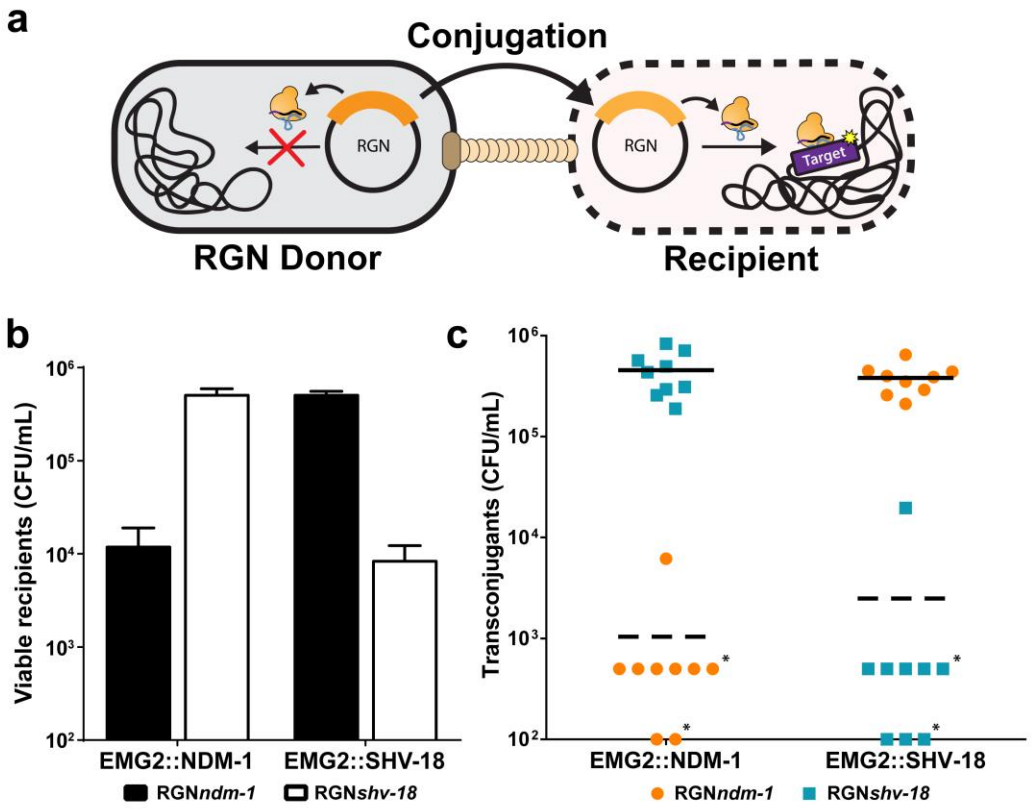
Supplementary Material for *RNA-Guided Nucleases as Programmable-Spectrum Antimicrobials and Microbial Population Sculptors*

Supplementary Discussion

Phage-based therapies are dependent on their ability to deliver nucleic acids into bacteria, which can be resisted through a variety of mechanisms[1]. These delivery vehicles can be limited to a subset of bacteria defined by the chosen phage, such that the design of programmable antimicrobials may require additional considerations as to the phage platform chosen. However, rational modification to phage host range through tail fiber alterations[2] or the use of bacteriophage cocktails[3] can mitigate the host range limitations of phage-based therapies. Although the use of bacteriophages in humans has been met with challenges[3], especially in the Western world, a renaissance in phage-based therapeutics has begun to address these challenges, such as demonstrating safety in humans[4], improving the persistence of phages remaining in circulation by reducing their clearance by the host[5] and minimizing endotoxin release by using non-lytic phage engineered with heterologous kill functions[6]. Additionally, we devised a complementary delivery strategy using a mobilizable broad-host-range system to introduce RGNs to recipient cells via conjugation. The use of conjugative delivery from probiotics into target bacteria would enable a platform where engineered cells could integrate complex environmental cues and execute lethal payload delivery, akin to previously described sentinel cells[7]. Future work is needed to improve the efficiency and spectra of delivery strategies for RGNs, which may include broad-host-range bacteriophages and more efficient conjugative strategies, as well as chemical delivery technologies.

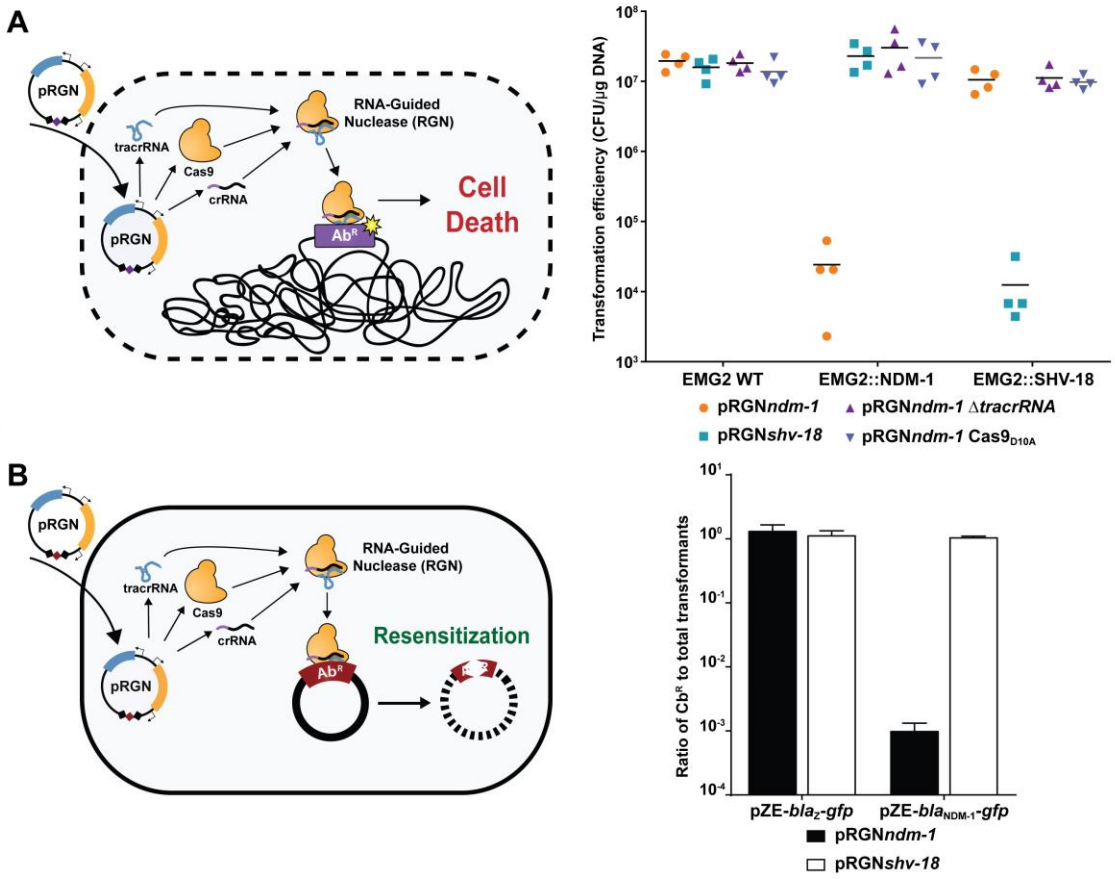
Supplementary Figures

Supplementary Figure 1



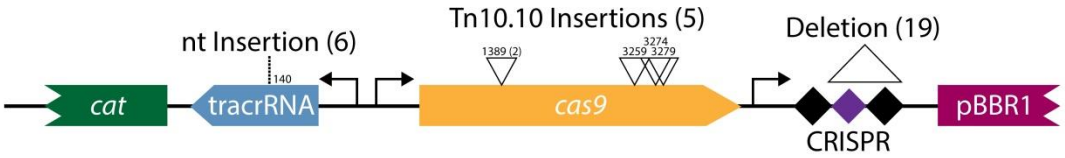
Supplementary Figure 1: Mobilizable RNA-guided nucleases (RGNs) can be conjugated into target bacteria for selective removal of multidrug resistance. (a) Schematic of mobilizable RGN-mediated cell killing. (b-c) S17-1 λpir donor cells possessing RGN*ndm-1* or RGN*shv-18* were mated at a donor:recipient ratio of 340±66:1 for 3 hours with EMG2 recipient cells that contain $bla_{\text{NDM-1}}$ (EMG2::NDM-1) or $bla_{\text{SHV-18}}$ (EMG2::SHV-18) integrated into the chromosome. Cultures were plated on LB + carbenicillin (Cb) to select for surviving recipient cells (b) and LB + chloramphenicol (Cm) + Cb to select for transconjugants (c) (Cm resistance is encoded by the RGN plasmids). (c) Transfer of a mobilizable RGN into cells containing the cognate target sequence (dashed line) reduced the number of viable transconjugants to the limit of detection (*) (100 CFU/mL or 500 CFU/mL for three or six of the biological replicates, respectively) in almost all cases. Error bars indicate s.e.m. of three independent experiments, each with three biological replicates (n=9).

Supplementary Figure 2



Supplementary Figure 2: Design and validation of programmable RGN constructs. **(A)** Plasmids pRGN*ndm-1*, pRGN*shv-18*, pRGN*ndm-1* Δ*tracrRNA*, and pRGN*ndm-1* $Cas9_{D10A}$ were transformed into competent wild-type EMG2 (EMG2 WT) as well as otherwise isogenic strains containing chromosomally integrated bla_{NDM-1} (EMG2::NDM-1) or bla_{SHV-18} (EMG2::SHV-18). Transformants were enumerated on LB+ chloramphenicol (Cm) to select for pRGN transformants and to determine transformation efficiencies, which demonstrated the specific incompatibility of an RGN construct and its cognate protospacer (n=4). **(B)** Plasmids pRGN*ndm-1* and pRGN*shv-18* were transformed into EMG2 cells containing either pZE-bla_{NDM-1}-*gfp* or pZE-bla_{Z}-*gfp* plasmids. Transformants, first selected in appropriate antibiotic media, were enumerated on LB+Cm or LB+Cm+carbenicillin (Cb) agar to calculate the ratio of transformants retaining Cb resistance (Cb^R) to total transformants. Error bars indicate s.e.m. of three independent experiments, each with three biological replicates (n=9).

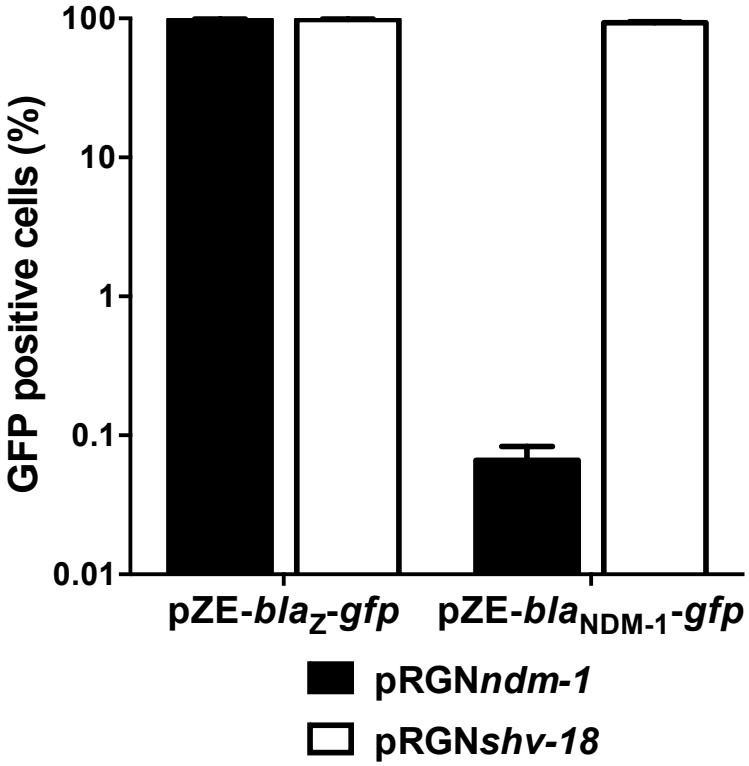
Supplementary Figure 3



Mutation	Number	%
Deletion of spacer and one repeat	19	63.3
tracrRNA Insertion (A at nt140/141)	6	20.0
Transposon Insertion (Tn10.10)	5	16.7

Supplementary Figure 3: Characterization of escape mutants that tolerated transformation of a cytotoxic RGN construct. EMG2::NDM-1 or EMG2::SHV-18 colonies that tolerated transformation of the pRGN*ndm-1* or pRGN*shv-18* plasmids (Supplementary Fig. 2b) were re-isolated and sequenced to identify escape mutations. Spacer deletion in the CRISPR locus, point mutations in *tracrRNA* and transposon insertions in *cas9* led to pRGN inactivation in successful transformants. Five escape mutants from three independent experiments were sequenced per strain (n=30).

Supplementary Figure 4



Supplementary Figure 4: Transformation of pRGN*ndm-1* leads to loss of a high-copy GFP-expressing plasmid as evidenced by flow cytometry. EMG2 cells containing either pZE-*bla*$_{Z}$-*gfp* or pZE-*bla*$_{NDM-1}$-*gfp* plasmids were transformed with pRGN*ndm-1* or pRGN*shv-18* plasmids and transformants were selected overnight in LB+chloramphenicol (no selection for *bla* was applied). Plasmid loss was determined by calculating the percentage of GFP-positive cells following gating by forward and side scatter. Error bars indicate s.e.m. of measurements from three independent experiments, each with three biological replicates (n=9).

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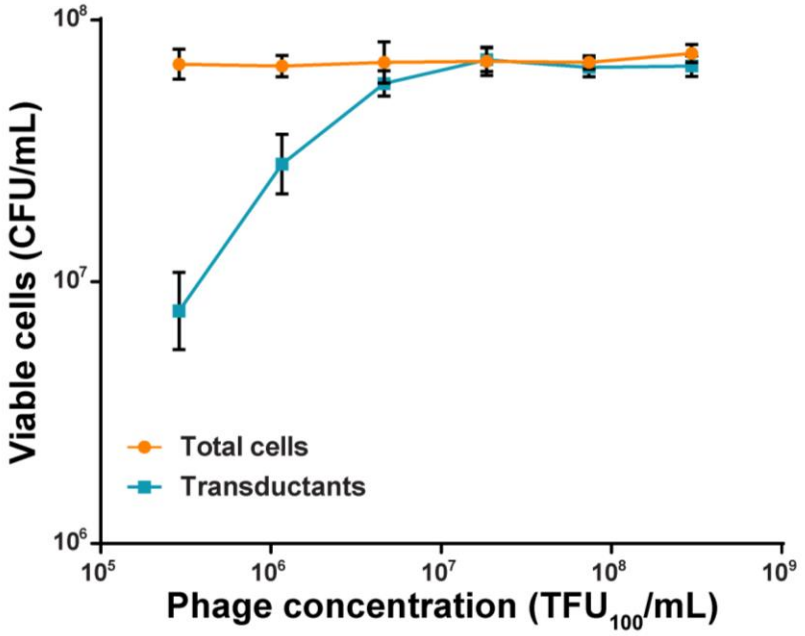
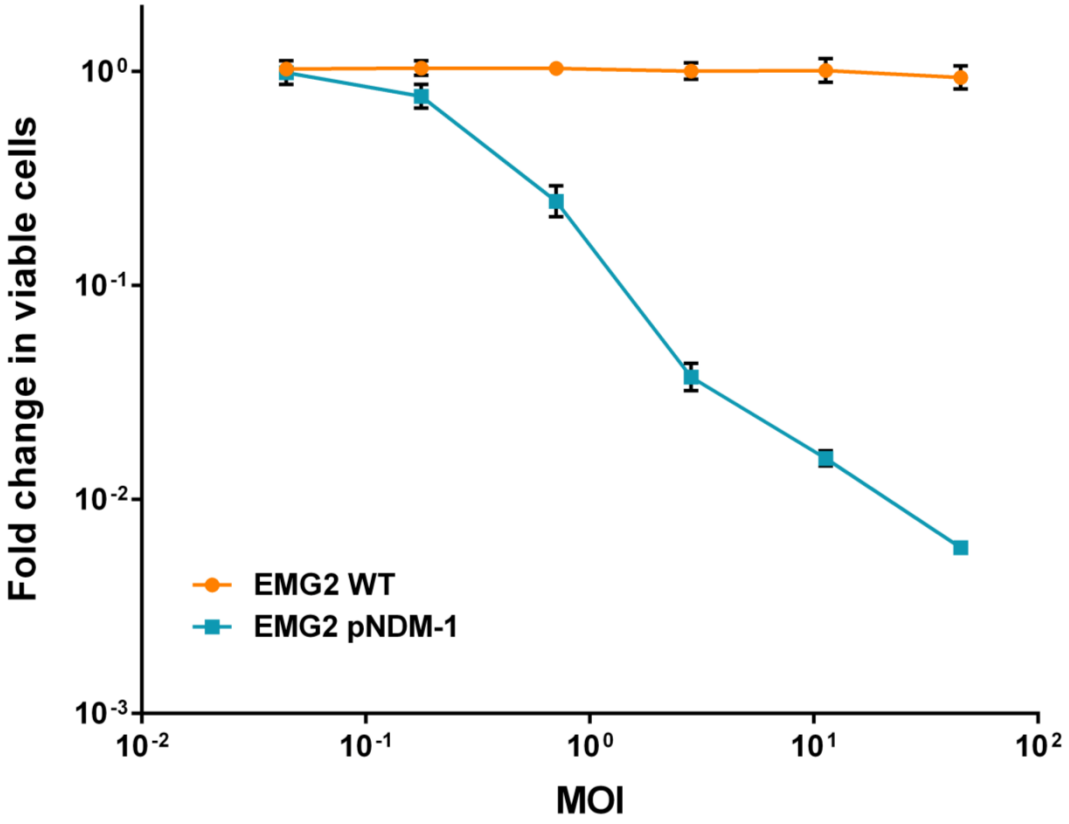
Supplementary Figure 5



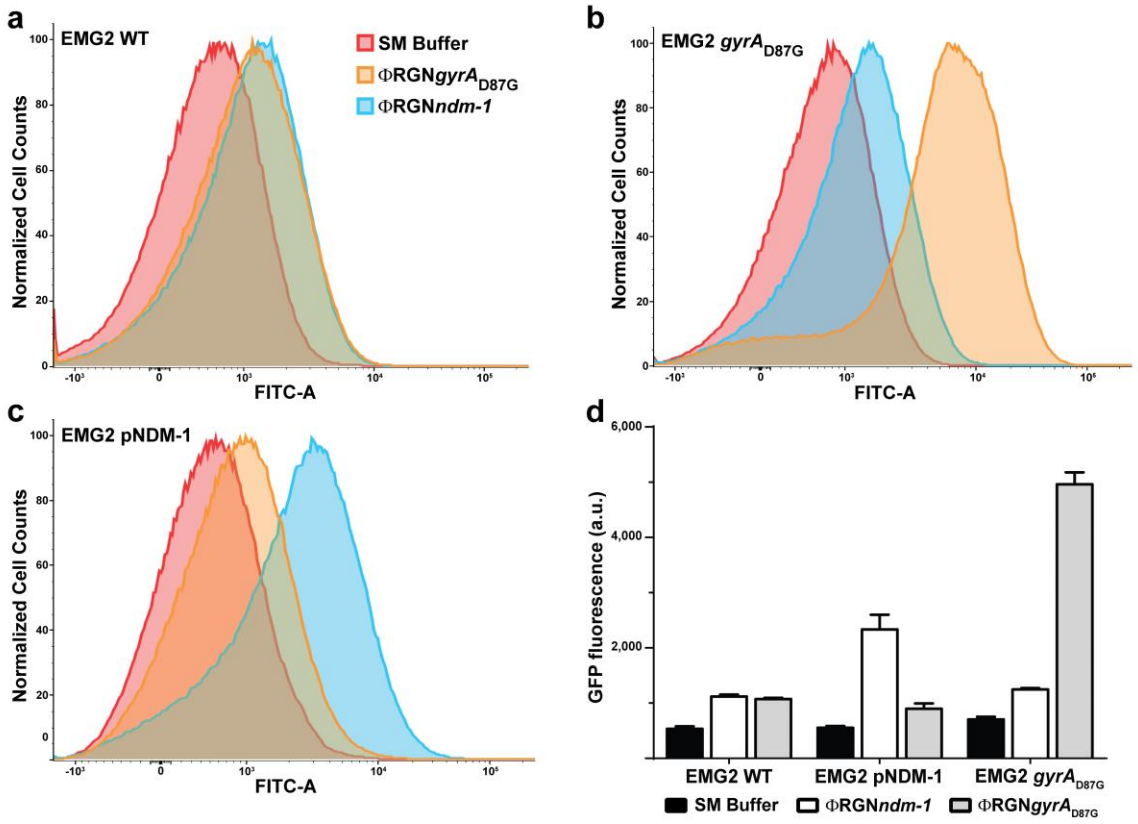
Supplementary Figure 5: Titrating delivery of ΦRGN*ndm-1*. Approximately 6.5x10^6 CFU/mL of EMG2 wild-type cells were incubated with dilutions of ΦRGN*ndm-1* phagemid for 2 hours and plated onto LB and LB+kanamycin to determine the highest dilution of the purified ΦRGN stock able to transduce approximately 100% of the recipient cell population (defined as TFU_{100}/mL). The ΦRGN*ndm-1* phagemid encodes a kanamycin resistance gene. Error bars indicate s.e.m. of three independent experiments (n=3).

Supplementary Figure 6



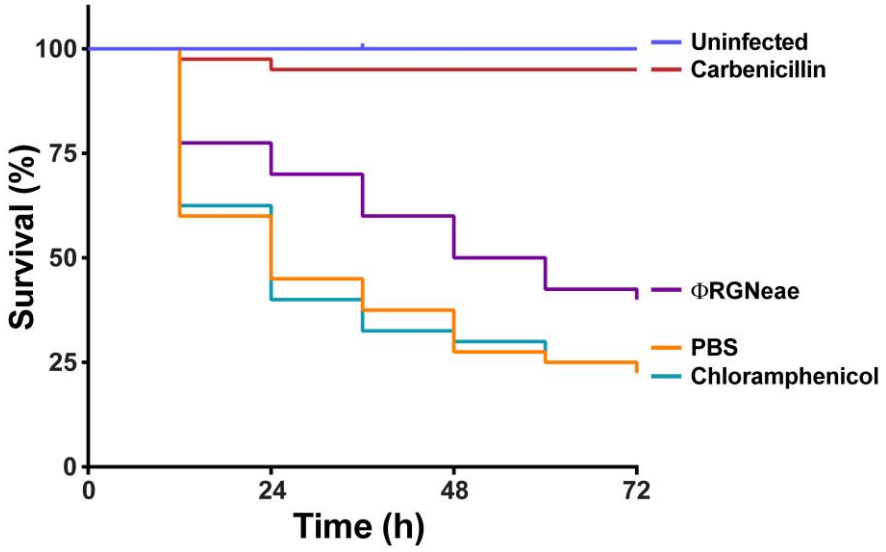
Supplementary Figure 6: Dose response of target cells to ΦRGN*ndm-1*. Approximately 6.5x10^6 CFU/mL of EMG2 wild-type (WT) or EMG2 pNDM-1 cells were treated with increasing concentrations of ΦRGN*ndm-1* particles for two hours. Viable cells were determined by plating onto non-selective LB agar. Error bars indicate s.e.m. of three independent experiments (n=3). MOI=multiplicity of infection.

Supplementary Figure 7



Supplementary Figure 7: Treatment of *E. coli* with ΦRGNs induces DNA damage and an SOS response in cells that possess a cognate target sequence. EMG2 wild-type (WT) (a), EMG2 *gyrA*$_{D87G}$ (b) and EMG2 pNDM-1 (c) containing the pZA3LG reporter plasmid were treated with either SM buffer, ΦRGN*ndm-1* or ΦRGN*gyrA*$_{D87G}$. GFP expression on pZA3LG is under the control of the SOS-responsive $P_{L(lexO)}$ promoter[8]. Injection of single-stranded phagemid DNA led to a mild induction of the SOS response, whereas RGN activity in cells that possessed a cognate target sequence led to stronger induction of SOS. Histograms were generated by combining data from four biological replicates and are normalized to the mode of the population. (d) Summary of flow cytometry histograms. The arithmetic means of the geometric mean fluorescence of populations in a–c were calculated across four independent biological replicates (n=4). Error bars represent s.e.m.

Supplementary Figure 8



Supplementary Figure 8: Comparison of ΦRGN*eae* to conventional antibiotic treatment of EHEC-infected *Galleria mellonella* larvae. *G. mellonella* larvae (n=40) were injected with PBS or approximately $3x10^5$ colony forming units (CFU) of enterohemorrhagic *E. coli* (EHEC). Larvae were subsequently treated with PBS, ΦRGN*eae* at MOI~30, chloramphenicol or carbenicillin. The antibiotics were both applied at a concentration of ~32mg/kg, corresponding to the CLSI resistance breakpoints for Enterobacteriaceae (32µg/mL)[9]. The minimum inhibitory concentration (MIC) values for the EHEC strain are 4µg/mL and >64µg/mL for carbenicillin and chloramphenicol, respectively (Supplementary Table 1). Administration of ΦRGN*eae* significantly improved survival compared to untreated and chloramphenicol treated larvae (Log-rank test, $p<0.05$).

Supplementary Tables

Supplementary Table 1: Minimum Inhibitory Concentration (MIC) (μg/mL)

Strain	AMP	CAZ	CTX	IPM	OFX	CIP	GEN	CAR	CHL
EMG2 Wild-Type	2	0.25	≤0.0625	0.25	0.125	≤0.03125	4	ND	ND
EMG2 *gyrA*$_{D87G}$	4	0.25	≤0.0625	0.25	0.5	0.125	4	ND	ND
EMG2 pNDM-1	>64	>64	>64	32	0.125	≤0.03125	>64	ND	ND
EMG2 pSHV-18	64	1	0.25	0.25	0.125	≤0.03125	>64	ND	ND
ATCC43888 F' (EHEC)	4	ND	ND	ND	ND	≤0.03125	8	4	>64

AMP=ampicillin; CAZ=ceftazidime; CTX=cefotaxime; IPM=imipenem; OFX=ofloxacin; CIP=ciprofloxacin; GEN=gentamicin; CAR=carbenicillin; CHL=chloramphenicol; ND=Not Determined

Supplementary Table 2: Bacterial Strains and Plasmids Used in This Study

Identifier	Strain/Plasmid	Relevant Features	Source/Reference
Bacterial Strains			
fRC149	*Escherichia coli* EMG2	F$^+$	CGSC #4401
fMM28	*E. coli* CJ236	FΔ*(HindIII)::cat* (Tra$^+$ Pil$^+$ Cm^R)	NEB #E4141S
fMM194	*E. coli* RFS289	F', *gyrA*$_{D87G}$ (Ofx^R)	CGSC #5742
fMM269	*E. coli* EMG2::NDM-1	EMG2 Δ*lacZYA*::*bla*$_{NDM-1}$	this study
fMM268	*E. coli* EMG2::SHV-18	EMG2 Δ*lacZYA*::*bla*$_{SHV-18}$	this study
fMM384	*E. coli* EMG2 *gyrA*$_{D87G}$	EMG2 *gyrA*$_{D87G}$ (Ofx^R)	this study
fRC275	*E. coli* EMG2 Sm^R	EMG2 *rpsL*$_{K43N}$ (Sm^R)	this study
fRC301	*E. coli* DH5αPRO	M13cp	Chasteen *et al.* 2006[10]
fMM425	*E. coli* CDC1001728	pNDM-1	ATCC BAA-2469
fMM278	*E. coli* S17-1 λ*pir*	RP4-2-Tc::Mu-Km::Tn7	Simon *et al.* 1983[11]
fMM362	*E. coli* EMG2 pNDM-1	pNDM-1 from ATCC BAA-2469	this study
fMM426	*Klebsiella pneumoniae* K6	pSHV-18	ATCC #700603
fRC280	*E. coli* EMG2 pSHV-18	pSHV-18 from ATCC #700603	this study
fMM427	*E. coli* O157:H7 43888	*eae*$^+$	ATCC #43888
fMM428	*E. coli* O157:H7 43888 F'	F' from CJ236	this study
RGN Plasmids			
pMM178	pRGN*ndm-1*	Cm^R, crRNA targeting *bla*$_{NDM-1}$	this study
pMM282	pRGN*ndm-1*(Cas9D10A)	Cm^R, RGN*ndm-1* (*cas*9 mutation)	this study
pMM281	pRGN*ndm-1*(Δ*tracrRNA*)	Cm^R, RGN*ndm-1* (deleted *tracrRNA* deletion)	this study
pMM228	pRGN*shv-18*	Cm^R, crRNA targeting *bla*$_{SHV-18}$	this study
pMM417	mRGN*shv-18*	Cm^R, Mobilizable RGN*shv-18*	this study
pMM441	mRGN*ndm-1*	Cm^R, Mobilizable RGN*ndm-1*	this study
RGN Phagemids			
pRC319	pΦRGN*ndm1*	Km^R, crRNA targeting *bla*$_{NDM-1}$	this study
pRC321	pΦRGN*shv18*	Km^R, crRNA targeting *bla*$_{SHV-18}$	this study
pRC323	pΦRGN*ndm1*/*shv18*	Km^R, crRNA targeting *bla*$_{NDM-1}$ and crRNA targeting *bla*$_{SHV-18}$	this study
pRC320	pΦRGN*gyrA*$_{D87G}$	Km^R, crRNA targeting *gyrA*$_{D87G}$	this study
pRC357	pΦRGN*eae*	Km^R, crRNA targeting *eae*	this study

Clinical Plasmids			
pMM425	pNDM-1	*bla*$_{NDM-1}$, multidrug resistance	ATCC BAA-2469
pMM426	pSHV-18	*bla*$_{SHV-18}$, *pemIK*, multidrug resistance	ATCC #700603
Other Plasmids			
pRC218	pMJ806 (Addgene 39312)	encodes Cas9 from *S. pyogenes*	Jinek *et al*. 2012[12]
pMM153	pZE-*bla*$_{Z}$-*gfp*	ColE1 *gfp* control plasmid, Cb^{R}	Lutz *et al*. 1997[13]
pMM234	pZE-*bla*$_{NDM-1}$-*gfp*	ColE1 *bla*$_{NDM-1}$, *gfp*	this study
pMM154	pZA-*bla*$_{z}$-gfp	p15A *gfp* control plasmid, Cb^{R}	Lutz *et al*. 1997[13]
pMM235	pZA- *bla*$_{NDM-1}$-*gfp*	p15A *bla*$_{NDM-1}$, *gfp*	this study
pMM253	pSIM9	recombineering machinery, Cm^{R}	Datta *et al*. 2006[14]
pRC306	pZEf-*gfp*	phagemid vector, Cm^{R}	this study
pMM395	pZA31-*gfp*	p15A *gfp* control plasmid, Cm^{R}	Lutz *et al*. 1997[13]
pMM398	pZA31-*pemI*	p15A *pemI* expression plasmid, Cm^{R}	this study
pMM444	pZE1LG	ColE1 P$_{L(lexO)}$-*gfp* SOS-responsive plasmid, Cb^{R}	Dwyer *et al.* 2007[8]
pMM447	pZA3LG	p15a P$_{L(lexO)}$-*gfp* SOS-responsive plasmid, Cm^{R}	this study
pMM364	R1162	Mobilizable wild-type plasmid	Richard Meyer

Abbreviations: Carbenicillin (Cb); Chloramphenicol (Cm); Kanamycin (Km); Ofloxacin (Ofx); Streptomycin (Sm)

Supplementary Table 3: Primers and Oligos Used in this Study

Identifier	Name	Sequence
Primers		
mmD3	SHV18-Chk-F	ATGCGTTATTTTCGCCTGTGTA
mmD4	SHV-18-Chk-R	TTAGCGTTGCCAGTGCTCG
mmD8	pNDM1-XhoI-F	CATGCGCTCGAGGCTCAGCTTGTTGATTATCATATG
mmD9	NDM1-SacI-R	CATAAGGAGCTCTCAGCGCAGCTTGTCGGCCA
mmD74	GpLtetO-SpCas9-F	CACTGACCGAATTCATTAAAGAGGAGAAAGGTGCGGCC GCATGCATCACCATCACCATCACATGGATAAGAAATAC TCAATAGGCTTAG
mmD75	GT1-SpCas9-R	TTCGACTGAGCCTTTCGTTTTATTTGATGCCTCTAGTCA GTCACCTCCTAGCTGACTCAA
mmD82	GSpCRISPR-T1-F	TATGCTGTTTTGAATGGTCTCCATTCTCTAGAGGCATCA AATAAAACGAAAGGCTCAGT
mmD83	GSptracrRNA-amp-R	AACCAAAAAAAACAAGCGCTTTCAAAACGCGTCGACAG GGTGAAGACGAAAGGGCCTCGTG
mmD98	mmS1-F	CTATAAAAATAGGCGTATCACGAGGCCC
mmD99	mmS1-R	TTGAGTATTTCTTATCCATGTGATGGTGATGG
mmD104	GGSpySpacer-F	GAGCATGAAGACCATTCAGCACACTGAGACTTGTTGAG TTC
mmD105	GGSpySpacer-R	GAGCATGAAGACCCCTCGCTCGTAGACTATTTTTGTCTA AAAAATTTCGT
mmD108	SpyCas9D10A-F	GCTATCGGCACAAATAGCGTCGGATG
mmD109	SpyCas9D10A-R	TAAGCCTATTGAGTATTTCTTATCCATCGAGG
mmD112	Cas9-400-Chk-R	GATAGATAGTTGGATATTTCTCATGATAAG

mmD113	Cas9-900-Chk-F	CTGAAATAACTAAGGCTCCCCTATC
mmD114	Cas9-1.8K-Chk-F	GATAGGGAGATGATTGAGGAAAGAC
mmD115	Cas9-2.8K-Chk-F	CGCATGAATACTAAATACGATGAAAATG
mmD151	NheI-tL17pBBR1-SpyCRISPR-F	CATGATGCTAGCACCCTGCAGAGCTTCTAGATGATGATTAAGGATCTATTTTTTTGGGCG
mmD153	Cas9-End-Chk-F	GTGCATATAACAAACATAGAGAC
mmD154	Lutz-BtwAbProm-R	CCTCGTGATACGCCTATTTTTATAGGTTAATG
mmD155	gyrA-F	CACCACTCATTGCCACATTCCTTGTG
mmD161	gyrA-R	CGGCTTCAAATTTAGCGATCTCTTC
mmD162	notracrRNA-ClaI-F	ATCGATATCGATTCCCTATCAGTGATAGAGATTGAC
mmD163	notracrRNA-ClaI-R	CATGATATCGATAGGGTGAAGACGAAAGGGCCTCG
mmD234	NDM-1-Start-F	GATGGAATTGCCCAATATTATGCACCC
mmD247	SpeI-catCRISPR-F	CACTCATCGCAGTCGGCCTATTGG
mmD248	XmaI-catCRISPR-R	CATGATcccgggGGACAGCAAGCGAACCATTTTTTTGGG
mmD253	KpnI-pemI-F	CATGATGGTACCATGCATACCACTCGACTGAAGAAGGTTG
mmD254	BamHI-pemI-R	CATGATGGATCCTCAGATTTCCTCCTGACCAGCCG
mmD266	XmaI-R1162-F	catgatcccgggGACAGACGTCATACTAGATATCAAGCGAC
mmD267	SpeI-R1162-R	catgatactagtGGAGCAGAAGAGCATACATCTGGAAG
rcD11	cam-3-F	ACGTCTCATTTTCGCCAGAT
rcD73	pSHV18-KIlacZYA-F	GGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTCTTTTGCTTTGCCACGGAAC
rcD74	SHV18-KIlacZYA-R	TTAAACTGACGATTCAACTTTATAATCTTTGAAATAATAGTGCTTATCCCGGTCGTTTATCTACGAGCCGGATAACGC
rcD77	pNDM1-KIlacZYA-F	GGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTGCTCAGCTTGTTGATTATCATATG
rcD78	NDM1-KIlacZYA-R	TTAAACTGACGATTCAACTTTATAATCTTTGAAATAATAGTGCTTATCCCGGTCGTTTATTCAGCGCAGCTTGTCGGCCA
rcD169	XmaI-CRISPR-R	CATGATCCCGGGGTTTTATGGACAGCAAGCGAACC
rcD183	AvrII-CRISPR-F	CATGATCCTAGGCGTCTAAGAAACCATTATTATCATGACATTAACC
rcD184	XmaI-pZEfS-noGFP-F	CATGATCCCGGGCAGAAGATCCTGCAGGGCCTTC
rcD185	AvrII-pZEfS-noGFP-R	CATGATCCTAGGAAATTGTAAACGTTAATATTTTGTTAAAATTCGC
Oligos		
mmO11	SP-T-GG-Cap-1	CATGAGGGTCTCACGAGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC
mmO14	SP-B-GGCap-5	CATGAGGGTCTCCTGAAGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAAC
mmO17	SP-T-ndm1-6	GGGCAGTCGCTTCCAACGGTTTGA
mmO18	SP-B-ndm1-6	TGACGATCAAACCGTTGGAAGCGA
mmO19	SP-T-ndm1R-7	TCGTCAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC

mmO20	SP-B-Rshv18-2	ATTTGCGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAAC
mmO21	SP-T-shv18-2	GCAAATTAAACTAAGCGAAAGCCA
mmO22	SP-B-shv18-2	GACAGCTGGCTTTCGCTTAGTTTA
mmO23	SP-T-shv18-2-3Cap	GCTGTCGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGGAGACCCTCATG
mmO58	SP-B-ndm1-1-Cap5	CTGCCCGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTGAGACCCTCATG
mmO59	SP-T-ndm1-1-Cap3	TCGTCAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGGAGACCCTCATG
mmO87	SP-B-gyrA111-Cap5	TGGTATGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTGAGACCCTCATG
mmO88	SP-T-gyrA111	ATACCATCCCCATGGTGACTCGGC
mmO89	SP-B-gyrA111	TAGACCGCCGAGTCACCATGGGGA
mmO90	SP-T-gyrA111-Cap3	GGTCTAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGGAGACCCTCATG
mmO92	SP-B-shv18-2-Cap5	ATTTGCGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTGAGACCCTCATG
mmO115	ndm1-1-T	TTCCAACGGTTTGATCGTCAGTTTTA
mmO116	ndm1-1-B	TGACGATCAAACCGTTGGAAGCTAGC
mmO121	shv18-2-T	CTAAGCGAAAGCCAGCTGTCGTTTTA
mmO122	shv18-2-B	GACAGCTGGCTTTCGCTTAGGCTAGC
mmO204	SP-B-eae1-Cap5	GAGTCAGTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTCGTGAGACCCTCATG
mmO205	SP-T-eae1	TGACTCCCGCTTTACGGCAAATTT
mmO206	SP-B-eae1	GCACCTAAATTTGCCGTAAAGCGG
mmO207	SP-T-eae1-Cap3	AGGTGCGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGGAGACCCTCATG

Supplementary Table 4: CRISPR Loci

RGN	Oligos (mmO---)
RGN*ndm-1*	11/58, 17/18, 59/14
RGN*shv-18*	11/92, 21/22, 23/14
RGN$gyrA_{D87G}$	11/87, 88/89, 90/14
RGN*ndm-1/shv-18*	11/58, 17/18, 19/20, 21/22, 23/14
RGN*eae*	11/204, 205/206, 207/14

Supplementary References

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