High-yield bioproduction of virus-free P4-EKORhE multi-lysin transducing particles as an antimicrobial gene therapeutic

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A description for the construction of the bioengineered P4-EKORhE as a means of producing transducing particles and a comprehensive method for the production of very high yields, of up to 10^{12} particles per milliliter, enables the possibility of using virus-like particles for the transduction of genetically encoded antimicrobials using a combination of synthetic biology and upstream and downstream processing optimization. The final product, a gene-delivered antimicrobial in the form of the multi-lysins cassette, is shown to be fully functional before and after being packaged within P4-EKORhE particles. The antimicrobial activity of the multi-lysins cassette is characterized by the activity of its contributor lysis proteins and tested *in vivo* in both pure bacterial *Escherichia coli* cultures and in a model of infection using A549 immortalized human epithelial tissue cell cultures. This work exemplifies several bioproduction methods and demonstrates how the virology of the P4 and P2 bacteriophages can be harnessed to establish a bioprocess for the production of transducing particles with very high yields, avoiding contamination of the natural virus and maintaining the antimicrobial effectiveness of the final product.

high-yield bioproduction| bioprocess engineering | virus-like particles| virus-free particles | transducing particles | antimicrobials | gene therapeutics | A549-model of infection | P4-EKORhE

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Introduction

Harnessing the natural mechanisms that bacteriophages use to repurpose its host target bacteria metabolism to produce their own viral structural material and package their own viral DNA for self-replication and propagation purposes is the way in which transducing particles are produced. The most characteristic protein-made parts of bacteriophages are the capsid, which acts as a structure to package and protect the genetic material, contiguous to a tail, which allows the genetic material to be ejected upon infection, and the tail-fibres, which allows the bacteriophage to specifically interact with a particular bacterial host[1]. For a general schematic of these three basic structures, see Figure 1.

These protein structures allow the bacteriophage to store, protect and, upon infection, release the genetic material within the target bacteria. If these protein components can be produced while at the same time blocking the capability of the virus to store its own viral DNA, it is possible to produce a transducing particle that can be used to contain and deliver an heterologous genetic payload within a target bacterial host. This is particularly straightforward if using bacteriophages from which a "cos-site" has been identified e.g., such as the case for λ or P2/P4 bacteriophages.

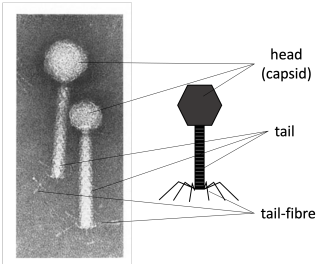

Fig. 1. The characteristic protein structures in bacteriophages. *Left:* An electron microscope image of bacteriophage P2 (with a larger head) and P4 (with a smaller head). *Right:* A schematic labelling of the main protein structures in tailed bacteriophages. The capsid (head), the tail, and the tail-fibres. The right side of the image is made by the author. The left side of the image is adapted from the literature[2].

Virus-free VLP production based on conditionally-replicating P4 bacteriophage. As described extensively in the literature[2][3][4][5], the bacteriophage P4 is a helper-dependent bacteriophage which can use the late gene products of temperate bacteriophage P2 to encapsulate its own P4 double-stranded DNA. The P4 bacteriophage capsid is about a third the volume size of the P2 bacteriophage[6] and harbours a genome which is also one-third of its size (i.e. 11.624 kb versus 33.574 kb, respectively). As mentioned before, the P4 bacteriophage is able to elicit the overexpression of the P2 prophage late genes [4] without the P2 prophage being excised or replicated [7]. An additional characteristic of P4 is that, when a P2 prophage is not present in the host bacterial cell, P4 can still be maintained as a plasmid[8], which allows P4 to hijack a subsequent infection of a virulent

P2 bacteriophage.

Therefore, the viral cycle of bacteriophage P4 is a very particular phenomenon whereas a bacteriophage parasites another bacteriophage in order to perpetuate itself, the one re-purposing the gene products of the latter.

While the bacteriophage P2 typically constructs an icosahedral capsid (i.e. the head) exhibiting T = 7 symmetry using the *gpN* capsid protein, the *gpO* scaffolding protein, and the *gpQ* portal protein, the presence of P4 bacteriophage, makes these structural proteins arrange themselves into a smaller capsid with T = 4 symmetry. The regulation of this size transformation is facilitated by the P4-encoded protein *Sid*, which creates an external scaffold around the compact P4 procapsids[9]. It is this reduction in the size of the bacteriophage capsid that grants the P4 bacteriophage the exclusive capacity to package its own genome while impeding the larger P2 genome to propagate.

The P4 bacteriophage viral cycle is very well described in the literature[10]. The P4 is a satellite virus that relies on the helper bacteriophage P2 for its propagation. The viral cycle begins when P4 infects a susceptible bacterial host such as *Escherichia coli*, in conjunction with P2. During infection, P4 utilizes the P2 machinery and structures to replicate its genome and produce viral proteins. Of particularity, P4 can also exist in a plasmid form within the host cell, where it can replicate independently of the P2 phage. This plasmid form allows P4 to remain within a bacterial population, even when P2 is not present. When conditions are favorable, such as when P2 infects the same host, P4 can integrate into the P2 life cycle, co-packaging its genome with P2 during lytic growth, and leading to the release of new virions that contain both P2 and P4 genetic material. This interplay allows P4 to exploit the resources of P2, enhancing its own propagation while maintaining its unique life cycle characteristics.

Therefore, knowing that P4 can be maintained as a plasmid does, and knowing that P4 is a virus dependent on the presence of P2 for its conditional propagation (i.e. for the production of its necessary protein material for its packaging, storage and subsequent infection), it should be possible to use the phenomenon for the production of transducing particles using the conditionally replicating capability of the P4 bacteriophage.

Based on the aforementioned, and accounting for the fact that a minimal essential region of P4 bacteriophage has been described in the literature[11][12], it is possible to devise a "conditionally-propagating transducing particle" with a P4-minimal, here termed as "P4-min", version, that has 7.190 kb in size, and allows for the expression of the necessary gene products to produce fully functional P4 bacteriophages while allowing to harbour a foreign genetic circuit of up to 4.249 kb (i.e. which makes it up to a size similar to the 11.439 kb of the complete P4 bacteriophage). Therefore, a system inspired in the P4-min would allow hosting a genetic device that could serve as a controllable means of genetically encoding genetic circuit that produces antimicrobial-like molecules. In this light this work exemplifies the use of a system of lysis proteins, termed *the multi-lysin cassette*, including the kanamycin-resistance marker, and a co-located P4-cos site, which spans a total cargo of 4.151 kb. In the context of this work, it is important to highlight that P4-min entails a "transducing particle" that can be propagated only in the presence of P2 bacteriophages and therefore enabled to reach the yields readily observed when replicating natural bacteriophages ($\geq 10^8$ cfu/mL).
Therefore, a transducing particle is here considered as a viral particle that, instead of having its own genome, contains a plasmid encoding heterologous or synthetic genetic material and a "cos-site" that acts as a "packaging signal". The following Figure 2 schematizes the main ways in which bacteriophages can be used to produce transducing particles using P2/P4.

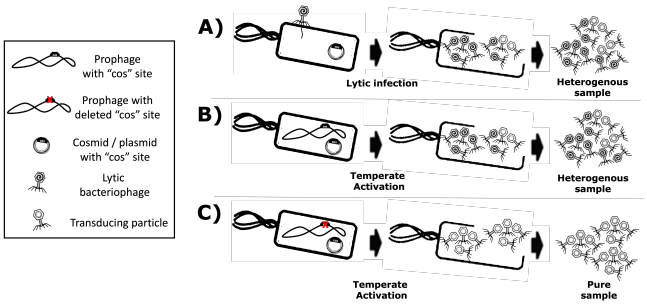

Fig. 2. Production of transducing particles using lytic or temperate bacteriophages. Transducing particles are bacteriophage-based particles harbouring cosmids, instead of bacteriophage genomes, that have been selected and maintained within host-producing strains. **A)** When using a lytic bacteriophage a heterogeneous sample of bacteriophages and transducing particles is obtained. **B)** When using a temperate bacteriophage with a functional cos-site a heterogeneous sample of bacteriophages and transducing particles is obtained. **C)** When using a temperate bacteriophage with a *knocked-out* cos-site a "virus-free", pure sample, of transducing particles is obtained.

The main disadvantage of using helper P2 bacteriophages for the production of transducing particles is the contamination of the lysate with wild-type bacteriophages (i.e. natural bacteriophages with self-replicating and propagating capabilities). The contamination with wild-type bacteriophages, present in the resulting lysate, can come from two main sources: non-adsorbed helper phage, and the production and packaging of competing viral genomes.

The production and packaging of competing viral genomes occur because both the DNA of the bacteriophage and the proteins necessary for the assembly of the virion are produced within the host bacterial cell. During the production of transducing particles, because the DNA of the virus contains a packaging signal, as well as the cosmid of interest, there is a competition for DNA packaging, and the resulting

lysate contains both transducing particles (i.e. virions with plasmid/cosmid) and viruses (i.e. virions with viral genome). The Figure 2.A and Figure 2.B, above, exemplify these scenarios.

When using temperate bacterial strains with prophages (i.e. lysogens) as helper bacteriophages for the production of transducing particles, the main disadvantage is that the spontaneous activation of the lysogenic bacterial strain so as to switch to a lytic state that will amount to the production of bacteriophage particles occurs at a very low frequency[13]. In this light, the activation of temperate strain after induction with a chemical[14], or after irradiation with UV-light[15][16], has been described in the literature. Nevertheless, each activator is rather strain-specific. In this sense, for example, UV-light irradiation can be used to activate bacteriophage λ lysogens, but not the bacteriophage P2 lysogens that are used in this work.

In this regard, it is important to see that it has been identified that the epsilon (ε) gene product, from the natural P4 bacteriophage, is able to effectuate the activation of the production of temperate strains of P2 bacteriophage[17]. The P4 bacteriophage is a satellite bacteriophage that predates on the P2 bacteriophage late gene products as a "helper" for its construction and lytic growth (as described thoroughly in the literature[10]). Such "late genes" are related to the production of the aforementioned bacteriophage structural proteins such as the capsid, the tail, and tail-fibres (i.e. as seen in Figure 1 and, as for the predator P4 bacteriophage, no other proteins are required for its complete constitution as a virus[18]. In this light, the literature describes that *the transactivation of P2 late gene expression by P4 requires the P4 delta (δ) gene product and works even in the absence of P2 DNA replication*[19] which is a strategy that facilitates the packaging of the P4 viral genome over competing P2 viral genomes.

Therefore, the possibility of using lysogenic bacterial strains of P2 bacteriophages for the production of bacteriophage-based transducing particles is facilitated by the fact that inducers have been identified, and there are means by which competition of cosmid packaging against natural viral genomes can be mitigated.

The means by which temperate lysates can be made free from viral contamination, as seen in Figure 2.C above), is by using a temperate strain with the cos-site *knocked-out*, by means of genome engineering techniques, so as to hamper the packaging of viral genomes, once these are activated, and allow cosmids containing the cos-site to be exclusively packed within the bacteriophage-based transducing particles.

In this work, the P4-EKORhE, a virus-free virus-like transducing particle production system based on the P4 bacteriophage's capacity to hijack the production of late structural proteins of P2 temperate prophages, will be demonstrated as a means to deliver a genetically-encoded antimicrobial in the form of a multi-lysins cassette.

Bacteriophage lysins as a gene-delivered antimicrobial. The multi-lysins cassette is a cominbation of genetically encoded lysins ready to be delivered using transducing particles. Bacteriophage lysins (i.e. lysis proteins) and lysin- or lysis-accessory proteins are bacteriophage-encoded proteins that are usually necessary to burst the bacterial host after infection, to release a progeny of viral bacteriophage particles. Lysins are among many other proteins present in nature, good candidates as component effectors in gene-delivered antimicrobial because these proteins can interfere with an essential element of a living system: individual compartmentalization[20]. These generally termed "lysins" will be considered as different effectors for the showcased antimicrobial activity of the multi-lysins cassette. Bacteriophage lysins, as other endolysins or lysis-accessory proteins such as holins and spanins, generally act by disrupting the membranes and cell walls of bacterial cells and therefore making these inviable for survival.

Because these proteins generally target bacterial cell membranes and cell walls, they have a similar mechanism of action to membrane-disrupting antibiotics in the sense that these actuate by disrupting bacterial cell membranes and/or cell walls.

In this sense, in order to design a gene-delivered antimicrobial that might be effective against potential infections of *Escherichia coli* in humans or animal clinical context. The advantages of targeting *Escherichia coli* are a low biosecurity risk and that it can serve as a model to other pathogenic bacteria with higher biosecurity risk, such as *Escherichia coli K1* strains for its capacity to cross the blood-brain-barrier in neonatal meningitis and because of its described potential for uropathogenic infections[21]. The approach taken in this work has been to combine different genetically encoded lysins, and lysis-accessory proteins, to ensure a potent antimicrobial effect. It has been observed before that, using different lysins in different combinations, allows to observe an added effect in the capability of using lysins as a bacteriostatic agent [22]. Nevertheless, these studies have been performed with lysins being added extracellularly to the cultures of bacterial cells, instead of being genetically-encoded within a biological genetic circuit and expressed within the target bacterial cells themselves, as this work with "genetically encoded multi-lysins" exemplifies for more details about the construction of the multi-lysins cassette).

Together, λ's LysS, LysR, and Rz, with the MS2's gpL, and the PhiX174's gpE single-gene lysis proteins, can act in synergy targeting bacterial cells by the different lysis mechanisms (as described in Supplementary Section SS4.4).

In this work, a rhamnose-inducible, controllable, and conditionally replicating bacteriophage, the P4-EKORhE (Figure 5), is utilized to encode, within its genetic cargo capacity, which is only limited by the *headful* size of its original genome, the multi-lysins cassette, as a means to test the use of a gene-delivered antimicrobial. The multi-lysins cassette is then characterized for its effectiveness as an antimicrobial (Figure 4, and a model of infection both using pure bacterial cell cultures (Figure 7.C) and a human model of bacterial infection using A459 immortalized human epithelial cells (Figure 7.D and 7.E) is used to further ascertain the effectiveness of gene-delivered antimicrobials using P4-EKORhE.

In this light, one of the novelties of the proposed approach is applying the concept of searching synergies among different lysins and lysin-accessory proteins that act as they are meant to act: intracellularly, taking advantage of transduction as a gene-delivery vehicle for the intracellular expression of effector proteins (i.e. the lysins, and lysis-accessory proteins, showcased in this work).

In Figure S11 is shown the first version of a functional cosmid, encoding the multi-lysins cassette, with a strong aTc-inducible promoter. In Figure 3 a conceptual scheme of how the P4-EKORhE multi-lysins is employed as an antimicrobial therapeutic.

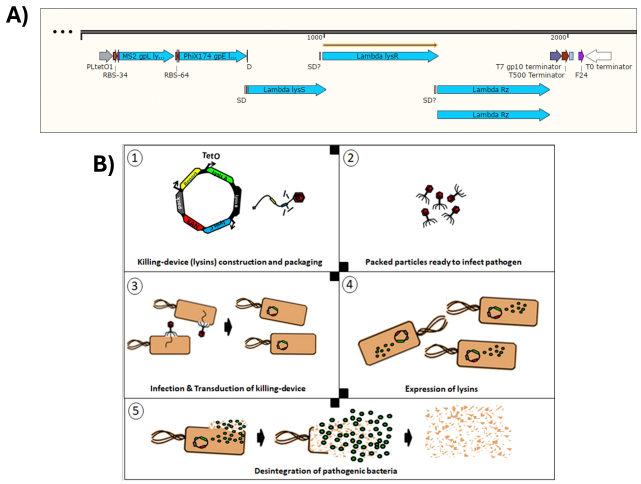

Fig. 3. Conceptual scheme of the P4-EKORhE multi-lysin antimicrobial. A) The multi-lysins cassette harbouring all lysins and lysis accessory proteins. The different encoded lysis and lysis accessory proteins used to construct the multi-lysins cassette. A study of the functionality of these circuits is presented in Figure 4. The "SD" and "SD?" labels contained within the construct are predicted "Shine-Delgarno" sequences that correspond to the multi-cistronic nature of the λ bacteriophage lysis operon (*lysS*, *lysR* and *Rz*) and the likely presence of unidentified *ghost promoters*. **B)** Conceptual scheme of the P4-EKORhE multi-lysins as a genetically-encoded antimicrobial therapeutic.

Results and discussion

R1. The multi-lysins cassette. There is an ample variety of promoters available for the study of promoter strength in *Escherichia coli*[23] and the construction of different biological genetic devices, such as the multi-lysins cassette. In this work, only two different promoters have been used: a strong anhydrous-tetracycline (aTc)-repressible promoter[24], for the strongly-repressible strong activation of the multi-lysins, and a weak rhamnose-inducible promoter[25][26], for the strongly-repressible weak activation of virus-like P4-EKORhE production of transducing particles.

Since the multi-lysins cassette is to be delivered via P4-EKORhE transducing particles, it is important that during production and packaging of this genetically-encoded antimicrobial, a strong enough promoter be used (e.g. the tetO1/aTc-inducible promoter when used in Z1 cells.) The tetO1/aTc-inducible promoter (PLtetO1 in Figure 3.A) would repress the encoded genetic circuit in "production cells" harbouring the tetR repressor. These can be safely used to produce high titers of transducing particles ready to package P4-EKORhE cosmids encoding the multi-lysins cassette without the risk of activating it. In Figure S11, the first functional version of the multi-lysins cassette is featured, with a "tetO1 promoter" (a strong aTc-inducible promoter) upstream of the multi-lysins cassette as a means to control the expression of lysins in order to prevent these from killing the bacterial cells used to produce transducing particles (see Figure 2). The multi-lysins cassette is also included in the final version of the high-yield producing P4-EKORhE, as depicted in the genomic map showcased in Figure 5.

As aforementioned, the multi-lysins cassette that is showcased in this work is a gene-delivered antimicrobial composed of three different lysis cassettes from three different bacteriophages, PhiX174, MS2 and λ bacteriophages[27][28][29][30][31] (see Supplementary Section SS4.4). These encoded lysis cassettes have been located contiguously (MS2's gpL single-gene lysis protein, PhiX174's gpE single-gene lysis protein and the "λ lysis cassette" (i.e. encoding the LysR endolysin, and the *lysS* and *Rz*) gene products, respectively) and expressed under a controlled promoter for facilitating packaging within transducing particle producing bacteria for bioproduction.

The effect of these lysins, and lysis-accessory proteins, expressed in combination as a multi-lysins cassette (i.e. the combination of MS2's gpL, PhiX174's gpE contiguous to the LysR-*lysS*-*Rz* cassette) is illustrated in Figure 4, below.

In regards to the effectiveness of the multi-lysins cassette, Figure 4 suggests that the activation of lysis shown leads to an antimicrobial effect that is shown over the period of 5 hours, which suggests this device as a powerful alternative to natural bacteriophages because of the observed decrease in bacterial cell optical densities.

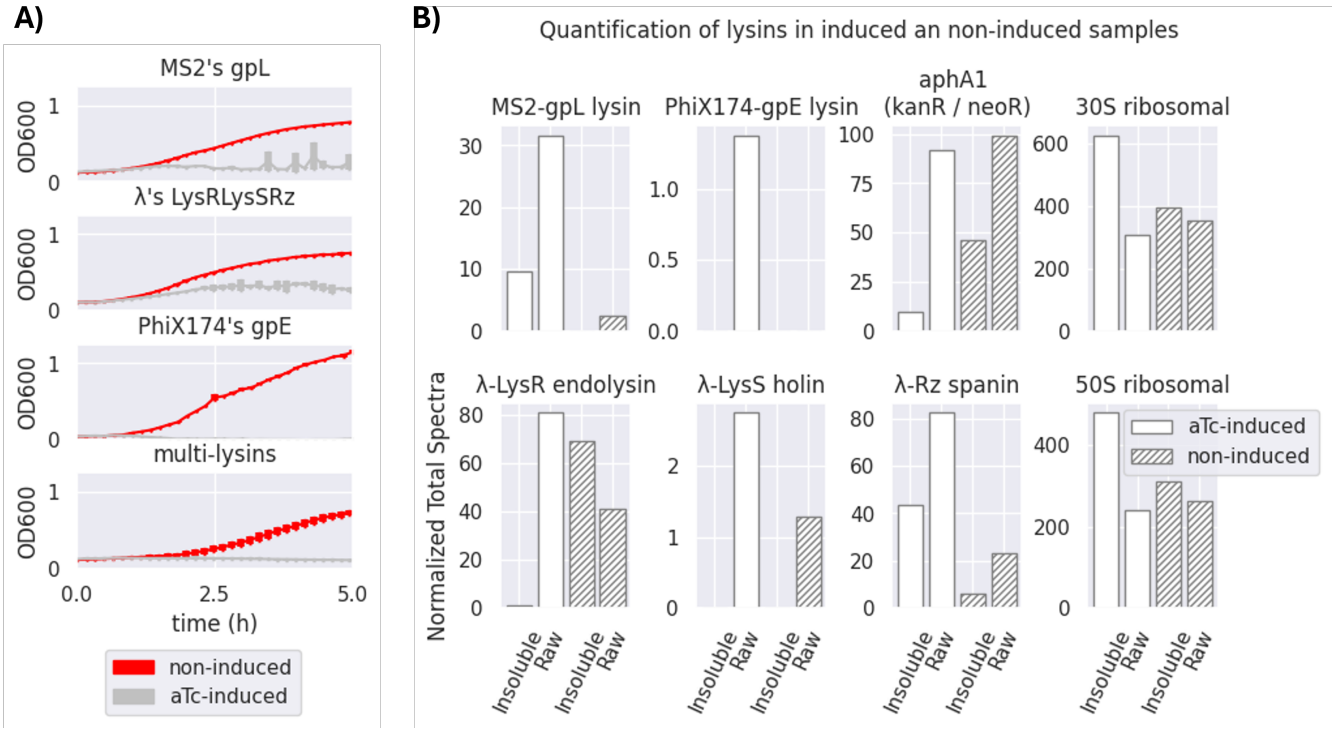

Fig. 4. Effectiveness of a transformed multi-lysins cassette A) Optical density (OD_{600}) measurements of bacterial survival profiles in pure bacterial cell cultures of *Escherichia coli* DH5α Z1 showing the antimicrobial effect of the multi-lysins cassette (the bottom-most plot) and each of its contributors (MS2's gpL, λ's lysis cassette, and PhiX174's gpE, the top-three-to-bottom plots). The "red lines" represent the bacterial cells harbouring the multi-lysins cassette that have not been induced with anhydrous tetracycline (aTc). The "silver lines" represent bacterial cells harbouring the lysins cassette that have been induced with anhydrous tetracycline at the start of the culture. ** A second replica is available in Figure S1 of the Supplementary Section SS2 for Replicas. The thickness of each line represents variability among the "n" samples using the standard deviation, where n = 3.* **B)** Normalized Total Spectra (NTS) obtained from a Mass-Spectrometry (MS) analysis of 4 different samples (2 induced and 2 non-induced, raw- and insoluble-phase) of bacteria containing the multi-lysins cassette. The Normalized Total Spectra includes data that computes the peptides found with over 95% probability that belong to each of the lysin sequences studied. **aTc-induced:** The protein extract comes from bacteria for which cosmids containing the multi-lysins cassette have been activated by adding aTc (anhydrous tetracycline) to the culture media. **Non-induced:** The protein extract comes from bacteria for which cosmids containing the multi-lysins cassette have not been externally induced with aTc. **Insoluble fraction:** Protein fraction extracted from the pellet of the lysed cells. **Raw fraction:** Protein fraction extracted from samples containing both, the supernatant and the pellet, of lysed cells. For more details see the Supplementary Section SS3.

Remarkably, both three systems of lysins (from MS2, λ and PhiX174 bacteriophages) appear to give an noticeable contribution to the multi-lysins cassette, while the most outstanding contributor is the encoding of the single-lysis gene gpE from the PhiX174 bacteriophage.

The results for the proteomics analysis for the presence of lysins and lysis-accessory proteins when inducing and non-inducing the multi-lysins cassette follow in Figure 4.B, where 4 different samples: two samples being aTc-induced, for the raw (i.e. both soluble and insoluble) and insoluble-only fractions, respectively, and an additional two samples in which the multi-lysins cassette was not aTc-induced (i.e. labelled as non-induced in the figure), for both the raw and the insoluble fractions, were tested. In Figure 4, the Normalized Total Spectra (NTS) for the presence of peptides belonging to the query sequences is demonstrated. The figure includes the aminoacid sequences from the lysins and lysis-accessory proteins used as target sequences, while the kanamycin-resistance *aphA1* gene product, together with the 30S and the 50S ribosomal proteins, are used as positive controls, for cosmid-borne and genome-borne expression, respectively.

Additional data, such as the peptide coverage obtained from the MS data, both generally among samples and per each of the samples analysed, is included in Figure S5 and Figure S6, respectively, that can be found in the Supplementary Section SS3 for Mass-spectrometry and proteomics.

Figure 4 confirms the presence of the aforementioned *ghost promoters* and Shine-Delgarno (SD) sequences (Figure 3.A and Figure S7) in the λ's lysis cassette, peptides from λ's lysis cassette were found in both induced and non-induced samples (despite a remarkable increase is observed upon activation with aTc compared to the non-induced samples) demonstrating through the analysis of Figure 4, that λ's lysis

cassette, and MS2's gpL, are noticeably, but not remarkably, effective upon activation but rather upon accumulation of its gene products, in an orchestration between LysS-holin/antiholin, and Rz and Rz1 spanins, with the LysR endolysin, over a period of time. Both MS2's gpL and PhiX174's gpE peptides were found only on aTc-induced samples, which is consistent with the observation that no *ghost promoters* nor suspicious Shine-Delgarno sequences were observed upstream its coding sequences (as shown in Figure S7).

Likewise, the constitutive gene expression of the gene products of *lysS* (and the LysR protein) and *Rz* can be justified by the presence of a ghost promoter upstream the possible SD-like sequence "GGAAGGAG" at the "end -3" position of PhiX174's gpE, or from a ghost promoter in any of each of the preceding sequences of *lysS* and *Rz* gene products (i.e. the only ones observed in the non-induced protein extract) as the most plausible explanation (see sequences in the Supplementary Section SS8). The MS results data demonstrated in Figure 4.B, above, shows factually that these "*ghost promoters*" and "hidden SD sequences" do exist, and corroborate their appearance when the cosmid is not activated with aTc. However, because their contribution to lysis is not remarkable (as is for PhiX174's gpE) their effect does not impede its use as a production system for transducing particles encoding the gene-delivered antimicrobial, as shown later in Figure 5.C). In contrast, MS2's gpL and PhiX174's gpE, as they have no potential *ghost promoters* or hidden SD sequences upstream of their own coding sequences, do only appear on aTc-induced samples.

The hypothetical synergistic lysis dynamics of the multi-lysins cassette is expected to be effectuated as follows (see Supplementary Section SS4.4 for comprehensive details about these action mechanisms of the chosen lysis proteins): (1) The λ's LysS[32] accumulates within the cytoplasm and, when reaching critical concentration, generates pores in the cytoplasm's side of the bacterial membrane[33]. This pore-formation in the cytoplasmic side of the membrane (2) facilitates the intrusion of λ's LysR endolysin[34], and single-gene lysins MS2's gpL[35] and PhiX174-gpE[36], which disrupt the membrane[37][38] and allow for (3) the λ-Rz[39] and Rz1[40] spanins to finally perform a final hit once the membrane is already disrupted[41][42] (which hit is also observed when aTc is not used in Figure 4 due to the presence of *ghost promoters* and SD sequences enabling the expression of the $lambda$'s lysis cassette regardless of the presence of anhydrous tetracycline). These results portray an indubitable correspondence between activation of the multi-lysins cassette with the inducer anhydrous tetracycline (aTc) and the presence of lysins (and lysis-accessory proteins), and with the antimicrobial effect demonstrated in Figure 4.

R2. The conditionally-replicating P4-EKORhE. The virus-free virus-like, conditionally replicating, P4-EKORhE (i.e. a *Latinised* transcription from P4-$\mathcal{E}$KO-Rh$\mathcal{E}$) which literally stands for "*epsilon (ε) knock-out rhamnose-inducible epsilon (ε) P4 bacteriophage*", is a bioengineered rhamnose-inducible P4-based system for the high-yield bioproduction of transducing particles presenting itself as a genome-minimised P4 (P4-min) bacteriophage with its epsilon (ε) gene *knocked-out*, and a degenerate, codon-optimised, version of the a synthetic epsilon (ε) put on a different region to be expressed under the control of a rhamnose-inducible promoter.

For the construction of the P4-EKORhE, a P4 bacteriophage was used as a starting material to retrieve the essential parts of P4 (see Figure 5) and combine it with a genetically-encoded cargo in the form of the multi-lysins cassette (Figure S11).

During construction, an essential part of the P4 bacteriophage[11] (i.e. from P4p05 til P4s04, see it in Figure 5.A, or in Supplementary Section SS8) was PCR-amplified and cloned together with the multi-lysins cassette to confer it with a selection marker (i.e. kanamycin resistance). Following successful selection with kanamycin, the now genome minimized P4 (P4-min) was further amplified by PCR to produce a *knock-out* within the natural epsilon (ε) coding sequence in P4 and include a degenerate, codon-optimized version of epsilon (ε) that cannot recombine with its natural one.

The whole epsilon (ε) gene, the target for the *knock-out*, was not fully removed and rather a minimal deletion was effectuated to avoid the potential disruption of P4 functionality. In the end, a final mutant that had the epsilon (ε) gene stably *knocked-out* was generated and a construct containing a degenerate version of the epsilon (ε) gene, downstream a rhamnose-inducible promoter, was inserted downstream the cos-site which is further away from the original position of the epsilon (ε) gene. Therefore, the bioengineered P4-EKORhE differs from a natural P4 bacteriophage in that it is a cosmid that is (1) genome-minimised, (2) that has a selection marker, and (3) two sequences of the epsilon (ε) gene that are degenerate to each other and therefore cannot recombine. The first of which is *knocked-out* and therefore dysfunctional, the second of which is fully functional and degenerate to the first.

The final construct is exemplified in Figure 5, which shows a display of the features of P4-EKORhE harbouring the multi-lysins cassette. The sequencing confirmation for the P4-EKORhE can be found by analysing the trace sequences (obtained after assembly of the P4-EKORhE cosmids shown in Figure 5.A and 5.B). A whole-plasmid sequencing service was used to obtain the full-sequence of the "stabilized form" of the P4-EKORhE (see Figure 5.B.B). Both sequences are compiled in the Supplementary Section SS8.5 and SS8.6, respectively.

A scheme showcasing how particles can be produced using

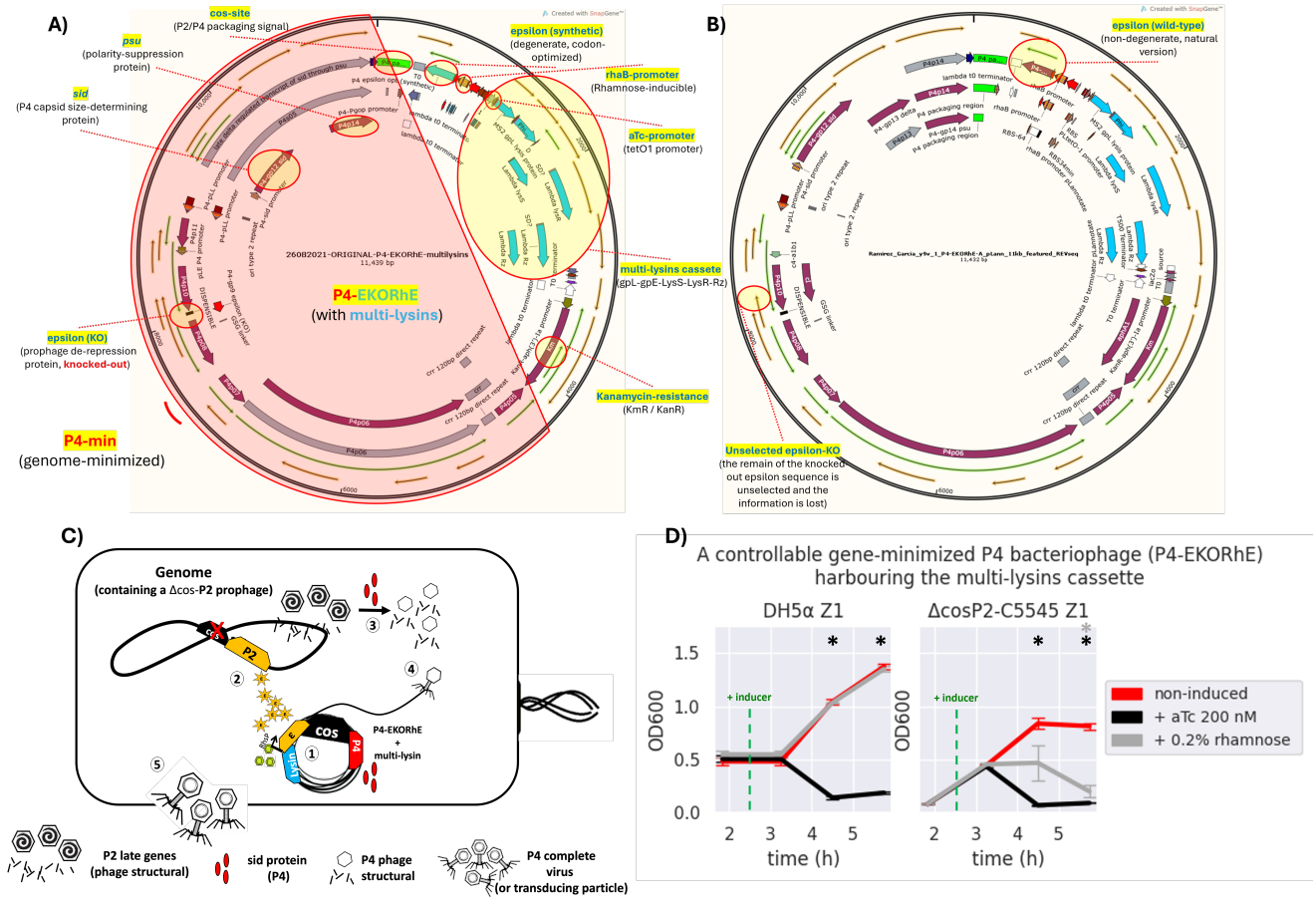

Fig. 5. The bioengineered P4-EKORhE harbouring the multi-lysins cassette. A) A kanamycin-selectable cosmid of 11,439 bp containing a genome-minimised P4 bacteriophage (P4-min, see highlighted in red) that has a *knocked-out*-version of the epsilon (ε) gene, an alternative functional synthetic, codon-optimised, and degenerate version of the epsilon (ε) gene, and the multi-lysins cassette harbouring the 3 systems of lysis form MS2, PhiX174 and λ bacteriophages. **B)** The same cosmid after a few passages loses the dysfunctional *knocked-out wild-type* version of the epsilon (ε) gene, while the synthetic degenerate version gets restored to the sequence observed in the wild-type epsilon (ε) protein commonly seen in the natural P4 bacteriophage (see red circles for "unselected epsilon-KO" and "epsilon (wild-type)"). The stable version of the P4-EKORhE is now 11,432 bp. Fully featured sequences of **A)** and **B)** can be found in the Supplementary Sections SS8.5 and SS8.6, respectively. **C)** A conditionally-replicating, and rhamnose-inducible, P4 bacteriophage as a transducing particle. **(1)** The P4-EKORhE is propagated as a cosmid harbouring the multi-lysin cassette, and expresses the *sid* protein upon activation. **(2)** a Δcos version of the P2 prophage is inhibited by the activity of P4 but activates its late genes, with the delta (δ) gene product, which are the necessary structural proteins for P2. Additionally, the rhamnose-inducible epsilon (ε) gene product activates the self-replication of the Δcos version of the P2 prophage. **(3)** The *sid* protein from P4 converts P2 heads into smaller P4 heads, thus further compromising P2 propagation. **(4)** P4 structural proteins package the P4 cosmid harbouring the multi-lysins cassette (i.e. or any other engineered circuit), or any other accessory cosmid. See Figure 5 for a display of the features of P4-EKORhE harbouring the multi-lysins cassette. **D)** Dynamics of of the activation of the P4-EKORhE-multi-lysins cosmids with inducer, aTc (anhydrous tetracycline) or rhamnose, in strains of *Escherichia coli*, transformed on *Escherichia coli* DH5α Z1, or the engineered lysogen Δcos:TriR-P2-c5545 Z1, respectively.The sample size (n) used is n = 3, * = *p-value* < 0.05, measured at the data points used after the addition of the inducer, using a standard t-test using SciPy libraries (see General Methods SS6.1).

this controllable, gene-minimized, P4-EKORhE is shown in Figure 5.C. The figure shows that the system is inducible because the P4-EKORhE encodes an synthetic epsilon (ε) coding sequence, downstream a rhamnose-inducible promoter.

A demonstration of the dynamics by which P4-EKORhE harbouring the multi-lysins cassette functions is shown in Figure 5.D. The figure shows that bioproduction with the P4-EKORhE system has the main advantage that bacteriophage production is fairly repressed when rhamnose is not present in the culture (maintained in all cases with kanamycin 50 µg/mL), and fully activated when 0.2% (w/v) rhamnose is added to the producing culture. As expected, the P4-EKORhE is only propagable in Δcos:TriR-P2-c5545 Z1 bacterial cells, as no effect on the activation of epsilon (ε), using 0.2% (w/v) rhamnose, is observed when tested on DH5α Z1 cells. Instead, the activity of P4-EKORhE, is

seen to be repressed to usable levels when rhamnose is not present in the a bacterial culture of Δcos:TriR-P2-c5545 Z1, and completely inducible when 0.2% (w/v) rhamnose is added to the producing bacterial culture. The addition of anhydrous tetracycline (aTc) to the producing bacterial culture elicits a remarkable drop in the observed OD_{600} due to the controllable activation of the multi-lysins cassette with anhydrous tetracycline (aTc).

The assessment of the capacity of P4-EKORhE for the production of transducing particles shown in Figure 5.D suggests that although the repression of P4-EKORhE is not 100% effective, it is enough to maintain a stable culture for high-yield bioproduction. This facilitates the possibility of maintaining the cosmid within a production strain (i.e. the engineered lysogen Δcos:TriR-P2-c5545 Z1), using a transformation protocol, and storing it in glycerol stocks at -80ºC, for later expansion. This future expansion can be done by regrowing the frozen strain, containing the maintained P4-EKORhE cosmid, and further inducing it with 0.2% (w/v) rhamnose, for the production of a new batch P4-EKORhE transducing particles. In this light, the added repressible capability in P4-EKORhE allows for an easier and more reproducible production of P4-based transducing particles.

R3. High-yield bioproduction of transducing particles with P4-EKORhE. Using the P4-EKORhE system for the production of transducing particles is facilitated by the controllable feature of its rhamnose-inducible synthetic epsilon (ε) cassette. However, the upstream optimization of the bacterial cell culture growing conditions together with a downstream processing that maintains the integrity of the particles while maximizing the yield of bioproduced transducing particles is an essential. Figure 6 is highlights the improvement obtained when changing the standard PEG8000 purification of bacteriophages for an ultrafiltration protocol[43] together with the changes in bacterial cell culture growth media from LB to TB broth.

In this way, the controllability of P4-EKORhE enables the implementation of a special bioporcess in which bacteriophage lysates are continuously enriched in a *fed-batch* fashion by overloading the growing lysates with different iterations of fresh incoming Δcos-P2-c5545 Z1 bacterial cells harbouring P4-EKORhE (as shown in the steps 5 and 6 of Figure 8. The aforementioned process shows in Figure 6 its capacity to reach yields of at least 10^9 infectious particles per each drop of 10 µL used for the spot assay (Figure S4), which translates to the lysate being about 10^{12} pfu/mL (i.e. with a transduction capacity of 10^{12} cfu/mL), according to the formula below:

$$titer = dilution_{lysate} \cdot count_{plaques} \cdot volume^{-1}{}_{sample} = pfu{\cdot}mL^{-1} \text{ or } cfu{\cdot}mL^{-1}$$

Therefore, Figure 6 shows how the P4-EKORhE system has

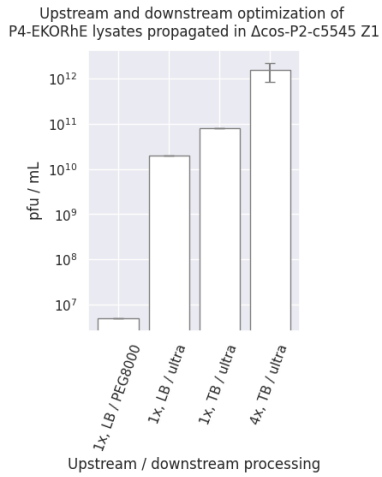

Fig. 6. Upstream and downstream optimization of P4-EKORhE. Different yields are obtained when using P4-EKORhE according to the upstream and downstream optimization. In regards to upstream optimization, lysates are produced using either one or four enrichment cycles (i.e. 1x or 4x), using LB or TB broth. In regards to downstream optimization, lysates are concentrated using either 10% PEG8000 or molecular weight cut-off ultrafiltration. The plaque-forming units are taken from the plates shown in Figure S4. The sample size (n) used is n = 1, except for "4x, TB / ultra" which has n = 3.

been optimized to maximize the final yield of 10^{12} cfu/mL P4-EKORhE-based transducing particles by a succession of enrichment cycles (up to four times, 4x), providing a nutrient rich media (Terrific Broth, TB) and using the ultrafiltration protocol as a physical means of separation and concentration (as depicted in Figure 8).

R4. Transduction of the multi-lysins cassette with P4-EKORhE. In Figure 7.A the antimicrobial potential of the multi-lysins cassette transduced with P4-EKORhE particles is demonstrated. The optical densities (OD_{600}) of a transduced bacterial cell culture are measured over a period of 5 hours and a comparison with the effectiveness of natural bacteriophages, T4 and K1F, using the same target bacteria is showcased in Figure 7.B and 7.C.

As the figure demonstrates, the P4-EKORhE-multi-lysins particles show an active effectiveness that is proportionate to the MOI (Multiplicity of Infection). Of particular interest is the case where MOIs of 10 are used. A distinctive activity is shown for the positive control (hampering the activity of the tetR repressor with 200 nM aTc) versus the negative control, where the tetR repressor seems to be able to inhibit to a significant degree the expression of lysins. The behaviour observed with the target strain is as good or better than with the positive control, demonstrating the antimicrobial effect, during the 5 hours of OD_{600} measurements shown.

To facilitate further discussion, in Figure 7.C, the relative are

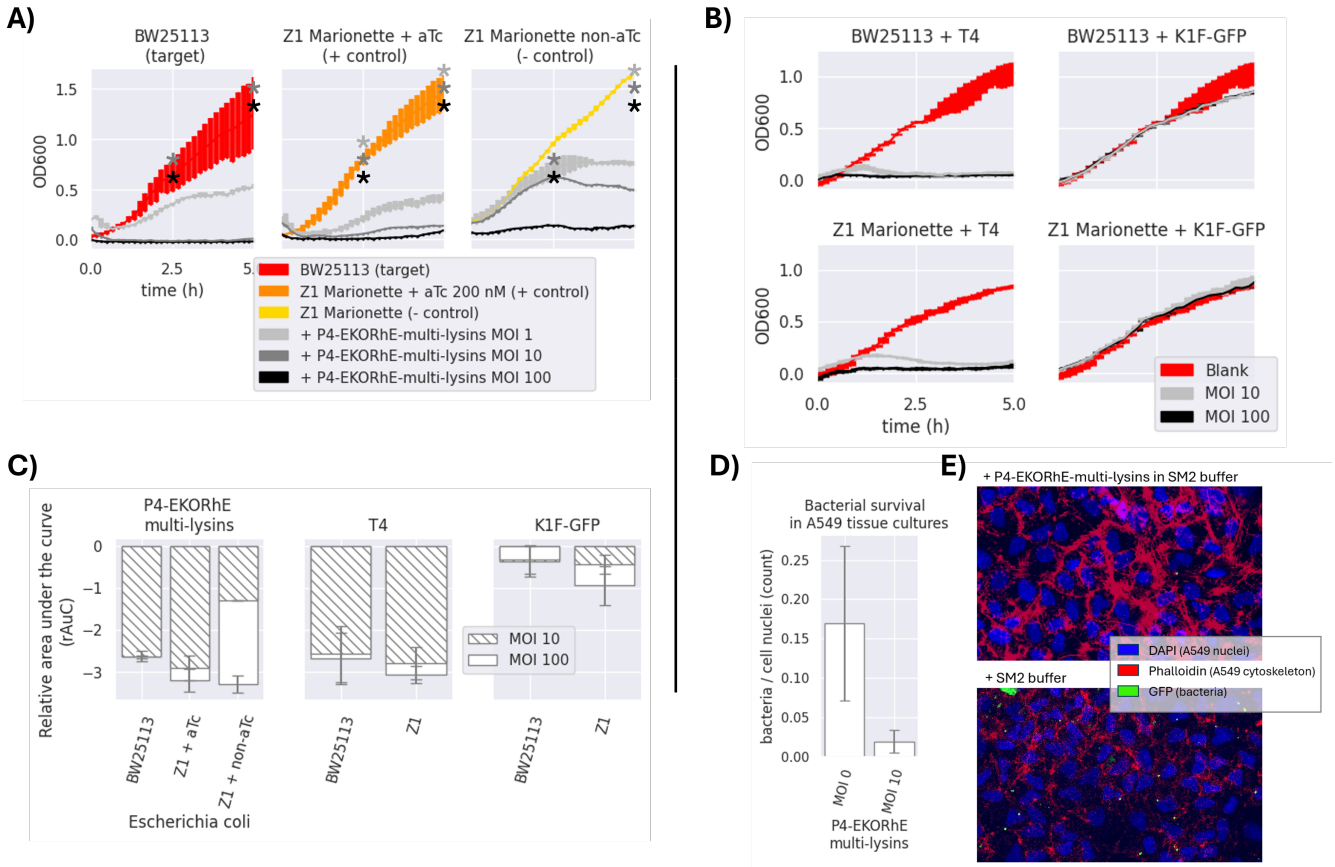

Fig. 7. **A) Antimicrobial activity of P4-EKORhE-multi-lysins.** Optical density measurements of the target and control *Escherichia coli* strains i.e., BW25113 and Z1 Marionette, respectively, when exposed to P4-EKORhE-multi-lysins particles at different MOIs. No selection antibiotics were used for the realization of these experiments. **B) Antimicrobial activity of natural enterobacteriophages to its respective hosts.** Antimicrobial activity of enterobacteriophages to its respective target and control E coli strains i.e., BW25113 and Z1 Marionette when exposed to natural enterobacteriophages containing a replicative virus at different MOIs. For a replica of the same set of experiments see Figure S2 in the Supplementary Section SS2 for Replicas. The thickness of each line represents variability among the "n" samples using the standard deviation, where n = 3.**C)** Relative area under the curve of P4-EKORhE-multi-lysins, T4 and K1F-GFP at MOIs of 10 and 100 on *E. coli* strains BW25113 (target) and Z1 Marionette (+/- control). rAuC(MOI = 0) = 0 $\pm$1. The sample size (n) is n = 2 from the results obtained in the two different replicas in Figure 7 and Figure S2 which contains n = 3 for each measure of time (total n=6). The rAuC has been integrated from 0 to 5 hours in all cases.. **D)** The differences in bacterial count following treatment with P4-EKORhE harbouring the multi-lysins cassette on A549 cell tissue at an MOI of 10 versus with a blank solution (MOI = 0) of SM2 buffer, following an immunohistochemistry fluorescence image analysis. **E)** Immunohistochemistry fluorescence image analysis on a sample infected with GFP-fluorescent bacteria treated with P4-EKORhE harbouring the multi-lysins cassette versus the blank solution of SM2 buffer. The sample size (n) used is n = 7 for the (- control), while n = 6 for the target was used. The *p-value* for the difference between the two samples is p = 0.31. The images used to count for the results shown in **D)** can be found in the Supplementary Section SS5 for Immunohistochemistry imaging.

under the curve (rAuC) corresponding to the growth curves shown in Figure 7.A and 7.B is used to demonstrate a more accurate way in which the antimicrobial effectiveness can be compared among samples, and the MOIs used, for both P4-EKORhE, and T4 and K1F-GFP bacteriophages. These results showing that infection of a pure bacterial cell culture of *Escherichia coli* BW25114 with T4 behaves similarly to the controllable P4-EKORhE.

Regarding the results obtained with K1F bacteriophages, it is important to mention that K1F is specialized to infect encapsulated strains of *Escherichia coli*[21] and therefore its effectiveness for infecting normal strains such as BW25113 is expected to be compromised, as shown in the figure and its corresponding rAuC values.

The results in Figure 7.A, 7.B and 7.C are consistent with that which was found during the initial characterisation of the multi-lysins cassette in Figure 4. The most remarkable observation in the figure is that, for natural bacteriophages, increasing the MOI from 10 to 100 does not translate to a significantly different result, despite it is clear that an MOI of 10 there is an observable momentaneous growth of the bacterial cell culture at about 1 hour post-culture, which is

delayed to after 2.5 hours with P4-EKORhE, which correlate with the results described with the relative area under the curve (rAuC). Of significance for further discussion, is the fact that when using MOIs of 100, certain antimicrobial effectiveness is also observed in negative control strains of Z1 Marionette to which tetR repressor has not been hampered using aTc.

In regards to the effect of the multiplicity of infection (i.e. MOI), on the bacteriostatic activity of P4-EKORhE encoding the multi-lysins cassette, it is found that, as demonstrated in Figure 7.A, and as described in the literature [44], at high MOIs of 100, the phenomenon of "lysis-from-without" should be considered attributive to the results obtained, although the multi-lysins cassette bears the most important significance, as observed at lower MOIs of 10. This phenomenon is clear when analysing the results of the negative control, when using high-MOIs of 100 on the non-aTc negative control samples.

The fact that the "lysis-from-without" effect is rather not remarkably contributing to bacteriostatic times is exemplified when comparing results shown in Figure 7.C. When comparing the results observed at different MOIs it can be seen that the differences in rAuC values obtained for MOIs of 10 versus MOIs of 100, for the P4-EKORhE, are not equally proportionate between BW25113 and the Z1 controls. Therefore it is unlikely that these are due to "lysis-from-without". Still, it is clear that, when testing the P4-EKORhE, the results obtained for non-induced Z1 strains highlight that, only at MOIs of 100, there is a notable increase in antimicrobial effectiveness observed which is necessarily due to "lysis-from-without", if considering the tetR repressor in Z1 cells is functional enough when aTc is not added.

An alternative hypothesis to this observation, although less likely, would be the on of "tetR repressor depletion", reliying on the possibility that, for MOIs that are high enough (e.g. of 100), it can be that a single bacterial cell can be transduced multiple times (i.e. interact with many transducing particles simultaneously and over time) and therefore can exhaust the available tetR repressors within the cell without compromising the integrity of the membrane. Therefore, the unavailability of tetR repressors would lead to leaky gene expression of the multi-lysins cassette which could be responsible for the observed bacteriostatic effect, which is not observed at lower MOIs of 10 (i.e. compare the rAuC and the bacteriostatic times for the "Z1 + aTc" versus the "Z1 non-aTc", when using the P4-EKORhE, in the rAuC plot of the Figure 7.C).

Regardless of whether the effect of high MOIs may be caused by "lysis-from-without" or "exhaustion of the tetR repressor" in Z1 cells infected with P4-EKORhE, these hypotheses are still speculative. Experimentally, it should be possible to further elucidate whether "lysis-from-without" or "exhaustion of the tetR repressor" is responsible for the differences observed by using P4-EKORhE encoding blank genetic sequences without antimicrobial activities so as to determine the effect of transduction on the phenomenon of "lysis-from-without". However, with the information available in this work, it is fair to maintain that the figure shows a difference that is significant between aTc-induced Z1 cells versus non-induced Z1 cells, and which has to be attributed to something other than the effect that can be attributed to the activity of the multi-lysins cassette. Therefore, effectiveness (although milder) is also observed at low MOIs of 10, although not to the level observed in all the other samples used, and certainly not on the positive control with the "Z1 Marionette + aTc".

If "lysis-from-without" were occurring at lower MOIs, the observed 2.5h delay in lysis on the negative control bacterial cell culture would not occur (i.e. see in Figure 7.A). On observing both replicas (Figure 7.A and Figure S2) for the non-activated Z1 cells after infection with P4-EKORhE, it is observed that at an MOI of 100, there is a mild antimicrobial activity (i.e. where a bump on bacterial growth is observed at 2.5 hours). This hampered activity is observed to start right after infection (i.e. at time = 0) and this observation suggests that the reason that negative control strains, when infected with P4-EKORhE, display some antimicrobial activity at high MOIs of 100 can be attributed more importantly to "lysis-from-without" or "tetR repressor depletion".

The figures above highlight the potential of P4-EKORhE particles as an antimicrobial alternative to natural bacteriophages. In Figure 7.B and 7.C it is shown that, for both BW25113 and Z1 Marionette, when infected with T4 bacteriophage, the bacterial culture is not able to grow during the the 5-hour measurement, similarly as when with transduction using P4-EKORhE. These results suggest the feasibility of using a transducing particle, as an alternative to a natural bacteriophage, with the advantage of being able to take full-control of the mechanisms of lysis and without the need to account for the viral replication of the transducing particle (because it cannot propagate on target bacteria).

The P4-EKORhE on A549-tissue cultures as a model of infection. The experiments with measuring optical cell densities using pure bacterial cell cultures provide a preliminary way in which to assess the effectiveness of transducing particles, alongside natural bacteriophages. However, when looking forward to the development of a potential antimicrobial therapeutic it is of added-value to attempt to reproduce experimental conditions that are more similar to a real scenario. Because of that, a model of infection using A549 human lung epithelial immortalized cells has been used to further assess the capacity of P4-EKORhE encoding the multi-lysins cassette to perform as an antimicrobial in a more complex condition.

In Figure 7.D, the ratio of bacteria/nuclei on the images taken

from two sets of experiments, one in which supplementation with 100 µL of an SM2-buffer solution containing P4-EKORhE transducing particles harbouring the multi-lysins cassette was added after 1h incubation of bacterial cells on the A549 cell tissue loan, versus another in which 100 µL of blank SM2 buffer was added, is demonstrated.

The figure shows that the differences in bacterial count after one hour of bacterial growth followed by two hours post-treatment with P4-EKORhE are indicative of the effectiveness of P4-EKORhE to deliver the multi-lysins cassette in a more complex environment. The Figure 7.D shows a remarkable difference in averages for bacterial counts obtained between samples with P4-EKORhE particles in SM2 buffer (target samples) versus samples with SM2 buffer only (negative control). However, the significance analysis for a sample size (n) of n = 6 is only p = 0.31, suggesting the need for further optimization or an increase in the number of samples to see the basis for the difference in averages observed. The images in Figure 7.E are also indicative of this remarkable difference in averages. The remaining images, with which the bacteria/cell nuclei count have been used can be found in Figure S12 for target samples, and Figure S13 for negative controls.

In the context of the effectiveness of the multi-lysins cassette as a gene-delivered antimicrobial, the results presented demonstrate the potential of P4-EKORhE as a system for the high-yield bioproduction of transducing particles to transduce a genetically-encoded antimicrobial, in the form of the multi-lysins cassette, in a model of infection based on human cell cultures as a means to corroborate the results obtained using pure bacterial cell cultures.

Conclusion

Effectiveness of the multi-lysins cassette. Regarding the effectiveness of using lysins, and lysis-accessory proteins, as antimicrobials, it can be stated that the multi-lysins cassette, encoding a combination of lysins and lysis-accessory proteins, such as holins and spanins, has demonstrated the capability of constructing genetically encoded antimicrobials with potential for synergistic effects by combining different effector proteins that can actuate through different lysis mechanisms. Another manner in which the multi-lysins cassette would be made more effectively is by incorporating other kill-switches of different potency that mechanistically target as divergent elements of bacterial survival as possible. This could entail the including of nuclease elements such as CRIPSR-Cas9, as both targeting and analytical biological devices[45], as well as different inhibitors of bacterial growth that do not necessarily have to be lysins or lysin-like proteins, but that would work effectively towards higher synergistic effect than those positively found by combining the three different lysin systems here shown (i.e. the multi-lysins cassette).

High-yield bioproduction with P4-EKORhE. The bio-engineered P4-EKORhE, as a minimized, controllable and conditionally replicating bacteriophage, that uses temperate P2 bacteriophages with their cos-site *knocked-out* to produce virus-free P4-like transducing particles at remarkably high yields is demonstrated as the final solution for a system for the production of high-yield (10^{-6} - 10^{-10} pfu/mL) transducing particles that is also free from infectious wild-type natural P2 or P4 viruses. Using biological activators such as epsilon (ε) demonstrate the possibility of using natural proteins from the P4 bacteriophage that enable the activation of temperate P2 bacteriophages, and allow for a more reproducible and controllable production of transducing particles. The further downstream processing optimization for the system based on P4-EKORhE demonstrates the possibility of using this system to achieve the highest yields which are greater than 10^{-12} pfu/mL, establishing P4-EKORhE, a nutritious media such as TB (Terrific Broth), and a bioprocess based on an hybrid fed-batch continuous supply of growing bacteria yielding virus-free P4-transducing particles, as the most optimal procedure for the production of high-yields of transducing particles.

Transduction of the multi-lysins cassette with P4-EKORhE. The transduction of the multi-lysins cassette via the P4-EKORhE is an effective system that shows that the antimicrobial activity of the multi-lysins cassette is not compromised, but rather enhanced, respective to the results obtained during the characterization of its antimicrobial activity using transformed bacterial cell cultures. The results obtained during transduciton in pure bacterial cell cultures are also similar to the ones found when infecting the bacterial cell cultures with natural bacteriophages such as T4 or K1F-GFP which highlights the potential of both P4-EKORhE and the multi-lysins cassette in a clinical context.

Of additional significance, these results also highlight that the antibiotic marker selection (i.e. to maintain the transduced cosmids within the bacterial hosts) has not been problematic when testing the performance of the P4-EKORhE-multi-lysins at the demonstrated MOIs of 100 and 10. Therefore, although a therapeutic of this kind could be delivered in conjunction with a selection antibiotic with an expected improvement in its effectiveness, it has been shown that it is not necessary to achieve similar, and better, bacteriostatic times as the ones observed during the initial characterization of the multi-lysins cassette, on transformed bacterial cells, where kanamycin (50 µg/mL) was added to the growth culture before activation of the multi-lysins cassette with anhydrous tetracycline.

The improved effectiveness of the transduced multi-lysins cassette is supported by the fact that multiple transducing particles may be able to effectively transduce cosmids multiple times during the initial hours of infection and aid in the antimicrobial effect against bacteria. Additionally, it has

been shown how transducing particles, at high MOIs of 100, could improve the effect by the aforementioned phenomenon of "lysis-from-without", as evidenced by comparing the results obtained with positive and negative controls, when transduced with P4-EKORhE.

When P4-EKORhE harbouring the multi-lysins cassette, is used in cell cultures of A549 contaminated with *Escherichia coli*, the results obtained, despite with a *p-value* of 0.31, demonstrate a tendency on a remarkable difference in averages for bacterial counts, that correlates with the results obtained when using pure bacterial cell cultures. The results also are inviting to perform similar experiments at higher MOIs where a better difference between treated and untreated samples would be observed, accounting for the fact that P4-EKORhE cannot propagate in target host bacteria.

Ultimately, the results shown in this work demonstrate encoding the multi-lysins cassette in cosmid packaged within a P4-EKORhE system for the production of transducing particles can be used as an antimicrobial agent that is as effective as a natural bacteriophage, but with the advantage that is non-replicative and, most importantly, fine-tunable.

Therefore, here is a comprehensive demonstration of a proof-of-concept gene-therapeutic where a "bacteriophage-based transducing particle with a genetically encoded antimicrobial" is an alternative to “natural bacteriophages", demonstrating the possibility to use the P4-EKORhE as gene-delivery vehicle to effectively produce high-yields of transducing particles to deliver the multi-lysins cassette into target bacterial cells in both pure bacterial cultures and in a model of infection using A549 human epithelial cell cultures infected with *Escherichia coli* and opens the door to finding new and effective antimicrobial therapeutics that target a range of pathogenic bacterial strains.

Materials and methods

MM1. General Materials. All chemicals were purchased from Sigma Aldrich (now Merck) or ThermoFisher Scientific unless stated otherwise. Primers and gene fragments were commercially synthesised by Integrated DNA Technologies (IDT) or GENEWIZ, US. The stock and working concentrations of antibiotics used in this work are stated in Table S1. All strains used throughout this work are stated in Table S3.

For the construction of genetic devices (Supplementary Section SS4 and SS8), Q5 High-Fidelity Polymerase, Phusion High-Fidelity DNA Polymerase and NEBiuilder HiFi DNA Assembly Master Mix were purchased from New England Biolabs (NEB) and used according to protocol. All restriction enzymes and ligases were purchased from New England Biolabs (NEB). All enzymes were stored at -20 °C and used with the buffers provided according to the manufacturer’s instructions.

MM2. Mass-spectrometry and proteomics. In order to ascertain that bacterial cells are eliminated upon activation of the multi-lysins cassette when using anhydrous tetracycline (aTc), it is useful to identify these lysins on a western blot, only when the circuits are activated, in order to relate the correspondence between gene expression and the decrease in the optical densities, as shown in Figure 4. However, because the encoded lysins included in the multi-lysins cassette are multiple (i.e. up to six different coding sequences, Figure S11), when constructing the system it was unfeasible to include Histidine-tags, which would otherwise facilitate its purification and identification, because having repetitive DNA sequences within the cassette would generate genetic stability problems in the cosmids encoding repetitive sequences, which would lead to the impossibility of gene-synthesising it using a commercial supplier.

Therefore, in order to demonstrate the presence of the lysins, upon activation with aTc, and to relate its expression to its antimicrobial effect, an SDS-PAGE gel for expression verification (Protocol SS6.12), and later extraction for Mass-Spectrometry (MS) analysis was employed after the protein extraction of experimental samples (Protocol SS6.11). However, because bacterial cells would be disrupted upon activation of the lysins cassette, an SDS-PAGE gel did not yield bands sharp enough to be distinguished by inspection by the eye. In this regard, to distinguish them among the remaining proteins in the lysate was not possible, but a lower fraction band of the gel was used for protein extraction and MS, confirming the presence of the lysins and lysis-accessory proteins showcased in this work (Figure 4).

To prepare samples for MS (Supplementary Section SS3), induced and non-induced protein lysates from a culture of Marionette Z1 bacterial cells containing a P4-EKORhE cosmid i.e., in Figure 5, which harbours the aTc-inducible multi-lysins cassette showcased in this work, was processed and purified using a standard protein extraction protocol (see Protocol SS6.11). Two types of lysates were produced, one coming from the pellet, or insoluble phase (i.e. as lysins are expected to act within cell walls and membranes), and another which was considered a "raw lysate" i.e., containing both soluble and insoluble phases of the lysate where proteins.

Four different samples of pure bacterial cell cultures (*Escherichia coli*) Z1 cells transformed with the multi-lysins cassette cosmid were grown in 1 mL Eppendorf at 37ºC, on shaking conditions, until reaching the exponential growth phase (about 0.4-0.5 OD_{600}). Subsequently two of the samples were aTc-induced with 200 nM[46] of anhydrous tetracycline and grown for a further two hours to wait for the lysis of the bacterial cell culture. The bacterial cell cultures were treated with Lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and 5 mM EDTA) and sonicated to make sure both induced and non-induced samples were treated equally and also so that non-induced samples are surely lysed (see

Protocol SS6.11 for more details).

MM3. Preparation of optical density measurements. In order to ascertain the antimicrobial effectiveness of the P4-EKORhE transducing particles encoding the multi-lysins cassette (Figure 7.A and 7.B), replicas of 96-well plates are utilized. For each well on a 96-well plate (Cellstar Multiwell Plates, Sigma-Aldrich), 100 µL of bacterial culture of *Escherichia coli* at an OD_{600} = 0.1 are dissolved in doubly concentrated LB media, and half diluted with an additional 100 µL of, either an SM2 buffer solution supplemented with P4-EKORhE-multi-lysin transducing particles or T4 or T7 bacteriophages (at MOIs of 10 or 100) or with an SM2 buffer only solution for blanks (i.e. for MOI = 0). The plates are measured on a plate reader measuring optical densities at about 600 nm to determine bacterial cell densities over time. When the bacterial cells being tested were the Z1 cells, and there was the intention to activate the multi-lysins cassette upon infection with P4-EKORhE, anhydrous tetracycline was added at a concentration of 200 nM[46]. For the transformed multi-lysins cassettes experiments (Figure 4 and Figure 5.D) the same methodology was employed where realized but without doubling LB media concentration and without adding any SM2 buffer solutions, but kanamycin (50 µg/mL) was used instead to recover the cells after cosmid transformation and selection before activation. Before activation, cosmid harbouring cells where resuspended in LB media and the multi-lysins cassette was activated using anhydrous tetracycline (aTc, 200 nM)[46] and L-rhamnose was added at a concentration of 0.2% (w/v)[25].

MM4. Relative area under the curve as antimicrobial effectiveness. For a more accurate comparison between the antimicrobial effect of the P4-EKORhE transducing particles harbouring the multi-lysins cassette with the antimicrobial effect of natural bacteriophages T4 and K1F-GFP, the rAuC from the OD_{600} plots over the time span that goes from 0 to 5 hours were extracted for all plots. The rAuC is plotted using the area under the curve (AuC), obtained using the scripted `def AuC(x, y):` function shown below, of the non-induced, or non-transduced (i.e. MOI = 0), sample and using it to subtract the mean values of the induced samples and the standard deviation normalized (by dividing its values) to the blank experiments with non-transduced or non-infected bacterial cells. It is understood that rAuC(MOI = 0) = 0±1 i.e. for MOI = 0, the rAuC = 0 with an absolute standard deviation of 1. Therefore MOI = 0 have not been included in the rAuC plots as these are a relative measure. For integration of each of the curves from the corresponding plots used to obtain the values of AuC, which can later be used to compute the rAuC as aforementioned, the Trapezoid Rule has been used to interpolate the area under the curve using the equation encoded in the following script:

```
def AuC(x, y):
    sum = 0
    for i in range(1, len(x)):
        h = x[i] - x[i-1]
        sum += h * (y[i-1] + y[i]) / 2
    return sum
```

Where `x` is time, and `y` are the point values of OD_{600} over time which form the basis of the total sum of the area under the curve against the x-axis from `x=0` (i.e. time = 0) until the final value of `x`.

MM5. Preparation of animal tissue cultures for transduction. In regards to the mammalian cell culture model of infection, cell lawns of A549 cells (A549 - ATCC CCL-185) are cultivated. The A549 cells are cultured using a base medium F12/K (Gibco/Invitrogen) with 10% fetal bovine serum (FBS) added to the base medium with a standard amount of p/s (i.e. penicillin/streptomycin), incubated at 37ºC with 5% CO_2. The A549 cells are seeded in T25 flasks until transferred to 6-well plates (Cellstar Multiwell Plates, Sigma-Aldrich).

When the growing A549 cells are transferred to the 6-well plates, using 2 mL of media per well, these are grown on top of microscopy coverslip slides that have been deposited on each of the 6-well, so as to be able to remove the coverslip slides for later preparation, following an immunohistochemistry protocol, for fluorescence imaging using DAPI, phalloidin and a GFP enhancer to count bacterial cells (i.e. harbouring a GFP plasmid) alongside cell nuclei (i.e. stained with DAPI) surrounded by the cell cytoskeleton (i.e. stained with phalloidin).

Once the 6-well plate with the lawn of A549 cells is grown to about 90%confluency the media is exchanged with 2 mL Leibovitz CO_2-supplemented media (L-15 Medium, Sigma-Aldrich) with 10% FBS only, without antibiotics, before adding bacteria to the media. Following it, 100 µL of bacteria at an OD_{600} of 0.1 is added to the media and grown incubated at 37ºC for one hour. After the hour, either a 100 µL of a particular SM2 buffer solution supplemented with P4-EKORhE transducing particles harbouring the multi-lysins cassette at an MOI of 10 or a 100 µL of a blank solution of SM2 buffer (i.e. for MOI = 0) is added to each well. Further incubation for one hour is effectuated before removing the media and starting the following immunohistochemistry protocol.

For the immunohistochemistry protocol, which is adapted from the literature[21], the liquid phase of the cell cultures grown in the 6-well plate is removed by aspiration. The 6-well plate is placed under the fume hood, and the cells are fixed by adding 500 µL of a solution of 4 % PFA (i.e. paraformaldehyde) before placing the plate on ice for 15 minutes. The liquid is again removed from the plate and its wells are washed 3 times with PBS for 5 minutes each time. The cells are then permeabilized with 500 µL of ice-cold PEM/0.05 % saponin (i.e. PEM Buffer: 0.1M PIPES/1,4-Piperazinediethanesulfonic acid,

Piperazine-1,4-bis(2-ethanesulfonic acid), Piperazine-N,N-bis(2-ethanesulfonic acid), 5 mM EGTA, 2mM Magnesium chloride. Brought to pH 6.8, using a NaOH solution) and later aspirated and washed three times with PBS for 5 minutes. The remains on each well are aspirated before being incubated for 15 minutes, at room temperature, with 500µL of a 50 mM solution of ammonium chloride. The wells containing the coverslip slides with the cells upon are then aspirated and washed one time, for 5 minutes, with PBS/0.05 % saponin. Subsequently, 40 µL of a mixture that contains 300 µL of 0.05% saponin, 7.5 µL of phalloidin and 1.5 µL of a GFP enhancer is added on each of the six wells of the plate and incubated for 45 minutes in dark conditions. The wells are then washed twice, for 5 minutes, with PBS/0.05% saponin and once with PBS. After aspiration, a drip of Fluoroschield (Fluoroshield™ with DAPI, Sigma-Aldrich) on each coverslip glass slide on each well of the 6-well plate. The coverslips are then delicately rinsed with water before being put on microscope slides. The microscope slides with the coverslips are then let dry in dark conditions and after sealing with CoverGrip (CoverGrip™, Biotium) sealant these are stored at 4ºC until microscopy imaging.

For the microscopy (ZEISS Celldiscoverer 7 - Automated Live Cell Imaging), images are taken at random and those images that can count more than 60 nuclei per image are taken to ensure the homogeneity of the samples from which imaging is performed. Following the obtaining of such images, bacteria are counted in each image respective to the number of nuclei (i.e. blue nuclei stained with DAPI) together with bacteria expressing GFP (i.e. green dots stained with GFP enhancer).

MM6. Upstream optimization and downstream processing with P4-EKORhE. For the production of P4-EKORhE-based transducing particles harbouring the multi-lysins cassette, several protocols have been tested which have led to different results. The outputs of such protocols are shown in Figure S3.

The standard protocol would be producing particles by growing an overnight (O/N) culture of *Escherichia coli* Δcos:TriR-P2-c5545, that has been transformed with a P4-EKORhE-multi-lysins cosmid, in a culture media of Luria-Bertrani (LB) with 50 µg/mL kanamycin (to select for the selection marker in the P4-EKORhE) and 10 µg/mL trimethoprim (to select for the Δcos:TriR cassette in the c5545 strain harbouring the P2 prophage) at 37ºC. Then, a 10% (v/v) of the O/N culture can be combined with LB media using 50 µg/mL kanamycin and 10 µg/mL trimethoprim. The culture is shaken for 3-4 hours at 150 rpm and 37°C grown until grown to exponential phase (OD_{600} to 0.2 - 0.5). During the exponential phase, the epsilon (ε) gene within the P4-EKORhE cosmid is activated by adding 0.2% (w/v) rhamnose to the media and further incubated at 150 rpm and 37°C for 3 hours until lysis is observed. The debris from the lysate is pelleted at 4000 G for 30 minutes. The lysate is then filter-sterilized, with the option to store the filter-sterilized lysates at 4°C if desired.

After the lysate is obtained, a PEG8000 concentration protocol (Protocol SS7.2), or an ultrafiltration protocol[43], is effectuated in order to purify and concentrate the lysate samples with the conditionally replicating transducing particles. When using the PEG8000 protocol, the yields would not improve over 10^6 transducing particles per millilitre (i.e. in Figure S3 measured as pfu/mL). An alternative strategy using ultrafiltration is shown to improve the yields to up to 10^{10} particles per millilitre (i.e. as it can be seen in the replica "P4-EKORhE, x1 LB broth + ultrafiltration" shown in Figure S3.

Fortunately, even higher yields of P4-EKORhE transducing particles can be obtained when using Terrific Broth (TB) as a culturing media, and using a 4-step enrichment technique, followed by ultrafiltration (as depicted in Figure 8), with observed yields of up to 10^{12} particles per millilitre, as it is seen in the 3 replicas on the top row of the spot assay shown in Figure 6.

The protocol, using the showcased enrichment technique, is characteristic of P4-EKORhE because its capacity to be repressed and activated (i.e. a controllable P4-EKORhE system). This characteristic allows for the multiplication of the Δcos:TriR-P2-c5545 Marionette Z1 *Escherichia coli* lysogenic bacteria in a *fed-batch* fashion while maintaining a "dormant cosmid" before its activation with 0.2% (w/v) rhamnose. A high concentration of producing bacteria harbouring the P4-EKORhE is thus possible, and the later activation of the P4-EKORhE rends the highest yields obtained throughout this work.

In this procedure, an O/N culture of Δcos:TriR-P2-c5545 E. coli lysogens, previously transformed with the P4-EKORhE cosmid, is grown in Luria-Bertani (LB) medium using 50 µg/mL kanamycin (i.e. to select for the selection marker in the P4-EKORhE) and 10 µg/mL trimethoprim (i.e. to select for the Δcos:TriR cassette in the c5545 strain harbouring the P2 prophage). Subsequently, a 10% (v/v) of the aforementioned overnight culture is combined with fresh LB media (with kanamycin and trimethoprim) and shaken for 2 hours at 150 rpm and 37°C until the culture is recalled at exponential growth phase (OD_{600} = 0.5). Concurrently, another 4% (v/v) portion of the overnight culture, pre-mixed with the necessary amount of $CaCl_2$ to make a final solution of 0.1 M in a flask containing a volume of Terrific Broth (TB) media, including kanamycin and trimethoprim at the aforementioned concentrations, and shaken for 3-4 hours at 150 rpm and 37°C, reaching the exponential growth phase. Pre-mixture of cells with $CaCl_2$ is essential to avoid the precipitation of phosphate salts in the TB broth used for the culture. After reaching the exponential growth phase, 0.2% (w/v) rhamnose is added to the TB culture to induce the production of the epsilon (ε) gene, to

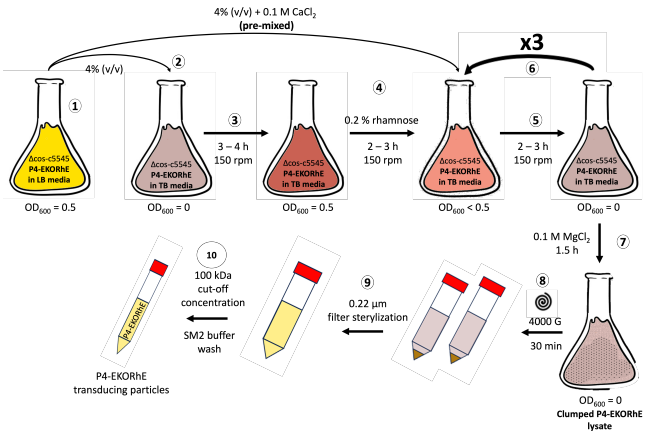

Fig. 8. High yield P4-EKORhE based transducing particle production protocol. Following steps from **(1)** to **(10)**: **(1)** A flask containing a Δcos:TriR-P2-c5545 Z1 culture maintaining the P4-EKORhE cosmid is grown in LB (Luria-Bertrani) media until reaching exponential phase (i.e. OD_{600} = 0.5). **(2)** A 4% (v/v) of the exponentially growing LB culture is transferred into a flask containing TB (Terrific Broth) media. **(3)** The TB culture is grown to an exponential phase. **(4)** The TB culture is supplemented with 0.2% (w/v) rhamnose for the activation of the P2 prophage to enable the production of P4 particles, and a pre-mixed solution of exponentially growing cells with $CaCl_2$ is prepared to reach a concentration of 0.1 M. **(5)** The TB culture is grown for 2 - 3 hours to accumulate lysed cells containing a proportion transducing particles. **(6)** The TB culture is re-supplemented at a 4% (v/v) with the initial exponentially growing LB culture of Δcos:TriR-P2-c5545 Z1 and re-balanced to maintain 0.1 M $CaCl_2$ and 0.2% (w/v) rhamnose for as many cycles as necessary (i.e. from **5** to **6**). **(7)** The TB culture is supplemented with 0.1 M $MgCl_2$ and further grown for 1.5 hours or until clumps are observed. **(8)** The clumped lysate is pelleted at 4000 G for 30 min to separate cell debris. **(9)** The supernatant is filter-sterylyzed. **(10)** The lysate is concentrated using a 100kDa molecular-weight cut-off ultrafiltration column and replaced with SM2 buffer to obtain a cleaned-up final lysate of P4-EKORhE transducing particles.

activate the Δcos:TriR-P2 prophage. After 2 hours of lysis in the TB culture, a 4% (v/v) sample of bacteria from the LB culture is introduced into the lysing TB-grown culture, again pre-mixing it with the necessary amount of $CaCl_2$ to maintain a final solution of 0.1 M in the growing TB culture, and compensating the rhamnose to maintain the 0.2% (w/v) proportion. Concurrently, a new exponentially growing LB culture is prepared by adding 10% (v/v) of the overnight culture, which is shaken in parallel for a further 2 hours at 150 rpm and 37°C until it reaches the recall stage at the exponential growth phase. This growing LB culture is also introduced into the ongoing lysis process of the TB culture, after 2 hours, pre-mixed with the necessary amount of $CaCl_2$ to make a final solution of 0.1 M, while maintaining the proportion to a final 0.2% (w/v) rhamnose. This re-lysing procedure is repeated as many times as necessary (e.g. 0 to 3), ideally up to three additional times, to maximize the yield of transducing particles obtained. The lysis procedure is finished by adding 0.1 M $MgCl_2$ and the culture is shaken at 37 ºC for a further 1.5 hours until visible clumps are observed. Afterwards, the debris from the lysate is pelleted at 4000 G for 30 minutes, followed by filter-sterilization of the lysate with 0.22 µm sterile syringe filters. After filtration, the lysate can be paused and stored at 4°C if desired, until further use. The lysate can then be ultrafiltrated using the Amicon 100 kDa cut-off columns by centrifuging at 3000 G. The recovered, the non-eluted, residue contains the highly concentrated P4-EKORhE based transducing particles.

Before titering, the lysate is pre-treated with DNAseI from NEB using 10µL lysate + 10µL DNAseI buffer + 1µL DNAseI + 79 µL of water, incubated for one hour. Because P4 bacteriophages are conditionally replicating, lysates are titered using a spot assay (Supplementary Section SS7.4.1), on lawned plates of Δcos:TriR-P2-c5545 Marionette Z1 *Escherichia coli* lysogenic bacteria with 5 mM Ca^{++}.

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DECLARATION OF CONFLICT OF INTEREST

R.R.G. is the founder of ACGTx (formerly Genova Tx). The other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

R.R.G. conceived the project, executed experiments, prepared and revised the manuscript. R.R.G. created the bioengineered P4-EKORhE system via gene assembly. R.R.G. designed plasmids and genetic circuits for the creation of P4-EKORhE. R.R.G. modified, characterized the antimicrobial effectiveness of the multi-lysins cassette and repurposed it as a gene-delivered antimicrobial. R.R.G. performed optical density measurements, immunohistochemistry and fluorescence microscopy imaging. Phage K1F-GFP, host strain E. coli EV36, and reagents for immunohistochemistry and microscopy were provided by A.P.S. Manuscript was revised by J.J.B.

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