Enabling genetic analysis of diverse bacteria with Mobile-CRISPRi

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The vast majority of bacteria, including human pathogens and microbiome species, lack genetic tools needed to systematically associate genes with phenotypes. This is the major impediment to understanding the fundamental contributions of genes and gene networks to bacterial physiology and human health. Clustered regularly interspaced short palindromic repeats interference (CRISPRi), a versatile method of blocking gene expression using a catalytically inactive Cas9 protein (dCas9) and programmable single guide RNAs, has emerged as a powerful genetic tool to dissect the functions of essential and non-essential genes in species ranging from bacteria to humans1–6. However, the difficulty of establishing effective CRISPRi systems across bacteria is a major barrier to its widespread use to dissect bacterial gene function. Here, we establish ‘Mobile-CRISPRi’, a suite of CRISPRi systems that combines modularity, stable genomic integration and ease of transfer to diverse bacteria by conjugation. Focusing predominantly on human pathogens associated with antibiotic resistance, we demonstrate the efficacy of Mobile-CRISPRi in gammaproteobacteria and Bacillales Firmicutes at the individual gene scale, by examining drug–gene synergies, and at the library scale, by systematically phenotyping conditionally essential genes involved in amino acid biosynthesis. Mobile-CRISPRi enables genetic dissection of non-model bacteria, facilitating analyses of microbiome function, antibiotic resistances and sensitivities, and comprehensive screens for host–microorganism interactions.

Clustered regularly interspaced short palindromic repeats interference (CRISPRi) is a programmable method for controlling gene expression that has enabled systematic interrogation of gene phenotypes in diverse organisms1–6. In bacterial CRISPRi, a single guide RNA (sgRNA)–catalytically inactive CRISPR-associated protein 9 (dCas9) complex binds to a target gene by base pairing and reduces gene expression by sterically blocking transcription elongation1,2 (Fig. 1a). New CRISPRi targets are easily programmed by substituting the first 20 nucleotides of the sgRNA sequence (spacer) to match the non-template strand of the target gene, making the design and construction of CRISPRi libraries that target specific sets of genes or the entire genome straightforward1,3. Genetic screens using CRISPRi libraries have contributed new insights to fundamental biology and molecular medicine, including identifying functions for uncharacterized essential genes7–9 and drug modes of action10.

CRISPRi provides several advantages over other methods for genetic manipulation in bacteria. CRISPRi knockdowns can be induced1–6 and titrated or tuned10,11, enabling depletion of essential gene products without complex strain construction strategies that remove genes from their native regulation. Dissecting genetic redundancy via multiplexed CRISPRi targeting of several genes in the same cell11 requires markedly less effort than construction of multiple-deletion strains. At the genome scale, CRISPRi expands on previous transposon–based gene perturbation methods, such as Tn-seq12, by allowing all genes—including essential genes that cannot be studied through deletion—to be systematically targeted so that a relatively small strain library provides comprehensive coverage of the genome. Moreover, the DNA sequences encoding sgRNAs serve as unique barcodes to differentiate CRISPRi strains mixed in a pool, allowing for competitive fitness measurements using next-generation sequencing13. CRISPRi blocks the expression of downstream genes in operons1,2, but this property can be used to further simplify libraries by targeting operons instead of genes.

Despite these advantages, CRISPRi has been used in only a few bacterial species both because CRISPRi has been transferred using species-specific or narrow host range strategies and because components need to be optimized for function in different species. To overcome this barrier, we developed ‘Mobile-CRISPRi’—a suite of modular and transferable CRISPRi components that can stably integrate into the genomes of diverse bacteria. The modularity of every component of Mobile-CRISPRi makes it straightforward to clone in organism-specific sgRNA libraries and other components (for example, promoters; Fig. 1b and Supplementary Figs. 1 and 2). Mobile-CRISPRi achieves transfer and genomic integration by distinct mechanisms for gammaproteobacteria and Firmicutes. For gammaproteobacteria, Mobile-CRISPRi is transferred from Escherichia coli using the broad host range RP4 plasmid conjugation machinery and is integrated into the recipient genome downstream of the highly conserved glmS gene using the extensively
characterized Tn7 transposition system\(^{4,11}\) (Fig. 1c, top). Consistent with previous reports\(^9\), this strategy was unsuccessful in Bacillales Firmicutes (Supplementary Fig. 3), leading us to transfer CRISPRi using an integrative and conjugative element in *Bacillus subtilis* (ICEBs1). Here, Mobile-CRISPRi is transferred from *B. subtilis* to other Bacillales Firmicutes (for example, *Staphylococcus aureus*) and integrated into *trnS-leu2* (ref. \(^9\)) (Fig. 1c, bottom). Notably, the ICEBs1 host range is probably broader than previously thought\(^9\). Critically, Mobile-CRISPRi integrations either downstream of *glmS* (Tn7) or into *trnS-leu2* (ICEBs1) do not disrupt those gene functions\(^4,11\), occur in a specified orientation and are stable (Fig. 2a) and functional (Supplementary Fig. 4) in the absence of selection for \(\geq 50\) generations, enabling studies of gene and antibiotic function in which maintaining selection is problematic or impossible.

We assessed the efficacy of Mobile-CRISPRi in multiple bacterial species, focusing on those involved in human disease. We first measured CRISPRi transfer by quantifying the number of recipient colonies (that is, transconjugants) on selective agar plates as a fraction of total recipients (from non-selective plates; Fig. 2b). Most species showed transfer efficiencies sufficient for genome-scale sgRNA library construction (for example, *Enterobacter* sp. \(\sim 10^{-2} - 10^{-3}\), and *Listeria monocytogenes* \(\sim 10^{-2}\), but some were more suited for single-gene knockdown approaches (for example, *Acinetobacter baumannii* \(\sim 10^{-4}\)). Transfer efficiencies can also vary significantly at the strain level (Supplementary Fig. 5).

We then assessed CRISPRi knockdown efficacy with a ‘test’ version of Mobile-CRISPRi consisting of *rfp* (encoding red fluorescent protein (RFP)) and either an sgRNA targeting *rfp* (to measure knockdown) or lacking an sgRNA (a control to normalize *rfp* expression). Quantification of *rfp* knockdown in single cells using flow cytometry indicated that knockdown efficiency ranged from \(\sim 8\)-fold in *Pseudomonas aeruginosa* (possibly due to dCas9 degradation; Supplementary Fig. 7) to \(\sim 150\)-fold in *S. aureus*, with a median knockdown of \(\sim 40\)-fold across all measured species (Fig. 2c...
and Supplementary Fig. 6). In addition, knockdown was titratable (Supplementary Fig. 8). We confirmed that CRISPRi was also functional against native genes by targeting P. aeruginosa pyocyanin production (Supplementary Fig. 9). In this visual assay, the culture appears yellow because the production of the blue pigment pyocyanin has decreased.

Our initial assessment of Mobile-CRISPRi used pathogenic strains possessing at least rudimentary tools for perturbing gene function. To determine whether Mobile-CRISPRi functions in an environmental isolate with no existing genetic system, we tested transfer and knockdown in V. casei, a gammaproteobacterium originally isolated from French wash-rind cheeses and broadly associated with cheese microbiomes18. We found that Mobile-CRISPRi is an effective genetic tool for gene knockdown in various species. Knockdown was tested using a Mobile-CRISPRi variant containing a constitutively expressed rfp reporter and an sgRNA targeting rfp. RFP expression was normalized to a strain lacking either dcas9 (for P. aeruginosa) or an sgRNA (all others; no sgRNA controls are recommended for future experiments). $n = 4$ for all strains except E. faecalis, P. mirabilis and V. casei for which $n = 3$. Data are represented as the mean ± s.d. The species for the strains used in panels b and c and their corresponding rfp fold knockdowns are: B. subtilis (182-fold), S. aureus (150-fold), L. monocytogenes (105-fold), E. faecalis (45-fold), E. coli (65-fold), E. cloacae (32-fold), E. aerogenes (40-fold), P. aeruginosa (8-fold), K. pneumoniae (34-fold), V. casei (8-fold), A. baumannii (10-fold), Salmonella enterica (54-fold) and P. mirabilis (35-fold). Dashed grey line in a and c indicates 100% stability (a) and 100% RFP expression (c).

We tested whether Mobile-CRISPRi could be used to explore MOA in pathogenic gammaproteobacteria associated with antibiotic resistance (that is, Gram-negative rods). We expected that strains with a small reduction in expression of the direct target of an antibiotic would be sensitized to low concentrations of that antibiotic (that is, drug–gene interaction or synergy). Partial knockdown of the essential gene, folA, which encodes the trimethoprim target dihydrofolate reductase19, increased sensitivity to trimethoprim, shifting the minimal inhibitory concentration (MIC) by 2–4-fold, depending on the species (Fig. 3 and Supplementary Table 7), indicating synergy. Even though CRISPRi knockdown in P. aeruginosa was at a lower efficiency than other strains (Fig. 2b), there was still a clear shift towards sensitivity (Fig. 3). Moreover, concentrations of trimethoprim below the MIC for the wild-type strain completely inhibited growth of the folA knockdown strains, clearly demonstrating synergy (Supplementary Fig. 10). As expected for an essential gene, fully induced CRISPRi targeting folA was lethal in Enterobacter aerogenes, Klebsiella pneumoniae and P. aeruginosa (Supplementary Fig. 10). We conclude that Mobile-CRISPRi targeting essential genes can be used to generate sensitized strains for antibiotic MOA studies.

A compelling feature of CRISPRi is the ease of pooled knockdown library construction, either for defined gene sets or at the genome scale1. As a proof of principle, we construct a 40-member library of selected Enterobacter cloacae genes (Supplementary Tables 1 and 2). In the pooled context, each sgRNA functions as a barcode, enabling quantification of each knockdown strain in the pool. We first evaluated strain representation in our pipeline by performing two different pooled experiments. In the first, all steps from initial cloning to analysis were performed in a pool (Fig. 4a). This revealed that all sgRNA strains were present and had reasonable representation in the pool (31 out of 40 sgRNA counts were within one standard deviation (s.d.) of the median, with a maximum 50-fold difference in representation). In the second, each sgRNA plasmid was constructed individually and an equimolar mixture of plasmids was used to transform E. coli and perform downstream steps (Fig. 4b).
Fig. 3 | CRISPRi knockdown of folA increases sensitivity to trimethoprim in multiple species. MIC assays for trimethoprim sensitivity in E. aerogenes (left), K. pneumoniae (middle) and P. aeruginosa (right) with or without Mobile-CRISPRi targeting folA (n = 3). Data are represented as the mean ± s.d.

Fig. 4 | A Mobile-CRISPRi library targeting auxotrophic genes in E. cloacae. a, Tn7 Mobile-CRISPRi library construction. sgRNAs were cloned as a pool, transformed and mated into E. cloacae. b, sgRNAs were cloned individually, mixed as a pool with equal representation and mated into E. cloacae as a pool. Representation of individual CRISPRi strains was determined by Illumina sequencing. c, Fitness of CRISPRi strains in glucose minimal media after 6 or 12 doublings with or without CRISPRi induction by IPTG, determined from the library constructed by pooled cloning (n = 2). The asterisks indicate that strains had no fitness change in the pooled screen, but decreased fitness in the arrayed screen. Putative auxotrophic or essential gene knockdown strains are indicated next to the sgRNA names. d, Correlation between fitness measurements in libraries constructed by pooled cloning or individual cloning (n = 2); a linear fit is shown.
This assessed the variability of all steps downstream of cloning and revealed a maximum twofold difference in representation. Thus, Mobile-CRISPRi transfer and integration is highly uniform, with essentially all variability derived from the initial cloning step. Further optimization or alternative cloning strategies may decrease the variability in sgRNA representation.

We next evaluated the fitness of our strain library, which includes knockdowns of ten amino acid biosynthesis genes, four putative essential genes and six well-characterized genes, each targeted by two sgRNAs (Supplementary Table 1). We grew our library in glucose minimal medium in competition with a 100-fold excess of wild-type E. coli and then measured the relative frequency of each strain in the library after 6 and 12 generations with or without CRISPRi induction to initiate knockdown. Using the fitness calculation of van Opijnen et al., we found that the fitness of strains with sgRNAs targeting amino acid biosynthesis and those targeting some putative essential genes decreased, whereas representation of non-essential genes that are unrelated to amino acid biosynthesis remained constant (Fig. 4c and Supplementary Table 2). The fitness for affected strains was more pronounced at 12 doublings than at 6 doublings, suggesting that a larger number of generations was required to detect existing protein products. In addition, both guides generally decreased the fitness of the essential and auxotrophic genes, but with more variability than previously observed. Finally, the fitness measurements from the completely pooled construction (Fig. 4a) and those in the equal representation library (Fig. 4b and Supplementary Table 2) were highly correlated (R² = 0.92), indicating that the initial frequency of the strain in the pooled library did not affect the measurement of the fitness (Fig. 4d).

We also screened an arrayed library of each individual knockdown strain. Importantly, we confirmed the auxotrophy of amino acid biosynthesis knockdown strains, finding that their poor growth in minimal medium was suppressed by relevant amino acids (Supplementary Fig. 11 and Supplementary Table 3). Thus, knockdown effects are specific to the targeted gene and do not represent off-target effects of CRISPRi. We found that knockdown of some putative essential genes (for example, mreD) showed no apparent phenotype, possibly because of limited growth after induction or low sgRNA efficacy. We conclude that Mobile-CRISPRi enables both pooled and arrayed library construction and straightforward assaying of phenotypes.

We anticipate that Mobile-CRISPRi will be a transformative technology for non-model bacteria lacking genetic tools and will facilitate cross-species genetic analysis, complementing existing Tn- seq technology. The advantages of CRISPRi over other technologies is its ability to interrogate essential genes and double-mutant combinations, as well as construction of parsimonious genome-scale knockdown libraries. Mobile-CRISPRi transfer and knockdown systems bring this technology to a broad array of organisms and its modularity makes it straightforward to expand host range (for example, combining different transfer and integration functions, and anti-restriction proteins) and increase knockdown efficiency (for example, use of ‘alternative’ dCas9 genes). The stability of Mobile-CRISPRi in the absence of selection suggests that it could be a valuable tool for dissecting the genetics of host–microorganism interactions in both pathogenic and microbiome contexts, and aid in MOA studies in relevant human pathogens. Interestingly, our approach for transferring CRISPRi mirrors the natural transfer of CRISPR systems by transposons related to Tn7 (ref. 23). We will continue to look to nature for future approaches to dissect the vast landscape of bacterial genetics.

Methods

Construction of the Mobile-CRISPRi vectors. A complete list of Mobile-CRISPRi vectors can be found in Supplementary Table 4. All plasmids were constructed by restriction enzyme digestion of vector DNA followed by either ligation or NEBuilder HiFi DNA Assembly with insert DNA (all enzymes were purchased from NEB). To generate the Mobile-CRISPRi vectors, the pUC origin of replication in the Tn7 transposon plasmid pT7 was replaced with the B6K origin that requires the tetracycline-resistance (encoded by the tetr gene) for expression. We engineered pT7-based plasmids pMP1050, pMP1055, and a backbone for all Tn7-based Mobile-CRISPRi derivatives. Derivatives were constructed by inserting components into the following modules/restriction sites: antibiotic markers/XhoI, reporter genes (for example, rfp)/PMel, sgRNA promoters and sgRNAs/EcoRI, sgRNA spacers (for creating sgRNA libraries)/BsAI, regulatory genes (for example, lacI)/Smal, dCas9 promoters and ribosome-binding sites/Spl, and dCas9/Spl-Ascl. To create a Mobile-CRISPRi plasmid that integrates into the ICEB1 element, two ~1-kb DNA fragments flanking the rapI gene were amplified from B. subtilis 168 genomic DNA and used to replace the Tn7 transposon ends in a pMP1055 derivative (pMP1096), generating pMP1290. pMP1290 served as a base for all ICE-based Mobile-CRISPRi derivatives and has the same unique restriction sites listed for the modules above. sgRNAs were cloned into the BsaI site of Mobile-CRISPRi derivatives by ligating annealed oligos. Oligos were designed to include overlaps that were complementary to the sticky ends generated by BsaI. Oligos were added to 1× NEB bufer 4 at 5 M sodium concentration, denatured for 5 min at 95 °C and then annealed by transferring the reactions to room temperature. Annealed oligos were then diluted 1:20 and 2 µl of the dilution was ligated to 100 ng BsaI-digested vector for 1 h at room temperature. sgRNAs were designed as previously described.

Construction of the Mobile-CRISPRi strains and mating assays. A complete list of constructs used in the study can be found in Supplementary Table 5. Tn7-based Mobile-CRISPRi strains were constructed by tri-parental or quad-parental mating as previously described in Choi et al., with several modifications. All Tn7 sites, generated MFdCas9 (a p65 gene that is dependent on diaminopimel (DAP) for growth and contains the RP4 transfer machinery) were inoculated with a Tn7 transposable plasmid (pMP1039; a derivative of pTNS3 (ref. 24)) with a spontaneous small deletion upstream of the P. promoter) to a transposon plasmid (various pMP1055 derivatives) as mating donors. Matings with B. amunii ATCC19606 required the presence of a third donor strain containing the self-mobilizing RP4 transfer plasmid pRK2013 (ref. 25) for unknown reasons. Cultures of the two E. coli donor strains (transposon and transposase donors) were grown overnight (~16 h) at 37 °C in lysogenic broth (LB) + 300 µg M DAP (Alfa Aesar B22391) + 100 µg ampicillin. Recipient strains assayed here also grew to saturation in LB after incubation at 37 °C for ~16 h. Each donor and recipient strain, Tn7, was added to 700 µl LB and mixed by pipetting. Mixes of donor and recipient strains were pelleted for 2 min at 7,000 g, washed twice with 1 ml LB, resuspended in 30 µl LB after the final wash, pipetted onto a cellulose filter (MF-Millipore HAWG01300) placed on a pre-warmed LB + 300 µg M DAP plate and incubated at 37 °C for ~6 h. Filters were then transferred to microcentrifuge tubes containing ~100 µl PBS and vortexed briefly. Cells were incubated at 37 °C on a shaking platform until they reached the density that the cells could use for induction into media that select for the Mobile-CRISPRi plasmid and recipient (for example, LB + kanamycin) without DAP (the absence of DAP will select against donor E. coli). Antibiotic concentrations used for selection were: 30 µg ml−1 kanamycin and 30 µg ml−1gentamicin (for P. aeruginosa). ICE-based Mobile-CRISPRi strains were constructed by bi-parental mating as previously described.2–3 With these modifications, ICE donor strains were generated by transformation of B. subtilis with Mobile-CRISPRi integration plasmids using natural competence as previously described. Expression of the ICE anti-repressor RapI induces conjugation genes found on the ICE element and promotes excision.3 Neutralization of the large insert size of Mobile-CRISPRi plasmids resulted in very few transformants. To produce a strain with a stable ICE element in the presence of an isopropyl-β-D-thiogalactoside (IPTG)-inducible rapI gene that transformed at high efficiency, a dCas9 gene linked to a chloramphenicol-resistance marker was integrated into ICE—selection for the chloramphenicol marker and the extra homology present in the dCas9 gene improved transformation efficiency. For mating, one 3 ml LB culture of each donor and recipient strain were inoculated with 100 µl LB and mixed by pipetting. Mixes of donor and recipient strains were pelleted for 2 min at 7,000 g, washed twice with 1 ml LB, resuspended in 30 µl LB after the final wash, pipetted onto a cellulose filter (MF-Millipore HAWG01300) placed on a pre-warmed LB + 300 µg M DAP plate and incubated at 37 °C for ~6 h. Filters were then transferred to microcentrifuge tubes containing ~100 µl PBS and vortexed briefly. Cells were incubated at 37 °C on a shaking platform until they reached the density at which they could use for induction into media that select for the Mobile-CRISPRi plasmid and recipient (for example, LB + kanamycin) without DAP (the absence of DAP will select against donor E. coli). Antibiotic concentrations used for selection were: 30 µg ml−1 kanamycin and 30 µg ml−1gentamicin (for P. aeruginosa).

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transconjugants (antibiotic-resistant Dap+ colonies for Tn7 matings and KanR StrR colonies for ICE matings) to viable cells (LB colonies for Tn7 matings and StrRe colonies for ICE matings). For Tn7 transfer to the B. subtilis attTn7 site in E. coli (Supplementary Table 1), the native attTn7 site at 12-DH10B was occupied by an unmarked Tn7 to prevent chromosomal transposition, whereas test attTn7 sites were cloned onto a chloramphenicol-resistant plasmid.

Mobile-CRISPRi stability assays. Four independently generated isolates (n = 4 isolates) of E. coli K-12 BW25133 and B. subtilis 168 containing Mobile-CRISPRi systems targeting rfp were grown to saturation at 37 °C in LB + kanamycin (30 µg ml⁻¹) for E. coli, E. cloacae and K. pneumoniae and 7.5 µg ml⁻¹ for B. subtilis) to select for retention of the Tn7 or ICE element containing CRISPRi. Each isolate was transformed at 6000 for 3 min and washed twice with LB to remove any residual kanamycin. The cells were then diluted 1:1000 in LB and grown to saturation. The procedure of dilution and growth to saturation was repeated a total of 5 times for ~50 generations of growth. Cells were then serially diluted and plated on selective (LB + kanamycin) and non-selective (LB) plates. The ratio between colony counts on LB and LB + kanamycin was used to determine the fraction of cells that retained the Tn7 or ICE element.

RFP knockdown assays. RFP knockdown was measured using flow cytometry or a plate reader (for A. baumannii and V. casei; n = 4 independently constructed isolates for all strains except Proteus mirabilis (n = 3 isolates) and V. casei (n = 3 isolates)). Flow cytometry was performed by diluting overnight cultures of Mobile-CRISPRi reporter strain in fresh medium (LB for all gammaproteobacteria and B. subtilis, brain heart infusion broth for S. aureus) containing CRISPRi inducer (1 mM IPTG for all gammaproteobacteria except P. aeruginosa, 1% arabinose for P. aeruginosa, and 0.1 µg ml⁻¹ anhydrotrehalose for Firmicutes) and incubating cultures at 37 °C with rotation until the cultures reached mid-log phase (A₅₇₀ nm = 0.3–0.6). Cultures were then crosslinked with 1% formaldehyde (final) for 10 min, followed by quenching for 10 min with 0.5 µg ml⁻¹ (final) crosslink. Cells were then diluted 1:10 in PBS and loaded on a 1D LSRRI using a 610/20 BP filter (PE-Texas-Red fluorochrome). Data for at least 10,000 cells were collected for four independently constructed strain isolates. In all cases, data for 100% of the cells collected were used in the analysis. For V. casei, overnight cultures were normalized to 2.0 A₅₇₀ nm and then diluted 1:200 in LB with or without 0.5 mM IPTG. After 6 h of growth post-induction, the strains were normalized to 0.2 A₅₇₀ nm and washed once in 1× PBS. The samples were then transferred to a 96-well plate (200 µl in each well) in triplicate and measured for dsRed fluorescence (excitation: 557 nm; emission: 592 nm) using a bottom-read plate reader (Tecan). For A. baumannii, overnight cultures were diluted 1:10,000 in fresh LB with or without 0.1 mM IPTG. Cells were grown in a 96-well plate with measurements of A₅₇₀ nm and RFP every 10 minutes. The values reported reflect the RFP knockdown at mid-log growth. The values reported are the mean values and the error bars reflect the s.d. from the mean.

Pyocyanin knockdown assays. Strains were grown overnight in Kings Medium A Base (HiMedia M1543) to induce pyocyanin and pyorubin production and 1% arabinose to fully induce dcas9 expression. Growth was repeated three times (n = 3); representative results are shown.

Antibiotic sensitivity assays. MIC assays were performed using the broth microdilution method as previously described, except that 0.1% arabinose for P. aeruginosa and 0.5% glycerol and stored at -80 °C. The average fitness from two biological replicates (n = 2) is presented in Supplementary Table 2. Pearson’s r was calculated from a linear fit of the data using Excel.

Ordered average Mobile-CRISPRi libraries for E. cloacae were constructed by following the procedure for single-gene CRISPRi strain construction with modifications for automation (Supplementary Fig. 1). Each donor Tn7-CRISPRi strains were prepared by transformation of individually cloned plasmids into the MFDpir strain and arrayed in a 96-well plate. An equal amount of transposase strain was added to each well and pinched to a LB + 300µg MDA 2% agar plate using a Singer ROBOT robot (four technical replicates per plate). Secondary PCR was performed with the plate, which was incubated for 6h. Kanamycin-resistant E. cloacae CRISPRi strains were selected on LB supplemented with kanamycin two times and stored at -80 °C as a glycerol stock. To screen the growth phenotype of each strain, cells were added to glycerol stocks onto rectangular LB agar plates in 384-format using a Singer ROBOT robot (four technical replicates on one plate in this screen). For each screen, exponentially growing cells in 384-format were then pinned to defined media plates and incubated for 16h at room temperature to avoid mucoid colony formation. Plates were imaged using a Powereshot G10 camera (Canon) at a time point at which fitness differences were apparent but growth had not saturated. The calculation of relative fitness was carried out as described in Koo et al. with minor modifications. Relative fitness was measured by the colony opacity of each mutant determined with Iris colony-sizing software. The relative fitness of each mutant was calculated as: Relative fitness = (average colony opacity of CRISPRi strain)/(average colony opacity of CRISPRi with no sgRNA strain); knockdown strains were grown in quadruplicate (n = 4). The average relative fitness calculated from two of the same media plates is presented in Supplementary Table 3.

dCas9 western blot. Cultures of P. aeruginosa were diluted back from the stationary phase and grown to saturation in the presence or absence of 1% arabinose at 37 °C and then 1 mL culture was added to 2.5 mL 5x SDS-PAGE sample buffer and boiled at 100 °C for 10 min before storage. Samples were boiled at 90 °C for 2 min before running on a 10% Bis-Tris Plus gel (Thermo Fisher) alongside the PagerRuler Plus Protein Ladder (Thermo Fisher) at 150 V for 1 h. Proteins were transferred to a nitrocellulose membrane (0.45 µm; Bio-Rad) at 100 V for 2 h at 4 °C using the Mini Trans-Blot Cell system (Bio-Rad). Protein amounts were checked by Ponceau staining (0.1% Ponceau S; 5% (v/v) acetic acid) for 25 min at room temperature, followed by washing in H₂O to destain. Membranes were blocked in Odyssey Blocking Buffer (Li-cor) at 4 °C overnight. Each primary antibody (anti-CRISPR-Cas9 (191468; Abcam) and Myc (9E10; Santa Cruz Biotechnology)) was used at 1:1,000 in PBS + 0.05% Tween 80 + 3% BSA at room temperature for 2h. Secondary antibody (IRDye 680RD goat anti-mouse IgG) was used at 1:10,000 in Odyssey Blocking Buffer (Li-cor) at room temperature for 1 h. All membrane washes were performed with PBS + 0.05% Tween 80. Blots were imaged on a Li-cor Odyssey Aries at 700 nm.

Data collection. Flow cytometry data were collected using BD FACSDIVA v8.0.1.

Data analysis. Data analysis was performed in Galaxy v18.01 (pooled sequencing data), FlowJo v10.4.2 and FCS Express 6 Plus (flow cytometry data), GraphPad Prism 7.0e (graphing and statistical analysis) and Excel v16.12. The plasma sediment maps were created using SnapGene v3.1.4.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. The sgRNA design code is available from GitHub (https://github.com/tracki/sgRNA_design).
Data availability
The data that support the findings of this study are available from the corresponding authors upon request.

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Author contributions
J.M.P., B.-M.K., M.M.H., A.D.G., J.E.P., I.N.E., R.J.D., C.A.G. and O.S.R. designed the study. J.M.P., B.-M.K., R.P., G.E.H., C.C.H., Y.F.I., C.H.S.L., J.Q. and M.R.S. performed the experiments. J.M.P., B.-M.K., R.P., G.E.H., Y.F.I. and J.S.H. analysed the data. J.M.P., B.-M.K., H.O., C.A.G. and O.S.R. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Software and code

Policy information about availability of computer code

Data collection Flow cytometry data was collected using BD FACSDIVA v8.0.1.

Data analysis sgRNA design code is available from Github (https://github.com/traeki/sgrna_design). Data analysis was performed in Galaxy v18.01 (pooled sequencing data), FlowJo v10.4.2 and FCS Express 6 Plus (flow cytometry data), GraphPad Prism 7.0e (graphing and statistical analysis) and Excel v16.12. Plasmid sequence maps were created using SnapGene v3.1.4. Iris was used to measure colony sizes from plate images (https://github.com/critichu/Iris).

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Sample sizes of three to four biological replicates are standard for the types of experiments shown here. Experiments with two biological replicates showed strong agreement. The use of three to four replicates is standard in the field of molecular microbiology.

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No data were excluded.

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In all cases except the library construction experiments, replicate data were from independently constructed strains and showed excellent agreement.

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Materials & experimental systems

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Methods

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| ☒   | Flow cytometry |
| ☒   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials are available upon request.

Antibodies

Antibodies used

Anti-CRISPR-Cas9 (AbCam 191468), c-Myc (Santa Cruz Biotechnology 9E10), IRDye 680RD Goat anti-Mouse IgG

Validation

Each primary antibody (Anti-CRISPR-Cas9 (AbCam 191468) and c-Myc (Santa Cruz Biotechnology 9E10)) were used at 1:1000 in PBS + 0.05% Tween80 + 3% BSA at room temperature for 2hr. Secondary antibody (IRDye 680RD Goat anti-Mouse IgG) was used
at 1:10000 in Odyssey Blocking Buffer (Licor) at room temperature for 1hr. Antibodies were validated by comparing western blot results to strains with no epitope.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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Methodology

Sample preparation
Bacterial cells from a mid-log culture were treated with 1% formaldehyde, diluted 1:10 in PBS, and run on the flow cytometer.

Instrument
BD LSRII

Software
Flow cytometry analysis was performed using FlowJo v10.4.2, FCS Express 6 Plus, and Excel v16.12.

Cell population abundance
RFP levels were measured for at least 10,000 cells and 100% of the data collected was used in the analysis.

Gating strategy
No gating strategy was used. Flow cytometry was only used to measure RFP levels in bacterial cells, not to separate out subpopulations for further analysis. Supplemental Figure 6 shows histograms of RFP levels (Texas-red or B-A) for all cells collected.

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