Summary

In one aspect, the disclosure provides an anti-bacterial protein complex comprising: (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; wherein the first and second immunity protein domains are non-covalently bound to the respective first and second PB molecules; and wherein the immunity protein domains of the scaffold are covalently coupled to one another.

In one aspect, the disclosure provides an anti-bacterial method, comprising contacting a bacterium or population of bacteria with an anti-bacterial complex as described herein.

In one aspect, the disclosure provides the use of the anti-bacterial complex as described herein in the manufacture of a medicament for preventing or treating a bacterial infection or a condition caused by or associated with a bacterial infection.

In one aspect, the disclosure 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, wherein the immunity protein domains of the scaffold are capable of covalently coupling to one another; (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(s), optionally 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.

In one aspect, the disclosure provides a method of producing an anti-bacterial complex comprising, providing a host cell as described herein, 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.

In one aspect, the disclosure 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 covalently coupled to one another, 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.

The disclosure provides an anti-bacterial protein complex comprising:

(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;

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.

The complexes of the present disclosure 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.

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

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 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 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 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 examples, 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.

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 examples, the heterologous moiety may 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 examples, 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 examples, 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 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 examples, 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 examples, 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 disclosure 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 disclosure is one which comprises:

a first PB comprising a colicin E9 effector portion;

a second PB comprising a colicin D effector portion; and

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 chimeric PBs, comprising targeting portions from PBs different to those of their effector portions.

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

a first PB comprising a colicin E9 effector portion;

a second PB comprising a colicin E3 effector portion;

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").

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 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");

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

The disclosure 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.

The disclosure 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 disclosure further provides a method of prophylaxis or treatment of a bacterial infection or a condition 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.

The disclosure 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 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 disclosure may 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 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 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;
(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:
(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.

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.

The disclosure 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.

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.

Thus the disclosure 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 disclosure.

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 disclosure includes the combination of the aspects and preferred features described except where 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:

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 linkers represented by dotted line. (**d**) Cartoon structure of the Im9-Im3-ImD fusion protein associated with its cognate bacteriocins.

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).

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.

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.

Claims:

1. An anti-bacterial protein complex comprising:

(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;

wherein the first and second immunity protein domains are non-covalently bound to the respective first and second PB molecules; and

wherein the immunity protein domains of the scaffold are covalently coupled to one another.

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; and/or

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.

3. An anti-bacterial complex according to claim 1 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.

4. An anti-bacterial complex according to claim 1 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.

5. An anti-bacterial complex according to claim 1 wherein the immunity protein scaffold is a fusion protein, optionally 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.

6. An anti-bacterial complex according to claim 1 comprising bacteriocins having two or more different effector portions, e.g. wherein the different effector portions have different enzymatic activities, optionally wherein each effector portion has a different enzymatic activity.

7. An anti-bacterial complex according to claim 1 comprising bacteriocins having two or more different targeting portions, e.g. wherein each of the bacteriocins has a different targeting portion.

8. An anti-bacterial complex according to claim 7 wherein each bacteriocin has a targeting portion specific for the same species or strain of bacterium.

9. An anti-bacterial complex according to claim 7 wherein said targeting portions bind to two or more different receptors or use two or more different translocation portals, optionally wherein each targeting portion binds to a different receptor and/or each targeting portion uses a different translocation portal, further optionally wherein the anti-bacterial complex comprises bacteriocins having targeting portions specific for two or more different species or strains of bacterium.

10. An anti-bacterial method, comprising contacting a bacterium or population of bacteria with an anti-bacterial complex according to any one of claims 1 to 9.

11. The anti-bacterial complex according to any one of claims 1 to 9 for use in therapy.

12. Use of the anti-bacterial complex according to any one of claims 1 to 9 in the manufacture of a medicament for preventing or treating a bacterial infection or a condition caused by or associated with a bacterial infection.

13. 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, wherein the immunity protein domains of the scaffold are capable of covalently coupling to one another;

(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(s), optionally 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.

14. A method of producing an anti-bacterial complex comprising, providing a host cell according to claim 13, 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.

15. 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 covalently coupled to one another, 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.