Enterobacter cloacae*, e.g. two or more bacteriocins having different targeting portions specific for *Enterobacter cloacae*, e.g. derived from cloacins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Enterobacter cloacae*, e.g. derived from cloacins. Cloacins include cloacin DF13.

- 30 A complex may comprise two or more bacteriocins having targeting portions specific for *Salmonella enterica*, e.g. two or more bacteriocins having different targeting portions specific for *Salmonella enterica*, e.g. derived from salmocins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Salmonella enterica*, e.g. derived from salmocins. Salmocins include
- 35 SalE1a, SalE1b, SalE2, SalE3 and SalE7.

A complex may comprise two or more bacteriocins having targeting portions specific for *Yersinia pestis*, e.g. two or more bacteriocins having different targeting portions specific for *Yersinia pestis*, e.g. derived from pesticins. For example, all of the bacteriocins in the complex may have different targeting portions specific for *Yersinia pestis*, e.g. derived from pesticins. Pesticins include pesticin 1.

- 40 specific for *Yersinia pestis*, e.g. derived from pesticins. Pesticins include pesticin 1.

In other embodiments, it may be desirable that two or more bacteriocins in a given complex each have targeting portions specific for a different bacterial species or strain, in order to provide a single complex having activity against two or more species or strains of bacterium. It will be understood that the bacteria in question are Gram-negative bacteria.

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For example a complex may comprise a bacteriocin having a targeting portion specific for *E. coli* (e.g. a colicin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion), *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*) (e.g. a klebacin targeting portion), *Enterobacter cloacae* (e.g. a cloacin targeting portion), *Salmonella enterica* (e.g. a salmocin targeting portion) or *Yersinia pestis* (e.g. a pesticin targeting portion).

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A complex may comprise a bacteriocin having a targeting portion specific for *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *E. coli* (e.g. a colicin targeting portion), *Klebsiella* (e.g. *K. pneumoniae*) (e.g. a klebacin targeting portion), *Enterobacter cloacae* (e.g. a cloacin targeting portion), *Salmonella enterica* (e.g. a salmocin targeting portion) or *Yersinia pestis* (e.g. a pesticin targeting portion).

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A complex may comprise a bacteriocin having a targeting portion specific for *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*) (e.g. a klebacin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *E. coli* (e.g. a colicin targeting portion), *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion), *Enterobacter cloacae* (e.g. a cloacin targeting portion), *Salmonella enterica* (e.g. a salmocin targeting portion) or *Yersinia pestis* (e.g. a pesticin targeting portion).

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A complex may comprise a bacteriocin having a targeting portion specific for *Enterobacter cloacae* (e.g. a cloacin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *E. coli* (e.g. a colicin targeting portion), *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion), *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*) (e.g. a klebacin targeting portion), *Salmonella enterica* (e.g. a salmocin targeting portion) or *Yersinia pestis* (e.g. a pesticin targeting portion).

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A complex may comprise a bacteriocin having a targeting portion specific for *Salmonella enterica* (e.g. a salmocin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *E. coli* (e.g. a colicin targeting portion), *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion), *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*) (e.g. a klebacin targeting portion), *Enterobacter cloacae* (e.g. a cloacin targeting portion), or *Yersinia pestis* (e.g. a pesticin targeting portion).

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A complex may comprise a bacteriocin having a targeting portion specific for *Yersinia pestis* (e.g. a pesticin targeting portion) and one or more bacteriocins having targeting portions specific for one or more of *E. coli* (e.g. a colicin targeting portion), *Pseudomonas* (e.g. *P. aeruginosa*) (e.g. an S-type pyocin targeting portion), *Klebsiella* (e.g. *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola* or *K. aerogenes*) (e.g. a klebicin targeting portion), *Enterobacter cloacae* (e.g. a cloacin targeting portion) or *Salmonella enterica* (e.g. a salmocin targeting portion).

As already noted, the effector portions used in bacteriocins the present invention are enzymatic effector portions, and typically nucleases, since only these typically interact with cognate immunity protein domains.

However, targeting portions from any PB may be employed, regardless of what kind of effector domain they are usually associated with in the corresponding wild type PB.

Pore-forming bacteriocins kill target cells by depolarisation of the cytoplasmic membrane. These include pyocin S5, colicins A, E1, K, N, U, B, Ia, Ib, 5, 10, S4 and Y, and klebicins KpneA, KaerA, Kpnela, Kvarla and KoxyY (see Denkovskiene *et al.*, 2019).

Enzymatic PB effector portions may have various activities.

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Many act as nucleases, as already described.

Some nuclease effector portions possess DNase activity, including pyocins G, S1, S2, SD2, S3 and AP41, and colicins E2, E7, E8 and E9. Klebicin G is also believed to be a DNase.

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Some nuclease effector portions possess RNase activity, e.g. rRNase or tRNase activity. Those with rRNase activity include colicins E3, E4 and E6, klebicin C, and cloacin DF13. Those with tRNase activity include pyocin S4, colicins E5 and D, and klebicin D).

30 Still other enzymatic effector portions have different modes of activity, including degradation of peptidoglycan or precursor molecules thereof, resulting in inhibition of cell wall synthesis. These include colicin M, PaeM1, PaeM4, and klebicins KpneM, KpneM2 and KvarM.

35 The targets on which the effector portions act tend to be highly conserved across the bacterial kingdom, and thus a given effector portion will generally be active against a broad spectrum of organisms. Species specificity is therefore determined primarily by the targeting portion of the bacteriocin molecule. Thus it is possible to exchange effector portions relatively freely between PBs to generate chimeric bacteriocins. For example, chimeric pyocins containing a targeting portion from an S1 or S2 pyocin linked to an effector portion from either an E2 or E3 colicin have been demonstrated to retain pseudomonad-killing activity (Kageyama *et al.*, 1996). Chimeric bacteriocins are also employed in the Examples below.

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Thus the bacteriocin may comprise any suitable effector portion having a cognate immunity protein which can be incorporated into an immunity protein scaffold. As already noted, these are typically enzymatic (nuclease) effector portions, which can be combined with targeting portions from any suitable PB.

- 5 Thus, within a given complex, each bacteriocin typically has an enzymatic effector portion with nuclease activity. The effector portions of the bacteriocins may be the same or different. Thus, a complex may comprise two or more bacteriocins having the same effector portion. For example, all of the bacteriocins within the complex may have the same effector portion. Alternatively, a complex may comprise two or more bacteriocins with different enzymatic effector portions. For example, all of the bacteriocins within
- 10 the complex may have different effector portions. When the effector portions are different, they may nevertheless have the same enzymatic activity, e.g. DNase or RNase (which may be rRNase or tRNase). Thus, a complex may comprise two bacteriocins with different effector portions but wherein those effector portions have the same activity. Alternatively, some or all of the effector portions may have different enzymatic activities. In some embodiments, each of the effector portions in a given complex is an
- 15 enzymatic effector portion, and each of those effector portions may have a different enzymatic activity. Examples of suitable combinations include:
- an effector portion having DNase activity and an effector portion having RNase activity (e.g. rRNase or tRNase);
 - an effector portion having rRNase activity and an effector portion having tRNase activity;
- 20 - an effector portion having DNase activity, an effector portion having rRNase activity, and an effector portion having tRNase activity.

- It will be understood from the discussion above that, within a given bacteriocin molecule, the effector and targeting domains may be derived from the same wild type bacteriocin. Alternatively, the bacteriocin may
- 25 be chimeric, comprising an effector portion and a targeting portion from different bacteriocins. For example, the effector portion and targeting portion may be from different wild type bacteriocins, although it will be understood that the invention is not restricted to wild type bacteriocin sequence, and that modified or engineered bacteriocin sequences may also be used. They may be modified in their effector portions, in their targeting portions, or both.

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- An effector portion of a bacteriocin for use according to the invention may have the amino acid sequence of a wild type PB effector portion, or a functional fragment thereof, or may have at least 70% sequence identity to a wild type effector portion sequence or functional fragment thereof, e.g. at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%
- 35 sequence identity to a wild type effector portion. Typically the effector portion (or fragment thereof) has the same effector activity (i.e. enzymatic activity, typically nuclease) as the wild type effector portion. The effector portion or fragment thereof is also capable of binding to the cognate immunity protein domain of

the associated immunity protein scaffold. For example, it may be capable of binding to the cognate wild type immunity protein.

Suitable effector portions include the effector portions of:

- 5 pyocin G, S1, S2, SD2, S3 and AP41, colicin E2, E7, E8 and E9, and klebicin G (believed to be DNases), colicin E3, E4 and E6, klebicin C, and cloacin DF13 (believed to be rRNase); and
pyocin S4, colicin E5 and D, and klebicin D (believed to be tRNase).

- 10 A targeting portion of a bacteriocin for use according to the invention may have the amino acid sequence of a wild type PB targeting portion or a functional fragment thereof (i.e. a fragment capable of binding to the appropriate receptor on the target cell and mediating translocation of the effector portion into the target cell cytosol). Alternatively it may have at least 70% sequence identity to a wild type targeting portion sequence or functional fragment thereof, e.g. at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to a wild
15 type targeting portion or functional fragment thereof. Typically the targeting portion binds the same receptor and uses the same translocation portal as the wild type targeting portion.

Suitable targeting portions include those from:

- colicin A, E1, E2, E3, E4, E6, E7, E8, E9, K, M, N, U, B, Ia, Ib, 5, 10, S4 and Y;
20 pyocin G, L1, L2, L3, M1, M2, M4, S1, S2, S3, S4, S5, S6, S8, SD1, SD2, SD3, AP41, Sn, SX1 and SX2;
klebicin C, D, G, KpneA, KaerA, Kpnela, Kvarla, KoxyY, KpneM, KpneM2 and KvarM;
cloacin DF13;
salmocin SalE1a, SalE1b, SalE2, SalE3 and SalE7; and
pesticin 1.

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Illustrative examples of chimeric bacteriocins comprise:

- a KlebC targeting portion and a colicin E9 effector portion (designated "KlebC-E9");
a CloDF13 targeting portion and a colicin E3 effector portion (designated "CloDF13-E3");
and
30 a KlebG targeting portion and a colicin D effector portion (designated "KlebG-D").

These are described in more detail below.

The broad-spectrum effectiveness of the effector portions makes it readily possible to adapt a complex of the invention for use against different bacterial strains or species, by retaining the immunity protein scaffold and bacteriocin effector portions, and simply changing the targeting portions. Thus, having constructed a host cell capable of expressing an immunity protein scaffold and one or more cognate bacteriocins, it is possible to change the target specificity of the complex produced by that cell by simply exchanging the targeting portion (or portions) of the bacteriocin (or bacteriocins) for other targeting portions specific for one or more different strains or species of bacterium. It is not necessary to construct an entirely new host cell having a new immunity protein scaffold and cognate bacteriocins.

10 Immunity protein scaffold

The immunity protein scaffold comprises multiple (i.e. two or more) immunity protein domains, each capable of binding a cognate bacteriocin effector portion.

In their natural environment, an immunity protein is a single domain protein. However, it will be understood that the term “immunity protein domain” is used in this specification to refer to a functional domain of an immunity protein scaffold. Thus an immunity protein scaffold comprises multiple immunity protein domains, each corresponding to a single individual immunity protein. Each domain may be referred to by the name of the corresponding immunity protein (e.g. as a “colicin E9 immunity protein domain”) or an appropriate abbreviation or shorthand (e.g. “Im9 domain”).

20

The immunity protein domains in a given scaffold molecule may be the same or different. Where an immunity protein scaffold contains multiple copies of the same immunity protein domain, the complex may nevertheless containing bacteriocins having two or more different targeting domains, but they will typically have the same effector portion, or at least will be sufficiently similar that they can bind to the same immunity protein domain. However, if it is desirable that a complex contains a plurality of different bacteriocins, it may be more straightforward to control the stoichiometry by using an immunity protein scaffold having multiple different immunity protein domains.

An immunity protein domain for use in an immunity protein scaffold according to the invention may have the amino acid sequence of a wild type PB immunity protein or a functional fragment thereof, or may have at least 70% sequence identity to a wild type immunity protein sequence or functional fragment thereof, e.g. at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to a wild type immunity protein sequence or functional fragment thereof. Each immunity protein domain (or fragment thereof) is capable of binding to the cognate effector portion of one of the bacteriocins that make up the complex along with the immunity protein scaffold. For example, it may be capable of binding to the cognate wild type effector portion.

Suitable immunity protein domains include those of:

pyocin G, S1, S2, SD2, S3 and AP41, colicin E2, E7, E8 and E9, and klebicin G, colicin E3, E4 and E6; klebicin C, and cloacin DF13; and

5 pyocin S4, colicin E5 and D, and klebicin D;

which, as explained above, also provide suitable cognate effector domains.

Illustrative examples of immunity protein scaffolds comprise:

10 a colicin E9 immunity protein domain ("Im9") and a colicin D immunity protein domain ("ImD") (together designated "Im9-ImD");

a colicin E9 immunity protein domain ("Im9"), a colicin E3 immunity protein domain ("Im3"), and a colicin D immunity protein domain ("ImD") (together designated "Im9-Im3-ImD").

15 It will be understood that "Im9-ImD" will form a complex with PBs having effector domains from colicin E9 and colicin D. "Im9-Im3-ImD" will form a complex with PBs having effector domains from colicin E9, colicin E3, and colicin D. The PBs may be chimeric, having targeting portions from other PBs, as described elsewhere in this specification.

20 Typically an immunity protein domain binds to its cognate bacteriocin effector portion with very high affinity. For example, an RNase-Im complex may have a K_d of 10^{-10} M or less, e.g. a K_d of 10^{-10} M or less, 10^{-11} M or less, or 10^{-12} M or less, e.g. as measured at pH 7 and at 25°C, e.g. by stopped-flow fluorescence, e.g. as described in Walker et al. (2003) Biochemistry 42, 4161. A DNase-Im complex may have a K_d of 10^{-10} M or less, e.g. a K_d of 10^{-10} M or less, 10^{-11} M or less, 10^{-12} M or less, 10^{-13} M or less, or 10^{-14} M or less, e.g. when measured as for an RNase-Im complex, or as described in Wallis et al. (1995) Biochemistry 34, 13743-13750.

30 Within a given scaffold, the immunity protein domains are covalently coupled to one another. In some embodiments, they may be expressed separately and conjugated together, e.g. by chemical linkers. However, it will typically be more convenient for the immunity protein scaffold to be expressed as a fusion protein.

To produce an immunity protein scaffold as a fusion protein, a nucleic acid expression vector is constructed comprising coding sequences for each immunity protein component in one continuous open

reading frame, so that the respective immunity protein components can be translated as part of the same polypeptide chain.

- Typically, a peptide linker is included between each component to allow them to interact freely with their respective bacteriocin cytotoxic domains without steric hindrance. The skilled person is perfectly capable of designing a suitable linker. Conventionally, such linkers are between 10 and 20 amino acids in length, and have a high proportion of small and hydrophilic amino acid residues (e.g. glycine and serine) to provide the required flexibility without compromising aqueous solubility of the molecule. In some embodiments, it may be desirable to use more rigid linkers including other residues such as proline and asparagine in order to provide more separation between the individual domains. In some embodiments, the linker may have a propensity to form defined secondary structure, such as an alpha-helix. Sequences which adopt such structures are well known. For example, repeating units of the sequence EAAAK (SEQ ID NO: 1) may be used to form helical linkers, but the skilled person will be well aware of others.
- For example, the immunity protein scaffolds described in the Examples below employ the linker sequences NINGGPTGIGVS (SEQ ID NO: 2) and NGGGNGNSGGGS (SEQ ID NO: 3) between the immunity protein domains, both intrinsically unstructured sequences derived from the N-terminal sub-region of colicin E9. The helical linker ((EAAAK)₃EL) (SEQ ID NO: 4) is used between an M-type bacteriocin (KvarM) and an immunity protein domain. The skilled person will be able to design other suitable linkers as required.

The immunity protein scaffold may further comprise a heterologous moiety, i.e. a moiety which is neither an immunity protein domain or a linker peptide.

- The heterologous moiety may be peptidic or non-peptidic. When the heterologous moiety is peptidic, it may be part of the same fusion protein as the rest of the immunity protein scaffold. Alternatively, it may be linked to the immunity protein scaffold by chemical conjugation.

- For example, the heterologous moiety may act to increase solubility and/or half-life in vivo (e.g. in plasma) and/or bioavailability in a subject, as compared to a corresponding scaffold (or complex) otherwise identical but lacking such a moiety. Such modifications are also known to reduce clearance (e.g. renal clearance) of therapeutic proteins and peptides. Suitable peptidic moieties include immunoglobulin Fc domains. Suitable non-peptidic moieties include polymeric moieties. A polymeric moiety is preferably water soluble (amphiphilic or hydrophilic), non-toxic, and pharmaceutically inert. Examples include polyethylene glycol (PEG), homo- or co-polymers of PEG, a monomethyl-substituted polymer of PEG (mPEG), or polyoxyethylene glycerol (POG). See, for example, Francis et al., (1998), *Int. J. Hematology* 68:1-18; Zalipsky (1995), *Bioconjugate Chem.* 6:150-165; and Delgado et al. (1992), *Crit. Rev. Therap. Drug Carrier Syst.* 9:249-304.

Other suitable polymeric moieties include poly-amino acids such as poly-lysine, poly-aspartic acid and poly-glutamic acid (see for example Gombotz, et al. (1995), Bioconjugate Chem., vol. 6: 332-351; Hudecz, et al. (1992), Bioconjugate Chem., vol. 3, 49-57; Tsukada, et al. (1984), J. Natl. Cancer Inst., vol 73,; 721-729; and Pratesi, et al. (1985), Br. J. Cancer, vol. 52: 841-848).

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The polymeric moiety may be straight-chain or branched. It may have a molecular weight of 500-40,000 Da, for example 500-10,000 Da, 1000-5000 Da, 10,000-20,000 Da, or 20,000-40,000 Da.

Alternatively, the heterologous moiety may comprise a cytotoxic domain (e.g. a protein bacteriocin cytotoxic domain) such that the immunity protein scaffold itself becomes a further toxin, in addition to the PBs with which it is associated. It may further comprise a portion capable of mediating translocation across the target cell outer membrane, such as a protein bacteriocin targeting portion. In preferred embodiments, the heterologous moiety is an M-type bacteriocin, such as colicin M (ColM), KpneM, KpneM2, KvarM, PaeM1 or PaeM4, or a functional variant thereof.

15

M-type bacteriocins act by degrading peptidoglycan precursors in the periplasm, leading to cell lysis (Schaller et al., 1982). They have been found in a range of different species including *E. coli*, *Pseudomonas*, *Pectobacterium*, *Klebsiella*, and *Bulkholderia* (Ch  rier et al., 2021). Colicin M from *E. coli* is the best studied example of this class of bacteriocins. KvarM (see Dekovskien   et al., 2019) is a 30.8 kDa protein, which binds to and translocates through the outer membrane ferrichrome receptor FhuA into the periplasm of the cell, in a process powered by the Ton system, and is of particular interest as it has been shown to be active against a wide spectrum of *Klebsiella* strains, including those that are multidrug resistant in plate, liquid, and biofilm killing assays.

KvarM and Colicin M are organized into the three regions characteristic of bacteriocins: an N-terminal unstructured translocation region (PilsI et al., 1993), a central globular section that interacts with its outer membrane receptor FhuA, and a C-terminal catalytic region which hydrolyses lipid II precursors (Sham et al., 2014). However, their comparatively small size and compact folding mean that these regions do not form independently folded domains, and attempts to truncate these molecules typically result in misfolded proteins (Barreteau et al., 2010).

30

When the immunity protein scaffold comprises an M-type bacteriocin, the M-type bacteriocin is typically located at the N-terminus of the scaffold molecule, with the immunity protein domains located C-terminal of the M-type bacteriocin. A suitable linker will typically be present between the M-type bacteriocin and the first immunity protein domain. It may be desirable to have a rigid linker between the M-type bacteriocin and the first immunity protein domain, such as a linker having alpha-helical secondary structure, e.g. as illustrated in the examples below.

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The incorporation of an M-type bacteriocin to the immunity protein scaffold has the potential to increase the receptor binding capacity and cytotoxic activity of the complex as a whole. The resulting complex is able to bind to the receptor for the M-type bacteriocin as well as those targeted by the PB components of

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the complex (non-covalently associated with the immunity protein domains), thus further increasing the potential avidity of its interaction with the target cell surface, and is cytotoxic against bacteria susceptible to the M-type bacteriocin.

5 Host cell

The invention extends to host cells capable of expressing the immunity protein scaffold.

Thus the invention provides a host cell comprising nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain, and wherein the host cell is capable of expressing said immunity protein scaffold.

An advantage of the invention is that the host cell may also express the PB components, enabling production of the full anti-bacterial complex with correct stoichiometry from a single cell, thus minimising production and purification costs.

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The invention thus further provides a host cell comprising:

- (i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising at least a first immunity protein domain and a second immunity protein domain;
 - (ii) nucleic acid encoding at least one bacteriocin, the or each bacteriocin having an effector domain capable of binding to at least one of said immunity protein domains;
- wherein the host cell is capable of expressing said immunity protein scaffold and said at least one bacteriocin.

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Where the immunity protein scaffold contains only one type of immunity protein domain, and hence the complex contains only one type of bacteriocin, the cell may encode and express just that single cognate bacteriocin. More typically, the immunity protein scaffold contains at least two different immunity protein domains, and the cell comprises nucleic acids encoding at least two bacteriocins, each having an effector capable of binding to at least one of said immunity protein domains, resulting in an anti-bacterial complex containing at least two different types of bacteriocin.

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Thus the host cell comprises nucleic acids encoding each of the respective bacteriocins. The nucleic acid(s) encoding the immunity protein scaffold and the bacteriocin or bacteriocins are typically provided as part of one or more nucleic acid expression constructs or vectors. Thus they may be provided on a single vector or on two or more separate vectors.

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The skilled person will be capable of designing suitable nucleic acid expression constructs or vectors to obtain expression of the immunity protein scaffold and also the bacteriocin molecules as required. Typically such a vector comprises suitable transcriptional and translational regulatory sequences operably linked to a sequence encoding the desired protein, to enable transcription and translation of the protein by the host cell. The vectors may contain other sequences such as selection marker genes as required,

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depending upon the particular host cell. The vectors may be intended to integrate into a host cell chromosome, or may exist and replicate independently of the host chromosomes as an episome, e.g. a plasmid.

5 Since the immunity protein scaffold and the respective bacteriocin molecules are separate molecules in the final complex, associated via the non-covalent interactions between the immunity protein domains and the effector portions of the bacteriocin molecules, it will be understood that they will typically be expressed as separate molecules within the host cell.

10 The host cell is typically a bacterial host cell, e.g. an *E. coli* host cell.

As discussed above, the specificity of the PBs is determined by their targeting portions. An anti-bacterial complex can thus be adapted for use against different bacterial strains or species by simply changing the targeting portions of the component PBs while retaining the immunity protein scaffold and PB effector
15 portions. This can readily be achieved by modifying an existing host cell. It is not necessary to construct an entirely new host cell having a new immunity protein scaffold and cognate bacteriocins.

A nucleic acid or expression construct encoding a given PB may thus be designed to facilitate exchange of the targeting portion. For example, a suitable restriction site may be provided between the sequence
20 encoding the targeting portion and the sequence encoding the effector portion. A further restriction site may be provided at the opposite end of the coding sequence for the targeting portion, e.g. 5' of the start of the open reading frame for the PB.

The invention further provides a method of producing an anti-bacterial complex comprising, providing a
25 host cell as described and culturing said cell under conditions suitable for expression of the immunity protein scaffold and bacteriocin molecule(s). The method may further comprise a step of isolating the anti-bacterial complex, and optionally further steps of purification.

Alternatively, it may be desirable not to express all of the components of the anti-bacterial complex in the
30 same host cell. Instead, components may be expressed in two or more different host cells, each comprising nucleic acid encoding one or more of the individual components, and being capable of expressing those components. For example, the immunity protein scaffold may be expressed in one host cell and one or more bacteriocin molecules in one or more other host cells.

35 Thus the invention further provides a method of generating an anti-bacterial complex, the method comprising contacting an immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain, with first and second bacteriocin molecules each having an effector portion capable of binding to a respective one of said immunity protein domains, to form a complex of the invention.

In such cases, if a bacteriocin molecule is expressed in a host cell which is sensitive to that bacteriocin and does not also express the immunity protein scaffold, the host cell will typically also express a cognate immunity protein for the relevant bacteriocin. In such cases, the bacteriocin may be dissociated from the immunity protein and isolated from it, before contacting the bacteriocin with the immunity protein scaffold.

Subjects and conditions for treatment

The complexes and methods of the present invention are suitable for prophylaxis and/or treatment of bacterial infections, typically Gram negative bacterial infections, especially by *E. coli*, *Pseudomonas* (especially *Pseudomonas aeruginosa*), *Klebsiella* (e.g. *Klebsiella pneumoniae*), *Enterobacter cloacae*, *Salmonella* (e.g. *Salmonella enterica*) and *Yersinia pestis*, as well as conditions caused by or associated with such infection.

As discussed above, the strain specificity of any given bacteriocin is generally determined by its targeting portion. Thus, for treatment of infection by a given bacterial species or strain, or a condition associated therewith, a complex will typically be employed comprising at least one bacteriocin having a targeting portion specific for that species or strain.

The infection may be acute or chronic.

For example, *P. aeruginosa* infection of the lower respiratory tract is particularly common in patients with cystic fibrosis (where it represents the leading cause of mortality) and chronic obstructive pulmonary disease (COPD). Other patients with compromised respiratory tract function and/or compromised immune function may also be susceptible to infection, including patients with congestive heart failure, AIDS patients, and patients taking immunosuppressive medications or undergoing other immunosuppressive therapy, e.g. for cancer (especially chemotherapy) rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, sarcoidosis, focal segmental glomerulosclerosis, Crohn's disease, Behcet's Disease, pemphigus, ulcerative colitis, etc..

Acute conditions associated with or caused by *Pseudomonas* infection include community-acquired pneumonia and nosocomial infections such as ventilator-associated pneumonia and hospital-acquired pneumonia.

Thus, for treating *Pseudomonas* (e.g. *P. aeruginosa*) infection, or a condition associated therewith, it will typically be desirable to use a complex comprising at least one bacteriocin having a targeting portion specific for *Pseudomonas* (e.g. *P. aeruginosa*), e.g. a targeting portion of an S-type pyocin. As discussed elsewhere in this specification, it may be desirable to use a complex having at least two such bacteriocins, or which exclusively contains such bacteriocins.

E. coli infections are implicated in various conditions. Pathogenic adherent-invasive *E. coli* (AIEC), which are capable of forming biofilms, and of invading and replicating within host cells, may be particularly significant. For example, the abnormal colonisation of the ileal mucosa by AIEC is believed to be a factor on the development of Crohn's disease. *E. coli* are also commonly involved in urinary tract infections and other conditions.

For treating *E. coli* infection, or a condition associated therewith, it will typically desirable to use a complex comprising at least one bacteriocin having a targeting portion specific for *E. coli*, e.g. a targeting portion of a colicin. As discussed elsewhere in this specification, it may be desirable to use a complex having at least two such bacteriocins, or which exclusively contains such bacteriocins.

Infection with *Klebsiella pneumoniae* may be associated, for example, with sepsis, or with bronchitis or pneumonia (often in the form of bronchopneumonia). Affected patients are prone to developing lung abscesses, cavitation, empyema or pleural adhesions.

For treating *Klebsiella pneumoniae* infection, or a condition associated therewith, it will typically desirable to use a complex comprising at least one bacteriocin having a targeting portion specific for *Klebsiella pneumoniae*, e.g. a targeting portion of a klebicin. As discussed elsewhere in this specification, it may be desirable to use a complex having at least two such bacteriocins, or which exclusively contains such bacteriocins.

Likewise, for treating infection with other bacterial types, such as *Salmonella enterica*, *Enterobacter cloacae* or *Yersinia pestis*, or a condition associated therewith, it will typically desirable to use a complex comprising at least one bacteriocin having a targeting portion specific for that bacterial species or strain, e.g. a targeting portion of a salmocin, cloacin or pesticin as appropriate. As discussed elsewhere in this specification, it may be desirable to use a complex having at least two such bacteriocins, or which exclusively contains such bacteriocins.

Typically the subject to be treated is a mammal. The subject is typically human, but may be any other primate (great ape, old world monkey or new world monkey), or a domestic, laboratory or livestock animal, such as a mouse, rat, guinea pig, lagomorph (e.g. rabbit), cat, dog, pig, cow, horse, sheep or goat.

Pharmaceutical Compositions

The complexes described in this specification can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, pulmonary, intramuscular, intraperitoneal routes or topical application. Oral, intravenous or pulmonary routes may be preferred.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or
5 synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has
10 suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

15 The term "pulmonary administration" is intended to encompass any suitable delivery method by which the active agent is delivered to the lungs via the respiratory tract. The most common methods of pulmonary administration are oral and/or nasal inhalation.

The complexes of the invention may be formulated for pulmonary administration in any suitable manner,
20 e.g. in a liquid or solid (typically powder) form. Formulations may be delivered by any suitable mechanism or delivery device including an inhaler (e.g. metered-dose inhaler, dry powder inhaler) nebuliser (e.g. ultrasonic nebuliser, jet nebuliser, vibrating mesh nebuliser), etc..

Thus the invention further provides a device for pulmonary administration of a therapeutic composition to
25 a subject, the composition comprising an S-type pyocin as described elsewhere in this specification. The device may be an inhaler (e.g. metered-dose inhaler, dry powder inhaler) or nebuliser (e.g. ultrasonic nebuliser, jet nebuliser, vibrating mesh nebuliser).

Liquid compositions generally include an aqueous carrier such as water or physiological saline solution.
30 Dextrose or other saccharide solutions or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

Emulsions and nano-particle encapsulations, both employing lipids, may also be employed.

35 Solid (e.g. powder) preparations may utilise carriers such as sugars, cyclodextrins, etc. They may be prepared by any suitable method including spray drying, spray freeze drying, solvent precipitation, jet milling, etc..

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount"
40 (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will

depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Sequences

The following sequences are employed in the Examples below. Deviations from the corresponding wild type sequences are indicated, typically resulting from incorporation of suitable restriction sites to facilitate exchange of targeting portions. It will be understood that either the wild type or variant sequence (or further variations) may be used in the context of the present invention.

15 ColE9: amino acid sequence

(E9 DNase domain underlined)

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSGWSENNPWGGGSGSGIHWGGGSGRGNG
GGNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFGVV
LSSLIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPLDKATVNVNVRVDDVKDERQNISVSGVPMSV
20 PVVDAKPTERPGVFTASIPGAPVLNISVNNSTPAVQTLSPGVNTNTDKDVRPAGFTQGGNTRDAVIRFPK
DSGHNAVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQE
RQAKAVQVYNSRKSELDAAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQA
AFDAAAKEKSDADAALSAAQERRKQKENKEKDAKDKLAMESKRNKPGKATGKGKPVGDKWLDDAGKD
SGAPIPDRIADKLRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQQVGGRKV
25 YELHHDKPISQGGGEVYMDNIRVTTPKRHIDIHRGK (SEQ ID NO: 5)

[In the wild type sequence, the last residue of the targeting portion is D (not A) and the first residue of the effector portion is K (not M).]

30 ColE9 targeting portion: amino acid sequence

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSGWSENNPWGGGSGSGIHWGGGSGRGNG
GGNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFGVV
LSSLIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPLDKATVNVNVRVDDVKDERQNISVSGVPMSV
PVVDAKPTERPGVFTASIPGAPVLNISVNNSTPAVQTLSPGVNTNTDKDVRPAGFTQGGNTRDAVIRFPK
35 DSGHNAVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQE

RQAKAVQVYNSRKSELDAAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQA
AFDAAAKEKSDADAALSAAQERRKQKENKEKDAKDKLA (SEQ ID NO: 6)

[In the wild type sequence, the last residue of the targeting portion is D (not A).]

5

ColE9 effector portion (DNase): amino acid sequence

MESKRNKP GKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKLRDKEFKSFDDFRKAVWEEVSKDPELS
KNLNPSNKS SVSKGYSPFTPKNQQVGGGRKVYELHHDKPISQGGEVYDMDNIRVTPKRHIDIHRGK
(SEQ ID NO: 7)

10

[In the wild type sequence, the first residue of the effector portion is K (not M).]

ColE9: coding sequence

(E9 DNase domain underlined, NcoI site in **bold**)

15 ATGAGCGGTGGAGATGGACGCGGCCATAACACGGGCGCGCATAGCACAAAGTGGTAACATTAATGGT
GGCCCGACCGGGATTGGTGTAAGTGGTGGTGCTTCTGATGGTTCAGGATGGAGTTCGGAAAATAAC
CCGTGGGGTGGTGGTTCGGGTAGCGGCATTCACTGGGGAGGTGGCTCCGGTCTGGTAATGGCGG
GGGTAATGGCAATTCGGTGGTGGCTCGGGAACAGGCGGTAATTTGTCAGCAGTAGCTGCGCCAGT
GGCATTGTTTTCCGGCTCTTCCACTCCAGGAGCTGGCGGTCTGGCTGTCAGTATTTCTGCAAGC
20 GAATTATCGGCAGCTATTGCTGGTATTATTGCTAAATTAAGTAAATCTTAAATCACTCCTTTT
GGGGTTGTCTTATCTTCATTAATTCCGTCGGAAATAGCGAAAGATGACCCCAATATGATGTCAAAGAT
TGTGACGTCATTACCCGAGATGATATTACTGAATCACCTGTCAGTTCATTACCTCTCGATAAGGCAA
CAGTAAACGTAAATGTTCTGTGTTGTTGATGATGTAAAAGACGAACGACAGAATATTTCCGGTTGTTCA
GGTGTTCGATGAGTGTTCCGGTGGTTGATGCAAAACCTACCGAACGTCCAGGTGTTTTTACGGCAT
25 CAATTCCAGGTGCACCTGTTCTGAATATTTAGTTAATAACAGTACGCCAGCAGTACAGACATTAAGC
CCAGGTGTTACAAATAATACTGATAAGGATGTTCCGCCGCGCAGGATTTACTCAGGTGGTAATACCA
GGGATGCAGTTATTCGATTCCCGAAGGACAGCGGTCATAATGCCGTATATGTTTCAGTGAGTGATGT
TCTTAGTCCTGACCAGGTAAACAACGTCAAGATGAAGAAAATCGCCGTCAGCAGGAATGGGATGCT
ACGCATCCGGTTGAAGCGGCTGAGCGAAATTATGAACGCGCGCGTGCAGAGCTGAATCAGGCAAAT
30 GAAGATGTTGCCAGAAATCAGGAGCGACAGGCTAAAGCTGTTCAAGTTTATAATTCGCGTAAAGCG
AACTTGATGCAGCGAATAAACTCTTGCTGATGCAATAGCTGAAATAAAACAATTTAATCGATTTGCC
CATGACCCAATGGCTGGCGGTACAGAATGTGGCAAATGGCCGGGCTTAAAGCTCAGCGGGCGCA
GACGGATGTAAATAATAAGCAGGCTGCATTTGATGCTGCTGCAAAAGAGAAGTCAGATGCTGATGCT
GCATTAAGTGCCGCGCAGGAGCGCCGCAACAGAAGGAAAATAAAGAAAAGGACGCTAAGGATAAA
35 TTAG**CCATGG**GAGAGTAAACGGAATAAGCCAGGGAAGGCGACAGGTAAAGGTAAACCAGTTGGTGAT
AAATGGCTGGATGATGCAGGTAAAGATTCAAGAGCGCCAATTCCAGATCGCATTGCTGATAAGTTGC
GTGATAAAGAATTTAAAGCTTCGACGATTTTCGGAAGGCTGTATGGGAAGAGGTGTCGAAAGATCC
TGAGCTTAGTAAAAATTTAAACCAAGCAATAAGTCTAGTGTTCAAAAGGTTATTCTCCGTTTACTCC

AAAGAATCAACAGGTCGGAGGGAGAAAAGTCTATGAACTTCATCATGACAAGCCAATTAGTCAAGGT
GGTGAGGTTTATGACATGGATAATATCCGAGTGACTACACCTAAGCGACATATCGATATTCACCGAG
GTAAGTAA (SEQ ID NO: 8)

5 **ColE9 immunity protein ("Im9"): amino acid sequence**

MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
QWRAANGKSGFKQG (SEQ ID NO: 9)

ColE9 immunity protein ("Im9") with C-terminal LE(His)₆ tag: amino acid sequence

10 (C-terminal LE(His)₆ tag underlined)

MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
QWRAANGKSGFKQGLEHHHHHH (SEQ ID NO: 10)

15 **ColE9 immunity protein ("Im9") with C-terminal LE(His)₆ tag: coding sequence**

(C-terminal LE(His)₆ tag underlined, XhoI site in **bold**)

ATGGAAGTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTACAACCTGTAACAACAATTTGT
AATGCGGACACTTCCAGTGAAGAAGAACTGGTTAAATTGGTTACACACTTTGAGGAAATGACTGAGC
ACCCTAGTGGTAGTGATTTAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGTATTGTA
20 AACACAGTAAACAATGGCGAGCCGCTAACGGTAAGTCAGGATTTAAACAGGGCC**CTCGAGCACCAC**
CACCACCACCACTGA (SEQ ID NO: 11)

ColD: amino acid sequence

(tRNase domain underlined)

25 MSDYEGSGPTEGIDYGHSMVWVPSTGLISGGDVKPGGSSGIAPSMPPGWGDYSPQGIALVQSVLFPGII
RRIILDKLEEGLDWSGWSVSVHSPWGNKVSAAARTVLENGLRGGLPEPSRPAAVSFARLEPASGNEQKII
RLMVTQQLQVTDIPASQLPAAGNNVPVKYRLMDLMQNGTQYMAIIGGIPMTVPVVDVAVPVPDRSRPGT
NIKDVYSAPVSPNLPDLVLSVGQMNTPLVLSNPEIQEEGVIAETGNYVEAGYTMSSNNHDVIVRFPEGSDV
SPLYISTVEILDSNGLSQRQEAENKAKDDFRVKKEEAVARAEAEKAKAELFSKAGVNPQPPVYTQEMMER
30 ANSVMNEQGALVLNNTASSVQLAMTGTGVWTAAGDIAGNISKFFSNALEKVTIPEVSPLLMRISLGALWF
HSEEAGAGSDIVPGRNLEAMFSLSAQMLAGQGVIIEPGATSVNLPVRGQLINSNGQLALDLLKTGNESIP
AAVPVLNAVVRTATGLDKITLPAVVGAPSRITLVNVPQPSVPTDTGNHQPVPVTPVHTGTEVKSVEMPV
TTITPVSDVGGLRDFIYWRPDAAGTGVEAMDVMLNDPLDSGRFSRKQLDKKYKHAGDFGISDTKKNRET
LTKFRDAIEEHLSDKDTVEKGTyrREKGSKVYFNPNTMNVVVIKSNGEFLSGWKINPDADNGRIYLETGEL
35 (SEQ ID NO: 12)

[In the wild type sequence, the first two residues of the effector portion are VY (not MD).]

ColD targeting portion: amino acid sequence

40 MSDYEGSGPTEGIDYGHSMVWVPSTGLISGGDVKPGGSSGIAPSMPPGWGDYSPQGIALVQSVLFPGII
RRIILDKLEEGLDWSGWSVSVHSPWGNKVSAAARTVLENGLRGGLPEPSRPAAVSFARLEPASGNEQKII

RLMVTQQLEQVTDIPASQLPAAGNNVPVKYRLMDLMQNGTQYMAIIGGIPMTVPVVDVAVPDRSRPGT
NIKDVYSAPVSPNLPDLVLSVGQMNTPVLSNPEIQEEGVIAETGNYVEAGYTMSSNNHVDVIRFPEGSDV
SPLYISTVEILDSNGLSQRQEAENKAKDDFRVKKEEAVARAEAEKAKAELFSKAGVNPQPPVYTQEMMER
ANSVMNEQGALVLNNTASSVQLAMTGTGVWTAAGDIAGNISKFFSNALEKVTIPEVSPLLMRISLGALWF
5 HSEEAGAGSDIVPGRNLEAMFSLSAQMLAGQGVVIEPGATSVNLPVRGQLINSNGQLALDLLKTGNESIP
AAVPLNAVRDTATGLDKITLPAVVGAPSRITLVNVPVQPSVPTDTGNHQPVPVTPVHTGTEVKSVEMPV
TTITPVSDVGGLRDFIYWRPDAAGTGVEA (SEQ ID NO: 13)

Cold effector portion: amino acid sequence

10 MDVMLNDPLDSGRFSRKQLDKKYKHAGDFGISDTKKNRETLTKFRDAIEEHLSDKDTVEKGTyrREKGS
KVYFNPNTMNVVIKSNGEFLSGWKINPDADNGRIYLETGEL (SEQ ID NO: 14)

[In the wild type sequence, the first two residues of the effector portion are VY (not MD).]

15 **Cold: coding sequence**

(tRNase domain underlined, NcoI site in **bold**)

ATGAGTGATTACGAAGGTAGTGGTCCGACAGAAGGTATTGATTATGGGCACTCGATGGTCGTGTGG
CCGTCAACAGGACTGATTTCCGGTGGTGATGTGAAACCAGGAGGCTCATCAGGTATCGCTCCATCC
ATGCCTCCGGGATGGGGGATTACAGCCACAGGGTATCGCACTTGTACAAAGTGTTCTTTTCCG
20 GGAATTATTCGCCGATTATTCTGGATAAGGAACCTGAAGAGGGAGACTGGTCGGGATGGTCTGTCA
GTGTGCATAGCCCTTGGGGAACGAGAAAGTTTCCGCTGCACGAACAGTTCTTGAGAATGGTTTACG
TGGTGGTTTGCCAGAACCGTCTCGCCCGGCTGCTGTTTCTTTGCCCGTCTGGAGCCTGCTTCCGG
AAATGAGCAAAAAATTATTCGTCTTATGGTTACACAGCAACTGGAACAGGTAACGGATATTCGGGCCA
GCCAGTTACCAGCAGCGGGTAATAATGTACCGGTGAAATATCGTCTGATGGACCTTATGCAGAACGG
25 TACGCAATATATGGCTATTATCGGAGGTATTCCGATGACAGTGCCGGTAGTTGATGCCGTTCCAGTT
CCGGACCGGAGTCGTCCGGGAACAAATATTAAGATGTTTACAGTGCCCTGTATCACCAATCTAC
CGGACCTGGTATTAAGTGTGGGTCAGATGAATACTCCGGTTCTGTCTAATCCCGAAATTCAGGAAGA
AGGAGTTATTGCTGAGACAGGTAATTATGTTGAGGCTGGTTATACGATGTCCAGTAATAATCATGATG
TCATTGTTCTGTTTTCTGAAGGCAGTGATGTTTCTCCGTTATATATTTCAACTGTAGAGATTCTGGACA
30 GTAATGGTCTGAGTCAGCGTCAGGAAGCCGAAAATAAGCAAAGGATGATTTAGAGTCAAGAAAGA
AGAAGCGGTAGCCCGGGCTGAAGCTGAAAAAGCAAAAGCCGAGTTATTTAGTAAAGCAGGTGTGAA
CCAGCCTCCTGTATATACACAGGAAATGATGGAAAGGGCTAATTCAGTAATGAATGAGCAGGGAGCT
CTTGTCTGAATAACACTGCCAGTTCTGTACAACCTGGCGATGACCGGAACTGGTGTCTGGACTGCTG
CTGGTGATATTGCAGGCAACATAAGCAAGTTTTTCAGTAATGCTCTGGAGAAAAGTAACCATACCCGA
35 AGTGAGTCCCCTGCTTATGCGGATTTCTCTTGGTGCTCTGTGGTTTCATTGAGAAGAAGCTGGAGCA
GGAAGTGATATCGTGCCGGGGCGAAATCTGGAGGCGATGTTTTCGCTGAGTGCTCAGATGTTGGCT
GGACAGGGCGTGGTCATTGAACCTGGTGCGACGAGTGTAATCTGCCTGTTCTGGACAATTGATA
AACAGTAACGGGCAATTAGCTCTGGATTTACTGAAAACAGGGGAATGAAAGTATCCCGGCTGCGGTTCT
CTGTTCTTAATGCTGTCCGTGATACAGCAACAGGACTGGATAAAATCACGTTACCAGCAGTAGTAGG
40 CGCACCTTCCCGGACGATTCTGGTTAATCCGGTACCACAACCTTCGGTGCCAACAGATACAGGTAAT
CATCAACCCGTTCCGGTTACACCAGTGCATACAGGAACGGAAGTAAATCGGTGCAATGCCAGTAA

CGACGATTACTCCTGTTTCTGATGTTGGTGGACTACGAGATTTTATTTACTGGCGTCCTGATGCTGCT
 GGGACTGGTGTGGAAGCCATGGATGTGATGCTTAATGATCCTCTGGATTACAGGGCGATTTTCGCGTA
AACAACCTTGATAAAAAATATAAACATGCTGGTGAATTTGGTATTAGTGATACAAAAAGAATCGTGAAA
CTCTTACTAAATTCAGGGATGCTATTGAGGAGCATTATCGGATAAGGATACAGTAGAGAAAGGAAC
 5 ATACCGAAGAGAAAAAGGTTCAAAAGTTTATTTTAATCCTAATACGATGAATGTGGTTATAATTAAGTC
AAATGGTGAGTTCTTATCTGGGTGGAAAATAATCCAGATGCGGATAATGGTCGAATTTATTTAGAGA
CAGGTGAACATGA (SEQ ID NO: 15)

CoID immunity protein ("ImD"): amino acid sequence

10 MNKMAMIDLAKLFLASKITAIEFSERICVERRRLYGVDLSPNINLNCGEELFMAAERFEPDADRANYEIDDN
 GLKVEVRSILEKFKL (SEQ ID NO: 16)

CoID immunity protein ("ImD") with C-terminal LE(His₆) tag: amino acid sequence

(LE(His₆) tag underlined)

15 MNKMAMIDLAKLFLASKITAIEFSERICVERRRLYGVDLSPNINLNCGEELFMAAERFEPDADRANYEIDDN
 GLKVEVRSILEKFKLLEHHHHHH (SEQ ID NO: 17)

CoID immunity protein ("ImD") with C-terminal His₆ tag: coding sequence

(LE(His₆) tag underlined, XhoI cloning site in **bold**)

20 ATGAATAAGATGGCAATGATCGATTTGGCGAAATTATTTTAGCTTCGAAAATTACAGCAATTGAGTTT
 TCAGAGCGAATTTGTGTTGAACGGAAGATTGTATGGTGTAAAGGATTTGTCTCCGAATATATTA
 TTGTGGGGAAGAGTTGTTTATGGCTGCTGAGCGATTTGAGCCTGATGCAGATAGGGCTAATTATGAA
 ATTGATGATAATGGACTTAAGGTTGAGGTCCGATCTATCTTGGAAAAATTTAAATTACT**CGAGCACCA**
CCACCACCACCACTGA (SEQ ID NO: 18)

25

KlebC targeting portion: amino acid sequence

MADNQPVPLTPAPPGMVSLGVNENGEEMTVIGGDGSGTGFSGNEAPIIPGSGSLQADLGKSLTRLQA
 ESSAAIHATAKWTTENLAKTQAAQAERAKAAMLSQQAQAKAKQAKLTQHLKDVDRALQNNKTRPTVIDLA
 30 HQNNQQMAAMAEFIGRQKAIEEARKKAEREAKRAEEAYQAALRAQEEQQRKQAEIERKLQEARKQEA
 KAKAEADRIA AEKAEAEARAKAEAEERRKAEARKALFAKAGIKDTPVYTLEMTKAATTLFLTPGVRLLNRA
 PAMIQLSALAAEINGVLTTAASAVMTATAEFGWIASALWRGVAGVATSTVGPMVAAASTLFFSPRAGG
 GSDSKVPGRDIEMLAQARLFTAGKLSIEPGMKSVNLPVRGFISSETDGRQSLMLVKTGSDGVPSTVPVL
 DAVRDSTTGLDKITVPAMSGAPSRITLVNPVPIGPAAPWHTGNSGPVPVTPVHTGTEVKQADSIVTTTLPI
 35 ADIPPLQDFIYWQPDASGTGVEPIYVMA (SEQ ID NO: 19)

KlebC-E9 chimera (KlebC targeting portion; E9 effector portion): amino acid sequence

(E9 effector portion (DNase) underlined)

MADNQPVPLTPAPPGMVSLGVNENGEEMTVIGGDGSGTGFSGNEAPIIPGSGSLQADLGKSLTRLQA
 40 ESSAAIHATAKWTTENLAKTQAAQAERAKAAMLSQQAQAKAKQAKLTQHLKDVDRALQNNKTRPTVIDLA
 HQNNQQMAAMAEFIGRQKAIEEARKKAEREAKRAEEAYQAALRAQEEQQRKQAEIERKLQEARKQEA
 KAKAEADRIA AEKAEAEARAKAEAEERRKAEARKALFAKAGIKDTPVYTLEMTKAATTLFLTPGVRLLNRA

PAMIQLSALAAEINGVLTTAASAVMTATAEFSGWIASALWRGVAGVATASTVGPMVAAASTLFFSPRAGG
GSDSKVPGRDIEMLAQARLFTAGKLSIEPGMKSVNLPVRGFISSETDGRQSLMLVKTGSDGVPSTVPVL
DAVRDSTTGLDKITVPAMSGAPSRITLVNPVPIGPAAPWHTGNSGPVPVTPVHTGTEVKQADSIVTTTLPI
ADIPPLQDFIYWQPDASGTGVEPIYVMAMESKRKNKPGKATGKGKPVGDKWLDDAGKDSGAPIPDRIADK
5 LRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQQVGGGRKVYELHHDKPISQG
GEVYDMDNIRVTTPKRHIDIHRGK (SEQ ID NO: 20)

KlebC-E9 chimera: coding sequence(E9 effector portion (DNase) underlined)

10 ATGGCCGACAACCAGCCGTTCCGCTGACGCCAGCCCCACCAGGCATGGTGAGCTTAGGTGTGAA
CGAGAACGGAGAGGAAGAAATGACGGTAATCGGTGGCGATGGATCGGGCACGGGTTTCTCAGGCA
ACGAAGCACCGATTATTCCAGGCAGCGGTAGTCTCCAGGCCGATCTGGGTAAGAAAAGCTTAACCTC
GCCTGCAAGCGGAATCATCTGCAGCCATTACGCTACCGCAAAATGGACCACGGAAAACCTGGCCA
AGACACAGGCCCGCACAAAGCCGAACGTGCCAAAGCCGCAATGCTGAGCCAACAAGCGGCGAAAGCT
15 AAACAAGCGAAACTGACCCAGCACCTTAAAGATGTAGTCGACCGCGCGCTTCAGAATAACAAAACGC
GTCCTACCGTGATCGATTTGGCGCATCAGAACAAATCAGCAAATGGCGGCTATGGCAGAGTTTATCGG
CCGCCAGAAAGCGATTGAAGAAGCTCGTAAGAAAGCAGAACCGCAAGCCAAACGTGCTGAAGAAGC
GTATCAGGCCGCGTTGCGTGCGCAAGAAGAGGAGCAGCGTAAACAGGCCGAAATCGAACGCAAGC
TGCAAGAAGCCCGCAAGCAGGAAGCTGCGGCGAAAGCGAAAGCAGAAGCAGATCGCATTGCCGCC
20 GAGAAAGCAGAGGCGGAAGCTCGTGCGAAAGCGGAGGCCGAACGCCGTAAAGCGGAAGAAGCAC
GCAAAGCGCTGTTTGCGAAAGCAGGGATCAAAGACACCCCTGTTTACACCTTGGAATGACTAAAGC
GGCGACAACACTGTTCTTACCCAGGGGTTCCGGCTGTTAAATCGCGCGCCTGCCATGATCCAGCT
GTCAGCCTTGGCTGCGGAGATCAATGGCGTCTTAACCACCGCAGCTTCTGCGGTGATGACGGCGAC
TGCCGAATTTTCGGGTTGGATTGCCTCTGCCTTATGGCGCGGAGTGGCGGGAGTCGCGACCGCTTC
25 GACCGTGGGACCGATGGTTGCAGCCGCAAGCACCCCTGTTCTTTCTCCGCGCGCTGGTGGCGGTA
GTGATTCGAAAGTTCCCGGCCGTGACATTGAAATGCTCGCGGCACAAGCTCGGCTGTTTACAGCGG
GTAAGCTGTCCATTGAGCCCGGTATGAAAAGCGTCAATCTGCCGTTTCGTGGCTTCATTTCTCCGA
AACCGACGGTCGTCAGTCGCTTATGCTCGTCAAACCGGCAGCGATGGTGTACCCAGTACTGTGCC
GGTGCTGGATGCCGTCCGCGATTCTACGACAGGGCTGGACAAAATTACCGTCCCCGCAATGTCCGGG
30 CGCACCGAGTCGCACAATTCTGGTGAATCCTGTACCGATTGGGCCGGCAGCGCCGTGGCATAACCG
GGAATAGTGGCCCTGTTCCGGTGACTCCTGTGCATACGGGCACTGAAGTCAAGCAGGCTGACTCCA
TCGTTACGACGACCCTGCCGATTGCTGATATTCCGCCGCTGCAAGACTTCATCTACTGGCAGCCGG
ACGCGTCAGGAACCGGCGTAGAACCGATCTACGTGATGGCCATGGAGAGTAAACGGAATAAGCCAG
GGAAGGCGACAGGTAAAGGTAAACCAGTTGGTGATAAATGGCTGGATGATGCAGGTAAAGATTTCAG
35 GAGCGCCAATTCCAGATCGCATTGCTGATAAGTTGCGTGATAAAGAATTTAAAAGCTTCGACGATTTT
CGGAAGGCTGTATGGGAAGAGGTGTCGAAAGATCCTGAGCTTAGTAAAAATTTAAACCCAAGCAATA
AGTCTAGTGTTCAAAAGGTTATTCTCCGTTTACTCCAAAGAATCAACAGGTTCGGAGGGAGAAAAGT
CTATGAACTTCATCATGACAAGCCAATTAGTCAAGGTGGTGAGGTTTATGACATGGATAATATCCGAG
TGACTACACCTAAGCGACATATCGATATTCACCGAGGTAAGTAA (SEQ ID NO: 21)

CloDF13 targeting portion: amino acid sequence

MSGGDGRGPGNSGLGHNGGQASGNVNGTSGKGGPSSGGGTDPNSGPGWGTTHTPNGDIHNYNPGE
 FGNGGSKPGNGGNSGNHSGSSGGGQSSATAMAFGLPALATPGAEG LALS VSGDALSAAVADVLAAL
 KGPFFKGLWGIAIYGVLPSEIAKDDPNMMSKIMTSLPADTVTETPVSTLPLEQATVRVRQRVVDVVKDER
 5 QHIAVVAGRPMSVPVVDKPTKRPGVFSVSIPGLPSLQVSVPKGVPAAKAPPKGIVA EKGDSRPAGFTAG
 GNSREAVIRFPKETGQKPVYVSVTDVLT PAQVKQRQEEEEKRRQQA WDAAHPEEGLKREYDKAKAELDA
 EDKNIATLNGRITSTEKTIPGARTAVQEADKKVKEAEANKDDFVTYNPPHEYGSGWQDQVRYLDKDIQNNQ
 NEKLKAAQASLNAMNESLSRDKAALSGAMESRKQKEKKAKDAENKLNEEA (SEQ ID NO: 22)

10 ColE3 effector portion (rRNase): amino acid sequence

MEKNKPRKGF KDYGH DYHPAPKTENIKGLGDLKPGIPKTPKQNGGGKRRKRWTDGDKGRKIYEWDSQHGE
 LEGYRASDGQHLGSFDPKTGNQLKGPDPKRNIKKYL (SEQ ID NO: 23)

ColE3 immunity protein ("Im3"): amino acid sequence

15 MGLKLDLTWFDKSTEDFKGEEYSKDFGDDGSVMESLGVPFKDNVNNGCFDVIAEWVPLLQPYFNHQIDI
 SDNEYFVSFDYRDGDW (SEQ ID NO: 24)

KvarM: amino acid sequence

MSDTMIVVATPTPGFSYASGLTYGGGAFAGAPANGPSEGQIFFQTVLPAYQSPNLCIGQLAWMTDYINKN
 20 GVGNPKTWEVISQNVLIFCSADTALVLNPRIAVYDGFHKT KWAPAKFNFKTQSQEKFSGNVTTPIAAFGH
 YLWGEKPRPTVDLSSVGLKIQANQIDPVMIAVKNNAAAGTYQISGNFNRTFIDGDIPGLYLGNITMKTEGT
 LKIDAKGNWNYNGVVRAFNDTYDANPSTHRSKSAEDLTLLRLTQGTPYEIRIPGELKVS GSGKK (SEQ
 ID NO: 25)

25 CloDF13-E3 chimera (CloDF13 targeting portion; ColE3 effector portion): amino acid sequence
 (ColE3 effector portion (rRNase) underlined)

MSGGDGRGPGNSGLGHNGGQASGNVNGTSGKGGPSSGGGTDPNSGPGWGTTHTPNGDIHNYNPGE
 FGNGGSKPGNGGNSGNHSGSSGGGQSSATAMAFGLPALATPGAEG LALS VSGDALSAAVADVLAAL
 KGPFFKGLWGIAIYGVLPSEIAKDDPNMMSKIMTSLPADTVTETPVSTLPLEQATVRVRQRVVDVVKDER
 30 QHIAVVAGRPMSVPVVDKPTKRPGVFSVSIPGLPSLQVSVPKGVPAAKAPPKGIVA EKGDSRPAGFTAG
 GNSREAVIRFPKETGQKPVYVSVTDVLT PAQVKQRQEEEEKRRQQA WDAAHPEEGLKREYDKAKAELDA
 EDKNIATLNGRITSTEKTIPGARTAVQEADKKVKEAEANKDDFVTYNPPHEYGSGWQDQVRYLDKDIQNNQ
 NEKLKAAQASLNAMNESLSRDKAALSGAMESRKQKEKKAKDAENKLNEEA MEKNKPRKGF KDYGH DYH
PAPKTENIKGLGDLKPGIPKTPKQNGGGKRRKRWTDGDKGRKIYEWDSQHGE
 35 LEGYRASDGQHLGSFDPK
TGNQLKGPDPKRNIKKYL (SEQ ID NO: 26)

CloDF13-E3 chimera (CloDF13 targeting portion; E3 effector portion): coding sequence

(E3 effector portion (rRNase) underlined)

40 ATGAGCGGCGCGATGGTCGTGGTCCGGTAATAGCGGTCTGGGTCATAACGGCGGTCAAGCGAG
 CGGCAATGTGAACGGCACCAGCGGTAAGGGTGGCCCGAGCAGCGGTGGCGGTACCGATCCGAACA

GCGGTCCGGGTTGGGGTACCACCCACACCCCGAACGGTGATATCCACAACCTACAACCCGGGCGAA
TTCGGTAACGGCGGTAGCAAACCGGGCGGTAACGGCGGTAACAGCGGTAACCATAGCGGTAGCAG
CGGCGGTGGCCAGAGCAGCGCGACCGCGATGGCGTTTGGTCTGCCGGCGCTGGCGACCCCGGGT
GCGGAAGGTCTGGCGCTGAGCGTTAGCGGTGATGCGCTGAGCGCGGCGGTTGCGGATGTGCTGG
5 CGGCGCTGAAGGGTCCGTTCAAATTTGGCCTGTGGGGTATCGCGATTTATGGCGTGCTGCCGAGCG
AGATTGCGAAGGACGATCCGAACATGATGAGCAAAATCATGACCAGCCTGCCGGCGGACACCGTTA
CCGAAACCCCGGTGAGCACCTGCCGCTGGAGCAGGCGACCGTGCGTGTTCTGTCACGTGTGGTT
GACGTGGTTAAGGATGAACGTCAGCACATTGCGGTGGTTGCGGGTCGTCCGATGAGCGTTCCGGTG
GTTGACGCGAAACCGACCAAGCGTCCGGGTGTTTTAGCGTGAGCATTCCGGGCGCTGCCGAGCCT
10 GCAAGTGAGCGTTCCGAAGGGCGTTCCGGCGGCGAAGGCTCCGCCGAAAGGCATTGTTGCGGAGA
AAGGTGACAGCCGTCCGGCGGGTTTACCGCGGGTGGCAACAGCCGTGAAGCGGTGATCCGTTTT
CCGAAGGAAACCGGTCAGAAACCGGTGTACGTTAGCGTGACCGATGTTCTGACCCCGGCGCAAGTG
AAGCAGCGTCAAGAGGAAGAGAAACGTCGTCAACAAGCGTGGGATGCGGCGCACCCGGAAGAGGG
TCTGAAACGTGAATATGATAAAGCGAAGGCGGAACTGGACGCGGAGGATAAGAACATCGCGACCCT
15 GAACGGTCGTATTACCAGCACCGAAAAAACATTCCGGGTGCGCGTACCGCGGTTCCAGGAAGCGGA
CAAGAAAGTGAAGGAAGCGGAGGCGAACAAGACGATTTTGTACCTACAACCCGCCGACGAGTA
TGGCAGCGGTTGGCAGGATCAAGTGCGTTACCTGGACAAGGATATTCAGAACCAAAACGAAAACT
GAAAGCGGCGCAGGCGAGCCTGAACGCGATGAACGAGAGCCTGAGCCGTGACAAAGCGGCGCTG
AGCGGTGCGATGGAAAGCCGTAAACAAAAGGAGAAGAAAGCGAAGGATGCGGAAAAACAACTGAAC
20 GAAGAGGCCATGGAAGAAATAAGCCCAGAAAAGGTTTTAAAGATTACGGGCATGATTATCATCCAG
CTCCGAAAACCTGAGAATATTAAGGGCTTGTTGATCTTAAGCCTGGGATACCAAAAACACCAAAGCA
GAATGGTGGTGGAAAACGCAAGCGCTGGACTGGAGATAAAGGGCGTAAGATTTATGAGTGGGATTC
TCAGCATGGTGAGCTTGAGGGGTATCGTGCCAGTGATGGTCAGCATCTTGGCTCATTTGACCCTAAA
ACAGGCAATCAGTTGAAAGGTCCAGATCCGAAACGAAATATCAAGAAATATCTTTGA (SEQ ID NO:
25 27)

KlebG targeting portion: amino acid sequence

MGGGFNYNGEGATGTGLDRDPYVRDSNGNAIGVKSQYHAESYGTSSPALGPNGAIQITAGVIAVPGDKP
30 RPDGSGSGGNTVNTGPAGQLLMNKGQLGYWETRSTGAGNNEHNTSVFVAVGPSEAEKTASAEKALK
EKQQAEEAAKDFAAKTAASATAEKERQQAIAAATAAGQHQSVDARNSLNNATSDVSRKLSAADSALQ
EAKAKRKAIDA VPVATQAENKYQELQKIKGLKLNGEYGTKEWEIIGSNKEHDHWGYRFYPSGITKAQ
VDVAQSDAVNKRNQATSLASQATAAEQDSLKATAAYNAAETRRQAAQAALNSAEQAAAAERKRQEAEA
AAAAAAEKKRQADAVAKAAEEARAAAEKARLMQERQAAADKLKSTDIQSVRGIPSTASPAASPISWAVAS
35 LGGISLDSVTAGKAWTQIAEVMALRGIAGASLVGPVVATAVGLFWSRDVGIGSDVVPGRDISGLMPGDA
LSLPDLATLIKAADSKTGVSMPVRGRIIVREGDYLESQFVRTPVAGSVPVVRATLDKATGYWGYTLPAIQG
VPGQTILVSPSDAPGVNAPLGLAGPVPLPETIHTGGQTTVPQGGTVTVSPVIGEVDFRDLILVFPPEGLK
PLYVMA (SEQ ID NO: 28)

KlebG-D chimera (KlebG targeting portion; ColD effector portion (tRNase)): amino acid sequence
(ColD effector portion underlined)

MGGGFNYNGEGATGTGLDRDPYVRDSNGNAIGVKSGYHAESYGTSSPALGPNGAIQITAGVIAVPGDKP
RPDGGSGGGNTVNTGTPAGQLLMNKGQLGYWETRSTGAGNNEHNTSVFVAVGPSEAEKTASAEKALK
5 EKQQAEAAAKDFAAKTAAASATAEKERQQAIAAATAAGQHQSVDARNSLNNATSDVSRLKSAADSALQ
EAKAKRKAIDA VPVATQAENKYQELQQKIKGLKLKNGEYGTEKWEIIGSNKEHDHWGYRFYPSGITKAQ
VDVAQSDAVNKRNQATSLASQATAAEQDSLKATAAYNAAETRRAQAALNSAEQAAAAERKRQEAEA
AAAAAAEKKRQADAVAKAAEEARAAAEKARLMQERQAAADKLKSTDIQSVRGIPSTASPAASPISWAVAS
LGGISLDSVTAGKAWTQIAEVMALRGIAGASLVGPVVATAVGLFWSRDVGIGSDVVPGRDISGLMPGDA
10 LSLPDLATLIKAADSKTGVSM PVRGRIIVREGDYLESQFVRTPVAGSVPVVRATLDKATGYWGYTLPAIQG
VPGQTILVSPSDAPGVNAPLGLAGPVPLPETIIHTGGQTTVPQGGTVTVSPVIGEVD FRDLILVFPPEGLK
PLYVMAMDMVMLNDPLDSGRFSRKQLDKKYKHAGDFGISDTKKNRETLTKFRDAIEEHLSDKDTVEKGTY
RREKGSKVYFNPNTMNVV IKSNGEFLSGWKINPDADNGRIYLETGEL (SEQ ID NO: 29)

15

KlebG-D chimera (KlebG targeting portion; ColD effector portion (tRNase)): coding sequence
(ColD effector portion underlined)

ATGGGTGGTGGCTTCAACTACAATGGGGAAGGAGCGACTGGGACCGGGTTAGATCGGGATCCGTAT
GTTCCGCGATAGCAATGGCAACGCCATTGGTGTGAAATCAGGCTACCATGCTGAGTCCTACGGCACT
20 AGCTCTCCGGCGTTAGGTCCAAACGGTGCCATT CAGATCACGGCGGGAGTCATTGCGGTACCTGGC
GATAAACCGCGTCCTGATGGTGGCTCTGGTGGCGGGAATACCGTGAACACAGGTCCAGCGGGCCA
ACTCCTGGTGATGAACAAAGGTCAGCTGGGGTATTGGGAAACGCGTAGCACTGGAGCAGGCAACAA
CGAACACAACACGAGTGCTTTGTGCGCGTGGGACCGTCGGAAGCCGAGAAAACCGCGTCAGCGG
AAAAGGCTTTGAAAGAGAAACAACAAGCGGAAGCCGCTGCGAAAGACTTTGCGGCGAAAACCGCCG
25 CAGCATCAGCGACCGCGGAGAAAGAACGCCAGCAGGCGATTGCTGCTGCAACAGCTGCGGGCCAG
CATCAGAGCGTTTTCGGATGCGCGTAATAGCCTCAATAATGCGACGTCCGACGTTAGTCGCCTGAAGT
CTGCTGCTGATAGTGCCCTTCAGGAAGCGAAAGCGAAACGCAAAGCAGCCATTGATGCGGTACCCG
TAGCGACGCAGGCGGAAAACAAATACCAGGAAC TTCAGCAGAAAATCAAAGGCCTGAACTGAAGA
ACGGCGAATATGGCACTGAGAAATGGGAGATCATTGGTTCAAACAAAGAGCACGACCATTGGGGTT
30 ATCGCTTCTATCCGTGCGGTATCACCAAAGCTCAAGTGGACGTGGCGCAAAGCGATGCCGTCAATA
AGCGCAATCAGGCCACCTCGCTGGCTAGTCAGGCAACAGCCGCGAGAACAAGACAGCCTGAAGGCA
ACCGCCGCTATAATGCAGCCGAAACCCGCCGTCAAGCGGCGCAAGCAGCCCTGAACTCCGCCGA
ACAGGCAGCGGCCGCGAAGCGCAAACGTCAGGAAGCGGAAGCCGCGAGCCGCGAGCCGCTGAA
AAGAAACGGCAGGCCGATGCCGTGGCAAAAGCAGCAGAAGAAGCCCGTGCTGCTGCTGAGAAAGC
35 GCGCTTGATGCAGGAGCGCCAGGCCGCGGCAGATAAGCTGAAAAGCACGGACATCCAATCCGTTC
GTGGCATTCCGTCTACCGCGTCGCCGGCAGCGAGCCCAATCAGTTGGGCGGTGGCAAGTCTGGGA
GGGATTTCTCTGGACAGCGTCACTGCTGGCAAAGCATGGACGCAGATTGCGGAAGTAATGGCCAAA
CTGCGTGGAATTGCCGGCGCAAGCTTGGTCGGCCCTGTTGTGGCAACGGCGGTAGGCCTGTTCTG
GAGCCGTGATGTGGGCATTGGTAGCGATGTGGTGCCTGGTCGCGATATTTGGGCGCTCATGCCCG
40 GAGATGCGCTGAGTCTGCCGATTTAGCGACCCTGATCAAGGCGGCTGATTCCAAAACCGGCGTG
GCATGCCGGTTCGCGGTCGCATTATTGTCCGCGAAGGCGACTACCTGGAATCCCAGTTTGTGCGTA

CTCCGGTTGCTGGTTCAGTGCCGGTCGTTCTGCTACGTTGGACAAAGCGACAGGCTATTGGGGTT
 ACACGCTTCCAGCCATTGAGGGTGACCTGGTCAAACCATCCTCGTTTCTCCGTCAGATGCCCCGG
 GAGTGAATGCACCGCTCGGTTTAGCTGGCCAGTTCGTTACCCGAGACTATTATCCACACGGGTG
 GTCAAACCACCGTTCCGCAAGGCGGCACAGTAACCGTATCACCGGTTATTGGGGAAGTGGACTTCC
 5 GCGATCTGATCCTGGTGTTCCTCCAGAATCGGGTCTGAAACCCCTGTATGTGATGGCCATGGATGT
GATGCTTAATGATCCTCTGGATTGAGGGCGATTTTCGCGTAAACAACCTTGATAAAAAATATAAACATG
CTGGTGATTTTGGTATTAGTGATACAAAAAGAATCGTGAAACTCTTACTAAATTCAGGGATGCTATT
GAGGAGCATTTATCGGATAAGGATACAGTAGAGAAAGGAACATACCGAAGAGAAAAAGGTTCAAAAG
TTTATTTTAATCCTAATACGATGAATGTGGTTATAATTAAGTCAAATGGTGAGTTCTTATCTGGGTGGA
 10 AAATAAATCCAGATGCGGATAATGGTGAATTTATTTAGAGACAGGTGAACTATGA (SEQ ID NO: 30)

Im9-ImD immunity protein scaffold: amino acid sequence (Im9 underlined, ImD double underlined)
MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
QWRAANGKSGFKQGNGINGPTGIGVSMNKMAMIDLAKLFLASKITAIEFSERICVERRRLYGVKDLSNII
 15 NCGEELFMAAERFEPDADRANYEIDDNGLKVEVRSILEKFKL (SEQ ID NO: 31)

Im9-ImD immunity protein scaffold with C-terminal LE(His6) tag: amino acid sequence (Im9 underlined, ImD double underlined, LE(His6) tag dashed underlined)
MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
 20 QWRAANGKSGFKQGNGINGPTGIGVSMNKMAMIDLAKLFLASKITAIEFSERICVERRRLYGVKDLSNII
NCGEELFMAAERFEPDADRANYEIDDNGLKVEVRSILEKFKLLEHHHHHHH (SEQ ID NO: 32)

Im9-ImD immunity protein scaffold with C-terminal LE(His6) tag: coding sequence (Im9 underlined, ImD double underlined, XhoI cloning site in **bold**, LE(His6) tag dashed underlined)
 25 ATGGAAGTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTACAACCTGTAACAACAATTTGT
AATGCGGACACTTCCAGTGAAGAAGAACTGGTTAAATTGGTTACACACTTTGAGGAAATGACTGAGC
ACCCTAGTGGTAGTGATTTAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGTATTGTA
AACACAGTAAACAATGGCGAGCCGCTAACGGTAAGTCAGGATTTAAACAGGGCAACATTAATGGTG
GCCCGACCGGGATTGGTGTAAGTATGAATAAGATGGCAATGATCGATTTGGCGAAATTATTTTAGC
 30 TTCGAAAATTACAGCAATTGAGTTTTAGAGCGAATTTGTGTTGAACGGAGAAGATTGTATGGTGTTA
AGGATTTGTCTCCGAATATATTAAATTGTGGGGAAGAGTTGTTTATGGCTGCTGAGCGATTGAGCCT
GATGCAGATAGGGCTAATTATGAAATTGATGATAATGGACTTAAGGTTGAGGTCCGATCTATCTTGA
AAAATTTAAATTACTCGAGCACCAACCACCACTGA (SEQ ID NO: 33)

Im9-Im3-ImD immunity protein scaffold: amino acid sequence (Im9 underlined, Im3 double underlined, ImD dashed underlined)
MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
QWRAANGKSGFKQGNGINGPTGIGVSMGLKLDLTFWFDKSTEDFKGEEYSKDFGDDGSVMESLGVPFKD
NVNNCGFDVIAEWWPLLQPYFNHQIDISDNEYFVSFDYRDGDWNGGGNGNSGGGSMNKMAMIDLAKLF
 40 LASKITAIEFSERICVERRRLYGVKDLSNIINCGEELFMAAERFEPDADRANYEIDDNGLKVEVRSILEKF
KL (SEQ ID NO: 34)

Im9-Im3-ImD immunity protein scaffold with C-terminal LE(His₆) tag: amino acid sequence (Im9 underlined, Im3 double underlined, ImD dashed underlined)

MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDDSPSGIVNTVK
 5 QWRAANGKSGFKQGNINGGPTGIGVSMGLKLDLTWFDKSTEDFKGEEYSKDFGDDGSVMESLGVPFKD
NVNNGCFDVIAEWWPLLQPYFNHQIDISDNEYFVSFDYRDGDWNGGGNGNSGGGSMNKMAMIDLAKLF
LASKITAIEFSERICVERRRLYGVKDLSPNINLNCGEELFMAAERFEPDADRANYEIDDNGLKVEVRSILEKF
KLLEHHHHHHH (SEQ ID NO: 35)

10 **Im9-Im3-ImD immunity protein scaffold with C-terminal LE(His₆) tag: coding sequence** (Im9 underlined, Im3 double underlined, ImD dashed underlined)

ATGGAACTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTTACAACTTGTAACAACAATTTGT
AATGCGGACACTTCCAGTGAAGAAGAACTGGTTAAATTGGTTACACACTTTGAGGAAATGACTGAGC
ACCCTAGTGGTAGTGATTTAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGTATTGTA
 15 AACACAGTAAACAATGGCGAGCCGCTAACGGTAAGTCAGGATTTAAACAGGGCAACATTAATGGTG
GCCCGACCGGGATTGGTGTAAGTATGGGACTTAAATTGGATTTAACTTGGTTTGATAAAAGTACAGA
AGATTTTAAGGGTGAGGAGTATTCAAAAGATTTTGGAGATGACGGTTCAGTTATGGAAAGTCTAGGT
GTGCCTTTTAAGGATAATGTTAATAACGGTTGCTTTGATGTTATAGCTGAATGGGTACCTTTGCTACA
ACCATACTTTAATCATCAAATTGATATTTCCGATAATGAGTATTTTGTTCGTTTGATTATCGTGATGGT
 20 GATTGGAATGGCGGGGGTAATGGCAATTCGGTGGTGGCTCGATGAATAAGATGGCAATGATCGAT
TTGGCGAAATTATTTTAGCTTCGAAAATTACAGCAATTGAGTTTTTCAGAGCGAATTTGTGTTGAACG
GAGAAGATTGTATGGTGTTAAGGATTTGTCTCCGAATATATTAAATTGTGGGGAAGAGTTGTTTATGG
CTGCTGAGCGATTTGAGCCTGATGCAGATAGGGCTAATTATGAAATTGATGATAATGGACTTAAGGTT
GAGGTCCGATCTATCTTGAAAAATTTAAATTACTCGAGCACCACCACCACCACCCTGA (SEQ ID
 25 NO: 36)

KvarM-Im9-ImD immunity protein scaffold with C-terminal LE(His₆) tag: amino acid sequence
 (KvarM *italics*, Im9 underlined, ImD double underlined, LE(His₆) tag dashed underlined)

MSDTMIVVATPTPGFSYASGLTYGGGAFAGAPANGPSEGQIFFQTVLPAYQSPNLCIGQLAWMTDYINKN
 30 *GVGNPKTWEVISQNVLIFCSADTALVLNPRIAVYDGFHKTKWAPAKFNFKTQSQEKFSGNVTTPIAAFGH*
*YLWGEKGKPRTVDLSSVGLKIQANQIDPVMIAVKNNNAAGTYQISGNFNRNTFIDGDIPGLYLGNI**TMKTEGT*
LKIDAKGNWNYNGVVRAFNDTYDANPSTHRSKSAEDLTLLRLTQGTPYEIRIPGELKVSGSGKKEAAAK
*EAAAKEAAAKEL*MELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEG
DDDDSPSGIVNTVKQWRAANGKSGFKQGNINGGPTGIGVSMNKMAMIDLAKFLASKITAIEFSERICVERR
 35 RLYGVKDLSPNINLNCGEELFMAAERFEPDADRANYEIDDNGLKVEVRSILEKFKLLEHHHHHHH (SEQ ID
 NO: 37)

KvarM-Im9-ImD immunity protein scaffold with C-terminal LE(His₆) tag: coding sequence (KvarM *italics*, Im9 underlined, ImD double underlined, XhoI cloning site in **bold**, LE(His₆) tag dashed underlined)

ATGTCTGATACAATGATTGTTGTTGCTACTCCGACTCCGGGTTTTCTTATGCAAGTGGTTAACCTAT
 GGTGGTGGTGCATTTGCCGGAGCGCCGGCAAATGGCCCGAGCGAAGGTCAAATCTTCTTCCAAACT
 GTGCTACCTGCATATCAATCACCTAATCTCTGTATTGGTCAGCTGGCATGGATGACTGACTATATTAA
 TAAAAATGGCGTAGGTAACCCGAAGACTTGGAAGTAATTTCTCAAAACGTACTCATCTTCTGTAGTG
 5 CTGATACCGCCCTGGTTTTGAATCTCGAATTGCCGTTTACGACGGTTTTCATAAACTAAATGGGCT
 CCGGCGAAGTTCAATTTCAAACGCAAAGTCAGGAGAAGTTTAGCGGTAACGTGACGACCCCGATC
 GCAGCATTGGTGATTATCTTTGGGGTGAAGGAAAGCCTCGTACCGTTGATTTGTCATCTGTTGGCC
 TTAAGATCCAGGCTAATCAGATTGACCCTGTGATGATTGCAGTCAAAAAAATACGCTGCAGGCACTTA
 CCAGATTAGCGGTAATTTTAATCGAAATACTTTCATTGATGGCGATATCCCTGGCCTCTATCTGGGCA
 10 ACATCACCATGAAGACCGAAGGTACTTTGAAAATTGATGCTAAGGGTAATTGGAATTATAACGGGGT
 AGTACGTGCATTTAACGATACCTATGATGCCAACCCTAGCACCCATCGAAGCAAATCTGCAGAAGAC
 CTCACAACCTCTACTGCGACTCACTCAAGGGACTCCTTATGAAATTCGCATCCCGGGCGAAGTCAAAG
 TGAGCGGCTCCGGTAAGAAAGAAGCTGCGGCAAAGGAGGCAGCTGCGAAAGAAGCGGCTGCCAAG
 GAGCTCATGGAAGTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTTACAACCTGTAACAAC
 15 AATTTGTAATGCGGACACTTCCAGTGAAGAAGAAGTGGTTAAATTGGTTACACACTTTGAGGAAATGA
 CTGAGCACCCCTAGTGGTAGTGATTTAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGT
 ATTGTAAACACAGTAAACAATGGCGAGCCGCTAACGGTAAGTCAGGATTTAAACAGGGCAACATTA
 ATGGTGGCCCGACCGGGATTGGTGTAAGTATGAATAAGATGGCAATGATCGATTTGGCGAAATTATT
 TTTAGCTTCGAAAATTACAGCAATTGAGTTTTAGAGCGAATTTGTGTTGAACGGAGAAGATTGTATG
 20 GTGTTAAGGATTTGTCTCCGAATATATTAATTTGTGGGGAAGAGTTGTTTATGGCTGCTGAGCGATT
 GAGCCTGATGCAGATAGGGCTAATTATGAAATTGATGATAATGGACTTAAGGTTGAGGTCCGATCTA
 TCTTGGAAAAATTTAAATTACTCTGAGCACCACCACCACCACCACTGA (SEQ ID NO: 38)

KvarM-Im9-Im7-ImD immunity protein scaffold with C-terminal LE(His₆) tag: amino acid sequence
 25 (KvarM *italics*, Im9 underlined, Im7 double underlined, ImD dashed underlined)

MSDTMIVVATPTPGFSYASGLTYGGGAFAGAPANGPSEGQIFFQTVLPAYQSPNLCIGQLAWMTDYINKN
 GVGNPKTWEVISQNVLFCSADTALVLNPRIAVYDGFHKTKWAPAKFNFKTQSQKEFSGNVTTPIAAGH
 YLWGEKPRTVDLSSVGLKIQANQIDPVMIAVKNNAAAGTYQISGNFNRNTFIDGDIPGLYLGNITMKTEGT
 LKIDAKGNWNYNGVVRAFNDTYDANPSTHRSKSAEDLTLLRLTQGTPYEIRIPGELKVS GSGKKEAAAK
 30 EAAAKEAAAKELMELKHSISDYTEAEFLQLVTTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEG
DDDSPSGIVNTVKQWRAANGKSGFKQGNINGGPTGIGVSMMELENSISDYTEAEFVQLLKEIEKENEAAATD
DVLVDVLEHFVKITEHPDGTDLIYPSDNRDDSPEGIVKEIKEWRAANGKPGFKQNGGGNGNSGGGSM
 NKMAMIDLAKLFLASKITAEFSERICVERRRLYGVKDLSPNILNCGEELFMAAERFEPDADRANYEIDDNG
LKVEVRSILEKFKLLEHHHHHH (SEQ ID NO: 39)

35

KvarM-Im9-Im7-ImD immunity protein scaffold with C-terminal LE(His₆) tag: coding sequence
 (KvarM *italics*, Im9 underlined, Im7 double underlined, ImD dashed underlined)

ATGTCTGATACAATGATTGTTGTTGCTACTCCGACTCCGGGTTTTCTTATGCAAGTGGTTAACCTAT
 GGTGGTGGTGCATTTGCCGGAGCGCCGGCAAATGGCCCGAGCGAAGGTCAAATCTTCTTCCAAACT

GTGCTACCTGCATATCAATCACCTAATCTCTGTATTGGTCAGCTGGCATGGATGACTGACTATATTAA
 TAAAAATGGCGTAGGTAACCCGAAGACTTGGGAAGTAATTTCTCAAAACGTACTCATCTTCTGTAGTG
 CTGATACCGCCCTGGTTTTGAATCTCGAATTGCCGTTTACGACGGTTTTCATAAACTAAATGGGCT
 CCGGCGAAGTTCAATTTCAAAACGCAAAGTCAGGAGAAGTTTAGCGGTAACGTGACGACCCCGATC
 5 GCAGCATTGGTTCATTATCTTTGGGGTGAAGGAAAGCCTCGTACCGTTGATTTGTCATCTGTTGGCC
 TTAAGATCCAGGCTAATCAGATTGACCCTGTGATGATTGCAGTCAAAAAATAACGCTGCAGGCACTTA
 CCAGATTAGCGGTAATTTAATCGAAATACTTTCATTGATGGCGATATCCCTGGCCTCTATCTGGGCA
 ACATCACCATGAAGACCGAAGGTACTTTGAAAATTGATGCTAAGGGTAATTGGAATTATAACGGGGT
 AGTACGTGCATTTAACGATACCTATGATGCCAACCCCTAGCACCCATCGAAGCAAATCTGCAGAAGAC
 10 CTCACAACCTCTACTGCGACTCACTCAAGGGACTCCTTATGAAATTCGCATCCCGGGCGAACTCAAAG
 TGAGCGGCTCCGGTAAGAAAGAAGCTGCGGCAAGGAGGCAGCTGCGAAAGAAGCGGCTGCCAAG
 GAGCTCATGGAAGTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTTACAACCTTGTAAACAAC
 AATTTGTAATGCGGACACTTCCAGTGAAGAAGAAGTGGTTAAATTGGTTACACACTTTGAGGAAATGA
 CTGAGCACCCCTAGTGGTAGTGATTAAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGT
 15 ATTGTAACACAGTAAACAATGGCGAGCCGCTAACGGTAAGTCAGGATTTAAACAGGGCAACATTA
 ATGGTGGCCCGACCGGGATTGGTGTAAAGTATGGAAGTGGAAAATAGTATTAGTGATTACACAGAGGC
 TGAGTTTGTTCAACTTCTTAAGGAAATTGAAAAAGAGAATGAAGCTGCAACTGATGATGTGTTAGATG
 TGTACTCGAACACTTTGTAAAAATTACTGAGCATCCAGATGGAACGGATCTGATTTATTATCCTAGT
 GATAATAGAGACGATAGCCCCGAAGGGATTGTCAAGGAAATTAAGAATGGCGAGCTGCTAACGGT
 20 AAGCCAGGATTTAAACAGGGCAATGGCGGGGGTAATGGCAATTCGGTGGTGGCTCGATGAATAAG
 ATGGCAATGATCGATTTGGCGAAATTATTTTAGCTTCGAAAATTACAGCAATTGAGTTTTCAGAGCG
 AATTTGTGTTGAACGGAGAAGATTGTATGGTGTAAAGGATTTGTCTCCGAATATATTAAATTGTGGGG
 AAGAGTTGTTTATGGCTGCTGAGCGATTTGAGCCTGATGCAGATAGGGCTAATTATGAAATTGATGAT
 AATGGACTTAAGGTTGAGGTCCGATCTATCTTGGAAAAATTTAAATTACTCGAGCACCACCACCACCA
 25 CCACTGA (SEQ ID NO: 40)

Im97 immunity protein scaffold with C-terminal LE(His₆) tag: amino acid sequence (Im9 underlined,
 Im7 double underlined, LE(His₆) dashed underlined)

MELKHSISDYTEAEFLQLVTICNADTSSEEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDSPSGIVNTVK
 30 QWRAANGKSGFKQGSASGSASMELKNSISDYTEAEFVQLLKEIEKENVAATDDVLDVLEHFVKITEHPD
 GTDLIYPSDNRDSDPEGIVKEIKEWRAANGKPGFKQGLEHHHHHH (SEQ ID NO: 41)

S5E9 chimera: amino acid sequence

Pyocin S5 outer membrane translocation domain *italics*; pyocin S5 receptor binding domain underlined;
 35 pyocin G inner membrane translocation domain double underlined; colicin E9 DNase domain dashed
underlined)
 MSNDNEVPGSMVIVAQGPDDQYAYEVPPIDSAAVAGNMFGLIQREIYLQKNIYYPVRSIFEQGTKEKKEI
 NKKVSDQVDGLLKQITQGKREATRQERVDVMSAVLHKMESDLEGYKKTFTKGPFDIYEKQSSLSIYEAW
 VKIWEKNSWEERKKYPFQQLVRDELERAVAYYKQDSLSEAVKVLRLQELNKQKALKEKEDLSQLERDYRT
 40 RKANLEMKVQSELDQAGSALPPLVSPTPEQWLERATRLVTQAIADKKQLQTTNNTLIKNSPTPLEKQKAIY
 NGELLVDEIASLQARLVKLNAETTRRRTEAERKAAEEQALQNTYAMPANGSVVATAAGRGLIQVAQGAAS

LAQAISDAIAVLGRVLASAPSVMAVG FASLTYSRTAEQWQDQTPDSVRYALGMDANKLGLPSSVNLNA
VAKAGGTVDLPMRLTNEARGSTTTLSVVSTDGVSVPKAVPVRMAAYNTATGLYEVTVPSTVAEAPPLILT
WTPASPPGNQNPSSSTTPVVPKPVAVYEGATLTPVKAKPETYPGVM TLPGLIICFPADSGIKPVYVMFNA
MESKRNKPGKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKL RDKEFKSFDDFRKAVWEEVSKDPELS
 5 KNLNPSNKS SVSKGYSPFTPKNQQVGGGRKVYELHHDKPISQGGEVYDMDNIRVTTPKRHIDIHRGK
 (SEQ ID NO: 42)

S5E7 chimera: amino acid sequence

Pyocin S5 outer membrane translocation domain *italics*; pyocin S5 receptor binding domain underlined;
 10 pyocin G inner membrane translocation domain double underlined; colicin E7 DNase domain dashed underlined)
MSNDNEVPGSMVIVAQGPDDQYAYEVPPIDSAAVAGNMFGLIQREIYLQKNIYYPVRSIFEQGTKEKKEI
NKKVSDQVDGLLKQITQGKREATRQERVDVMSAVLHKMESDLEGYKKTFTKGPFIDYEKQSSLSIYEAW
VKIWEKNSWEERKKYPFQQLVRDELERAVAYYKQDSLSEAVKVL RQELNKQKALKEKEDLSQLERDYRT
 15 RKANLEMKVQSELDQAGSALPPLVSPTEQWLERATRLVTQAIADKKQLQTTNNTLIKNSPTPLEKQKAIY
NGELLVDEIASLQARLVKLNAETRRRRTAERKAAEEQALQNTYAMPANGSVVATAAGRGLIQVAQGAAS
LAQAISDAIAVLGRVLASAPSVMAVG FASLTYSRTAEQWQDQTPDSVRYALGMDANKLGLPSSVNLNA
VAKAGGTVDLPMRLTNEARGSTTTLSVVSTDGVSVPKAVPVRMAAYNTATGLYEVTVPSTVAEAPPLILT
WTPASPPGNQNPSSSTTPVVPKPVAVYEGATLTPVKAKPETYPGVM TLPGLIICFPADSGIKPVYVMFNA
 20 MESKRNKPGKATGKGKPVNNKWLNNAGKDLGSPVPDRIANKLRDKEFKSFDDFRKKFWEEVSKDPELS
KQFSRNNNDRMKVGKAPKTRTQDVSGKRTSFELHHEKPISQNGGVYDMDNISVVTTPKRHIDIHRGK
 (SEQ ID NO: 43)

ColE7 immunity protein ("Im7") with C-terminal LE(His₆) tag: amino acid sequence

25 (LE(His₆) tag underlined)
 MELKNSISDYTEAEFVQLLKEIEKENVAATDDVLDVLLLEHFVKITEHPDGTDLIYYPDNRDDSPGIVKEIK
 EWRAANGKSGFKQGLEHHHHHH (SEQ ID NO: 44)

Examples

30 To illustrate the flexibility and applicability of the approach described, the inventors constructed a heterotrimeric complex (containing 2 bacteriocin molecules and a cognate divalent immunity protein scaffold, described in Example 1) and a heterotetrameric complex (containing 3 bacteriocin molecules and a cognate trivalent immunity protein scaffold, described in Example 2). These complexes are illustrated in Figure 1.

Example 1**Components of trimeric complex**

The bacteriocin components of the trimeric complex are colicin E9 (ColE9) and colicin D (ColD). The immunity protein scaffold is a fusion protein containing their cognate immunity proteins "Im9" and "ImD", separated by a flexible linker peptide. The scaffold is referred to by the designation "Im9-ImD".
5 Complexes of the scaffold with ColE9, ColD or both are referred to by the shorthand notations:

[ColE9:Im9-ImD];

[ColE9:Im9-ImD:ColD]; and

[Im9-ImD:ColD] respectively.

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Colicin E9 and Colicin E3 are group A bacteriocins which bind to the vitamin B₁₂ receptor, BtuB, with nanomolar affinity before passing their intrinsically unstructured N-terminus through trimeric porins such as OmpF in the outer membrane. This intrinsically unstructured N-terminus contains the TolB box which binds to TolB, part of the energised Tol-Pal complex which spans the periplasm. Parasitisation of the Tol-
15 Pal system leads to the immunity protein being jettisoned and translocation of the cytotoxic C-terminal domain into the cytoplasm. Colicin E9 and Colicin E3 are highly conserved except for their cytotoxic domains which in E9 is a DNase and in E3 is a rRNase.

Colicin D is a group B bacteriocin which binds to the ferric-enterobactin receptor, FepA, with high affinity.
20 Colicin D passes through FepA and interacts with TonB of the Ton system within the periplasm which results in immunity protein release and translocation of the C-terminal tRNase domain to the cytoplasm. The uptake pathways of Colicin E9 and Colicin D are summarised in Figure 2.

In vitro assembly of trimeric complex

25 The individual bacteriocins were expressed in *E. coli* BL21 (DE3) as heterodimeric complexes with their respective immunity proteins. The immunity proteins were engineered to carry a His₆ tag at their C-terminus. Cell pellets were lysed by sonication, clarified by centrifugation and loaded onto 5 ml HisTrap HP columns. In each case the free bacteriocin was eluted from nickel affinity column using 6 M guanidinium hydrochloride. Eluted bacteriocins were refolded by dialysis into 25 mM Tris-HCl, pH 7.5,
30 150 mM NaCl and purified by gel filtration on a 26/60 S200 column equilibrated in the same buffer. The immunity protein scaffold Im9-ImD, also carrying a C-terminal His₆ tag, was expressed separately and purified by nickel affinity chromatography (eluting with imidazole) followed by gel filtration on a 26/60 S200 column equilibrated in 25 mM Tris-HCl, pH 7.5.

500 µl samples of (i) 20 µM Im9-ImD + 40 µM ColE9 + 40 µM ColD, 20 µM Im9-ImD + 40 µM ColD, 20 µM Im9-ImD + 40 µM ColE9, and 20 µM Im9-ImD were prepared in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl and were purified on a Superdex 200 increase 10/300 GL analytical gel filtration column (Cytiva) equilibrated in the same buffer.

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Good baseline separation was observed between the ColE9:Im9-ImD:ColD and the free colicins, which is essential to ensure that any killing activity observed is due to the heterotrimeric complex rather than contaminating free colicin. (Figure 4.)

10 **Cell killing by trimeric complex**

15 ml LB-0.7 % Agar was inoculated with either 200 µl *tolA*- BW25113, BL21 (DE3) (*btuB*-), *tonB*- BW25113 or *fepA*- BW25113 culture which was then overlaid onto an LB-1.5 % agar plate. Serial dilutions of ColD, ColE9 and [ColE9:Im9-ImD:ColD] were prepared over the concentration range of 100 nM to 137 pM and 3 µl of each dilution was spotted onto each of the inoculated agar plates. Plates were grown overnight at 37 °C and bacteriocin activity was seen as zones of clearance in the bacterial lawn. Results are shown in Figure 5A-C. In a similar experiment, serial dilutions of ColD, ColE9, a mixture of ColE9 + ColD, and [ColE9:Im9-ImD:ColD] were spotted onto a lawn of *tolA*- BW25113. Results are shown in Figure 5D.

20 As expected, ColD was not active against *tonB*- or *fepA*- cells and ColE9 was not active against *tolA*- or *btuB*- cells. The heterotrimeric [ColE9:Im9-ImD:ColD] retained activity against all strains tested showing that both ColE9 and ColD are active within the complex. ColD and ColE9 were also both tested as complexes with the Im9-ImD fusion protein ([ColE9:Im9-ImD] and [Im9-ImD:ColD]), which had no negative impact on the activity of either ColD or ColE9 alone (not shown).

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Interestingly, the activity of ColD against *tolA*- cells is impaired indicating that there is a decreased level of FepA expression in these cells. However, [ColE9:Im9-ImD:ColD] shows better activity against *tolA*- cells than either ColD alone or the mixture of ColE9 + ColD, suggesting that although ColE9 cannot enter and kill, the presence of the ColE9 receptor binding domain can bind the heterotrimeric complex onto the surface of the cell and aid FepA dependent activity of the ColD moiety.

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Trimeric complex avoids development of bacterial resistance

In addition to increasing strain coverage, the combination of multiple bacteriocins targeting independent uptake pathways drastically decreases the chances of resistance emerging in cells which are susceptible to more than one of the bacteriocin components. Resistance would require simultaneous mutation of multiple components to impact both uptake pathways. In vitro, resistant mutants will likely be much more prevalent than in vivo, due to little impact of nutrient receptors when grown on rich medium. Bacteriocin receptors are often virulence factors, upregulated during infection, and their loss impacts on the colonisation ability of the bacterium.

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Three-fold serial dilution of [ColE9:Im9-ImD:ColD], [Im9-ImD:ColD] and [ColE9:Im9-ImD] were prepared between 1 μ M and 5.6 pM in LB in a 96 well plate. MG1655 *E. coli* overnight was used to inoculate each well of the plate at a dilution of 1 in 125. Cultures were grown at 37 °C with shaking at 190 rpm and the OD660nm was measured after 6 hours and 24 hours (Figure 6).

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After 6 hours, 460 pM, 460 pM and 37 nM is sufficient to inhibit growth for [ColE9:Im9-ImD:ColD], [ColE9:Im9-ImD] and [Im9-ImD:ColD] respectively. However, after 24 hours 1.4 nM, 1 μ M or 0.33 μ M of [ColE9:Im9-ImD:ColD], [ColE9:Im9-ImD] and [Im9-ImD:ColD] respectively is required to prevent growth. This likely reflects domination of the overnight cultures of ColE9 or ColD treated cultures by resistant mutants. To test this the frequency of resistant mutants was calculated by plating out serial dilutions of MG1655 overnight culture onto plates containing 25 nM [ColE9:Im9-ImD:ColD], 25 nM [ColE9:Im9-ImD], 50 nM [Im9-ImD:ColD] or no bacteriocin. The original culture contained 1.8×10^9 c.f.u. / ml, which was reduced to 775 c.f.u. / ml by 50 nM [Im9-ImD:ColD], 5750 c.f.u. / ml by 25 nM [ColE9:Im9-ImD] and no detectable colonies for the sample plated onto 25 nM [ColE9:Im9-ImD:ColD].

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In an attempt to determine a value for [ColE9:Im9-ImD:ColD], 5 ml overnight culture of MG1655 *E. coli* containing 1.75×10^{10} c.f.u. was treated with 25 nM [ColE9:Im9-ImD:ColD] and the entire sample was plated out and grown overnight at 37 °C. No colonies were obtained. For [ColE9:Im9-ImD] and [Im9-ImD:ColD] the frequency of resistant mutants was 1 in 3.0×10^5 and 1 in 2.3×10^8 respectively. As resistance to [ColE9:Im9-ImD:ColD] would require mutation of both the ColE9 and ColD uptake pathways it would make sense that the resistant mutant frequency would be the product of the frequencies of resistance to the individual components (1 in 6.9×10^{11}) consistent with no resistant mutants observed experimentally.

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25 *In vivo expression of trimeric complex*

Genes encoding colicin E9, Im9-ImD and colicin D were cloned into pET21a and transformed into *E. coli* BL21 (DE3). Upon expression and purification only colicin E9 and Im9-ImD were apparent. A second copy of the colicin D gene was cloned into pACYCDuet1, which was co-transformed with colicin E9, Im9-ImD and colicin D cloned into pET21a, into BL21 (DE3) cells. Expressed protein was initially purified using a His-tag on the C-terminus of Im9-ImD and colicin D, colicin E9, and Im9-ImD were apparent in the nickel column elution (Figure 7).

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The [ColE9:Im9-ImD:ColD] complex was further purified on a 26/60 S200 gel filtration column, separating the intact complex from [ColE9:Im9-ImD] and [Im9-ImD:ColD] sub-complexes. An initial elution peak at 110 ml (fractions A1 to A5) corresponded to aggregated material eluting in the void volume of the column. A second elution peak at 145 ml (fractions A11-B2) was the [ColE9:Im9-ImD:ColD] complex. A third elution peak at 174 ml was a mixture of [ColE9:Im9-ImD] and [Im9-ImD:ColD]. SDS-PAGE analysis of

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representative fractions is shown in Figure 8. Initial purification of the [ColE9:Im9-ImD:ColD] gave a yield of ~10 mg/litre of culture.

The activity of the purified [ColE9:Im9-ImD:ColD] was tested against *E. coli* BW25113, *btuB*- BL21 (DE3), *fepA*- BW25113, *tolA*- BW25113 and *tonB*- BW25113 over a concentration range of 100 nM down to 137 pM. As with the *in vitro* assembled [ColE9:Im9-ImD:ColD], the *in vivo* assembled complex was active against *E. coli* strains lacking components of either the colicin E9 or colicin D uptake pathways (Figure 9).

Example 2

Components of tetrameric complex

The bacteriocin components of the tetrameric complex are chimeric proteins containing the targeting portions (receptor binding and translocation domains) of klebicin C (KlebC), cloacin DF13 (CloDF13) and klebicin G (KlebG), linked to the effector portions (cytotoxic domains) of colicin E9, colicin E3 and colicin D, respectively. The resulting chimeric proteins are designated KlebC-E9, CloDF13-E3 and KlebG-D.

The immunity protein scaffold is a fusion protein containing their cognate immunity proteins for ColE9 ("Im9"), ColE3 ("Im3") and ColD ("ImD"), separated by flexible linker peptides. The scaffold is referred to by the designation "Im9-Im3-ImD". The complex between the scaffold and the three chimeric bacteriocins is referred to by the shorthand notation [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D].

The protein-protein interactions involved in the uptake of cloacin DF13 (Krone et al., 1983; Thomas & Valvano, 1993; Wooldridge & Williams, 1991), klebicin G (unpublished) and klebicin C (Housden et al., 2021) are summarised in Figure 3.

***In vitro* assembly of tetrameric complex**

The chimeric bacteriocins were expressed in *E. coli* BL21 (DE3) as heterodimeric complexes with the immunity proteins for their respective cytotoxic domains (Im9, Im3 and ImD). The immunity proteins were engineered to carry a His₆ tag at their C-terminus. Cell pellets were lysed by sonication, clarified by centrifugation and loaded onto 5 ml HisTrap HP columns. In each case the free bacteriocin was eluted from nickel affinity column using 6 M guanidinium hydrochloride. Eluted bacteriocins were refolded by dialysis into 25 mM Tris-HCl, pH 7.5, 150 mM NaCl and purified by gel filtration on a 26/60 S200 column equilibrated in the same buffer. The immunity protein scaffold Im9-Im3-ImD, also carrying a C-terminal His₆ tag, was expressed separately and purified by nickel affinity chromatography (eluting with imidazole) followed by gel filtration on a 26/60 S200 column equilibrated in 25 mM Tris-HCl, pH 7.5.

A 500 µl sample containing 20 µM Im9-Im3-ImD, 30 µM KlebC-E9, 30 µM CloDF13-E3 and 30 µM KlebG-D prepared in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl was purified on a Superdex 200 increase 10/300 GL analytical gel filtration column (Cytiva) equilibrated in the same buffer. SDS -PAGE analysis of representative fractions is shown in Figure 10.

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Microbial killing activity of tetrameric complex

Serial dilutions of the tetrameric complex [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D] were prepared at concentrations between 6.1 µM and 25 nM. 5 µl spots were applied to nutrient broth (Merck) soft agar lawns inoculated with SG62, SR3 or SR6 *Klebsiella pneumoniae*. Plates were grown overnight at 37 °C and bacteriocin activity was seen as zones of clearance in the bacterial lawn. (Figure 11.)

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The activities of KlebC-E9 against SR3, CloDF13-E3 against SG62 and KlebG-D against SR6 are retained in the tetrameric [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D] complex. Furthermore, the activity of the tetrameric [Im9-Im3-ImD:KlebC-E9:CloDF13-E3:KlebG-D] complex against SR3 is enhanced relative to the individual component bacteriocins, illustrating that the increased receptor avidity due to the multiple targeting portions contributes to the killing activity of the complex.

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Example 3

Immunity protein scaffolds were designed that incorporate the M-type bacteriocin KvarM. The first contained KvarM linked to the two immunity protein domains Im9 and Im D (KvarM-Im9-ImD) and the second contained KvarM linked to three immunity protein domains, Im9, Im7 and ImD (KvarM-Im9-Im7-ImD). In both of these scaffolds, KvarM (at the N-terminal end of the fusion protein) is linked to Im9 using a rigid helical linker ((EAAAK)₃EL), and the immunity protein domains are linked by intrinsically unstructured sequences derived from the N-terminal sub-region of colicin E9. A His₆ tag used for nickel affinity purification was added to the C-terminal end of the fusion protein (after the last immunity domain). The scaffolds are illustrated schematically in Figure 12[A] & [B].

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KvarM-Im9-ImD and KvarM-Im9-Im7-ImD were expressed in *E. coli* BL21(DE3) cells and purified by nickel affinity chromatography followed by gel filtration. Serial dilutions of wild type KvarM, KvarM-Im9-ImD, and KvarM-Im9-Im7-ImD spanning concentrations of 2.5 µM to 4.9 nM were spotted onto a soft agar lawn of *Klebsiella quasipneumoniae* SG96 in nutrient broth. Once dry, the plates were incubated overnight at 37°C, with zones of clearance indicating bacteriocin mediated killing.

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Both KvarM-Immunity protein scaffolds showed cytotoxic activity against *K. quasipneumoniae* SG96 cells, with KvarM-Im9-ImD showing zones of clearance at concentrations ranging from 2.5 µM – 156 nM, and KvarM-Im9-ImD killing occurring down to 312 nM (Figure 12[C]). This demonstrated that the KvarM-immunity protein scaffolds can bind to KvarM's outer membrane receptor FhuA, and that the covalent fusion of two and three immunity protein domains to the C-terminus of the KvarM cytotoxic region does not abolish its enzymatic peptidoglycan precursor degrading activity. Interestingly, these results suggest that the immunity protein scaffold, which typically dissociates from the complexed nuclease bacteriocins

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on their binding to the target cell surface receptor, is being translocated into the periplasm of the target cell.

As with the other immunity protein scaffolds described herein, the KvarM-immunity protein scaffold fusions can be complexed with protein bacteriocins having effector portions (cytotoxic domains) cognate for the immunity protein domains, illustrated schematically in Figure 13. The addition of KvarM increases the complex's receptor binding and cytotoxic ability, as the KvarM component provides binding activity against its own receptor (in addition to those recognised by the other PB components of the complex) and cytotoxic activity against KvarM-susceptible strains.

Example 4

Components of trimeric complex

The bacteriocin components of the complex are chimeric proteins based on the pore-forming pyocin S5. These chimeric pyocins were constructed by replacing the pore-forming cytotoxic domain of pyocin S5 with a fusion of the inner membrane transport domain of pyocin G and the DNase-type cytotoxic domain of either colicin E9 or E7. Thus the chimeric pyocins contain the outer membrane transport (T_{om}) and receptor binding (R) domain of pyocin S5, followed by the inner membrane transport domain (T_{im}) of pyocin G, and the DNase domain of either colicin E9 or E7. The resulting chimeric proteins are designated S5E9 and S5E7 respectively.

The immunity protein scaffold is a fusion protein containing the immunity proteins of colicin E9 and E7, (Im9 and Im7, respectively), linked by a 7 amino acid linker having the sequence SASGSAS. The scaffold is referred to by the designation "Im97" (but could be designated "Im9-Im7") and the trimeric complex is referred to by the shorthand notation "S5E9-Im97-S5E7" (equivalent to "[S5E9:Im9-Im7:S5E7]"). The scaffold and chimeric pyocins are illustrated schematically in Fig. 15A.

In vitro assembly of trimeric complex

Proteins were expressed in *E. coli* BL21(DE3) and purified by nickel affinity chromatography. The immunity protein scaffold Im97 was isolated by nickel affinity chromatography by virtue of a C-terminal His₆-tag and further purified by gel filtration (Superdex 75). The chimeric pyocins S5E9 and S5E7 were co-expressed with their cognate immunity proteins Im9 and Im7, respectively. The pyocin-immunity protein complexes were isolated via nickel affinity chromatography by virtue of a C-terminal His₆ tag on the immunity protein and further purified by gel filtration (Superdex S200). To prepare uncomplexed pyocin the purified pyocin-immunity protein complexes were loaded onto a nickel affinity column and the pyocin eluted with 6 M guanidine hydrochloride. The application of guanidine hydrochloride unfolds the proteins allowing for dissociation of the pyocin with the immunity protein remaining bound to the column. Pyocins were refolded through dialysis in 50 mM Tris, 200 mM NaCl pH 7.5.

To construct and isolate the trimeric complex scaffold Im97 and chimeric pyocins S5E9 and S5E7, the component proteins were mixed in 50 mM Tris, 200 mM NaCl pH 7.5 in a 1:4:4 ratio, respectively, and incubated for 1 hour at room temperature. The complex was isolated by nickel affinity chromatography and eluted with imidazole. Further purification of the complex by gel filtration (Superdex S200) was performed to remove aggregated protein to give monodisperse S5E9-Im97-S5E7 complex.

SDS PAGE was performed to demonstrate the purity and integrity of the S5E9-Im97-S5E7 complex and the component pyocins in their free and immunity protein complex forms (Figure 15B). All proteins were shown to be highly purified with little evidence of degradation. However, multimeric pyocin was observed in samples of the refolded uncomplexed pyocins S5E9 and S5E7.

Cell killing by trimeric complex

Activity of the S5E9-Im97-S5E7 trimeric complex and the individual chimeric pyocins (in their free and immunity protein complex forms) against *P. aeruginosa* is shown in Fig. 15C.

Conclusions

The generation of the Im9-ImD fusion protein enables a heterotrimeric [ColE9:Im9-ImD:ColD] complex to be assembled *in vitro*. This complex retains all the activities of its component protein bacteriocins. The components of the trimeric [ColE9:Im9-ImD:ColD] complex have also been successfully co-expressed in *E. coli*, allowing purification of the assembled complex, thereby simplifying the production and purification process.

The heterotetrameric complex [Im9-Im3-ImD:KleB-C-E9:ColDF13-E3:KleB-G-D] has been assembled *in vitro* and retains activities of the three component protein bacteriocins.

A heterotrimeric complex containing chimeric pyocins has further been shown to be effective against *P. aeruginosa*. This construct also demonstrates that targeting portions from pore-forming PBs may be combined with nuclease effector portions from other PBs in the context of the complexes described in this specification.

The features disclosed in the foregoing description, or in the following claims, or in the accompanying drawings, expressed in their specific forms or in terms of a means for performing the disclosed function, or a method or process for obtaining the disclosed results, as appropriate, may, separately, or in any combination of such features, be utilised for realising the invention in diverse forms thereof.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without
5 departing from the spirit and scope of the invention.

For the avoidance of any doubt, any theoretical explanations provided herein are provided for the purposes of improving the understanding of a reader. The inventors do not wish to be bound by any of
10 these theoretical explanations.

Any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word “comprise” and “include”, and variations such as “comprises”, “comprising”, and “including” will be
15 understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range
20 is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent “about,” it will be understood that the particular value forms another embodiment. The term “about” in relation to a numerical value is optional and means for example +/- 10%.

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References

A number of publications are cited above in order to more fully describe and disclose the invention and the state of the art to which the invention pertains. Full citations for these references are provided below. The entirety of each of these references is incorporated herein.

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Claims:

1. An anti-bacterial protein complex comprising:
- (a) a first protein bacteriocin (PB) molecule and a second PB molecule;
- 5 and
- (b) an immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain;
- wherein the first and second immunity protein domains are non-covalently bound to the respective first
- 10 and second PB molecules.
2. An anti-bacterial complex according to claim 1 wherein each bacteriocin comprises a cell targeting portion and an effector portion, and binds via its effector portion to the corresponding immunity protein domain.
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3. An anti-bacterial complex according to claim 1 or claim 2 wherein the immunity protein scaffold comprises a third immunity protein domain, and the complex comprises a third bacteriocin molecule non-covalently bound to the third immunity protein domain.
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4. An anti-bacterial complex according to any one of the preceding claims wherein the immunity protein scaffold comprises two or more repeats of the same immunity protein domain, e.g. wherein each of the immunity protein domains is the same.
5. An anti-bacterial complex according to any one of claims 1 to 4 wherein the immunity protein scaffold contains two or more different immunity protein domains, e.g. wherein each of the immunity protein domains is different to each of the other component immunity protein domains.
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6. An anti-bacterial complex according to any one of the preceding claims wherein the immunity protein scaffold is a fusion protein.
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7. An anti-bacterial complex according to claim 6 wherein the immunity protein scaffold fusion protein further comprises an M-type bacteriocin moiety, such as colicin M (ColM), KpneM, KpneM2, KvarM, PaeM1 or PaeM4.

8. An anti-bacterial complex according to any one of the preceding claims comprising bacteriocins having two or more different effector portions, e.g. wherein the different effector portions have different enzymatic activities.

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9. An anti-bacterial complex according to claim 8 wherein each effector portion has a different enzymatic activity.

10. An anti-bacterial complex according to any one of the preceding claims comprising bacteriocins having two or more different targeting portions, e.g. wherein each of the bacteriocins has a different targeting portion.

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11. An anti-bacterial complex according to claim 10 wherein each bacteriocin has a targeting portion specific for the same species or strain of bacterium.

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12. An anti-bacterial complex according to claim 10 or claim 11 wherein said targeting portions bind to two or more different receptors or use two or more different translocation portals.

13. An anti-bacterial complex according to claim 12 wherein each targeting portion binds to a different receptor and/or each targeting portion uses a different translocation portal.

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14. An anti-bacterial complex according to claim 13 comprising bacteriocins having targeting portions specific for two or more different species or strains of bacterium.

15. An anti-bacterial method, comprising contacting a bacterium or population of bacteria with an anti-bacterial complex according to any one of the preceding claims.

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16. An anti-bacterial complex according to any one of claims 1 to 14 for use in a method of medical treatment.

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17. An anti-bacterial complex according to any one of claims 1 to 14 for use in the prophylaxis or treatment of a bacterial infection or a condition caused by or associated with a bacterial infection.

18. A host cell comprising:

(i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain;

(ii) nucleic acid encoding at least one bacteriocin, the or each bacteriocin having an effector domain capable of binding to at least one of said immunity protein domains;

wherein the host cell is capable of expressing said immunity protein scaffold and said bacteriocin.

19. A host cell according to claim 18 wherein the cell comprises first and second nucleic acids encoding respective first and second bacteriocins, each bacteriocin having an effector domain capable of binding to a respective one of said immunity protein domains;

wherein the host cell is capable of expressing said immunity protein scaffold and said bacteriocins.

20. A host cell according to claim 18 or claim 19 comprising:

(i) nucleic acid encoding an immunity protein scaffold, the immunity protein scaffold comprising first, second and third immunity protein domains;

(ii) first, second and third nucleic acids encoding respective first, second and third bacteriocins, each bacteriocin having an effector domain capable of binding to a respective one of said immunity protein domains;

wherein the host cell is capable of expressing said immunity protein scaffold and said bacteriocins.

21. A method of producing an anti-bacterial complex comprising, providing a host cell according to any one of claims 18 to 20, culturing said cell under conditions suitable for expression of the immunity protein scaffold and bacteriocin molecule(s), and optionally further comprising isolating the anti-bacterial complex.

22. A method of generating an anti-bacterial complex, the method comprising contacting an immunity protein scaffold comprising a first immunity protein domain and a second immunity protein domain, with first and second bacteriocin molecules each having an effector portion capable of binding to a respective one of said immunity protein domains, to form an anti-bacterial complex.