Supporting Information

Yosef et al. 10.1073/pnas.1500107112

SI Materials and Methods

Reagents, Strains, and Plasmids. LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and agar were from Acumedia. 2YT medium contained 1.6% (wt/vol) Bacto-tryptone (Acumedia), 1% (wt/vol) Bacto-yeast extract (Acumedia), and 0.5% (wt/vol) NaCl (Acumedia) in distilled water. Antibiotics, lysozyme, L-arabinose, and maltose were from Calbiochem. Sodium chloride and magnesium sulfate were from Merck. Restriction enzymes, ligation enzymes, and Phusion High-Fidelity DNA Polymerase were from New England Biolabs. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1.

Plasmid Construction. Plasmids were constructed using standard molecular biology techniques. DNA segments were amplified by PCR. Standard digestion of the PCR products and vector by restriction enzymes was carried out according to the manufacturer's instructions.

pIYEC1 plasmid, synthesized by GenScript, encodes a CRISPR array transcribed by a T7 promoter encoding three spacers targeting the *ndm-1* gene (N_1, N_2, N_3), three spacers targeting the *ctx-M-15* gene (C_1, C_2, C_3), and three spacers targeting the T4 phage genome (irrelevant for this study). pIYEC2 is similar to pIYEC1 except that it also encodes a chloramphenicol resistance marker. To construct pIYEC2, the chloramphenicol resistance marker from pKD3 (1) was amplified using oligonucleotides IY344F and IY344R. The amplified DNA and pIYEC1, both digested by HindIII, were ligated to yield pIYEC2. pNDM and pCTX plasmids were constructed by ligating PCR fragments encoding *ndm-1* or *ctx-M-15* to another PCR fragment containing an origin of replication and a str^r marker derived from plasmid pCas1+2 (2). pNDM* was constructed by ligating PCR fragments encoding *ndm-1* to another PCR fragment containing an origin of replication and a gentamicin^r marker. Plasmid pVEC was constructed by ligating an irrelevant DNA fragment to the origin of replication and the str^r marker derived from plasmid pCas1+2. Plasmids pTRX1, pTRX2, pTRX3, pTRX4, and pTRX5 were constructed to insert protospacers into the T7 genome (Table S1). The plasmids encode the *trxA* gene, a positive selection marker for T7 grown on hosts lacking *trxA*, flanked by desired protospacers and followed by 50 bp upstream and downstream of a DNA sequence corresponding to the end of T7 gene 1.3 and the beginning of T7 gene 1.4, respectively. The plasmids were constructed by PCR amplification of T7 phage encoding a *trxA* gene flanked by Flippase recognition target sites using the primers indicated in Table S2. The resulting PCR product was used as a template for PCR using primers IY260F and IY260R (Table S1). The final PCR fragment was ligated into pGEM-T vector (Promega). Constructed plasmids were validated as encoding the desired fragments by DNA sequencing.

Homologous Recombination-Based Genetic Engineering. Homologous recombination using short-homology flanking ends was carried out as previously described (3). To insert the six *cas* genes required for CRISPR interference under T7 control, we first cloned the T7 promoters upstream of the *cas3* and *cse1* genes in *E. coli* K-12. An overnight culture of *E. coli* RE1001 (Table S1) harboring the pSIM6 plasmid was diluted 1:100 in 50 mL fresh LB supplemented with 100 μg/mL ampicillin at 32 °C and aerated until the OD_{600} reached 0.4–0.6. The culture was then heat-induced for expression of the *red* recombination enzymes at 42 °C for 15 min in a shaking water bath followed by incubation in ice water for 10 min. The culture was then centrifuged at 4,600 × *g* for 10 min at 4 °C. The supernatant was removed, and the pellet was washed three times in ice-cold ddH_2O. The pellet was resuspended in 200 μL of ice-cold ddH_2O and kept on ice. The cultures were then electroporated with ∼500 ng of PCR products encoding a T7 promoter fused to either kanamycin or chloramphenicol resistance markers flanked by 50 bp of sequences flanking the original promoters of *cas3* (fragment T7*cas3::kan*) and *cse1* (fragment T7*cse1::cm*) genes. T7*cas3::kan* fragment was constructed by PCR amplification of the kanamycin resistance gene encoding flippase recognition target (FRT) sites from BW25113Δ*yeeX* (Table S1) by using primers RK41F and a primer encoding the T7 promoter in its 5′ end, RK41R (Table S1). The PCR fragment was then amplified with RK42F and RK42R (Table S1), encoding 50-bp homology to the immediate 5′ region of *cas3*. The T7*cse1::cm* fragment was constructed by PCR amplification of the chloramphenicol fragment encoding FRT sites from the pKD3 plasmid (Table S1) by using primers RK43F and a primer encoding the T7 promoter in its 5′ end, RK43R (Table S1). The PCR fragment was then amplified with RK44F and RK44R (Table S1), encoding 50-bp homology of the immediate 5′ region of *cse1*. Electroporation of these fragments was carried out using a 50-μL aliquot of electrocompetent bacteria in a 0.2-cm cuvette at 25 μF, 2.5 kV, and 200 Ω. After electroporation, 1 mL 2YT medium was added to the cuvette, followed by aeration at 32 °C for 3 h. The cultures were then inoculated on LB agar plates supplemented with 25 μg/mL kanamycin and 17.5 μg/mL chloramphenicol and incubated overnight at 32 °C. Recombinant colonies were streaked on 25 μg/mL kanamycin and 17.5 μg/mL chloramphenicol plates and incubated at 42 °C to eliminate the temperature-sensitive pSIM6 plasmid. A single colony was validated as encoding the desired substitutions by DNA sequencing using RK33R and RK29R. The entire manipulated cassette encoding *cas3* and *cse1* under the T7 promoters was transduced to the RE1001 strain and selected using both antibiotic markers to yield the RK6471 strain.

The *cas* genes were deleted as described previously (4). Briefly, *E. coli* DY378 was electroporated with ∼500 ng of PCR product generated by amplifying plasmid pKD3 using primers IY80F and IY80R (Table S2). This amplified DNA encoded a chloramphenicol resistance marker flanked on one end by 50 bp of sequences of the *cas3* promoter and on another end by 50 bp of the CRISPR leader sequence. Desired recombinants were selected on LB agar plates supplemented with 17 μg/mL chloramphenicol. The deletion was then transferred to IYB5101 using P1 transduction as previously described (5), yielding IYB5666.

To construct a λ phage encoding the *cas* genes under T7 promoters, an overnight culture of IYB5297/pSIM6 was diluted 50-fold in 25 mL LB medium with appropriate antibiotics and grown at 32 °C to an OD_{600} of 0.5. The culture was then heat induced for expression of recombination enzymes from both the λ prophage and the plasmid at 42 °C for exactly 4 min in a shaking water bath. The induced samples were immediately cooled on an ice slurry and then pelleted at 4,600 × *g* at 4 °C for 10 min. The pellet was washed twice in ice-cold ddH_2O, resuspended in 200 μL of ice-cold ddH_2O, and kept on ice until electroporation with ∼1,600 ng of a gel-purified PCR product obtained by amplifying the genomic DNA of RK6471 using primers IY333F and IY333R. A 25-μL aliquot of electrocompetent cells was used for each electroporation in a 0.2-cm cuvette at 25 μF, 2.5 kV, and 200 Ω. After electroporation, the bacteria were grown in 1 mL LB for 1 h in a 32 °C shaking water bath and inoculated on selection plates containing 17 μg/mL chloramphenicol. The chloramphenicol resistance marker was removed using the Flippase recombination

enzyme encoded by plasmid pCP20 (1), and chloramphenicol-sensitive colonies were used for phage induction at 42 °C. The resulting phage, λ_{cas}, encoding the six *cas* genes transcribed from T7 promoters but lacking a CRISPR array, was used to lysogenize BL21-AI, yielding IYB5614. The engineered CRISPR array was inserted into IYB5614/pSIM6 as described above by using a PCR fragment obtained from amplifying pIYEC2 by primers IY347F and IY347R. The resulting strain, IYB5656, harbors $\lambda_{cas\text{-}CRISPR}$, which encodes the six *cas* genes transcribed from T7 promoters and the CRISPR array encoding spacers against *ndm-1*, *ctx-M-15*, and the T4 phage genome.

Homologous Recombination of Bacteriophage T7. T7 phages encoding desired protospacers were constructed as previously described (6) by using plasmids pTRX1, pTRX2, pTRX3, pTRX4, and pTRX5.

1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
2. Yosef I, Goren MG, Qimron U (2012) Proteins and DNA elements essential for the CRISPR adaptation process in *Escherichia coli*. *Nucleic Acids Res* 40(12):5569–5576.
3. Sharan SK, Thomason LC, Kuznetsov SG, Court DL (2009) Recombineering: A homologous recombination-based method of genetic engineering. *Nat Protoc* 4(2): 206–223.
4. Yu D, et al. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 97(11):5978–5983.
5. Yosef I, Goren MG, Kiro R, Edgar R, Qimron U (2011) High-temperature protein G is essential for activity of the *Escherichia coli* clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system. *Proc Natl Acad Sci USA* 108(50):20136–20141.
6. Kiro R, et al. (2013) Gene product 0.4 increases bacteriophage T7 competitiveness by inhibiting host cell division. *Proc Natl Acad Sci USA* 110(48):19549–19554.

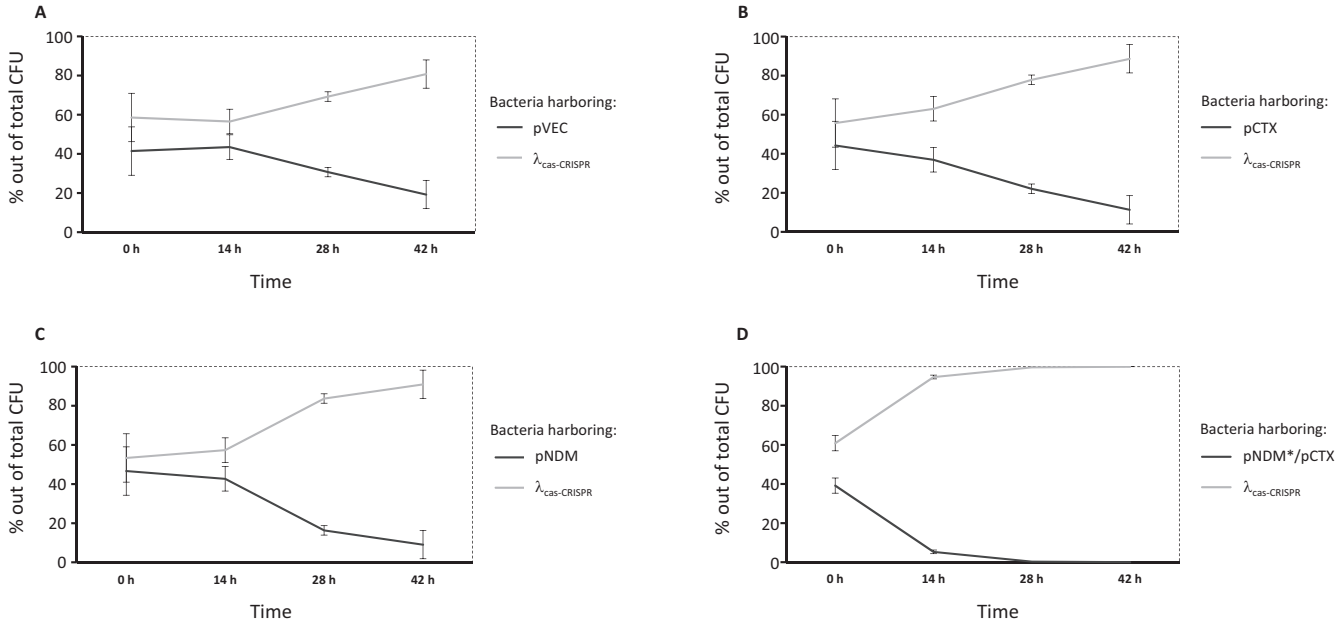

Fig. S1. Competitive fitness of a lysogen compared with bacteria harboring resistance plasmids. Bacteria encoding the $\lambda_{cas\text{-}CRISPR}$ prophage and (*A*) pVec, (*B*) pCTX, (*C*) pNDM, and (*D*) pNDM*+pCTX plasmids were mixed at a 1:1 ratio. They were then cultured together in LB at 32 °C for 14 h. The cells were then diluted 1/800 in LB and grown for an additional 14 h at 32 °C; this procedure was repeated once more. Samples from the mixed cultures were taken at the indicated time points and plated on either kanamycin or streptomycin or streptomycin+gentamicin agar plates to differentiate between lysogens (kanamycin^r) and plasmid-harboring bacteria (streptomycin^r for *A*–*C* or streptomycin^r+gentamicin^r for *D*). The CFU ratio of each strain was then determined by calculating the number of each type of resistant CFU out of the total resistant CFU.

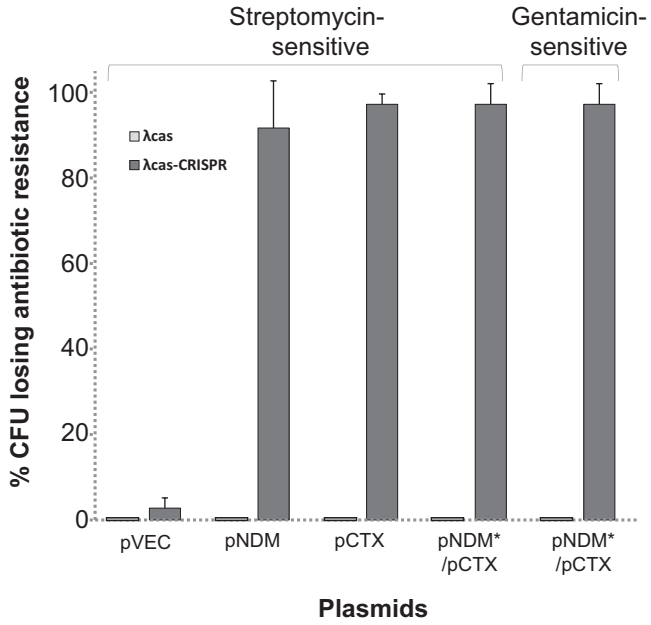

Fig. S2. Sensitization of antibiotic-resistant bacteria by lysogenization. *E. coli* K-12 harboring a control (pVEC), *ndm-1* (pNDM), *ctx-M-15* (pCTX), or *ndm-1* + *ctx-M-15* (pNDM*/pCTX) encoding plasmids were treated with λ_{cas} (light gray bars) or $\lambda_{cas\text{-}CRISPR}$ (dark gray bars) and plated on LB plates supplemented with 5 μg/mL tetracycline and 0.2% arabinose. Colonies (24 of each strain) were then inoculated on plates supplemented with 5 μg/mL tetracycline and 0.2% arabinose and having or lacking streptomycin or gentamicin. Bars represent percentage and SD from three independent experiments of streptomycin- or gentamicin-sensitive bacteria scored as CFUs unable to grow on plates with streptomycin or gentamicin out of the total number of CFUs able to grow on plates lacking these antibiotics.

Table S1. Bacterial strains, plasmids, and oligonucleotides used in this study

Bacteria, phages, plasmids, and oligonucleotides	Description/sequence	Source or reference
Bacterial strains		
NEB5α	F⁻ ϕ80*lacZ*ΔM15Δ*(lacZYA-argF)* U169 *deoR recA1 endA1 hsdR17* (r_k^-, m_k^+) *gal* ⁻ *phoA supE44* λ⁻ *thi* ⁻*1 gyrA96 relA1*	New England Biolabs
DY378	W3110 *λcI857 Δ(cro-bioA)*	(1)
BW25113ΔyeeX	F⁻, Δ*(araD-araB)567,* Δ*yeeX::kan,* Δ*lacZ4787(::rrnB-3),* λ⁻, *rph-1,* Δ*(rhaD-rhaB)568, hsdR514*	(2)
RE1001	K12-*araB::*T7*RNAP-tetA*	(3)
RK6471	K12-*araB::*T7*RNAP-tetA,* T7*cas3::kan,* T7 *cse1::cm*	This study
IYB5101	BW25113 *araB*::T7-RNAp-tetA	(3)
IYB5666	IYB5101 Δ*(cas3-cas2)*:: *cm*	This study
IYB5670	BW25113 *araB*::T7-RNAp-tetA, tet^r . harbors λcas prophage	This study
IYB5671	BW25113 *araB*::T7-RNAp-tetA, tet^r . harbors λcas-CRISPR prophage	This study
BL21-AI	F⁻ *ompT hsdSB*(rB–, mB–) *gal dcm araB*::*T7RNAP-tetA*, tet^r	Invitrogen (1)
IYB5297	F⁻ *ompT hsdSB*(rB–, mB–) *gal dcm araB*::*T7RNAP-tetA*, tet^r. harbors λcI857-kan prophage	This study
IYB5614	F⁻ *ompT hsdSB*(rB–, mB–) *gal dcm araB*::*T7RNAP-tetA*, tet^r. harbors λcas prophage	This study
IYB5656	F⁻ *ompT hsdSB*(rB–, mB–) *gal dcm araB*::*T7RNAP-tetA*, tet^r. harbors λcas-CRISPR prophage	This study
Phages		
λcI857-kan	Lambda phage carrying the temperature sensitive C-I allele *cI857*, Kan^R	(4)
λcas-cm	cI857 Kan^R, cm^R. Contains *cas3* under T7 promoter and *casABCDE* under T7 promoter	This study
λcas	cI857 Kan^R. Contains *cas3* under T7 promoter and *casABCDE* under T7 promoter	This study
λcas-CRISPR	cI857 Kan^R, cm^R. Contains *cas3* under T7 promoter, *casABCDE* under T7 promoter and engineered CRISPR array under T7 promoter	This study
$T7_{FRTtrxA}$	T7 with trxA flanked by FRT sites	(5)
Plasmids		
pCas1+2	pCDF-1b (Novagen) cloned with *cas1,2* under T7 promoter, Str^r.	(6)
pIYEC1	pUC57 cloned with anti NDM-1, CTX-M-15 and T7 phage spacers under T7 promoter, Amp^R	This study
pIYEC2	pUC57 cloned with anti NDM-1, CTX-M-15 and T7 phage spacers under T7 promoter, Cam^R, Amp^R	This study
pNDM	pCDF-1b (Novagen) based containing New Delhi Metallo-beta-lactamase-1 (NDM-1), Str^r, Carbapenem^r	This study
pCTX	pCDF-1b (Novagen) based containing CTX-M-15 beta-lactamase, Str^r, Carbapenem^r	This study
pVEC	pCDF-1b (Novagen) based plasmid, Str^r	This study
pNDM*	pBIL2C based containing New Delhi Metallo-beta-lactamase-1, Gentamicin^r, Carbapenem^r	This study
pTRX1	pGEM T-vector (promega) cloned with gp8 proto-spacer (7)	This study
pTRX2	pGEM T-vector (promega) cloned with NDM-1 proto-spacer	This study
pTRX3	pGEM T-vector (promega) cloned with CTX-M-15 proto-spacer	This study
pTRX4	pGEM T-vector (promega) cloned with NDM-1 and CTX-M-15 proto-spacer	This study
pTRX5	pGEM T-vector (promega) cloned with NDM-1 and CTX-M-15 proto-spacer	This study
pKD3	pSC101 encoding chloramphenicol resistance marker flanked by FRT sites	(8)
Oligonucleotides	5′→3′	
IY344F	ACCGAAGCTTTGAATATCCTCCTTAGTTCC	
IY344R	CGCCAAGCTTACGGGGCAACCTCATGTCAAGTGTAGGCTGGAGCTGCTTC	
IY246F	ATGGAATTGCCCAATATTAT	
IY246R	TCAGCGCAGCTTGTCGGCCA	
IY247F	GAACTAAATCAGGCACTTGAGCATCAAGATTGGTG	
IY247R	CACCAATCTTGATGCTCAAGTGCCTGATTTAGTTC	
IY346F	ATGGTTAAAAAATCACTGCGCCAGT	
IY346R	TTACAAACCGTCGGTGACGA	
IY142Fb	CACACGGTCACACTGCTTCC	
MG110R	CGATGCCCTTGAGAGCCTTC	
MG17F	ATAAGTCGGACACCATGGCA	
IY80F	AATAGCCCGCTGATATCATCGATAATACTAAAAAAACAGGGAGGCTATTAGTGTAGGCTGGAGCTGCTTC	
IY80R	ACCTTAATGTAACATTTCCTTATTATTAAAGATCAGCTAATTCTTTGTTTTGAATATCCTCCTTAGTTCC	
IY333F	ATGCGTAATGTGTGTATTGCCGTTGCTGTCTTTGCCGCACTTGCGGTGACCCGGAATGAAATTAATACGACTC	
IY333R	AACCTGTCGCACTCCAGAGAAGCACAAAGCCTCGCAATCCAGTGCAAAGCTCACAGTGGAGCCAAAGATA	
IY347F	GGCCAGCTAAATCGATGGGATGTGGCTTGCTATCTTTGGCTCCACTGTGAGGGATGTGCTGCAAGGCGAT	
IY347R	AACCTGTCGCACTCCAGAGAAGCACAAAGCCTCGCAATCCAGTGCAAAGCACGGGGCAACCTCATGTCAA	
IY309F	ACCCTCAAGAGAAAATGTAAAAGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCGATCCGTCAGCCTGCAGTTC	
IY309R	CCGAAGGTGAGCCAGTGTGAAAGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCTGTAGGCTGGAGCTGCTTCG	
IY340Fa	ACCCTCAAGAGAAAATGTAAAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCTGATCCGTCAGCCTGCAGTTC	
IY340Fb	ACCCTCAAGAGAAAATGTAAAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTGATCCGTCAGCCTGCAGTTC	
IY340Ra	CCGAAGGTGAGCCAGTGTGAGTACGTCCGCCGTTTGCGCATACAGCGGCACACTTTGTAGGCTGGAGCTGCTTCG	
IY340Rb	CCGAAGGTGAGCCAGTGTGAACCGCCAGCGCGACCGGCAGGTTGATCTCCTGCTTTGTAGGCTGGAGCTGCTTCG	
IY260F	TGGCTCTTTGCGGCACCCATCGTTCGTAATGTTCCGTGGCACCGAGGACAACCCTCAAGAGAAAATGTAA	

Table S1. Cont.

Bacteria, phages, plasmids, and oligonucleotides	Description/sequence	Source or reference
IY260R	CCAACCTTCTTAAACATAAAGTGTCTCCTTATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGA	
RK41F	GGAATTACTTCGCTTCGCC	
RK41R	CCTCCTTATCTCCCTATAGTGAGTCGTATTAATTTCATTCCGGGGATCCGTCGACC	
RK42F	AAACGCGTTTCTTTGGCTTAAAAAGGGAATGTGGGTTACACGAAGGGTAATGTAGGCTGGAGCTGCTTCG	
RK42R	GGATTTTCCCCAGTAATGGCATATATATTTAAAAGGTTCCATTAATAGCCCCTCCTTATCTCCCTATAGT	
RK43F	GCAGCATTACACGTCTTGAG	
RK43R	CTCCTTATCTCCCTATAGTGAGTCGTATTAATTTCTGAATATCCTCCTTAGTTCC	
RK44F	TTCGGGAATGATTGTTATCAATGACGATAATAAGACCAATAACGGTTTATGTGTAGGCTGGAGCTGCTTC	
RK44R	CGCGGGCGTACAGGGATCCAGTTATCAATAAGCAAATTCATTTGTTCTCCCTCCTTATCTCCCTATAGTG	
RK29R	GACTCTCGAGGCCACTGATCTCTACTGCAG	
RK33R	GACTCTCGAGGCAACAGCAGCAACATCAAG	

1. Yu D, et al. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 97(11):5978–5983.
2. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:0008.
3. Yosef I, Goren MG, Kiro R, Edgar R, Qimron U (2011) High-temperature protein G is essential for activity of the *Escherichia coli* clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system. *Proc Natl Acad Sci USA* 108(50):20136–20141.
4. Edgar R, Qimron U (2010) The Escherichia coli CRISPR system protects from λ lysogenization, lysogens, and prophage induction. *J Bacteriol* 192(23):6291–6294.
5. Kiro R, et al. (2013) Gene product 0.4 increases bacteriophage T7 competitiveness by inhibiting host cell division. *Proc Natl Acad Sci USA* 110(48):19549–19554.
6. Yosef I, Goren MG, Qimron U (2012) Proteins and DNA elements essential for the CRISPR adaptation process in *Escherichia coli*. *Nucleic Acids Res* 40(12):5569–5576.
7. Datsenko KA, et al. (2012) Molecular memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system. *Nat Commun* 3:945.
8. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.

Table S2. Oligonucleotides and templates used for construction of bacteria, phages, and plasmids

Constructed phage/plasmid	Oligonucleotides for PCR	DNA template
IYB5300	IY80F, IY80R	pKD3
RK6471	RK41F, RK41R, RK42F, RK42R, RK43F, RK43R, RK44F, RK44R	pKD3 and Genomic DNA of BW25113ΔyeeX
λcas-cm	IY333F, IY333R	Genomic DNA of RK6471
λcas-CRISPR	IY347F, IY347R	pIYEC2
T7-gp8	IY309F, IY309R	$T7_{FRTtrxA}$ (1)
T7-N_1N_2	IY340Fa, IY340Rb	$T7_{FRTtrxA}$ (1)
T7- C_2C_1	IY340Fb, IY340Ra	$T7_{FRTtrxA}$ (1)
T7- N_1C_1	IY340Fa, IY340Ra	$T7_{FRTtrxA}$ (1)
T7- C_2N_2	IY340Fb, IY340Rb	$T7_{FRTtrxA}$ (1)
pTRX1	IY309F, IY309R	$T7_{FRTtrxA}$ (1)
pTRX2	IY340Fa, IY340Rb	$T7_{FRTtrxA}$ (1)
pTRX3	IY340Fb, IY340Ra	$T7_{FRTtrxA}$ (1)
pTRX4	IY340Fa, IY340Ra	$T7_{FRTtrxA}$ (1)
pTRX5	IY340Fb, IY340Rb	$T7_{FRTtrxA}$ (1)

1. Kiro R, et al. (2013) Gene product 0.4 increases bacteriophage T7 competitiveness by inhibiting host cell division. *Proc Natl Acad Sci USA* 110(48):19549–19554.