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RESEARCH ARTICLE

Modulating Pathogenesis with Mobile-CRISPRi

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ABSTRACT Conditionally essential (CE) genes are required by pathogenic bacteria to establish and maintain infections. CE genes encode virulence factors, such as secretion systems and effector proteins, as well as biosynthetic enzymes that produce metabolites not found in the host environment. Due to their outsized importance in pathogenesis, CE gene products are attractive targets for the next generation of antimicrobials. However, the precise manipulation of CE gene expression in the context of infection is technically challenging, limiting our ability to understand the roles of CE genes in pathogenesis and accordingly design effective inhibitors. We previously developed a suite of CRISPR interference-based gene knockdown tools that are transferred by conjugation and stably integrate into bacterial genomes that we call Mobile-CRISPRi. Here, we show the efficacy of Mobile-CRISPRi in controlling CE gene expression in an animal infection model. We optimize Mobile-CRISPRi in *Pseudomonas aeruginosa* for use in a murine model of pneumonia by tuning the expression of CRISPRi components to avoid nonspecific toxicity. As a proof of principle, we demonstrate that knock down of a CE gene encoding the type III secretion system (T3SS) activator ExsA blocks effector protein secretion in culture and attenuates virulence in mice. We anticipate that Mobile-CRISPRi will be a valuable tool to probe the function of CE genes across many bacterial species and pathogenesis models.

IMPORTANCE Antibiotic resistance is a growing threat to global health. To optimize the use of our existing antibiotics and identify new targets for future inhibitors, understanding the fundamental drivers of bacterial growth in the context of the host immune response is paramount. Historically, these genetic drivers have been difficult to manipulate precisely, as they are requisite for pathogen survival. Here, we provide the first application of Mobile-CRISPRi to study conditionally essential virulence genes in mouse models of lung infection through partial gene perturbation. We envision the use of Mobile-CRISPRi in future pathogenesis models and antibiotic target discovery efforts.

KEYWORDS CRISPRi, conditionally essential genes, type III secretion, *Pseudomonas aeruginosa*, infection model, virulence lifestyle genes

All pathogenic bacteria require essential and conditionally essential (CE) genes for survival in the host environment (1). Essential genes are typically defined as genes that are indispensable for growth in rich culture media, whereas CE genes are required only in specific conditions, such as maintenance in a host niche (2). Next-generation sequencing of bacterial transposon (Tn)-mutant libraries (e.g., transposon sequencing [Tn-Seq] [3] and insertion sequencing [INSeq] [4]) from infected animals has enabled the comprehensive identification of essential and CE genes in a single experiment, rapidly increasing our knowledge of which genes are required for pathogenesis (5–16). There are two major limitations of using Tn-Seq to study CE genes, both arising from the complete loss of function usually caused by Tn mutagenesis. First, core essential

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genes are by definition excluded from the analysis of environment-specific essentiality. Second, all-or-nothing mutations preclude our ability to observe the relationship between expression levels of the gene product and fitness in the host environment; this information could be valuable in identifying CE genes for which the organism is highly sensitive to slight perturbations, which would be ideal candidates for inhibitors. Thus, methods that can partially perturb CE gene function in the context of pathogenesis are highly valuable.

Gene repression tools that are currently used to study CE genes during infection have provided numerous insights into gene function but have key technical limitations. Antisense RNAs (17, 18) have variable efficacy, substantial off-target effects (19–21), and cannot be rationally designed (22). Methods to trigger protein degradation (i.e., degrons) [23–26] require each gene of interest to be tagged at its native locus and suffer from toxicity due to interference with protein function and stability (26). Gene depletion from inducible promoters also requires the insertion of the promoter upstream of all genes of interest and is limited by the inability to optimize both the control of noninduced promoter expression (leakiness) and the maximal amount of induced gene product (27).

In contrast, CRISPR interference (CRISPRi)—the use of a catalytically inactive variant of the Cas9 nuclease (dCas9) to repress transcription (28)—is highly efficacious and specific in bacteria (29), is easily programmable by substituting the first 20 nucleotides of the guide RNA (sgRNA) (30), does not require modification of the chromosome at each targeted gene, and maintains the native regulation of targeted genes. We previously developed Mobile-CRISPRi, (31) a technology that enables the transfer and stable integration of CRISPRi systems into diverse bacteria (Fig. 1A). Here, we optimize Mobile-CRISPRi for targeting CE genes in a *Pseudomonas aeruginosa* PA14 murine pneumonia model of infection.

RESULTS

Optimized dCas9 expression eliminates toxicity and allows for graded knockdowns. dCas9 overexpression often causes nonspecific toxicity in bacteria (32), which would likely complicate the interpretation of our CRISPRi experiments in infection models. Indeed, we found that the full induction of an arabinose-inducible promoter (P_{BAD}) driving the expression of dCas9 variants from *Streptococcus pyogenes* ($dCas9_{Spy}$) (28) or *Streptococcus thermophilus* ($dCas9_{sth}$) (33) resulted in reduced growth of PA14 in rich culture medium, whereas partial induction showed no apparent toxicity (Fig. 1B). We reasoned that titrating chemical inducers (e.g., arabinose) in a murine infection model could be impractical due to variable tissue penetration (34–38). Instead, we focused on expressing $dCas9_{Spy}$ from a series of weak constitutive promoters from the BioBrick Registry (http://parts.igem.org/Main_Page) to reduce toxicity (see Fig. S1 in the supplemental material) and achieve partial knock down. To assess Mobile-CRISPRi efficacy using the BioBrick promoter strains, we employed a "test" version of Mobile-CRISPRi expressing monomeric red fluorescent protein (mRFP) and an sgRNA targeting the *mRFP* gene (31). Knockdown levels were quantified for each promoter through comparing the mutants' fluorescence normalized to growth over time. After 12 hours, we found stable fluorescence ratios between mutants without and with *mRFP*-targeting sgRNA (see Fig. S2 in the supplemental material). The gradient of knockdown ranged from 10- to 17-fold at the 14-hour timepoint, which roughly corresponded to the BioBrick promoter strength used to express dCas9 (Fig. 1C). We performed RNA sequencing (RNA-seq) on cells expressing dCas9 from the strongest of the three BioBrick promoters in our set and confirmed that CRISPRi retained specificity for RFP (Fig. 1D). We imaged cells expressing $dCas9_{Spy}$ from all three promoters and found no apparent defects in morphology (see Fig. S3 in the supplemental material). We conclude that Mobile-CRISPRi optimized with BioBrick promoters driving $dCas9_{Spy}$ enables a nontoxic gradient of constitutive knockdowns in *P. aeruginosa* PA14.

Mobile-CRISPRi targeting of CE genes in a murine pneumonia model. A major goal for developing Mobile-CRISPRi in infection models is to identify CE genes for which

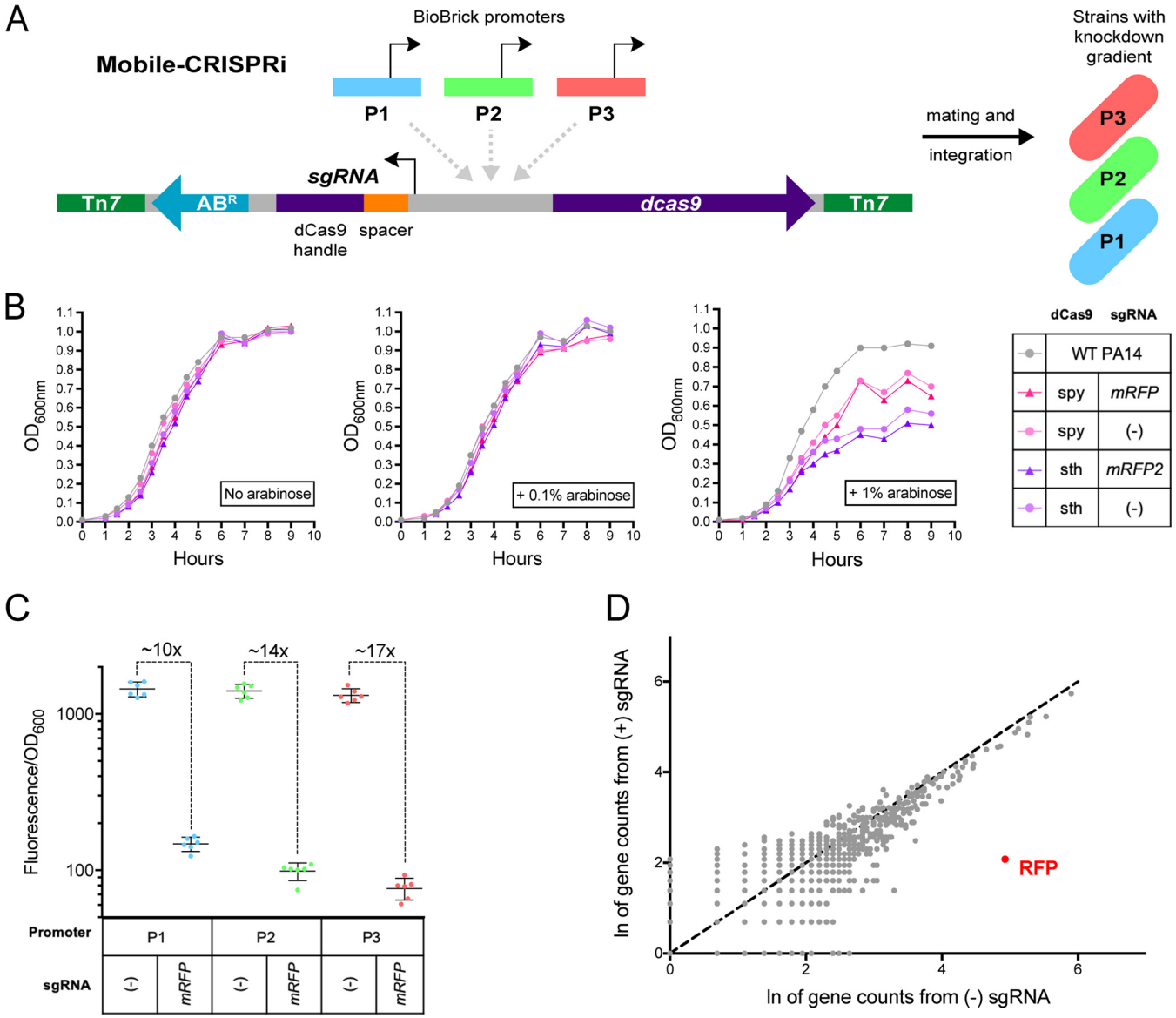

FIG 1 Toxicity, efficacy, and specificity of Mobile-CRISPRi in *Pseudomonas aeruginosa*. (A) Mobile-CRISPRi is comprised of an antibiotic resistance cassette (AB^R), sgRNA spacers specific to the gene of interest, a promoter driving *dcas9* expression, and *dcas9*. These components can be substituted before chromosomal integration into a pathogen to generate a knockdown strain. (B) Wild-type PA14 growth was compared to that of Mobile-CRISPRi PA14 strains featuring arabinose-inducible promoters driving dCas9 activity, two different variants of dCas9 (*S. pyogenes* and *S. thermophilus*), and the presence or absence of *mRFP*-targeting sgRNA. To induce the promoter, these strains were incubated with no arabinose, 0.1% arabinose, or 1% arabinose. (C) *mRFP* was cloned into Mobile-CRISPRi strains with constitutive promoters driving dCas9 expression. The median fluorescence of strains without sgRNA was compared to that of strains with *mRFP*-targeting sgRNA after 14 h of growth. The 10× knockdown associated with P1 is statistically different from the 14× (**, significant *P* value) and 17× knockdown (****, significant *P* value) associated with P2 and P3, respectively. (D) RNA was extracted from mutants featuring P3 with and without *mRFP*-targeting sgRNA. Gene counts from RNA-seq are plotted for each strain with a dashed line of slope = 1 for reference.

a modest perturbation has a substantial impact on pathogenesis. To do so, the system must enable the stable repression of the gene of interest over the course of infection. As a test case, we targeted *exsA*, which encodes the key activator of type III secretion system (T3SS) genes that are required for pathogenesis in *P. aeruginosa*. Because the *exsA* gene is positively autoregulated by the ExsA protein (39), we reasoned that modest knockdown would cause a large reduction in the transcription of T3SS genes, resulting in a loss of effector secretion and impaired virulence. Consistent with this, we found that CRISPRi knockdown of *exsA* reduced the expression of T3SS genes by more than 100-fold (Fig. 2A), similar to the expression levels observed in a strain with an *exsA* disruption (*exsA*::Tn; the Tn insertion position is shown in Fig. S4 in the supplemental

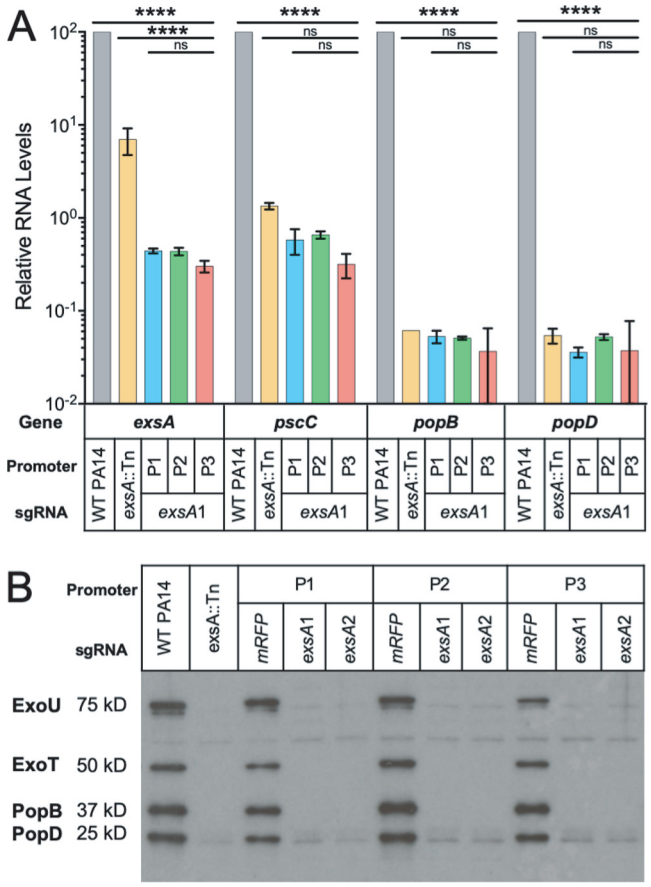

FIG 2 T3SS-associated gene transcription, protein secretion profiles, and clearance of PA14 strains in infection model. (A) Reverse transcriptase quantitative PCR (RT-qPCR) analysis for T3SS-related genes across PA14 strains, normalized to WT PA14 RNA levels. (B) Immunoblot analysis of exoenzyme U and exoenzyme S secretion for PA14 strains grown in MinS medium (for type III protein secretion induction).

material) (40). We found that all three BioBrick promoters driving dCas9$_{Spy}$ expression were equally effective at reducing *exsA* transcript levels, likely because even modest reductions in ExsA protein levels disrupt positive autoregulation. The *esxA*::Tn transposon mutation is insertional rather than deletional, which may have led to high levels of the nonfunctional *exsA* transcript, as measured by quantitative PCR (qPCR) (see Table S1 in the supplemental material). CRISPRi appeared to be slightly more effective at reducing *exsA* transcript levels than the *exsA*::Tn allele, possibly because CRISPRi can repress both ExsA-dependent transcription from the *exsC* promoter and ExsA-independent transcription from the *exsA* promoter (41). Knock down of *exsA* also eliminated the detectable production of T3SS pilus (PopB/D) and effector (ExoT/U) proteins (Fig. 2B). Neither the *exsA* knockdown nor the nontargeting control sgRNA strains showed a growth defect in rich culture medium (Fig. S1).

The loss of *exsA* function is known to strongly attenuate virulence in a murine pneumonia model (42, 43). To test whether Mobile-CRISPRi can be used to probe the functions of CE genes, such as *exsA*, in a host environment, we intratracheally instilled C57BL/6 mice with a range of 10^5 to 10^7 CFU of wild-type (WT) *P. aeruginosa* PA14, an isogenic *exsA*::Tn mutant, or Mobile-CRISPRi strains containing dCas9$_{Spy}$ driven by the P3 BioBrick promoter, and either an *exsA*-targeting sgRNA or a nontargeting control sgRNA. Although CRISPRi using all three BioBrick promoters resulted in similar levels of *exsA* knockdown, we chose P3 because we reasoned that it would serve as the most stringent test of potential dCas9$_{Spy}$ toxicity in the context of a mouse infection. We collected the lungs 18 hours after infection and plated lung homogenates to estimate the number of viable bacteria (44) (see Fig. S5A in the supplemental material). Strains

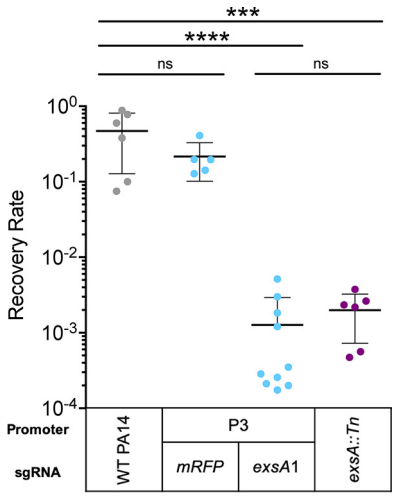

FIG 3 Recovery rates following murine lung infection. Following infection, lung homogenate serial dilutions were plated to estimate the CFU of bacteria recovered from the lung. Recovery rate is output CFU relative to input CFU.

with the *exsA*::Tn allele or Mobile-CRISPRi-targeted *exsA* were highly attenuated for virulence and yielded similar recovery rates (Fig. 3). This demonstrates that Mobile-CRISPRi is an effective tool to knock down CE genes in PA14 during a mouse infection and implies that Mobile-CRISPRi is as stable during *in vivo* infection as it is during growth in culture (31). Furthermore, levels of CFU recovery were similar between WT and nontargeting Mobile-CRISPRi, suggesting that the nonspecific toxicity of dCas9 was mitigated by reduced expression. Other general indicators of infection, including hypothermia and leukopenia, were observed for the nontargeting and WT controls (Fig. S5B and S5C). In contrast, both the *exsA*::Tn and Mobile-CRISPRi-targeted *exsA* strains produced similar levels of white blood cell counts (equivalent or higher than those seen in the phosphate-buffered saline [PBS] control) and similar body temperatures, altogether indicative of reduced virulence (Fig. S5B and S5C). Consistent with this, WT and nontargeting strains showed severe lung injury not seen in the *exsA*::Tn and *exsA*-targeting strains (Fig. S5D). We conclude that Mobile-CRISPRi can probe CE gene phenotypes in infection models.

DISCUSSION

A lack of genetic tools that enable facile and precise control over CE and essential gene expression has severely hampered our progress toward understanding bacterial pathogenesis past the point of simply identifying virulence factors. Our work demonstrates that Mobile-CRISPRi is a valuable genetic tool for characterizing CE genes in the context of an animal infection. We establish a synthetic biology approach for generating CRISPRi knockdown gradients and mitigating nonspecific toxicity by using promoters from the BioBrick Registry to control dCas9 expression. Furthermore, we show that Mobile-CRISPRi repression remains stable despite the stringent fitness constraints imposed during growth in a murine infection model—a key prerequisite to large-scale CRISPRi screens for bacterial gene function in pathogenesis. Finally, as a proof of principle, we successfully use Mobile-CRISPRi to modulate the pathogenesis of *P. aeruginosa* in a mouse pneumonia model by targeting the CE gene *exsA*. Our studies lay the groundwork for future CRISPRi screens that probe the molecular details of pathogenesis.

Mobile-CRISPRi is an excellent complement to established methods of gene function analysis during pathogenesis. Tn-based techniques (e.g., Tn-Seq [3]/INSeq [4]), transposon site hybridization (45), and signature-tagged mutagenesis (46) have been enormously successful at identifying genes required for growth in mouse models of infection. Mobile-CRISPRi partial knockdowns can be used to further characterize these

gene sets by modulating expression to determine the amount of gene product required for virulence. Multiplexed CRISPRi can be used to dissect the genetic pathways by which CE genes operate (29) and will be particularly valuable for characterizing synergies between partially redundant secreted effector proteins. CRISPRi is currently the only method of systematically perturbing essential gene function that can be rationally designed and involves only a single step of strain construction. Thus, a combination of Mobile-CRISPRi partial knockdowns and Tn libraries will enable a comprehensive characterization of all genes required for pathogenesis—including essential genes.

Both Tn mutagenesis and CRISPRi screens have potential pitfalls that should be considered when interpreting single-gene data. When passaged, Tn-mutagenized strains can accumulate second-site suppressors that distort phenotypic analysis. Relatedly, CRISPRi can be inactivated by mutation; the most frequent type spontaneously occurs in the *dcas9* gene and is enriched in a population of strains when CRISPRi causes a strong fitness defect (47). To circumvent these fitness defects, the weak P1 version of Mobile-CRISPRi can be used to target "sensitive" genes, for which a modest knock down causes a strong fitness defect (48). Finally, Tn mutagenesis and CRISPRi can both alter the expression of downstream genes in an operon (i.e., polarity), but the CRISPRi effect is much more predictable because the knock down of downstream genes is generally proportional to that of the targeted gene (29).

We previously demonstrated that Mobile-CRISPRi could be used to repress gene expression in a number of bacterial pathogens associated with antibiotic resistance (e.g., the *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species [ESKAPE] pathogens) (31, 49). Our optimized Mobile-CRISPRi system opens the door to systematic analysis of CE genes in these pathogens during infection, enabling drug-gene interaction studies and, in principle, a screen for new inhibitors that synergize with the host immune system.

MATERIALS AND METHODS

Construction of Mobile-CRISPRi plasmids and strains. Plasmids encoding nuclease-null *Streptococcus pyogenes* and *Streptococcus thermophilus* dCas9s were gifted by Lei Qi and Sarah Fortune, respectively. The vectors containing a Tn7-based Mobile-CRISPRi system were constructed as previously described by Peters (31). dCas9 was expressed from the arabinose-inducible P_{BAD} promoter and three constitutive promoters, namely, Anderson BBa_J23117 (P1), Anderson BBa_J23114 (P2), and Anderson BBa_J23115 (P3). The chimeric sgRNA was expressed by a constitutive derivative of the P_{trc} promoter with no LacI operator site. In this study, all *Pseudomonas aeruginosa* UCBPP-PA14 Mobile-CRISPRi strains were constructed by tri-parental mating as previously described (31). Complete lists of plasmids and strains used in the study can be found in Tables S2 and S3, respectively, in the supplemental material. The PA14 *exsA*::Tn strain was obtained from a transposon insertion library (40).

Toxicity measurements. For dCas9 toxicity measurements, WT PA14 and the mutants were streaked onto *Pseudomonas* isolation agar (PIA) plates and incubated for 20 hours at 37°C. On the second day, one colony from each plate was cultured in 2 ml LB and incubated at 37°C with shaking at 350 rpm for 12 hours. Then, cultures were diluted in 100 μl LB medium with no inducer, 0.1% arabinose, or 1% arabinose to yield a mixture with an optical density at 600 nm (OD_{600nm}) of 0.05 in a 96-well plate (catalog no. 351177; Corning, NY). These cultures were grown with a lid for 9 to 10 hours on a plate shaker (OrbiShaker MP, Benchmark Scientific, NJ) at 37°C and 900 rpm. The OD_{600nm} of the plate cultures was measured every hour using a microplate reader (SpectraMax 340PC; Molecular Devices, CA).

RFP knockdown efficiency. Following triparental mating, two *P. aeruginosa* colonies were picked from each strain to serve as biological replicates and were incubated overnight in 3 ml of LB with 100 μg/ml gentamicin selective medium at 37°C with shaking. These cultures were diluted to 0.01 OD_{600nm} into fresh LB medium, and 200 μl of this culture was added in triplicate to a clear bottom, black, 96-well plate (Corning Costar). This plate was covered with an optically clear seal, and a needle was used to poke holes in each of the wells. Fluorescence (excitation, 557 nm; emission, 592 nm) and OD_{600nm} were monitored during incubation in a microplate reader (Synergy H1; BioTek Instruments, VT) with continuous, fast, double orbital shaking. Samples were blanked with a well containing LB medium. For each replicate, the fluorescence value was divided by OD_{600nm} values at each time point and plotted in 30-minute intervals.

RNA extraction. PA14 Mobile-CRISPRi strains, *exsA*::Tn, and WT were streaked onto Vogel Bonner minimal medium (VBMM) or LB agar plates and incubated overnight at 37°C. One colony from each plate was grown in MinS (T3SS-inducing minimal medium supplemented with nitrotriacetic acid and lacking calcium medium) (50) or LB medium at 37°C for 16 hours with shaking at 250 rpm. Then, the strains were subcultured in 400 μl fresh MinS or LB medium until the OD_{600nm} reached 1.0. Total RNA was extracted

from cell pellets using the RNeasy minikit (Qiagen) according to the manufacturer's instructions with on-column DNase I digestion (Qiagen). The RNA extracts were aliquoted and stored at −80°C.

Reverse transcriptase quantitative PCR. cDNA was synthesized using random hexamer primers and a RevertAid first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA). To check the amplification efficiency of the primers, a 1:50 dilution of WT PA14 cDNA was mixed with PowerUp SYBR green master mix (Thermo Scientific) and detected by the MX3000P qPCR System (Stratagene, La Jolla, CA). Primers for T3SS-related genes (see Table S3 in the supplemental material) had amplification efficiencies between 90% and 110%. A PA14 housekeeping gene, *nadB*, was used as internal control for normalization of total RNA levels (51). The relative efficiency of each primer pair was tested and compared with that of *nadB*, and the threshold cycle ($2^{-\Delta\Delta CT}$) data analysis was used (52). All reactions were performed in triplicates and repeated at least twice using independent cultures, with average values of biological replicates and error bars representing standard deviation of $\Delta\Delta CT$.

cDNA library preparation and RNA-seq. The RNA concentration for each sample was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific). A total of 10 ng of RNA of each sample was fragmented for 6 min, cDNA libraries were prepared using a NEBNext Ultra RNA library prep kit for Illumina (New England BioLabs [NEB] number E7770S). Libraries were sequenced in collaboration with the Chan Zuckerberg Biohub in San Francisco on an Illumina MiSeq instrument in 150-bp paired-end runs. Approximately 1,000,000 reads were collected for each of the two samples, with ~94% alignment to PA14 WT by Bowtie2 (53), and transcripts were counted with HTSeq (54). Only genes with a nonnormalized read count greater than 1 in both samples were included in analysis, with a coverage of 1,286 genes (~20% genome).

Type III secretion profile of *Pseudomonas aeruginosa* by immunoblotting. To knock down the *exsA* gene, two specific sgRNAs, *exsA*1 and *exsA*2, were designed. PA14 Mobile-CRISPRi mutants, *exsA*::Tn, and WT were streaked onto VBMM agar plates and incubated at 37°C overnight. One colony from each plate was grown at 37°C for 16 hours in a shaking incubator at 250 rpm in MinS medium (50). Bacteria were removed by centrifugation at 6,000 × *g* for 15 min. Then, the supernatant was collected and the secreted proteins were precipitated by the addition of ammonium sulfate. The protein pellets were dissolved in sample buffer. After boiling, samples were loaded onto ExpressPlus 4% to 20% PAGE gels (Genscript, Piscataway, NJ) and run under denaturing conditions. PAGE gels were transferred to a polyvinylidene difluoride (PVDF) membrane and immunoblotted with polyclonal rabbit antiserum against ExoU, ExoT/ExoS, PopB, and PopD proteins, as previously described (50).

Murine infection model. Pathogen-free male C57BL/6J mice, 8 weeks of age, were purchased from Jackson Laboratories. Animal experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at UCSF. A total of 29 mice were randomly assigned in the following 5 groups: G1, WT PA14, 6 mice; G2, P3 with *mRFP*-targeting sgRNA, 5 mice; G3, P3 with *exsA*1 targeting sgRNA, 10 mice; G4, *exsA*::Tn, 6 mice; and G5, saline control, 2 mice. Mice were anesthetized with isofluorane prior to intratracheal instillation with bacteria at a range of 1×10^5 to 1×10^7 CFU/animal in a volume of 50 μl, per an established protocol (44). Animal weights and rectal temperatures were measured prior to euthanasia. The lungs were collected in 1 ml of sterile PBS and processed with a handheld homogenizer (Polytron PT1200E; Kinematica). A total of 50 μl of lung homogenate with appropriate dilutions were spread onto PIA plates with and without gentamicin to count output CFU. The bacterial recovery rate was calculated as the ratio of output CFU to input CFU. For whole-blood analysis, blood was collected by cardiac puncture into acid citrate dextrose (Sigma-Aldrich), and white blood cells (WBCs) were measured by a hematology analyzer (Genesis; Oxford Science).

Microscopy. An overnight culture was diluted 1:100 and grown to mid-log phase in LB medium. This culture was diluted to an OD_{600nm} of 0.1 before being added to an agar pad composed of 1% agarose and LB medium. No. 1 coverslips were used, and the slide was sealed with Valap. Thereafter, 250 μl of LB medium was added to the agar pad to prevent desiccation. Sample slides were mounted in the stage top of a Nikon Ti microscope warmed to 37°C. All images were collected on a Nikon Ti-E inverted microscope equipped with a Plan Apo VC 100×/1.4. Images were acquired with an Andor Zyla 4.2 sCMOS camera controlled with MicroManager. Multiple stage positions were collected using an ASI XYZ stage. Brightness and contrast were adjusted (identically for compared image sets) using Fiji software.

Statistical analysis. GraphPad Prism (v. 7.0) was used for the statistical analysis of all the data. For log-transformed bacterial recovery rate, temperature, WBC counts, and weight changes, all groups were analyzed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. For the transcription data, two-way ANOVA followed by Tukey's multiple-comparison test was implemented. To compare changes in fluorescence at 14 hours, data points associated with *mRFP*-targeting sgRNAs were normalized to median fluorescence of the respective strain without sgRNAs. One-way ANOVA followed by Tukey's multiple-comparison test was used to assess the significance of the knockdown levels.

Data availability. All RNA-seq data have been deposited in the National Center for Biotechnology Information's GEO database (55) and are accessible through accession number GSE134771.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00304-19.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLS file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

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We declare no competing interests.

Author contributions were as follows: conceptualization, J.M.P. and O.S.R.; methodology and investigation, J.Q., N.K.P., M.A.Y., S.C., N.H., A.L., E.C., and M.R.S.; supervision, E.C., M.R.L., and O.S.R.; draft writing and revision, J.Q., N.K.P., J.M.P., and O.S.R.; funding acquisition, O.S.R.

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