Quorum sensing controls the *Pseudomonas aeruginosa* CRISPR-Cas adaptive immune system

Nina M. Høyland-Kroghsbo[a], Jon Paczkowski[a], Sampriti Mukherjee[a], Jenny Broniewski[b], Edze Westra[b], Joseph Bondy-Denomy[c], and Bonnie L. Bassler[a,d,1]

[a]Department of Molecular Biology, Princeton University, Princeton, NJ 08544; [b]Biosciences, Environment and Sustainability Institute, University of Exeter, Penryn TR10 9FE, United Kingdom; [c]Department of Microbiology and Immunology, University of California, San Francisco, CA 94158; and [d]Howard Hughes Medical Institute, Chevy Chase, MD 20815

Contributed by Bonnie L. Bassler, October 20, 2016 (sent for review September 22, 2016; reviewed by Magnus Lundgren and Luciano Marraffini)

CRISPR-Cas are prokaryotic adaptive immune systems that provide protection against bacteriophage (phage) and other parasites. Little is known about how CRISPR-Cas systems are regulated, preventing prediction of phage dynamics in nature and manipulation of phage resistance in clinical settings. Here, we show that the bacterium *Pseudomonas aeruginosa* PA14 uses the cell–cell communication process, called quorum sensing, to activate *cas* gene expression, to increase CRISPR-Cas targeting of foreign DNA, and to promote CRISPR adaptation, all at high cell density. This regulatory mechanism ensures maximum CRISPR-Cas function when bacterial populations are at highest risk for phage infection. We demonstrate that CRISPR-Cas activity and acquisition of resistance can be modulated by administration of pro- and antiquorum-sensing compounds. We propose that quorum-sensing inhibitors could be used to suppress the CRISPR-Cas adaptive immune system to enhance medical applications, including phage therapies.

quorum sensing | CRISPR | immunity | phage | phage defense

Many bacteria and almost all Archaea carry CRISPR-Cas (clustered regularly interspaced short palindromic repeats; CRISPR-associated) adaptive immune systems, which provide sequence-specific immunity against previously encountered viruses and plasmids (1, 2). Genomic CRISPR arrays are composed of repetitive sequences alternating with spacers derived from parasitic genomes (viruses, plasmids, transposons). The process of spacer acquisition, known as adaptation, results in heritable immunization (1). Upon reinfection, processed CRISPR RNAs (crRNAs) guide Cas proteins to cleave complementary parasite genomes, which provides the bacterium with immunity (3, 4). Thus, CRISPR-Cas, by patrolling the cell, combats viral attacks and also enables the cell to avoid acquisition of foreign plasmids to which its ancestors have been exposed (5, 6).

Expression of CRISPR-Cas adaptive immune systems is costly (7, 8), possibly because of autoimmunity (9, 10) and deployment of resources that could otherwise be invested in growth. To limit fitness costs, some CRISPR-Cas systems are induced upon infection (11–13). Other environmental cues regulate CRISPR-Cas. In *Escherichia coli*, membrane stress activates CRISPR-*cas* (14) and CRISPR-*cas* is repressed by the DNA binding protein H-NS (histone-like nucleoid structuring protein). H-NS–mediated repression is relieved by the transcription factor LeuO (15, 16). CRISPR-*cas* is repressed by glucose and activated by cAMP receptor protein–cAMP in *Pectobacterium atrosepticum* (17). In addition to these mechanisms, theory and data suggest phage proliferation—and therefore risk of infection—increases with increasing bacterial cell density (18, 19). Bacteria monitor cell density using a cell–cell communication mechanism known as quorum sensing (QS). QS involves the production, release, and detection of extracellular signal molecules, called autoinducers (AI). QS controls behaviors that require cells to act in synchrony to achieve effective outcomes (20).

Results

We explored the idea that bacteria could use high AI levels at high cell density as an indicator of high risk of phage infection. To do this, we investigated whether QS controls CRISPR-Cas in the pathogen *Pseudomonas aeruginosa* (21). We used *P. aeruginosa* UCBPP-PA14 (denoted PA14), which has a type I-F CRISPR-Cas system (22) that provides phage resistance (8, 23, 24). In PA14, two CRISPR regions flank the *cas* genes: *cas1*, *cas3*, and *csy1–4*. Csy1–4 form a complex with a mature crRNA (22). Cas3, which is a nuclease and a helicase, cleaves DNA bound by the Csy1–4 complex. The two primary QS AI-receptor pairs in PA14 are called LasIR and RhlIR. LasI produces the AI 3-oxo-C12-homoserine lactone (3OC12-HSL), which is bound by LasR. The LasR–3OC12-HSL complex activates target genes, including *lasI*, resulting in autoinduction, as well as genes required for virulence (25–27). LasR–3OC12-HSL also activates *rhlI* and *rhlR* (28). RhlI synthesizes the AI C4-homoserine lactone (C4-HSL) that, in conjunction with RhlR, activates a second wave of QS genes (20, 29).

As a readout of CRISPR-Cas, we followed expression of *cas3*, encoding the nuclease that cleaves target DNA. *cas3* expression tracks with cell density: minimal *cas3* expression could be detected at low cell density, and activation of expression occurred in exponential phase (Fig. 1*A*) ($P = 0.00005$). With respect to QS control, single Δ*lasR*, Δ*rhlR*, Δ*lasI*, and Δ*rhlI* mutants and the double Δ*lasR* Δ*rhlR* mutant showed no change or a modest reduction in *cas3* expression compared with the WT (Fig. 1*B*). The Δ*lasI* Δ*rhlI* double-synthase mutant, however, exhibited pronounced reductions in expression of *cas1* and *-3* and *csy1–4* relative to WT (Fig. 1*B* and Fig. S1) ($P = 0.0004$). Addition of AIs to the Δ*lasI* Δ*rhlI* mutant

Significance

The cell–cell communication process, called quorum sensing, activates all three key aspects of the prokaryotic adaptive immune system (termed CRISPR-Cas): expression, activity, and adaptation in the pathogen *Pseudomonas aeruginosa*. We show that pro- and antiquorum-sensing compounds activate and repress CRISPR-Cas, respectively, suggesting the exciting possibility of a combination quorum-sensing–inhibition-phage therapy cocktail. In *P. aeruginosa*, quorum-sensing inhibitors repress virulence, making *P. aeruginosa* more susceptible to elimination by the human immune system, while simultaneously making *P. aeruginosa* more prone to killing by phage therapy through inhibition of the CRISPR-Cas defense mechanism. Finally, because we show that quorum sensing activates adaptation by the CRISPR-Cas immune system, a quorum-sensing inhibitor should also reduce acquisition of resistance against the administered phage.

Author contributions: N.M.H.-K., J.P., S.M., and B.L.B. designed research; N.M.H.-K., J.P., and S.M. performed research; N.M.H.-K., J.P., and S.M. contributed new reagents/analytic tools; N.M.H.-K., J.P., S.M., J.B., E.W., J.B.-D., and B.L.B. analyzed data; and N.M.H.-K. and B.L.B. wrote the paper.

Reviewers: M.L., Uppsala University; and L.M., The Rockefeller University.

The authors declare no conflict of interest.

See Commentary on page 15.

[1]To whom correspondence should be addressed. Email: bbassler@princeton.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1617415113/-/DCSupplemental.

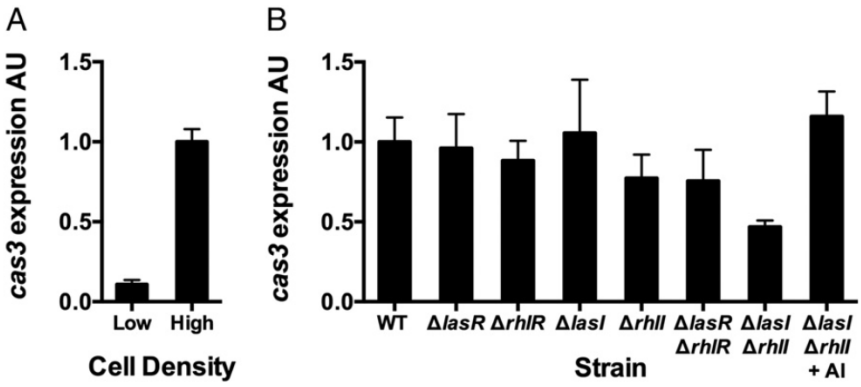

Fig. 1. QS activates *cas3* expression. (*A*) Relative *cas3* expression normalized to 5S RNA measured by qRT-PCR in PA14 at low and high cell density (OD_{600} = 0.1 and 1.0, respectively). (*B*) Relative *cas3* expression at high cell density measured as in *A* for PA14 (WT) and the designated QS mutants. AI indicates 2 μM 3OC12-HSL + 10 μM C4-HSL. Error bars represent SD from $n = 3$ replicates (*A*) and $n = 6$ replicates (*B*).

restored expression to WT levels (Fig. 1*B*). It is possible that the double-AI synthase mutant showed a more dramatic reduction in *cas* gene expression compared with the single mutants and the mutant lacking both receptor genes because of compensatory effects from the orphan QS receptor QscR, which promiscuously binds AIs (30). Another possibility is that the AIs regulate *cas* and *csy* expression through a pathway that operates independently of LasR, RhlR, and QscR, as has been discovered for a few genes in *P. aeruginosa* PAO1 (31).

To examine the consequences of QS on CRISPR-Cas activity, which is also called interference, we assayed the effectiveness of CRISPR-Cas in eliminating the CRISPR-targeted plasmid, called pCR2SP1 (23). This plasmid contains a protospacer targeted by CRISPR 2 spacer 1 flanked by a protospacer-adjacent motif (PAM) that is required for CRISPR interference (32). We used a plasmid rather than a phage because QS regulates phage adsorption in *P. aeruginosa* and may also affect other aspects of phage–host dynamics, complicating the analysis (33). We quantified retention of the control plasmid pHERD30T and the CRISPR-targeted plasmid pCR2SP1 over time in WT PA14 and in the Δ*lasI* Δ*rhlI* double-QS AI synthase mutant. No loss of the control plasmid occurred over the course of the experiment in either strain (Fig. 2*A*). With respect to the CRISPR-targeted plasmid, no loss occurred in either strain during low cell-density growth, and addition of AI had no effect (Fig. 2*B*) (0–3 h). However, after 5 h of growth, conditions under which QS has initiated, plasmid loss occurred in WT cells. In contrast, at $T = 5$ h, minimal loss occurred in the Δ*lasI* Δ*rhlI* double-QS AI synthase mutant. Addition of AI restored plasmid loss to WT levels in the Δ*lasI* Δ*rhlI* mutant (Fig. 2*B*). At 6.5 h, when QS is highly induced, although modest plasmid loss occurred in the Δ*lasI* Δ*rhlI* mutant, over 20-fold more of the Δ*lasI* Δ*rhlI* mutant cells retained the plasmid than did WT cells or Δ*lasI* Δ*rhlI* mutant cells supplemented with AI (Fig. 2*B*). This result shows that QS is required to potently induce CRISPR-Cas activity in PA14. There is residual CRISPR-Cas activity in the Δ*lasI* Δ*rhlI* mutant, suggesting a LasI RhlI-independent CRISPR-Cas activation mechanism exists in PA14.

The above results show that QS enhances plasmid loss during growth; that is, when the plasmid has already generated copies of itself (Fig. 2*B*). We next examined the influence of QS on the ability of CRISPR-Cas to eliminate a single incoming genetic element, which would resemble an attack by a single phage. For this assessment, we measured efficiency of transformation (EOT) at high cell density in WT PA14, the Δ*lasI* Δ*rhlI* mutant, and the Δ*lasI* Δ*rhlI* mutant supplemented with AI. In the ΔCRISPR Δ*cas* mutant, lacking both CRISPR arrays and all *cas* and *csy* genes, the EOT of the pCR2SP1 plasmid compared with the control plasmid pHERD30T was 100% because the ΔCRISPR Δ*cas* mutant is incapable of targeting either plasmid (Fig. 2*C*). In contrast, in WT PA14 the EOT was 2% because CRISPR-Cas is functional and efficiently cleaves the targeted plasmid DNA (Fig. 2*C*). The EOT was 14% in the Δ*lasI* Δ*rhlI* double-QS mutant, showing that—in the absence of QS—the CRISPR-Cas immune system is sevenfold less effective than in the WT ($P = 0.0006$). Addition of AIs to the Δ*lasI* Δ*rhlI* double-mutant restored CRISPR-Cas activity, reducing the EOT to 4%. Thus, QS regulation of CRISPR-Cas activity in PA14 is essential for high-level CRISPR-Cas–dependent immunity against infecting elements.

Our results reveal that 2% of CRISPR-Cas–proficient PA14 colonies survived antibiotic selection despite CRISPR-targeting of the plasmid conferring antibiotic resistance. It is unlikely that this result could be a consequence of spontaneous loss of CRISPR-Cas activity, because loss occurs with a frequency of ∼0.001% (34). One possibility is that, in these colonies, plasmid mutations had been acquired that prevented CRISPR targeting. To test this idea, we sequenced the pCR2SP1 plasmids in 10 WT and 10 Δ*lasI* Δ*rhlI* colonies that had retained pCR2SP1. No mutations were present in the protospacer or PAM sequences of any of the 20 plasmids (Fig. S2). This result suggests that QS regulation of CRISPR-Cas activity at the point of transformation is responsible for the altered EOT shown in Fig. 2*C*. We suggest that, if the CRISPR-Cas system fails to rapidly eliminate an incoming plasmid, plasmid replication occurs. Only one of the 35 CRISPR spacers in the two PA14 CRISPR arrays targets the protospacer in our plasmid. Replicating plasmids could titrate out all available CRISPR-Cas complexes possessing the matching crRNA, possibly explaining how a CRISPR-targeted plasmid persists despite an active CRISPR-Cas system.

Population-level CRISPR spacer diversity is crucial for bacteria to survive phage attack because phage cannot readily acquire point mutations, enabling them to simultaneously escape multiple crRNA spacers (24). Synchronized QS-mediated activation of CRISPR-Cas could boost population-wide acquisition of diverse spacers. The frequency of spacer acquisition is higher when a bacterium is challenged with a protospacer to which it already has immunity, a process called primed adaptation (35). We introduced a plasmid harboring a protospacer with an adaptation-priming seed mutation into the WT, the Δ*cas3* mutant, and the Δ*lasI* Δ*rhlI* mutant, and assayed individual colonies for expansion of the CRISPR2 locus. We investigated CRISPR2 because higher frequency adaptation occurs to the CRISPR2 locus than to the CRISPR1 locus (8). In the absence of Cas3, which is required for adaptation, incorporation of new spacers into the CRISPR2 array did not occur (Fig. 3). In contrast, 26.9% of the WT cells had incorporated one or two spacers. Significantly fewer Δ*lasI* Δ*rhlI* mutant cells underwent adaptation and acquired spacers (11.4%). Addition of AI to the Δ*lasI*

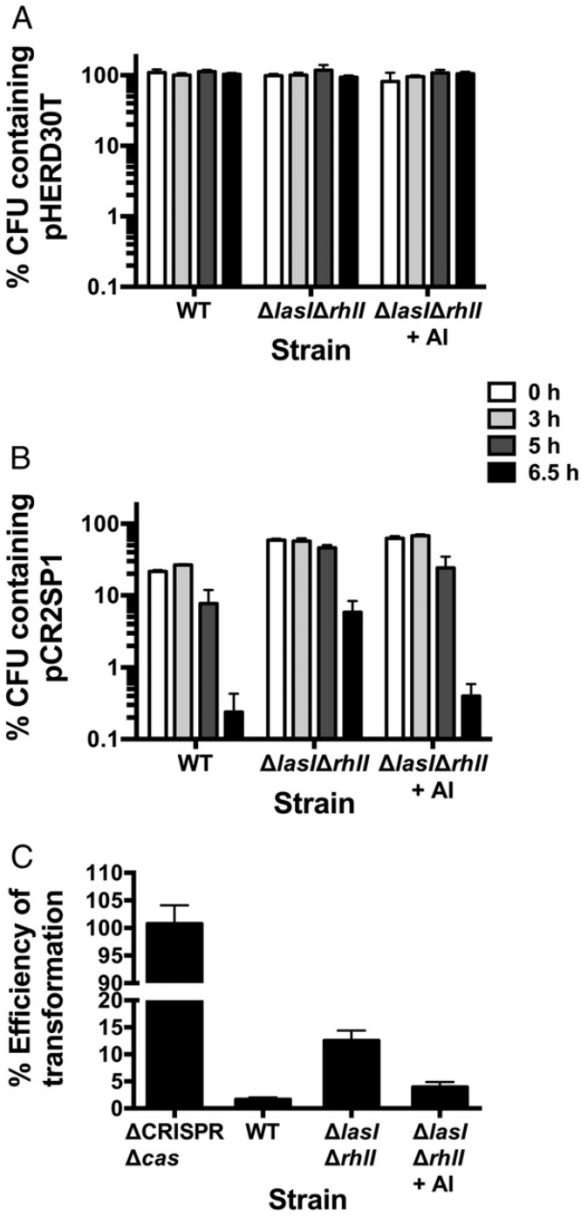

Fig. 2. QS regulates CRISPR-Cas activity. Retention of the control plasmid pHERD30T (*A*) and the CRISPR-targeted plasmid pCR2SP1 (*B*) in WT and in the Δ*lasI* Δ*rhlI* mutant during growth (100% denotes no plasmid loss). (*C*) EOT of WT PA14 and designated mutants at high cell density (OD_{600} = 1) quantified as the percentage transformation by the CRISPR-targeted plasmid pCR2SP1 compared with that of the parent vector pHERD30T lacking the targeted sequence (23). Here, 100% denotes an EOT ratio of 1 for the two plasmids. In all panels, AI indicates 2 μM 3OC12-HSL + 10 μM C4-HSL. Error bars denote SD from $n = 3$ replicates.

Δ*rhlI* mutant increased the total fraction of cells that acquired new spacers to 26% (i.e., to the level of the WT) (Fig. 3). The QS inhibitor Baicalein (36) prevents production of the virulence factor pyocyanin, the canonical QS-readout in PA14 (Fig. S3*A*). Baicalein also blocked the positive effect of AI on *cas3* expression (Fig. S3*B*) and inhibited the AI-mediated enhancement of CRISPR adaptation in WT PA14 and in the Δ*lasI* Δ*rhlI* mutant supplemented with AI (Fig. 3).

Our results demonstrate that *P. aeruginosa* uses QS to activate expression, activity, and adaptation of its CRISPR-Cas immune defense system. Consistent with our findings, microarrays and quantitative RT-PCR (qRT-PCR) in *Burkholderia glumae* PG1 showed altered expression of *cas* genes in a QS mutant, suggesting that QS regulation of CRISPR-Cas activity may be a general phenomenon for bacterial species harboring both QS and CRISPR-Cas systems (37). Our discovery of QS-mediated activation of CRISPR-Cas aligns well with previous studies showing QS-mediated phage defense via down-regulation of phage receptors, reducing infection rates (38, 39). QS repression of phage surface receptors at high cell density could be the first line of defense, effectively enabling bacteria to prevent infection. If this initial strategy fails, the second line of defense becomes crucial: QS activation of CRISPR-Cas immune defense enables pursuit of phages that make it into the cytoplasm. Although we have not yet shown both defense mechanisms operate in a single bacterial species, the capacity to do so certainly exists.

High cell-density induction of *cas* gene expression could, in addition to providing optimal phage defense, minimize the danger of autoimmunity, especially at low cell density when autoimmunity is expected to be particularly deleterious to a bacterial population. In *Streptococcus thermophilus*, Cas9 is constitutively produced and it confers a fitness cost that is most pronounced during lag phase and at low cell density, when the risk of phage infection is low (7, 13, 19). Experiments show that CRISPR-Cas–mediated autoimmunity results in deleterious effects, ranging from genome rearrangements and cell filamentation to suicide (9). Integration of spacers against the bacterial genome occurs at high frequencies in *S. thermophilus*, but these events are eliminated through autoimmunity (10). Consistent with this finding, computational analysis of CRISPR arrays in 330 bacterial and archaeal species found that only 0.4% of all spacers are self-targeting, suggesting strong selection against these events in nature (40). In *E. coli*, a mechanism exists to ensure preferential incorporation of spacers targeting foreign DNA (41), and in *P. atrosepticum*, selection against self-targeting occurs (42). We suggest that CRISPR-Cas systems may require tight regulation to properly balance the danger of autoimmunity with the risk of phage infection. We propose that, because QS correlates with a high probability of phage infection, placing the CRISPR-Cas system under QS control allows efficient activation of the system when the relative risk is high, while additionally enhancing the benefit-to-cost ratio for maintaining a functional CRISPR-Cas system by minimizing autoimmunity at low cell density when infection risk is low. Whether QS regulates *cas* expression directly, or whether the effect of QS on *cas* expression occurs via intermediates, awaits further study.

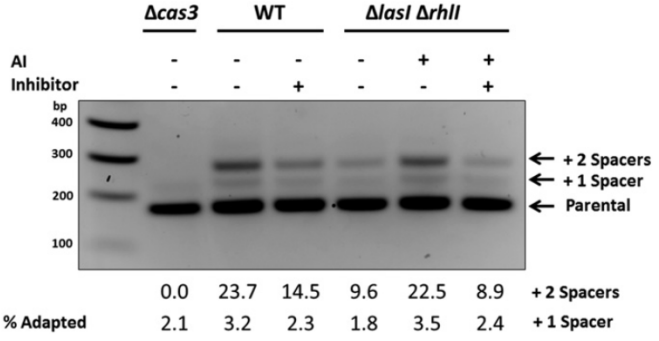

Fig. 3. QS controls CRISPR-Cas–mediated immunity by increasing spacer acquisition. Integration of new CRISPR spacers into the CRISPR2 locus was measured by PCR of single colonies of WT PA14, the Δ*cas3* mutant, and the Δ*lasI* Δ*rhlI* mutant. Each of the strains harbored the CRISPR-targeted plasmid, pCR2SP1 seed, containing a seed mutation to promote adaptation. Each adaptation event results in acquisition of a new spacer and CRISPR repeat, which is exhibited by a 60-bp expansion of the CRISPR locus. Quantitation of the spacer population present in each colony is shown below each lane of the gel. Data are shown for representative colonies. AI indicates 2 μM 3OC12-HSL + 10 μM C4-HSL and inhibitor indicates 100 μM Baicalein (36).

P. aeruginosa is a major pathogen that affects cystic fibrosis sufferers and causes hospital-acquired infections (21). The heavy use of antibiotics for *P. aeruginosa* control has led to widespread antimicrobial resistance (43). Thus, alternatives to conventional treatments for *P. aeruginosa* infection are urgently needed. QS inhibitors reduce *P. aeruginosa* virulence (44). Similarly, phage therapy targeting clinical *P. aeruginosa* isolates enhances survival of mice (45). Our findings suggest the exciting possibility of synergistic efficacy through a combination QS–inhibition-phage therapy mixture. QS inhibitors would repress virulence, making *P. aeruginosa* more susceptible to elimination by the host immune system, while simultaneously making *P. aeruginosa* more prone to killing by phage therapy through inhibition of the CRISPR-Cas defense mechanism. Finally, because QS activates CRISPR-dependent adaptation, a QS inhibitor should also reduce acquisition of resistance against the administered phage.

Materials and Methods

Bacterial Strains and Plasmids. Strains and plasmids used in this study are listed in Table S1. To construct chromosomal deletions in *P. aeruginosa* PA14, DNA fragments flanking the gene of interest were amplified, stitched together by Gibson assembly, and cloned into pEXG2 (a generous gift from Joseph Mougous, University of Washington, Seattle) (46, 47). The resulting plasmids were used to transform *E. coli* SM10, and subsequently mobilized into PA14 via biparental mating. Exconjugants were selected on LB (Luria–Bertani) containing gentamicin (30 μg/mL) and irgasan (100 μg/mL), followed by recovery of deletion mutants on M9 medium containing 5% (wt/vol) sucrose. Candidate mutants were confirmed by PCR. The pCR2SP1 seed plasmid was constructed by inserting a protospacer, targeted by CRISPR 2 spacer 1 containing a single base mutation in the seed region, between the HindIII and EcoRI sites in pHERD30T. PCR primers are listed in Table S2.

Growth Conditions. PA14 and mutants were grown overnight at 37 °C with shaking in LB broth. Cultures were back-diluted 1:1,000 and grown to $OD_{600} = 0.1$ for low cell density, and back-diluted 1:100 and grown to $OD_{600} = 1.0$ for high cell density in the presence or absence of the solvent control DMSO, 3OC12-HSL, C4-HSL (Sigma), or Baicalein (Cayman Chemical) at the specified concentrations. LB was supplemented with 50 μg/mL gentamicin where appropriate.

qRT-PCR. Cells were harvested at the indicated OD_{600}. RNA was purified using TRIzol (Ambion), and subsequently, DNase-treated (TURBO DNA-free, Thermo Fisher). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and quantified using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biociences).

Plasmid Retention Assay. PA14 or the Δ*lasI* Δ*rhlI* mutant was transformed with the CRISPR-targeted plasmid pCR2SP1 or the parent vector pHERD30T lacking the targeted sequence, as described below. Single colonies were suspended in LB and grown at 37 °C with shaking for 6.5 h. Colony forming units were enumerated on LB agar with and without appropriate antibiotics at time 0 and 3 h (low cell density) and at 5 and 6.5 h (high cell density). The percentage of plasmid retention was calculated.

EOT Assay. PA14 was grown to the appropriate OD_{600}, washed twice at room temperature in 300 mM sucrose, and electroporated with 1 μg pHERD30T or pCR2SP1 plasmid DNA. One milliliter of LB was added, and the cells were grown for 1 h at 37 °C with shaking, after which they were plated on LB medium containing 50 μg/mL gentamicin and incubated overnight at 37 °C. Colonies were counted using Image Quant Las 4000 and Image Quant TL software (GE Healthcare). Colony forming units per milliliter were quantified and the EOT was calculated as the percentage colonies transformed by pCR2SP1 compared with those transformed by pHERD30T.

Sequencing. WT and Δ*lasI* Δ*rhlI* mutant cells were transformed with the CRISPR-targeted pCR2SP1 plasmid at $OD_{600} = 1$, as described above. Ten WT and 10 Δ*lasI* Δ*rhlI* colonies were chosen for colony PCR. The region in pCR2SP1 containing the targeted protospacer and PAM was amplified as described below, using primers designed to anneal upstream and downstream of this region. The PCR fragments were separated by agarose gel electrophoresis and purified. Sequencing was performed by Genewiz.

Adaptation Assay. WT PA14, the Δ*cas3* mutant, and the Δ*lasI* Δ*rhlI* mutant were transformed with pCR2SP1 seed as described above. Single colonies were restreaked on LB medium containing 50 μg/mL gentamicin and either DMSO (control), 2 μM 3OC12-HSL + 10 μM C4-HSL (AI), or 100 μM Baicalein (inhibitor) and incubated at 37 °C overnight. Single colonies were tested for population-wide integration of new immunity spacers against the CRISPR-targeted plasmid by PCR using DreamTaq Green PCR Master Mix (Thermo Fisher), with primers designed to anneal upstream of the CRISPR2 array and in the second spacer, which enabled detection of expansion of this array. The PCR products were subjected to agarose gel electrophoresis and band intensities were analyzed using Image Quant TL software (GE Healthcare).

Statistical Analysis. *P* values were calculated using a Student *t* test, except for the data in Fig. 2*B*, which were analyzed using one-way ANOVA for multiple comparisons.

ACKNOWLEDGMENTS. We thank Dr. Julie S. Valastyan, Dr. Chari D. Smith, and other members of the B.L.B. group for helpful suggestions; Dr. Sine Lo Svenningsen for encouraging initial development of the hypothesis; and Dr. George O'Toole and Dr. Joseph Mougous for providing strains. This work was supported by the Howard Hughes Medical Institute, NIH Grant 2R37GM065859, and National Science Foundation Grant MCB-0948112 (to B.L.B.); a Danish Council for Independent Research, Postdoctoral Research Fellowship DFF-4090-00265, administered by the University of Copenhagen (to N.M.H.-K.); and a Jane Coffin Child Memorial Fund for Biomedical Research Postdoctoral fellowship (to J.P.). E.W. was supported by the Natural Environment Research Council, the Wellcome Trust, and the Biotechnology and Biological Sciences Research Council. J.B.-D. was supported by the University of California, San Francisco Program for Breakthrough Biomedical Research, funded in part by the Sandler Foundation, and an NIH Office of the Director Early Independence Award (DP5-OD021344).

1. Barrangou R, et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315(5819):1709–1712.
2. Marraffini LA, Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322(5909):1843–1845.
3. Brouns SJ, et al. (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321(5891):960–964.
4. Garneau JE, et al. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468(7320):67–71.
5. van der Oost J, Westra ER, Jackson RN, Wiedenheft B (2014) Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nat Rev Microbiol* 12(7):479–492.
6. Marraffini LA (2015) CRISPR-Cas immunity in prokaryotes. *Nature* 526(7571):55–61.
7. Vale PF, et al. (2015) Costs of CRISPR-Cas-mediated resistance in *Streptococcus thermophilus*. *Proc Biol Sci* 282(1812):20151270.
8. Westra ER, et al. (2015) Parasite exposure drives selective evolution of constitutive versus inducible defense. *Curr Biol* 25(8):1043–1049.
9. Vercoe RB, et al. (2013) Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. *PLoS Genet* 9(4):e1003454.
10. Wei Y, Terns RM, Terns MP (2015) Cas9 function and host genome sampling in Type II-A CRISPR-Cas adaptation. *Genes Dev* 29(4):356–361.
11. Agari Y, et al. (2010) Transcription profile of *Thermus thermophilus* CRISPR systems after phage infection. *J Mol Biol* 395(2):270–281.
12. Quax TE, et al. (2013) Massive activation of archaeal defense genes during viral infection. *J Virol* 87(15):8419–8428.
13. Young JC, et al. (2012) Phage-induced expression of CRISPR-associated proteins is revealed by shotgun proteomics in *Streptococcus thermophilus*. *PLoS One* 7(5):e38077.
14. Perez-Rodriguez R, et al. (2011) Envelope stress is a trigger of CRISPR RNA-mediated DNA silencing in *Escherichia coli*. *Mol Microbiol* 79(3):584–599.
15. Westra ER, et al. (2010) H-NS-mediated repression of CRISPR-based immunity in *Escherichia coli* K12 can be relieved by the transcription activator LeuO. *Mol Microbiol* 77(6):1380–1393.
16. Dorman CJ (2014) H-NS-like nucleoid-associated proteins, mobile genetic elements and horizontal gene transfer in bacteria. *Plasmid* 75:1–11.
17. Patterson AG, Chang JT, Taylor C, Fineran PC (2015) Regulation of the type I-F CRISPR-Cas system by CRP-cAMP and GalM controls spacer acquisition and interference. *Nucleic Acids Res* 43(12):6038–6048.
18. Knowles B, et al. (2016) Lytic to temperate switching of viral communities. *Nature* 531(7595):466–470.
19. Kasman LM, et al. (2002) Overcoming the phage replication threshold: A mathematical model with implications for phage therapy. *J Virol* 76(11):5557–5564.
20. Papenfort K, Bassler BL (2016) Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol* 14(9):576–588.
21. Rybtke M, Hultqvist LD, Givskov M, Tolker-Nielsen T (2015) *Pseudomonas aeruginosa* biofilm infections: Community structure, antimicrobial tolerance and immune response. *J Mol Biol* 427(23):3628–3645.
22. Wiedenheft B, et al. (2011) RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. *Proc Natl Acad Sci USA* 108(25):10092–10097.

23. Cady KC, Bondy-Denomy J, Heussler GE, Davidson AR, O'Toole GA (2012) The CRISPR/Cas adaptive immune system of *Pseudomonas aeruginosa* mediates resistance to naturally occurring and engineered phages. *J Bacteriol* 194(21):5728–5738.
24. van Houte S, et al. (2016) The diversity-generating benefits of a prokaryotic adaptive immune system. *Nature* 532(7599):385–388.
25. Latifi A, et al. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* 17(2):333–343.
26. Gambello MJ, Kaye S, Iglewski BH (1993) LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infect Immun* 61(4):1180–1184.
27. Seed PC, Passador L, Iglewski BH (1995) Activation of the *Pseudomonas aeruginosa* lasI gene by LasR and the *Pseudomonas* autoinducer PAI: An autoinduction regulatory hierarchy. *J Bacteriol* 177(3):654–659.
28. Pesci EC, Pearson JP, Seed PC, Iglewski BH (1997) Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179(10):3127–3132.
29. Pearson JP, Passador L, Iglewski BH, Greenberg EP (1995) A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92(5): 1490–1494.
30. Chugani S, Greenberg EP (2014) An evolving perspective on the *Pseudomonas aeruginosa* orphan quorum sensing regulator QscR. *Front Cell Infect Microbiol* 4:152.
31. Chugani S, Greenberg EP (2010) LuxR homolog-independent gene regulation by acyl-homoserine lactones in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 107(23): 10673–10678.
32. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155(Pt 3):733–740.
33. Glessner A, Smith RS, Iglewski BH, Robinson JB (1999) Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of twitching motility. *J Bacteriol* 181(5):1623–1629.
34. Jiang W, et al. (2013) Dealing with the evolutionary downside of CRISPR immunity: Bacteria and beneficial plasmids. *PLoS Genet* 9(9):e1003844.
35. Datsenko KA, et al. (2012) Molecular memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system. *Nat Commun* 3:945.
36. Luo J, et al. (2016) Baicalein attenuates the quorum sensing-controlled virulence factors of *Pseudomonas aeruginosa* and relieves the inflammatory response in *P. aeruginosa*-infected macrophages by downregulating the MAPK and NFκB signal-transduction pathways. *Drug Des Devel Ther* 10:183–203.
37. Gao R, et al. (2015) Genome-wide RNA sequencing analysis of quorum sensing-controlled regulons in the plant-associated *Burkholderia glumae* PG1 strain. *Appl Environ Microbiol* 81(23):7993–8007.
38. Høyland-Kroghsbo NM, Maerkedahl RB, Svenningsen SL (2013) A quorum-sensing-induced bacteriophage defense mechanism. *MBio* 4(1):e00362-12.
39. Tan D, Svenningsen SL, Middelboe M (2015) Quorum sensing determines the choice of antiphage defense strategy in *Vibrio anguillarum*. *MBio* 6(3):e00627.
40. Stern A, Keren L, Wurtzel O, Amitai G, Sorek R (2010) Self-targeting by CRISPR: Gene regulation or autoimmunity? *Trends Genet* 26(8):335–340.
41. Levy A, et al. (2015) CRISPR adaptation biases explain preference for acquisition of foreign DNA. *Nature* 520(7548):505–510.
42. Staals RH, et al. (2016) Interference-driven spacer acquisition is dominant over naive and primed adaptation in a native CRISPR-Cas system. *Nat Commun* 7:12853.
43. Jain K, Smyth AR (2012) Current dilemmas in antimicrobial therapy in cystic fibrosis. *Expert Rev Respir Med* 6(4):407–422.
44. O'Loughlin CT, et al. (2013) A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc Natl Acad Sci USA* 110(44):17981–17986.
45. Alemayehu D, et al. (2012) Bacteriophages φMR299-2 and φNH-4 can eliminate *Pseudomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway cells. *MBio* 3(2):e00029-12.
46. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343–345.
47. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102(22): 8006–8011.
48. Cady KC, O'Toole GA (2011) Non-identity-mediated CRISPR-bacteriophage interaction mediated via the Csy and Cas3 proteins. *J Bacteriol* 193(14):3433–3445.
49. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR (2013) Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* 493(7432):429–432.