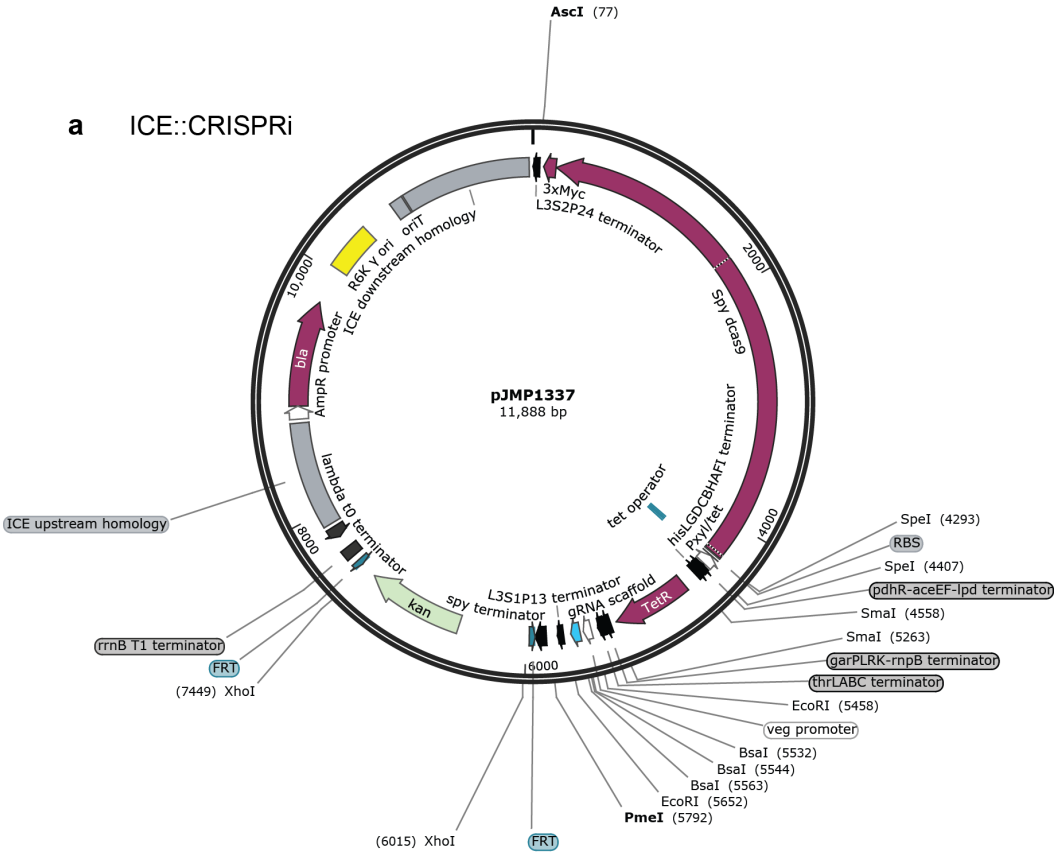
a
ICE::CRISPRi
AscI (77)
3xMyc
L3S2P24 terminator
2000
Spy dcas9
4000
pJMP1337
11,888 bp
tet operator
hisLGDCBHAFI terminator
PxylTet
SpeI (4293)
RBS
SpeI (4407)
pdhR-aceEF-lpd terminator
SmaI (4558)
SmaI (5263)
garPLRK-rnpB terminator
thrLABC terminator
EcoRI (5458)
veg promoter
BsaI (5532)
BsaI (5544)
BsaI (5563)
EcoRI (5652)
PmeI (5792)
6000
L3S1P13 terminator
gRNA scaffold
TetR
spy terminator
kan
8000
lambda t0 terminator
AmpR promoter
bla
10,000
R6K γ ori
oriT
ICE downstream homology
ICE upstream homology
rrnB T1 terminator
FRT
(7449) XhoI
(6015) XhoI
FRT

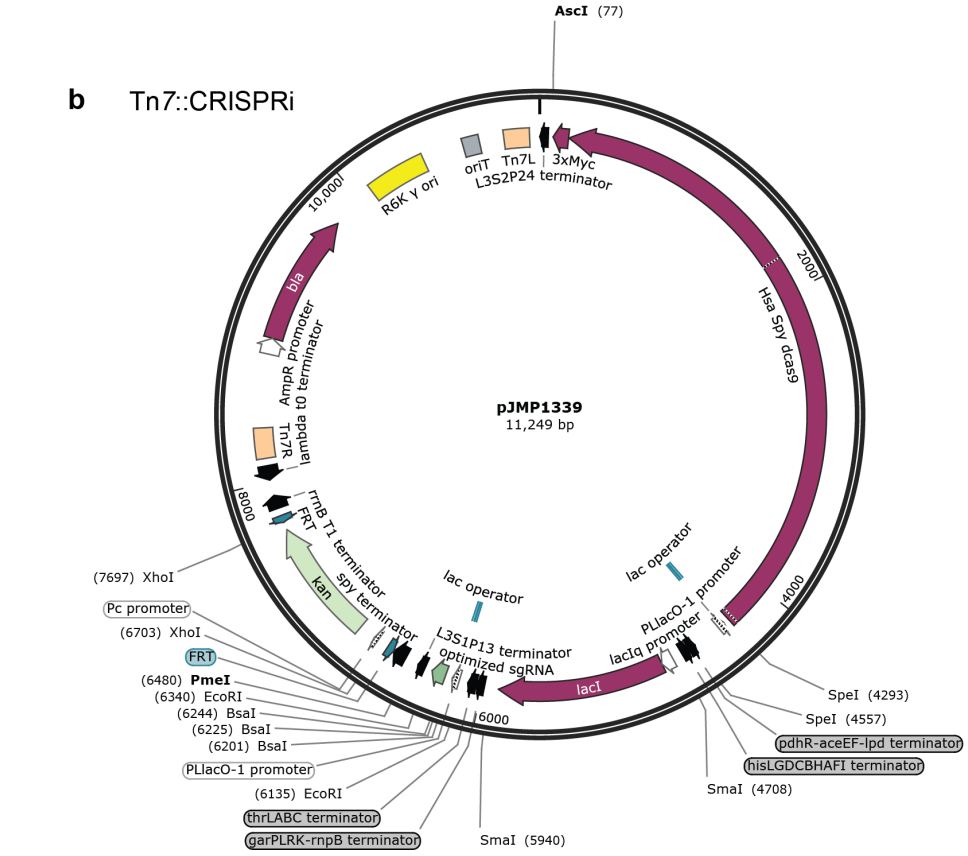
b
Tn7::CRISPRi
AscI (77)
oriT
Tn7L
3xMyc
L3S2P24 terminator
R6K γ ori
10,000
2000
Hsa Spy dcas9
4000
pJMP1339
11,249 bp
lac operator
PLlacO-1 promoter
lacIq promoter
lacI
SpeI (4293)
SpeI (4557)
pdhR-aceEF-lpd terminator
hisLGDCBHAFI terminator
SmaI (4708)
6000
L3S1P13 terminator
optimized sgRNA
spy terminator
kan
rrnB T1 terminator
FRT
8000
Tn7R
lambda t0 terminator
AmpR promoter
bla
(7697) XhoI
Pc promoter
(6703) XhoI
FRT
(6480) PmeI
(6340) EcoRI
(6244) BsaI
(6225) BsaI
(6201) BsaI
PLlacO-1 promoter
(6135) EcoRI
thrLABC terminator
garPLRK-rnpB terminator
SmaI (5940)

Fig. S1 | Modular design of Mobile-CRISPRi plasmids. Mobile-CRISPRi has a modular design such that new parts can be inserted by cutting with restriction enzymes followed by Gibson assembly. Antibiotic resistance markers and associated promoters are located between two XhoI sites—these markers can be removed from recipient cells by Flp-mediated recombination after selection[15]. PmeI is a unique site that can be used for inserting reporters, such as the *rfp* gene that was used in this study. The sgRNA and associated promoter is located between two EcoRI sites (see zoomed in version in Supplementary Fig. 2), and regulatory genes (e.g., *tetR* and *lacI*) can be cloned between two SmaI sites. The promoter and ribosome binding site (RBS) for *dcas9* is located between two SpeI sites (see zoomed in version in Supplementary Fig. 2). The unique AscI site is used in conjunction with SpeI to insert *dcas9* variants, with subsequent cloning of a different promoter and RBS for *dcas9* into SpeI. All sites are transcriptionally-insulated by strong terminators. **a,** pJMP1337 (ICE::CRISPRi) and **b,** pJMP1339 (Tn*7*::CRISPRi) are prototypical Mobile-CRISPRi plasmids for cloning new sgRNAs into BsaI sites (see zoomed in version in Supplementary Fig. 2).

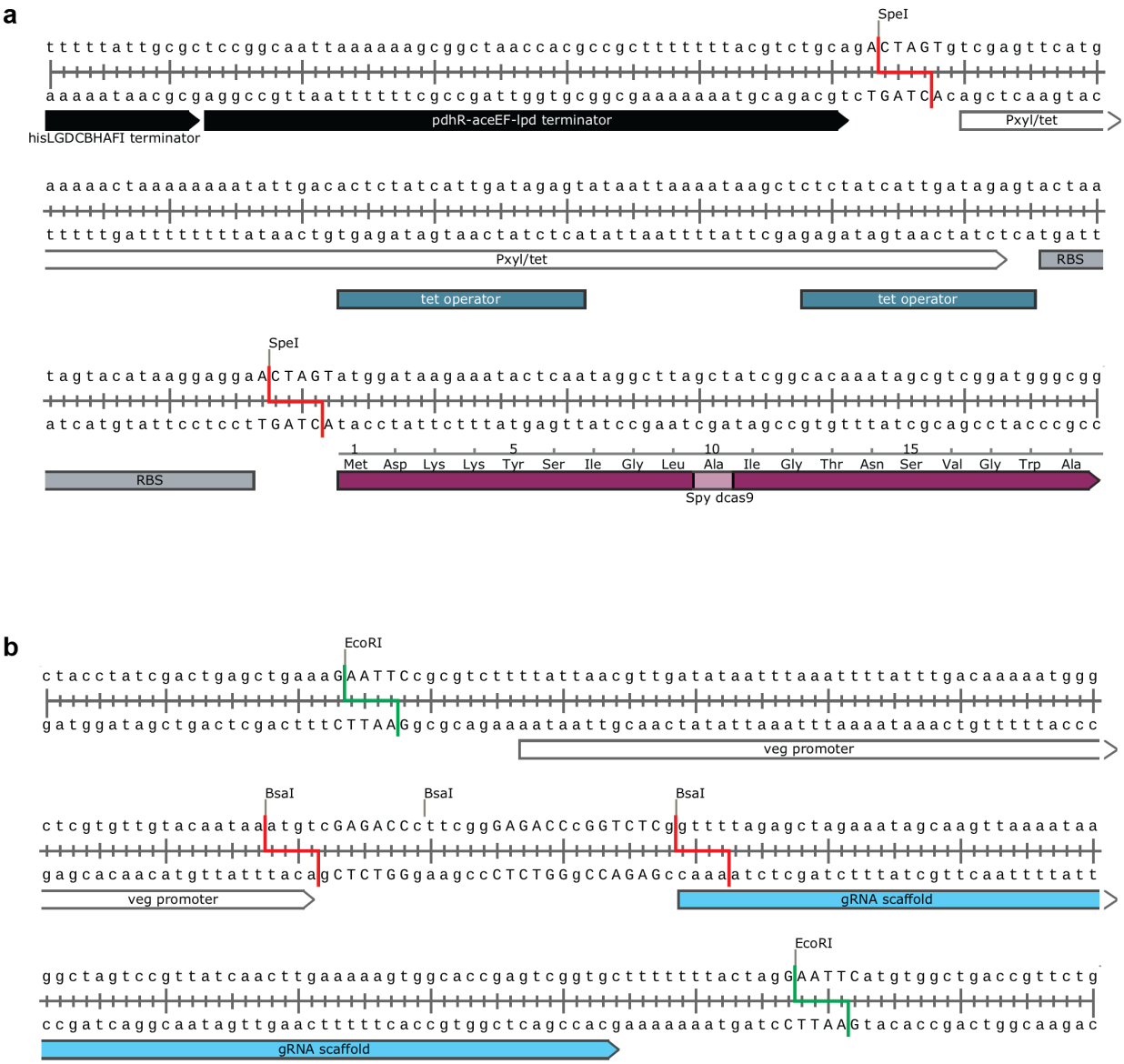

Fig. S2 | Cloning new promoters and sgRNAs into Mobile-CRISPRi. a, New promoters and RBSs can be cloned between the SpeI sites (red) upstream of dcas9. This example shows the *xyl/tet* promoter upstream of *dcas9* in pJMP1337. b, New sgRNAs and promoters are cloned between EcoRI sites (green)—typically by ordering a small piece of synthetic DNA. New 20-nt sgRNA sequences can be cloned into the BsaI sites (red; note that the middle, uncolored BsaI site is only present to reduce cloning background) using annealed oligos with complementary

sticky-ends to the BsaI cuts. This example shows the sequence of the sgRNA module in pJMP1337.

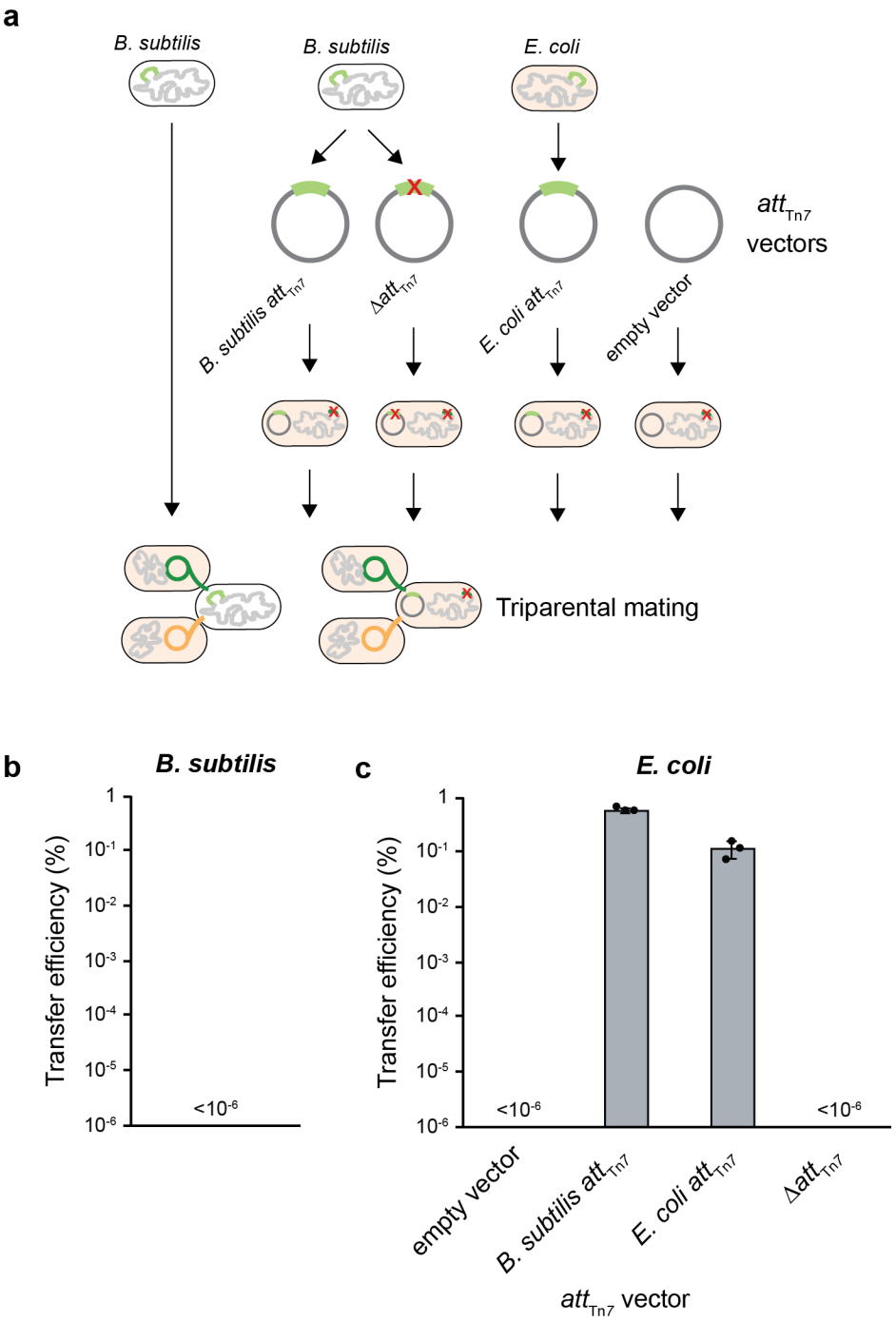

Fig. S3 | The *B. subtilis* att_{Tn7} site is not responsible for failure of Tn*7* methodology to transfer. a, Schematic of the transfer experiments. **b,** No transconjugants were obtained from mating a Tn*7* transposon with a *B. subtilis*-compatible kanamycin resistance marker into *B. subtilis*. **c**, Test to determine if the *B. subtilis* att_{Tn7} site is responsible for failure of Tn7

methodology to transfer. The *B. subtilis* att_{Tn7} site with ~1kb flanking DNA was cloned into a replicative plasmid and transformed into an *E. coli* strain in which integration into the chromosomal att_{Tn7} site is blocked. *E. coli* strains with variants of the attTn7 plasmid were used as recipients for a Tn7 transposon containing a kanamycin resistance marker. Kan^R transconjugants were obtained from matings with *E. coli* strains containing the *B. subtilis* att_{Tn7} site and *B. subtilis* flanking DNA with an *E. coli* att_{Tn7} site but were not obtained from an empty vector or *B. subtilis* flanking DNA with a precise deletion of att_{Tn7} (n=3), demonstrating that the *B. subtilis* att_{Tn7} site is compatible with Tn*7* integration and suggesting that other factors (such as transfer efficiency or transposon gene expression) are limiting. Data are represented as mean ± s.d.

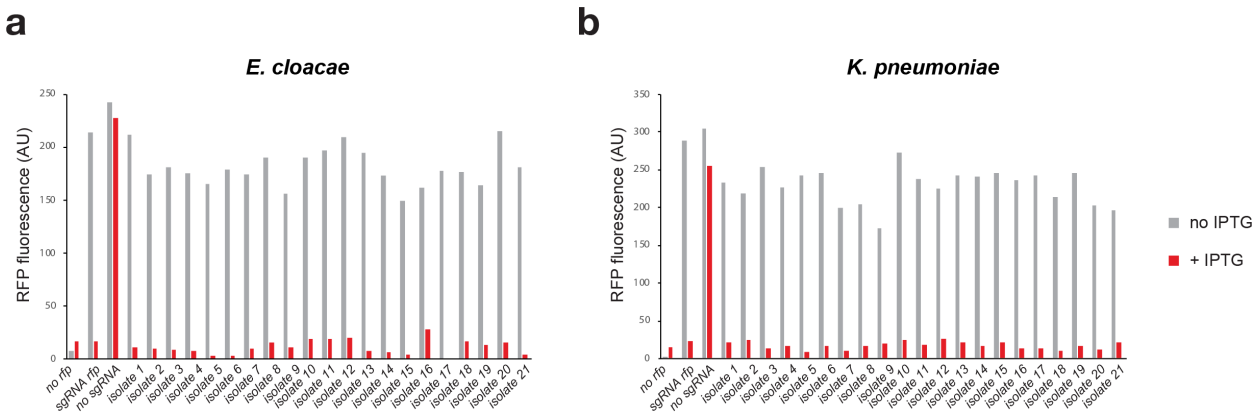

Fig. S4 | Mobile-CRISPRi knockdown remains active after non-selective growth. Mobile-CRISPRi knockdown of *rfp* after >50 generations of non-selective growth for **a,** *E. cloacae* **b,** *K. pneumoniae*. "*sgRNA rfp*" is a non-passaged positive control for knockdown.

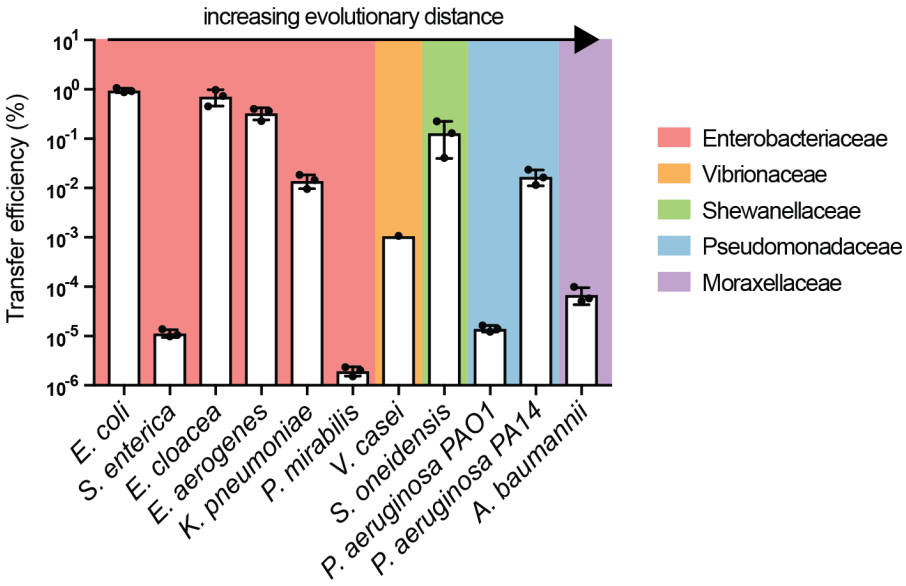

Fig. S5 | Tn*7* Mobile-CRISPRi transfer efficiency with increasing evolutionary distance from *E. coli*. Tn*7* Mobile-CRISPRi transfer efficiency varies significantly at the strain level (e.g., *P. aeruginosa* PAO1 vs PA14). Data are represented as mean ± s.d. (n=3, except *V. casei* for which n=1).

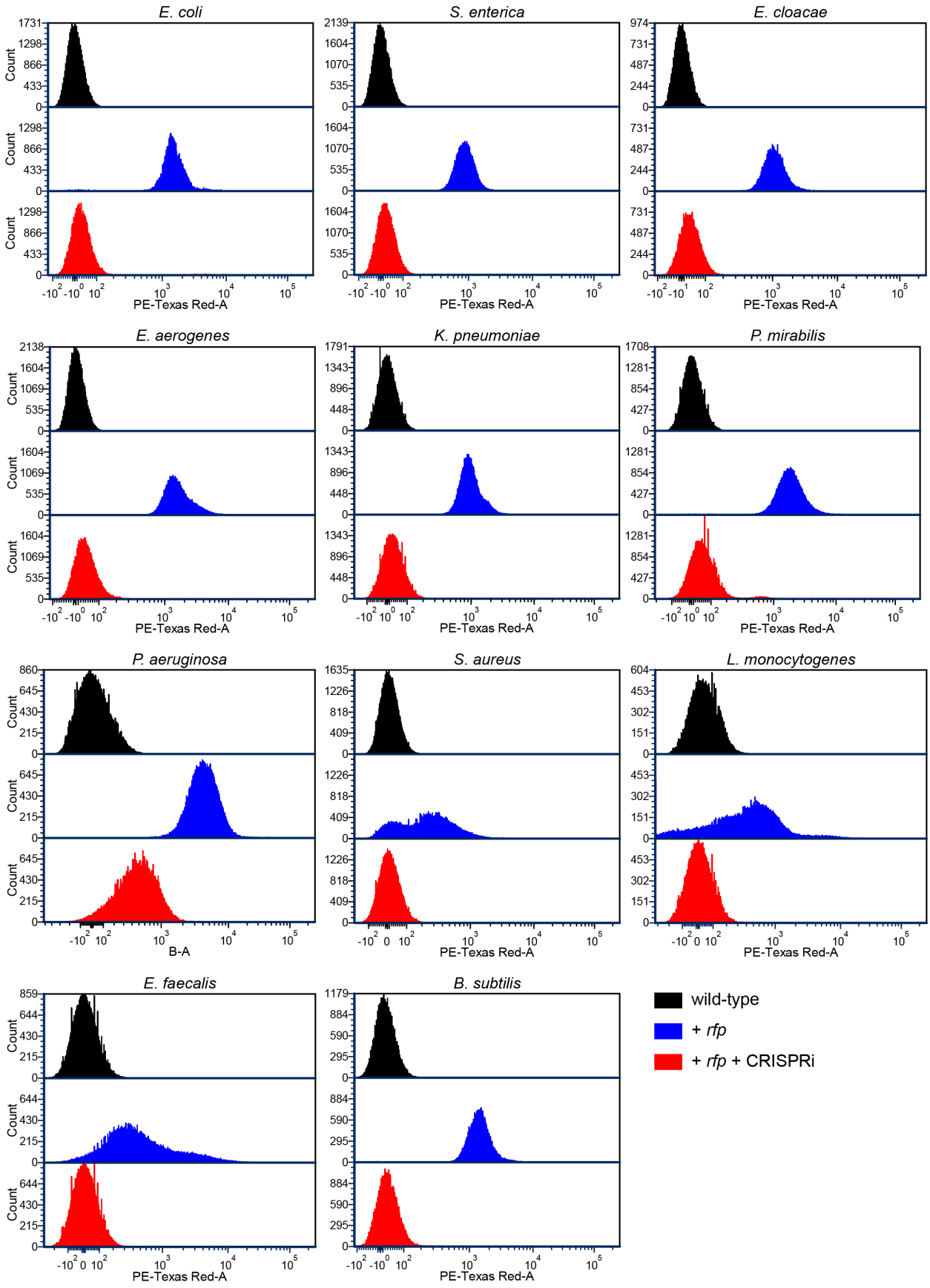
E. coli
S. enterica
E. cloacae
E. aerogenes
K. pneumoniae
P. mirabilis
P. aeruginosa
S. aureus
L. monocytogenes
E. faecalis
B. subtilis
Count
PE-Texas Red-A
B-A
wild-type
+ rfp
+ rfp + CRISPRi

Fig. S6 | Flow histograms for *rfp* knockdowns shown in Fig. 2c. "+ *rfp*" cells contain either no sgRNA or no *dcas9* (*P. aeruginosa*) and "+ *rfp* + CRISPRi" cells contain a functional CRISPRi system targeting *rfp*. *V. casei* and *A. baumanni rfp* knockdowns were measured by a plate reader and thus do not appear in the figure. These are representative histograms taken from experiments with four independent replicates, except *P. mirabilis* and *E. faecalis* for which n=3.

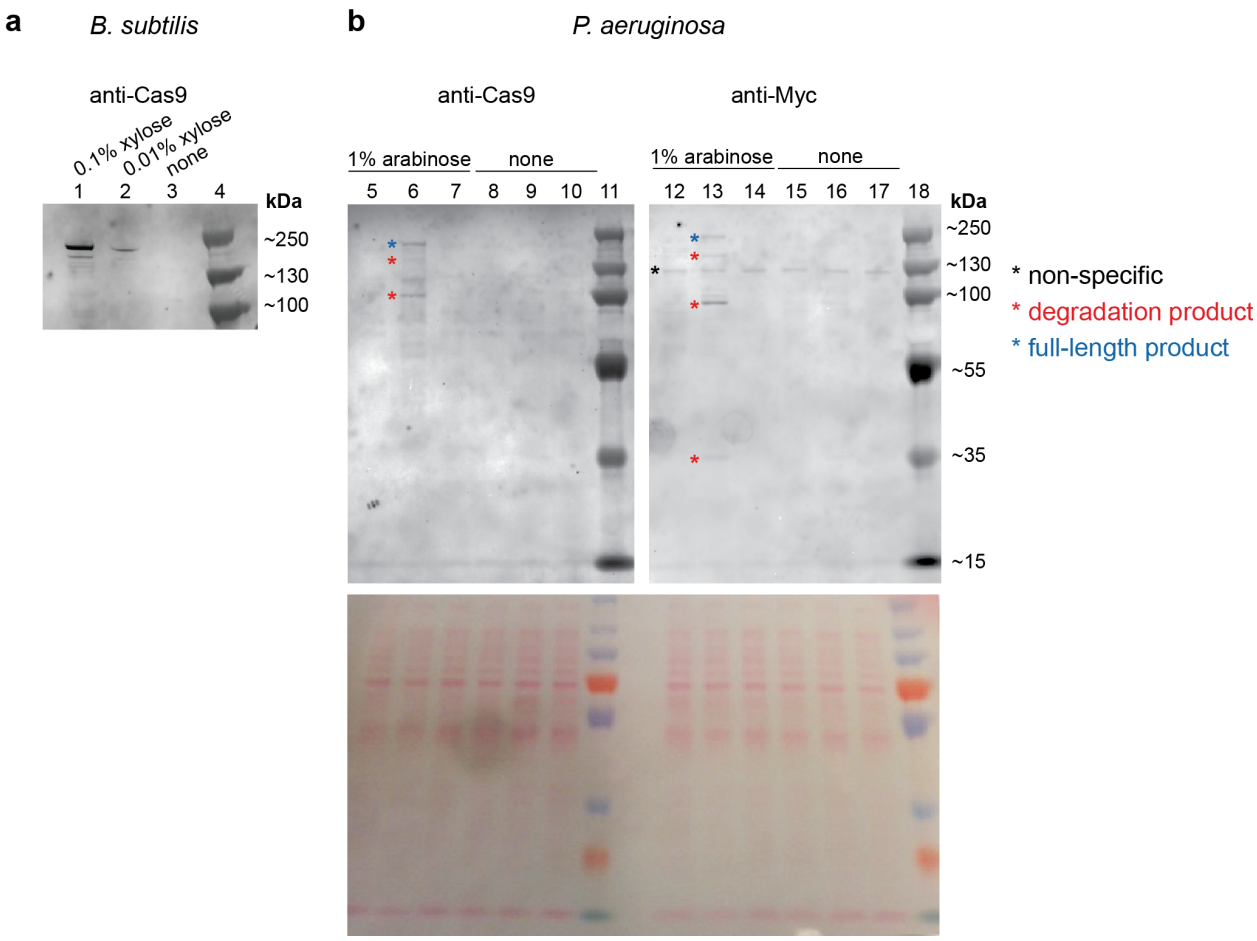

Fig. S7 | dCas9 is degraded in *P. aeruginosa*. Anti-dCas9 and anti-Myc (3X Myc tag was fused to the C-terminus of dCas9) western blots. Lanes: 1-2. S. pyogenes dCas9 expressed in *B. subtilis*; 3. no dCas9 in *B. subtilis*; 4. ladder; 5, 8, 12, and 15. GC codon optimized *S. pyogenes* dCas9 in *P. aeruginosa*; 6, 9, 13, and 16. Human codon optimized *S. pyogenes* dCas9 in *P. aeruginosa*; 7, 10, 14, and 17. No dCas9 in *P. aeruginosa*, and 11, and 18. ladder. The bottom gel is Ponceau stained and shows total protein. These experiments were independently repeated twice with similar results.

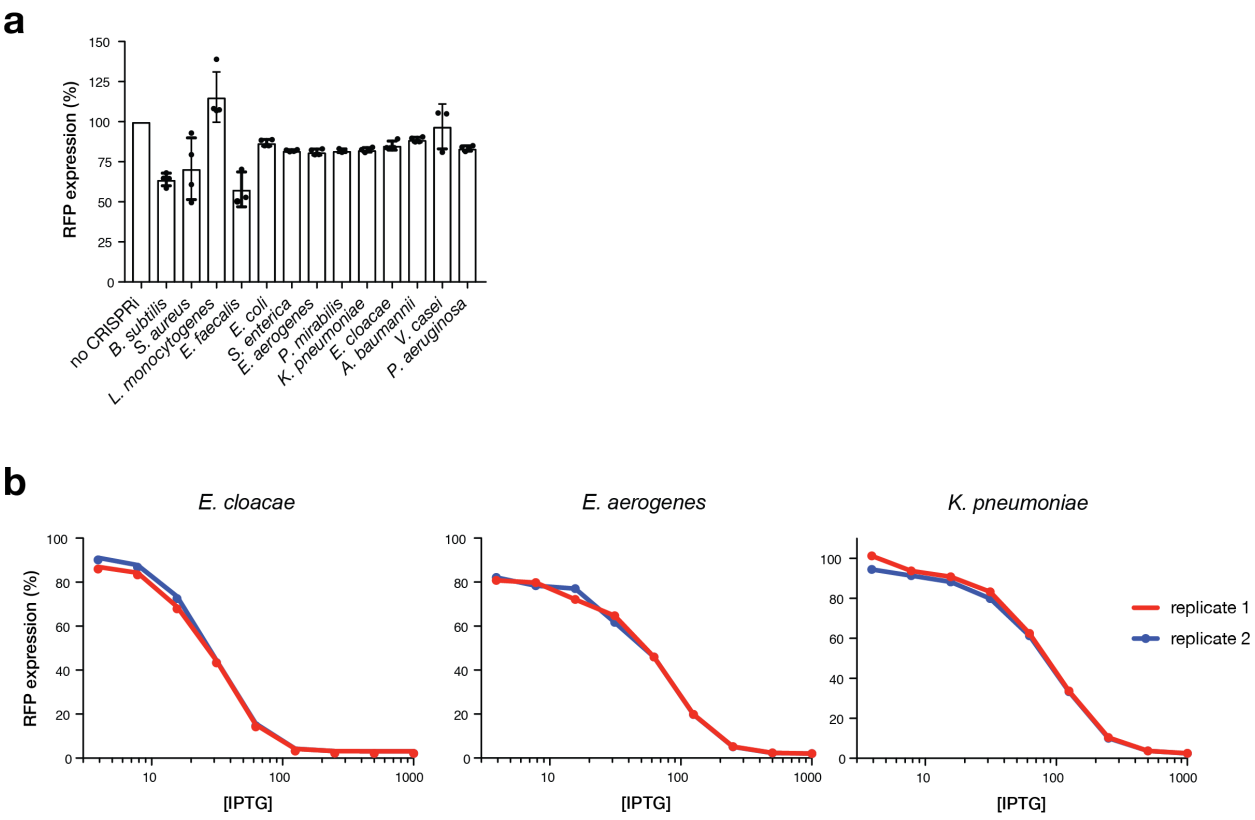

Fig. S8 | Mobile-CRISPRi shows mild uninduced knockdown and is titrable. a, Uninduced repression of *rfp* normalized to the no CRISPRi control for all strains shown in Fig. 2c. **b,** Mobile CRISPRi knockdown can be titrated with IPTG in *E. cloacae*, *E. aerogenes*, and *K. pneumoniae*. RFP expression is normalized to the no sgRNA control. n=4 for all strains except *E. faecalis*, *P. mirabilis*, and *V. casei* for which n=3. Data are represented as mean ± s.d.

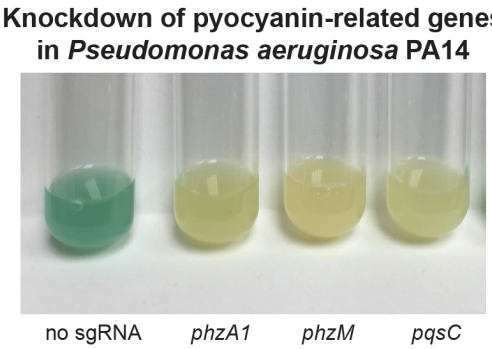

Fig. S9 | Mobile-CRISPRi knockdown of native genes in *P. aeruginosa*. Mobile-CRISPRi was used to target genes involved directly (*phzA1* and *phzM*) or indirectly in pyocyanin biosynthesis (*pqsC*). The loss of blue pigment indicates knockdown of the pyocyanin pathway. These experiments were independently repeated twice with similar results.

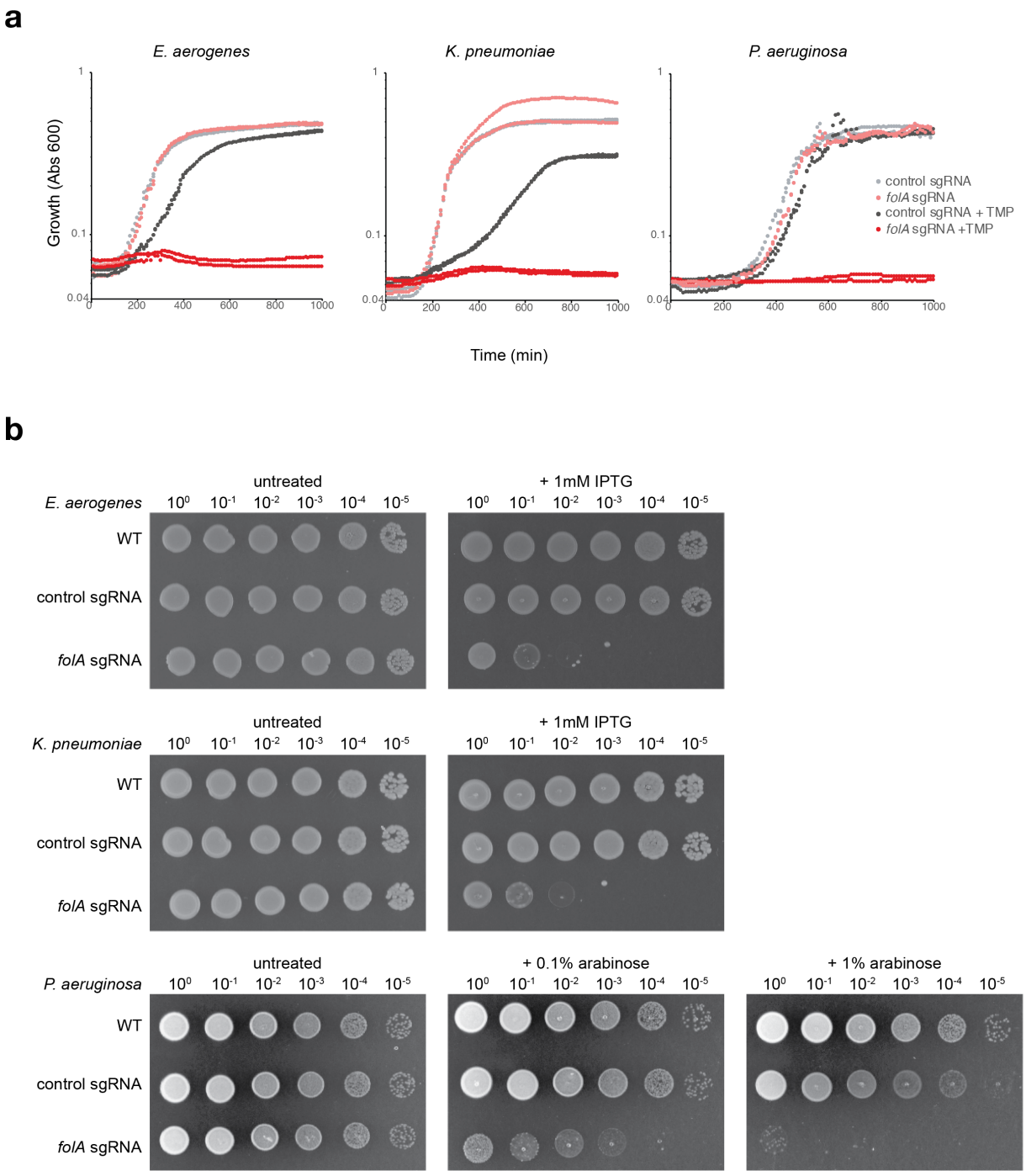

Fig. S10 | Mobile-CRISPRi reveals drug synergy and essentiality. a, Mobile-CRISPRi targeting *folA* or with a non-targeting control guide in various species were grown with or without trimethoprim (0.125, 0.5, and 8 μg/ml trimethoprim for *E. areogenes*, *K. pneumoniae*, and *P.*

aeruginosa, respectively), and with partial induction of CRISPRi (100 μM IPTG for *E. areogenes*, *K. pneumoniae*, 0.1% arabinose for *P. aeruginosa*); growth was monitored by absorbance at 600 nm. Two biological replicates are plotted for each strain. These experiments were independently repeated twice with similar results. **b,** Spotting dilution assays for Mobile-CRISPRi targeting the essential *folA* gene. CRISPRi knockdown was induced with IPTG (*E. aerogenes* and *K. pneumoniae*) or arabinose (*P. aeruginosa*). Full induction of *dcas9* in *P. aeruginosa* reduces growth (see the small colonies in "+ 1% arabinose) but retains plating efficiency. These experiments were independently repeated twice with similar results.

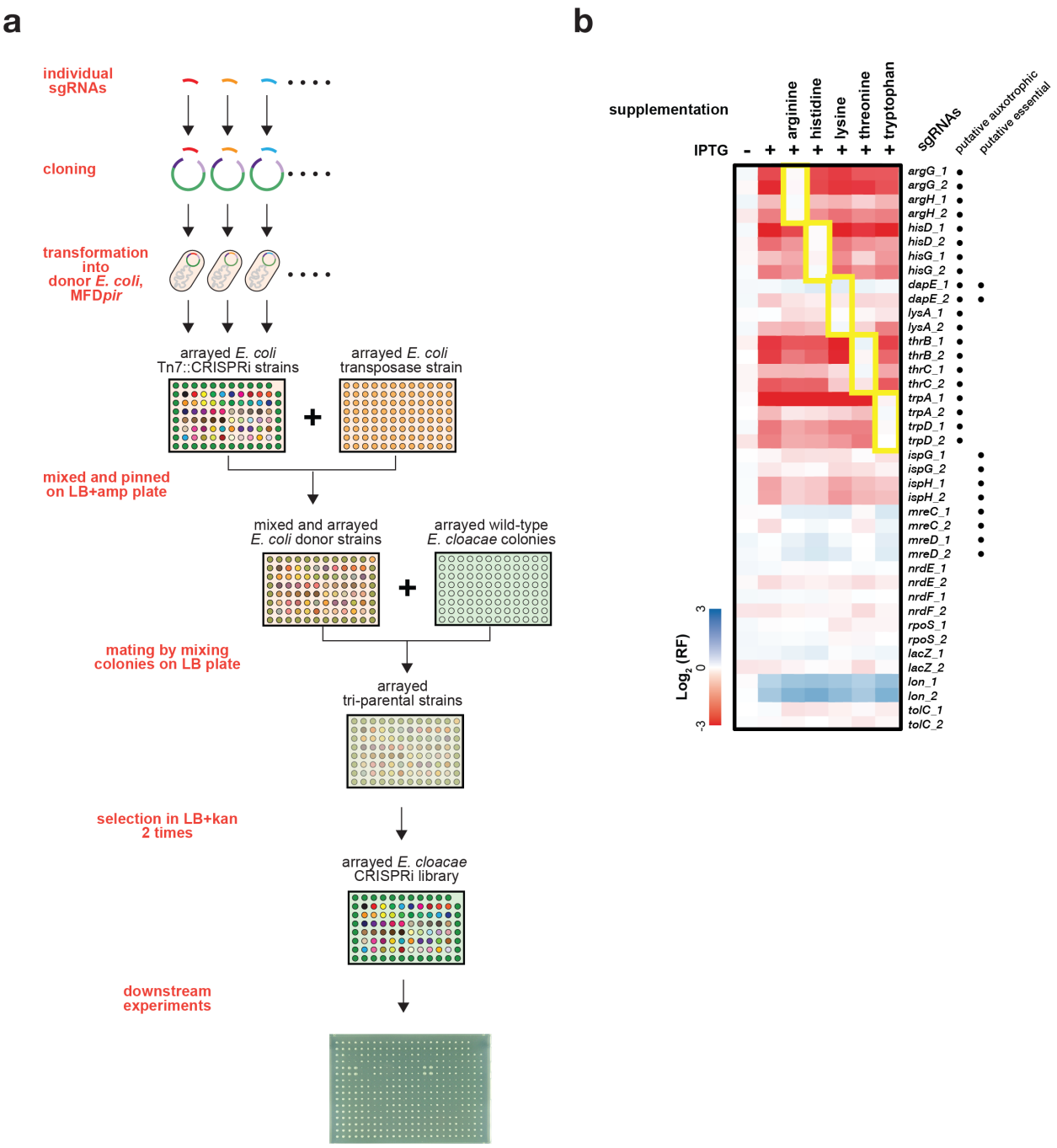

Fig. S11 | Validation of knockdown-induced auxotrophies by arrayed screen. a, Construction of an ordered CRISPRi library for *E. cloacae*. sgRNAs were cloned individually, transformed into the *E. coli* donor strain, MFD*pir*. Donor strains were arrayed in 96 well plate and then mating and selection were performed on LB agar plate using a Singer ROTOR robot. **b,** Heat map representation of relative fitness (RF) of 40 *E. cloacae* CRISPRi strains (y-axis) in

7 glucose minimal media conditions with various supplementation (x-axis). Yellow rectangles indicate complementation of auxotrophy of strains by relevant amino acids. Putative auxotrophic or essential gene knockdown strains are indicated next to sgRNA names.