

A Blueprint for Broadly Effective Bacteriophage-Antibiotic Cocktails Against Bacterial Infections

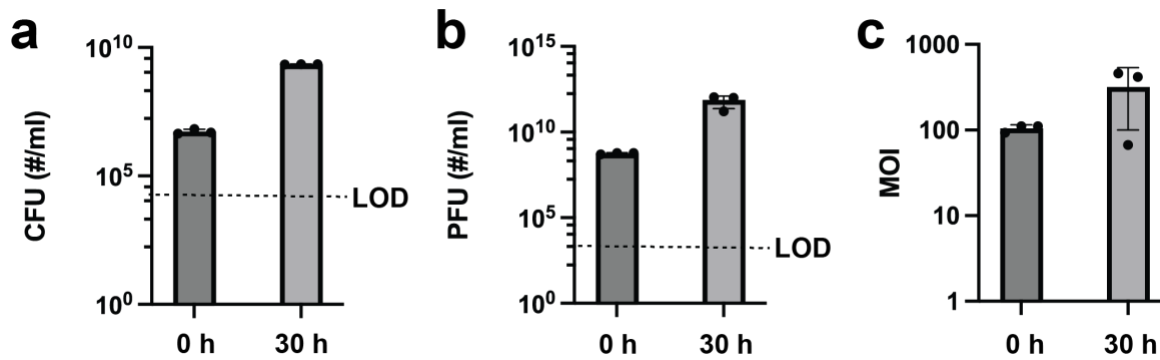
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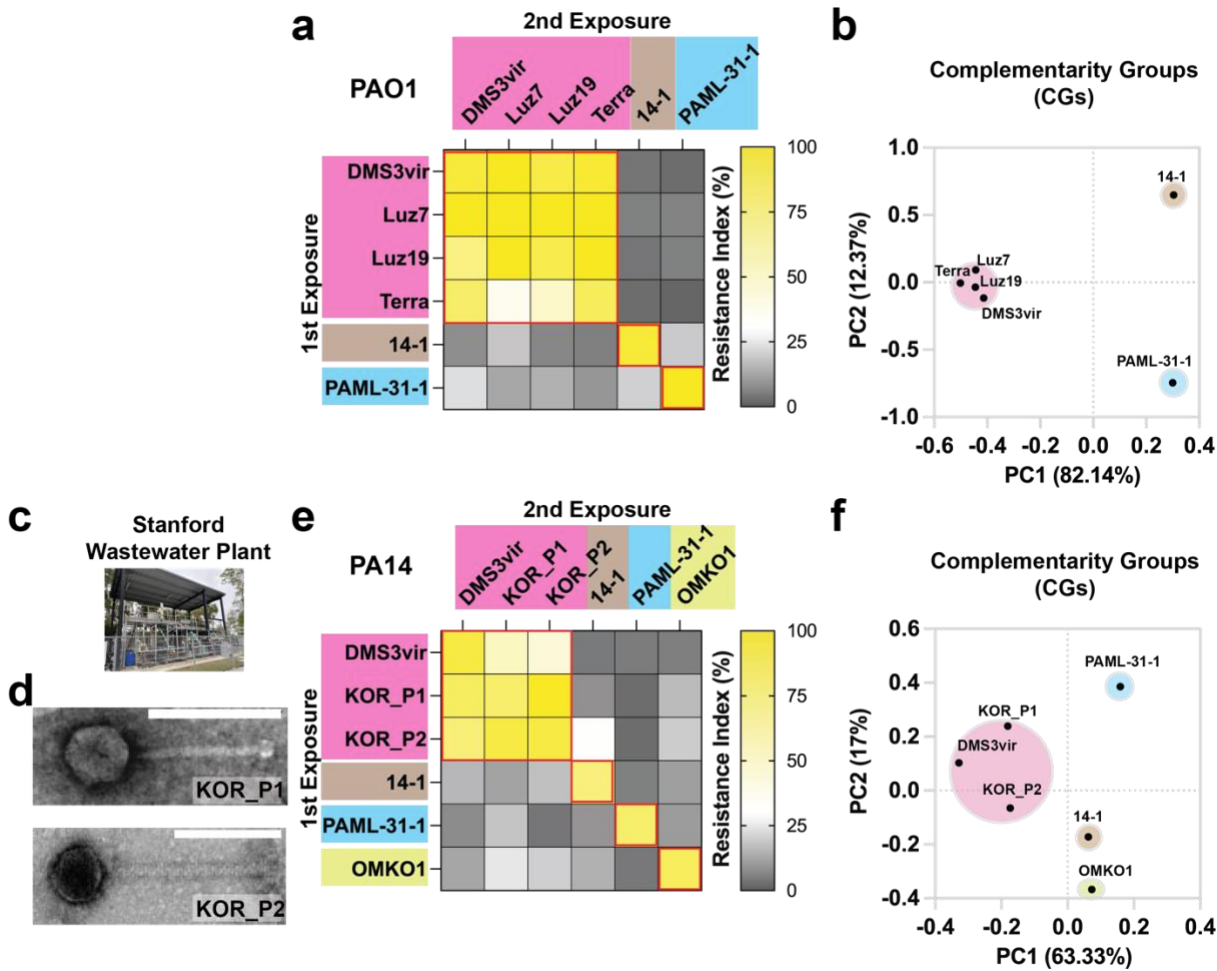
Supplementary Figures S1-S6

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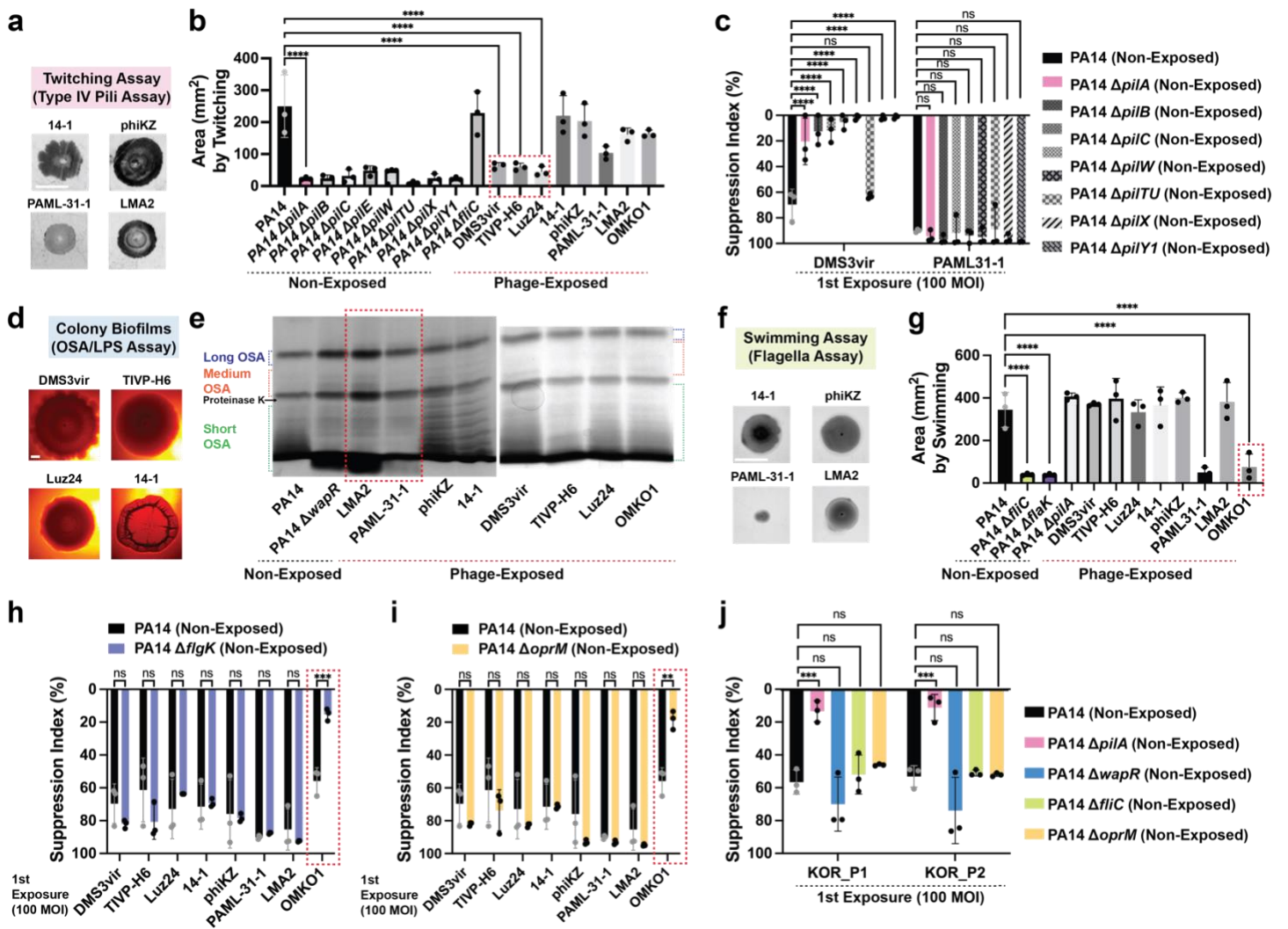
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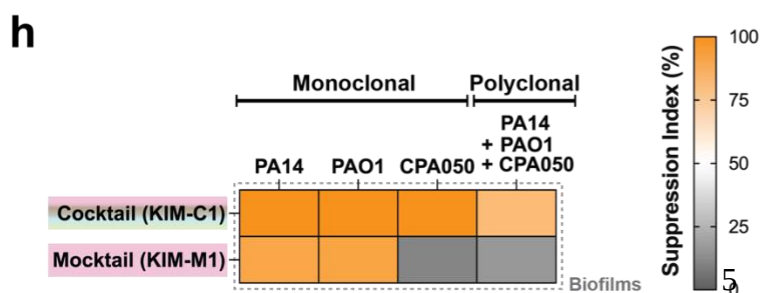
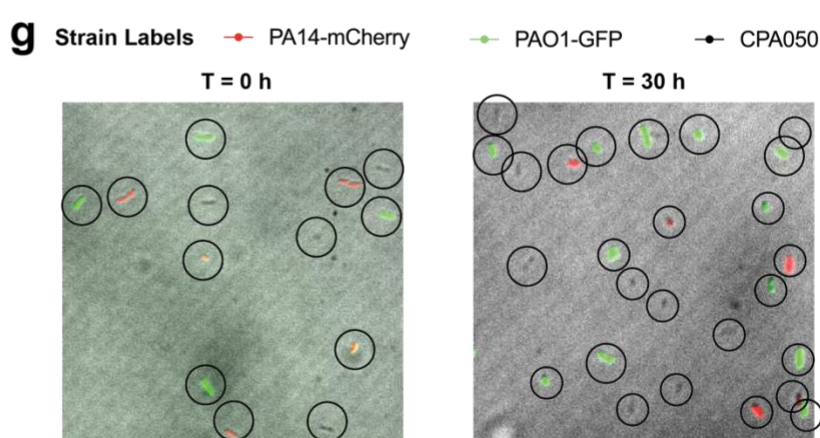
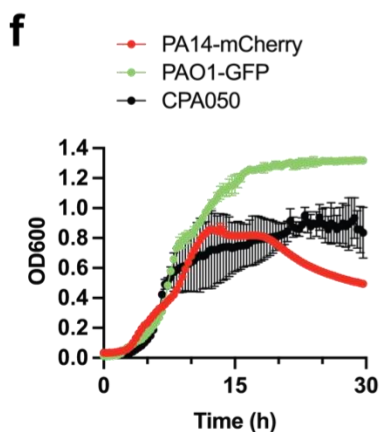
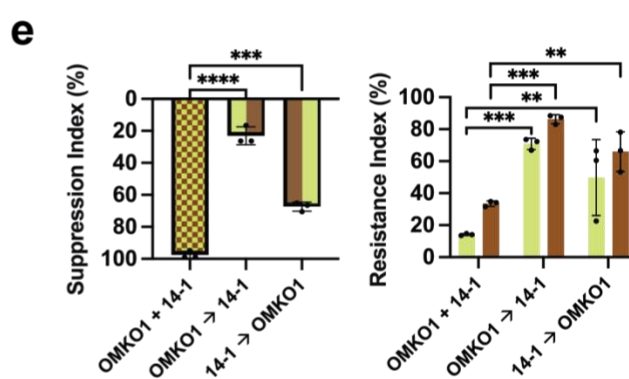
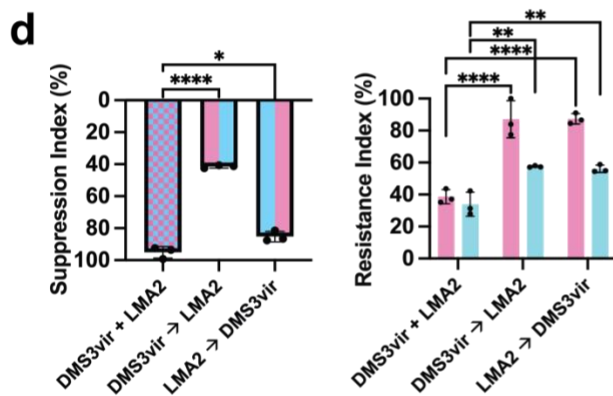
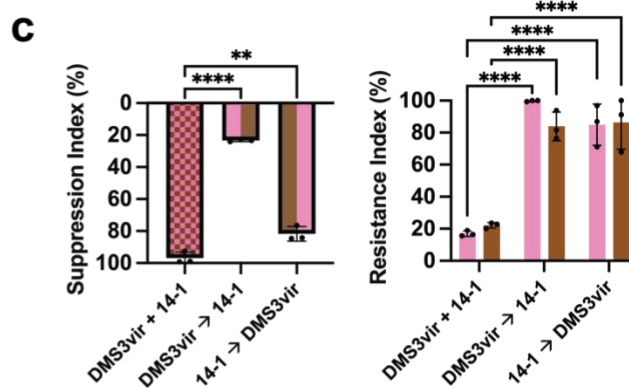
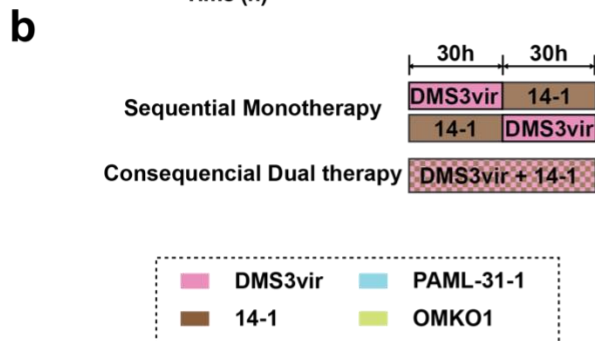
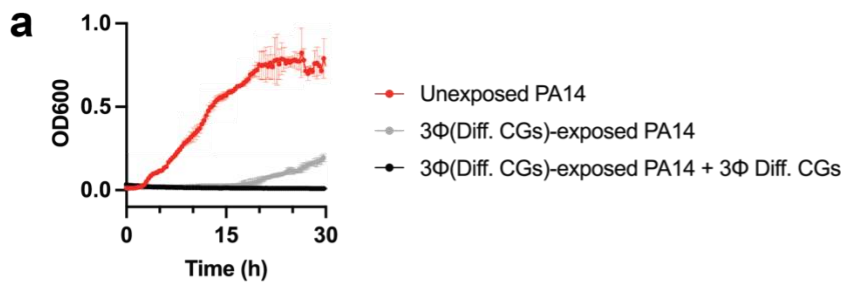
Supplementary Figure 1 | Repeated exposure to lytic bacteriophages leads *Pseudomonas aeruginosa* to develop constitutive, high-level resistance to those phages. **a**, colony-forming unit (CFU) of the wild-type *P. aeruginosa* strain PA14 in the presence of DMS3vir phage at 0 and 30 h. **b**, plaque-forming unit (PFU) of DMS3vir phage at 0 h and 30 h. **c**, MOI of DMS3vir phage was measured at 0 and 30 h. The number of replicates in each condition is three ($n = 3$). Error bars in these figures denote standard deviations from triplicate independent experiments. LOD indicates the limit of detection. Source data are provided as a Source Data file.



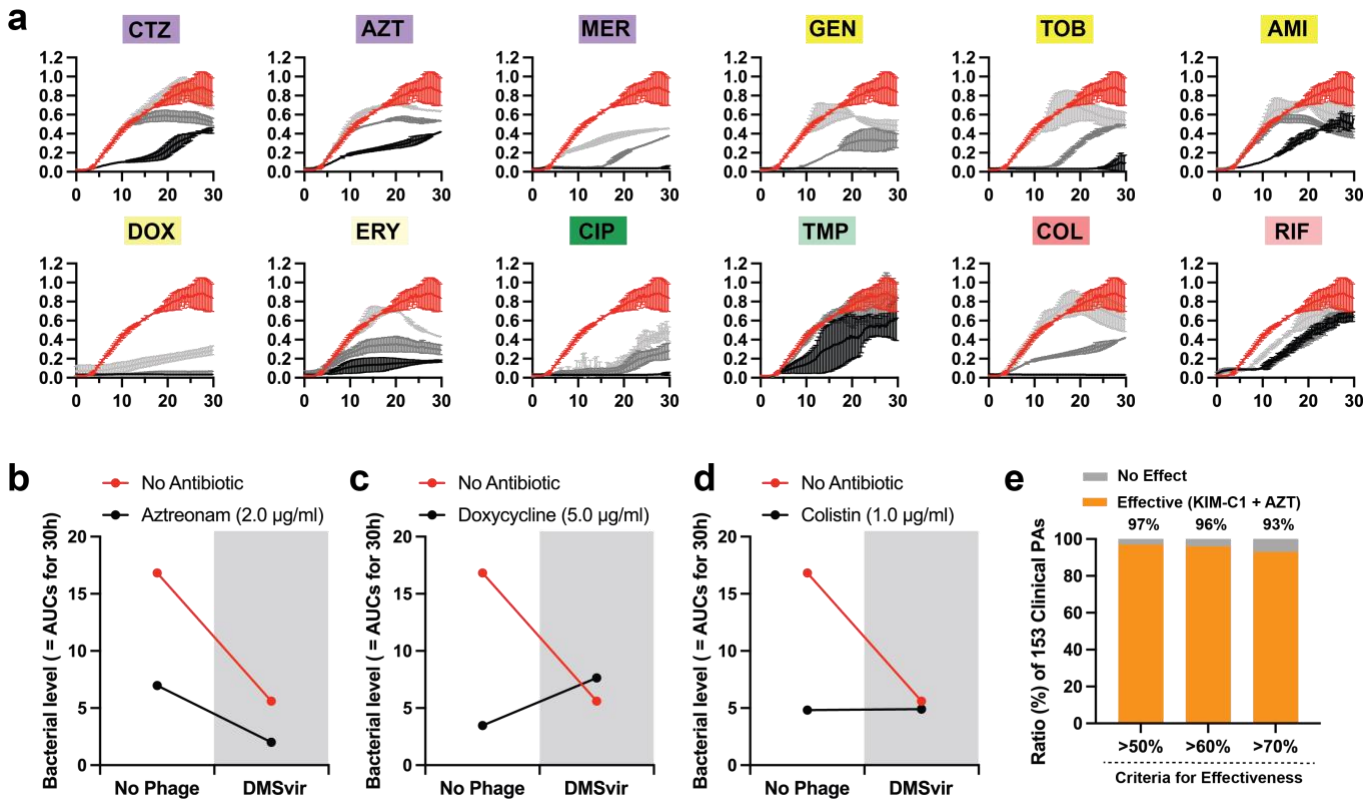
Supplementary Figure 2 | The same CGs observed for *P. aeruginosa* strain PA14 can be observed for *P. aeruginosa* strain PAO1 and also accommodate novel phages. a, Resistance Index matrix for phages targeting *P. aeruginosa* strain PAO1 demonstrating the presence of the same complementarity groups seen for PA14. PAO1 was initially exposed to one of a set of six phages and then subsequently “re-exposed” to each of the same set of six phages, all with an MOI of 100. Data for the Resistance Indices in the chart denote the means from triplicate independent experiments ($n = 3$). **b**, PCA analysis of the chart description of **2a**. **c**, The Stanford Wastewater Plant from which we isolated two “previously uncharacterized” phages. **d**, Transmission electron microscopy (TEM) images of the two phages called KOR_P1 (top) and KOR_P2 (bottom) isolated at the plant. Images are based on triplicate independent replicates. Scale bars, 100 μm . **e**, Resistance Index matrix for the four major complementarity groups including phages KOR_P1 and KOR_P2. Each row shows the Resistance Index from each designated phage-exposed PA14 that was re-challenged by six different phages with MOI = 100. Data represent the means from triplicate independent experiments ($n = 3$). **f**, PCA analysis of the chart description of **2e**. Source data are provided as a Source Data file.



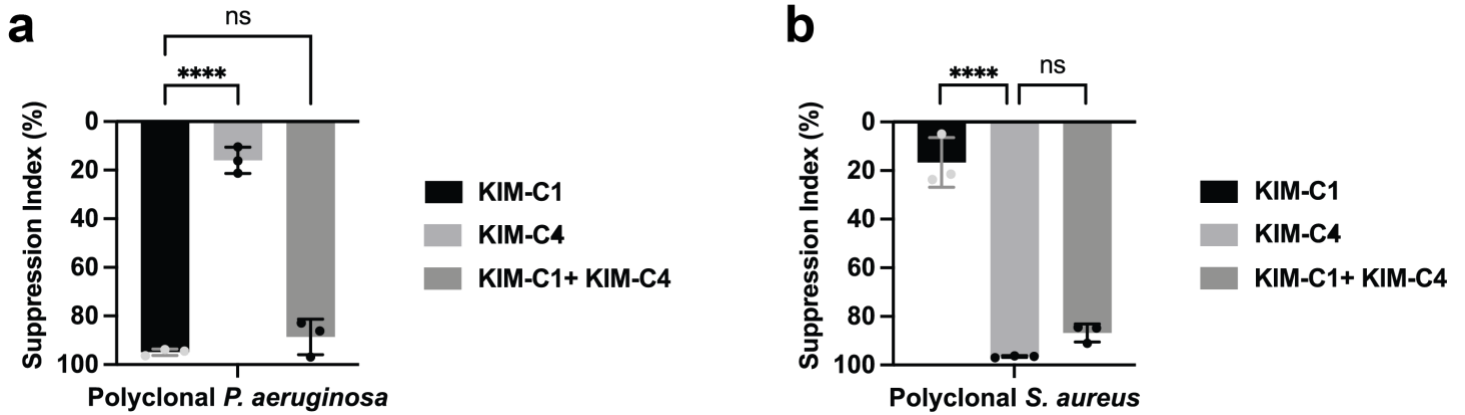
Supplementary Figure 3 | CGs of phages target the same *P. aeruginosa* receptor structures. **a**, Representative images of twitching motility assay by 14-1-, phiKZ-, PAML-31-1-, and LMA2-exposed *P. aeruginosa* strain PA14. **b**, Surface area (mm²) covered by twitching motility of wild-type, the designated mutants, and phage-exposed PA14 on 2.0% agar plates. **c**, Suppression Index measured for PA14 and PA14 Type IV mutants (PA14 $\Delta pilA$, PA14 $\Delta pilB$, PA14 $\Delta pilC$, PA14 $\Delta pilW$, PA14 $\Delta pilTU$, PA14 $\Delta pilX$, and PA14 $\Delta pilY1$) by DMS3vir and PAML31-1, each at MOI = 100. **d**, Colony biofilm phenotypes of the DMS3vir-, TIVP-H6-, Luz24-, and 14-1-exposed PA14 strains on Congo red agar medium after 100 h of growth (scale bar = 2 mm). **e**, Characterization of the phenotype of oligosaccharide antigen (OSA)-containing LPS by silver-stained SDS-PAGE gel for PA14, PA14 $\Delta wapR$, and the designated phage-exposed PA14. Images are based on triplicate independent replicates (n = 3). **f**, Representative images of swimming motility assay by 14-1-, phiKZ-, PAML-31-1-, and LMA2-exposed *P. aeruginosa* strain PA14. **g**, Surface area (mm²) covered by swimming motility of wild-type, the designated mutants, and phage-exposed *P. aeruginosa* on 0.3 % agar plates. **h**, Suppression Index was measured for PA14 (black) and the PA14 flagella mutants, PA14 $\Delta flaK$ (purple) treated with eight different phages, each with MOI = 100. **i**, Suppression Index was measured for PA14 (black) and the PA14 $\Delta oprM$ (yellow) by eight different types of phages, each with MOI = 100. **j**, Suppression Index of KOR_P1 and KOR_P2, each at MOI = 100, measured following treatment of PA14, PA14 $\Delta pilA$, PA14 $\Delta wapR$, PA14 $\Delta flhC$, and PA14 $\Delta oprM$. Images are based on a minimum of triplicate independent replicates. Scale bars = 10 mm. All reported values for panels 3b, 3c, 3g, 3h, 3i, and 3j are from three independent experiments with n = 3 and associated error bars represent standard deviation from the mean. Statistical significance in panels 3b and 3g is derived from One-way analysis of variance (ANOVA), while in panels 3c, 3h, 3i, and 3j is derived from Two-way ANOVA, and significance levels include **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Source data are provided as a Source Data file.



Supplementary Figure 4 | Cocktails containing phages from different CGs effectively kill bacteria without the emergence of phage resistance. **a**, Growth curves for the wild-type *P. aeruginosa* strain PA14 (red) and PA14 previously exposed with a cocktail of three phages with different CGs (Luz24+OMKO1+PAML-31-1) in the absence (gray) or presence (black) of the identical phage cocktails upon 2nd exposure. Remarkably, the secondary challenge with the three-phage cocktail effectively suppressed bacterial growth, highlighting the potential of phage cocktails in suppressing the development of phage resistance. Error bars in this figure denote standard deviations from triplicate independent experiments ($n = 3$). **b**, Schematic of sequential monotherapy versus consecutive dual therapy over 60 hours. For sequential monotherapy, treatment is initially with one phage (DMS3vir, pink) with MOI = 100 followed by a phage from a different CG (14-1, brown) with MOI = 100 after 30 hours. For consecutive dual therapy, two phages from different CG (DMS3vir + 14-1) each at MOI = 100 are added at both 0 and 30 hours. **c**, Suppression Index for consecutive dual therapy (DMS3vir, 14-1) versus two sequential monotherapies. Resistance Index from PA14 that was isolated after 60 hours of exposure and subsequently re-challenged with each constituent of the cocktail pattern as shown, each with MOI = 100. **d**, Suppression Index for consecutive dual therapy (DMS3vir, LMA2) versus two sequential monotherapies. Resistance Index from PA14 that was isolated after 60 hours of exposure and subsequently re-challenged with each constituent of the cocktail pattern as shown, each with MOI = 100. **e**, Suppression Index for consecutive dual therapy (OMKO1, 14-1) versus two sequential monotherapies. Resistance Index from PA14 that was isolated after 60 hours of exposure and subsequently re-challenged with each constituent of the cocktail pattern as shown, each with MOI = 100. All reported values for panels **4c**, **4d**, and **4e** are the mean of three independent experiments with $n = 3$ and associated error bars represent standard deviation from the mean. Statistical significance in panels **4c**, **4d**, and **4e** is derived from Two-way analysis of variance (ANOVA) and significance levels include $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. **f**, Growth curves for PA14-mCherry, PAO1-GFP, and CPA050. Error bars in this figure denote standard deviations from triplicate independent experiments. **g**, Representative images from triplicate experiments of individually discernable, fluorescently labeled cells (Red: PA14, Green: PAO1, and Black: CPA050), which were sampled from mixed cultures ($T = 0$ h, left and $T = 30$ h, right). **h**, Suppression Index for monoclonal and polyclonal *P. aeruginosa* biofilm cultures treated with the KIM-C1 cocktail (a mixture of three phages with different CGs: Luz24, OMKO1, and PAML-31-1) or the KIM-M1 mocktail (a mixture of three phages with same CG: DMS3vir, KOR_P1, and KOR_P2). Data are shown as an average of triplicate independent results. The phage dose used here is a combined MOI of 100. Source data are provided as a Source Data file.



Supplementary Figure 5 | Predictable, class-dependent interactions of conventional antibiotics with phage CGs. **a**, Growth curves (OD600) from wild-type PA14 grown under different sub-MICs of 12 conventional antibiotics used in these experiments: Red curves = no antibiotics in all growth curves. Error bars in this figure denote standard deviations from triplicate independent experiments (n = 3). Ceftazidime (CTZ) responses. Black, dark grey, and grey = 1, 0.5, 0.25 µg/ml CTZ, respectively. Aztreonam (AZT) responses. Black, dark grey, and grey = 2, 1, 0.5 µg/ml AZT, respectively. Meropenem (MER) responses. Black, dark grey, and grey = 0.5, 0.25, 0.125 µg/ml MER, respectively. Gentamycin (GEN) responses. Black, dark grey, and grey = 2.0, 1.0, 0.5 µg/ml GEN, respectively. Tobramycin (TOB) responses. Black, dark grey, and grey = 0.5, 0.25, 0.125 µg/ml TOB, respectively. Amikacin (AMI) responses. Black, dark grey, and grey = 1.5, 0.5, 0.25 µg/ml AMI, respectively. Doxycycline (DOX) responses. Black, dark grey, and grey = 20.0, 10.0, 5.0 µg/ml DOX, respectively. Erythromycin (ERY) responses. Black, dark grey, and grey = 40.0, 20.0, 10.0 µg/ml ERY, respectively. Ciprofloxacin (CIP) responses. Black = 2.0, 1.0, and 0.5 µg/ml CIP, respectively. Trimethoprim (TMP) responses. Black = 200, 100 and 50 µg/ml TMP, respectively. Colistin (COL) responses. Black = 2.0, 1.0, and 0.5 µg/ml COL, respectively. Rifampin (RIF) responses. Black = 2.0, 1.0, and 0.5 mg/ml RIF. **(b, c, d)**, Representative synograms showing the impact on PA14 bacterial levels (defined as the AUCs of OD600 for a 30-hour period) in the presence (black) or absence (red) of sub-MIC antibiotics, with (grey zone) or without (white zone) DMS3vir phage at MOI = 10. Data are presented for **(b)** synergistic, **(c)** antagonistic, and **(d)** non-additive (or independent) phage-antibiotic interactions. The scale of bacterial level is expressed in arbitrary unit (a.u.). **e**, The KIM-C1 phage cocktail plus aztreonam mediated suppression at different thresholds of efficacy (>50, >60, and >70% suppression). Source data are provided as a Source Data file.



Supplementary Figure 6 | Cocktails containing phages for multiple species demonstrate efficacy in the treatment of polymicrobial infections. (a, b), Suppression Index was determined for **a**, polyclonal *P. aeruginosa* clinical isolates (CPA077, CPA086, and CPA149) and **b**, polyclonal *S. aureus* clinical isolates (CSA007, CSA009, and CSA010), which are treated individually with the *P. aeruginosa*-specific cocktail (KIM-C1) that includes phages Luz24, PAML-31-1 and OMKO1, or the *S. aureus*-specific cocktail (KIM-C4) that includes phage vFB433 and vFB009, as well as a combined treatment with both cocktails administered simultaneously. All reported values for panels **6a** and **6b** are from three independent experiments with $n = 3$ and associated error bars represent standard deviation from the mean. Statistical significance in panels **6a** and **6b** is derived from One-way analysis of variance (ANOVA) and significance levels include $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Source data are provided as a Source Data file.

Supplementary Table 1 | A single *P. aeruginosa* PA14 strain with receptor mutation(s) conferring phage-resistance.

Phage Treatment	Gene(s)	Role of Gene(s)	Mutation Type	Amino acid Change
DMS3vir	<i>pilT</i>	Twitching motility	Frame Shift	-
TIVP-H6	<i>pilN</i>	Twitching motility	Substitution	61: F → I
			Substitution	58: Q → V
			Substitution	56: Q → L
			Substitution	50: RK → QF
			Substitution	47: Q → L
			Substitution	43: E → K
Luz24	<i>pilF</i>	Twitching motility	Substitution	243: P → L
	<i>pilJ</i>		Substitution	315: V → A
	<i>pilK</i>		Substitution	140: A → V
	<i>pilU</i>		Substitution	362: N → S
PAML-31-1	<i>wapR</i>	Rhamnosyltransferases involved in LPS CPA and OSA biosynthesis	Substitution	85: A → T
	<i>wbpZ</i>	Rhamnosyltransferases involved in LPS CPA and OSA biosynthesis	Substitution	338: A → T
	<i>migA</i>	LPS core biosynthesis	Substitution	92: Q → R
LMA2	<i>wapR</i>	Rhamnosyltransferases involved in LPS CPA and OSA biosynthesis	Substitution	78: K → R
	<i>wbpZ</i>	Rhamnosyltransferases involved in LPS CPA and OSA biosynthesis	Substitution	181: A → T
	<i>migA</i>	LPS core biosynthesis	Substitution	92: Q → R
			Substitution	170: M → V
OMKO1	<i>flgL</i>	Swimming motility	Frame Shift	-

Supplementary Table 2 | Detailed information on the phage cocktails used shown in Fig. 4a, 4b, 4c, 4d, and 4e. Suppression Index measured for 1st Phage (cocktails). Resistance Index from PA14 that was isolated after 30 hours-exposure from the 1st Phage (cocktails) treatment, and subsequently re-challenged with each constituent of the cocktail pattern as indicated, each at combined MOIs of 100.

Groups	1st Phage Treatment (for Suppression Index shown in Fig 4A-ii)	2nd Phage Treatment (for Resistance Index shown in Fig 4A-iii)
1Φ	DMS3vir	DMS3vir
	TIVP-H6	TIVP-H6
	Luz24	Luz24
	14-1	14-1
	phiKZ	phiKZ
	PAML-31-1	PAML-31-1
	LMA2	LMA2
	OMKO1	OMKO1
2Φ Same CG	DMS3vir + TIVP-H6	DMS3vir
		TIVP-H6
	DMS3vir + Luz24	DMS3vir
		Luz24
	TIVP-H6 + Luz24	TIVP-H6
		Luz24
	14-1 + phiKZ	14-1
		phiKZ
2Φ Diff. CGs	DMS3vir + 14-1	PAML-31-1
		LMA2
	DMS3vir + LMA2	DMS3vir
		14-1
	DMS3vir + OMKO1	DMS3vir
		LMA2
	14-1 + PAML-31-1	DMS3vir
		OMKO1
	14-1 + OMKO1	14-1
		LMA2
	LMA2 + OMKO1	14-1
		OMKO1
3Φ Diff. CGs	DMS3vir + 14-1 + LMA2	LMA2
		DMS3vir
		14-1
	DMS3vir + 14-1 + OMKO1	OMKO1
		DMS3vir
		14-1
	DMS3vir + LMA2 + OMKO1	OMKO1
		LMA2
		DMS3vir
	14-1 + PAML-31-1 + OMKO1	OMKO1
		14-1
		PAML-31-1
	Luz24 + PAML-31-1 + OMKO1 (= Cocktail KIM-C1)	OMKO1
		Luz24
		PAML-31-1

Supplementary Table 3 | The minimum inhibitory concentration (MIC) value of antibiotics for the bacterial strains (*P. aeruginosa* strain PA14 and *S. aureus* strain 1203) used in this study.

Strains	Antibiotic	MIC
<i>P. aeruginosa</i> PA14	Ceftazidime (CTZ)	0.75 µg/ml
<i>P. aeruginosa</i> PA14	Aztreonam (AZT)	2.0 µg/ml
<i>P. aeruginosa</i> PA14	Meropenem (MER)	0.375 µg/ml
<i>P. aeruginosa</i> PA14	Gentamycin (GEN)	2.0 µg/ml
<i>P. aeruginosa</i> PA14	Tobramycin (TOB)	0.75 µg/ml
<i>P. aeruginosa</i> PA14	Amikacin (AMI)	3.0 µg/ml
<i>P. aeruginosa</i> PA14	Doxycycline (DOX)	12.0 µg/ml
<i>P. aeruginosa</i> PA14	Erythromycin (ERY)	32.0 µg/ml
<i>P. aeruginosa</i> PA14	Ciprofloxacin (CIP)	0.38 µg/ml
<i>P. aeruginosa</i> PA14	Trimethoprim (TMP)	0.3 mg/ml
<i>P. aeruginosa</i> PA14	Colistin (COL)	1.0 µg/ml
<i>P. aeruginosa</i> PA14	Rifampin (RIF)	3.0 mg/ml
<i>S. aureus</i> 1203	Vancomycin (VAN)	1.5 µg/ml
<i>S. aureus</i> 1203	Doxycycline (DOX)	0.094 µg/ml
<i>S. aureus</i> 1203	Erythromycin (ERY)	3.0 µg/ml
<i>S. aureus</i> 1203	Ampicillin (AMP)	0.19 µg/ml
<i>S. aureus</i> 1203	Trimethoprim (TMP)	1.5 µg/ml
<i>S. aureus</i> 1203	Rifampin (RIF)	0.32 µg/ml

Supplementary Table 4 | Bacterial strains used in this study.

Strains	Genotype/description	Sources
<i>P. aeruginosa</i>		
PA14	UCBPP-PA14m, a human clinical isolate	Gitai Lab
PA14- <i>gfp</i>	PA14 <i>attTn7::[PA1/04/03-gfp]</i>	Gitai Lab
PA14- <i>mCherry</i>	PA14 <i>attTn7::[PA1/04/03-mCherry]</i>	Gitai Lab
PA14 Δ <i>pilA</i>	PA14 Δ <i>pilA aacc1::FRT</i>	Gitai Lab
PA14 Δ <i>pilB</i>	PA14 Δ <i>pilB aacc1::FRT</i>	Gitai Lab
PA14 Δ <i>pilC</i>	PA14 <i>pilC::Tn5</i>	Gitai Lab
PA14 Δ <i>pilW</i>	PA14 Unmarked in-frame deletions of <i>pilW</i>	Gitai Lab
PA14 Δ <i>pilTU</i>	PA14 Δ <i>pilTU::FRT</i>	Gitai Lab
PA14 Δ <i>pilX</i>	PA14 Unmarked in-frame deletions of <i>pilX</i>	Gitai Lab
PA14 Δ <i>pilY1</i>	PA14 Δ <i>pilY1 aacc1::FRT</i>	Gitai Lab
PA14 Δ <i>fliC</i>	PA14 Δ <i>fliC aacc1::FRT</i>	Gitai Lab
PA14 Δ <i>flaK</i>	PA14 <i>flgK::Tn5</i>	Gitai Lab
PA14 Δ <i>oprM</i>	PA14 <i>oprM::MAR2xT7</i>	Hancock Lab
PA14 Δ <i>wapR</i>	PA14 <i>wapR::MAR2xT7</i>	Hancock Lab
PAO1	mPAO1, a wild type	Secor Lab
PAO1 Δ <i>pf4</i> Δ <i>pf6</i>	mPAO1 Δ <i>pf4</i> Δ <i>pf6</i>	Secor Lab
PAO1- <i>gfp</i>	mPAO1, pUCP- <i>gfp</i>	Secor Lab
<i>P. aeruginosa</i> Br257	<i>P. aeruginosa</i> Br257, an environmental isolate	Lavigne Lab
<i>P. aeruginosa</i> Li010	<i>P. aeruginosa</i> Li010, a hospital sewage sample	Lavigne Lab
<i>P. aeruginosa</i> GHB15	<i>P. aeruginosa</i> GHB15, an environmental isolate	Lavigne Lab
CPA001 to CPA153	153 human clinical isolates from Stanford Health Care	This study
<i>S. aureus</i>		
<i>S. aureus</i> 1203	<i>S. aureus</i> FB1203	Felix Biotechnology, Inc.
CSA001 to CSA015	15 human clinical isolates from Stanford Health Care	This study

Supplementary Table 5 | Bacteriophage strains used in this study.

Phage strains (a host)	Lineage/description	Sources
<i>P. aeruginosa</i> Lytic Phages		
DMS3vir (<i>P. aeruginosa</i> PA14)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Casadabanvirus; A lytic mutant of <i>P. aeruginosa</i> Phage DMS3	O'Toole Lab
Luz7 (<i>P. aeruginosa</i> Br257)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Schitoviridae; Migulavirinae; Luzseptimavirus;	Lavigne Lab
Luz19 (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Autographiviridae; Krylovirinae; Phikmvirus;	Lavigne Lab
Luz24 (<i>P. aeruginosa</i> Li010)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Bruynoghevirus	Lavigne Lab
LMA2 (<i>P. aeruginosa</i> GHB15)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Pbunavirus; Pbunavirus LMA2	Lavigne Lab
PAML-31-1 (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Pbunavirus; Pbunavirus SN >99% identical to <i>P. aeruginosa</i> Phage NP3	Felix Biotechnology, Inc.
Terra (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Unidentified.	Felix Biotechnology, Inc.
TIVP-H6 (<i>P. aeruginosa</i> PA14)	Unidentified.	Felix Biotechnology, Inc.
14-1 (<i>P. aeruginosa</i> Li010)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Pbunavirus; Pbunavirus pv141	Lavigne Lab
phiKZ (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Phikzvirus; Phikzvirus phiKZ	Lavigne Lab
OMKO1 (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Phikzvirus; unclassified Phikzvirus	Felix Biotechnology, Inc.
KOR_P1 (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Unidentified.	This study
KOR_P2 (<i>P. aeruginosa</i> PAO1 $\Delta pf4\Delta pf6$)	Unidentified.	This study
<i>S. aureus</i> Lytic Phages		
<i>S. aureus</i> bacteriophage K (<i>Sa</i> 1203)	Myoviridae	ATCC (ATCC 19685-B1)
vFB009 (<i>Sa</i> 1203)	Unidentified.	Felix Biotechnology, Inc.
vFB433 (<i>Sa</i> 1203)	Unidentified.	Felix Biotechnology, Inc.
vFB468 (<i>Sa</i> 1203)	Unidentified.	Felix Biotechnology, Inc.
Romulus (<i>Sa</i> 1203)	Twortlikevirus	Lavigne Lab