A self-lysis pathway that enhances the virulence of a pathogenic bacterium

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In mammalian cells, programmed cell death (PCD) plays important roles in development, in the removal of damaged cells, and in fighting bacterial infections. Although widespread among multicellular organisms, there are relatively few documented instances of PCD in bacteria. Here we describe a potential PCD pathway in *Pseudomonas aeruginosa* that enhances the ability of the bacterium to cause disease in a lung infection model. Activation of the system can occur in a subset of cells in response to DNA damage through cleavage of an essential transcription regulator we call AlpR. Cleavage of AlpR triggers a cell lysis program through derepression of the *alpA* gene, which encodes a positive regulator that activates expression of the *alpBCDE* lysis cassette. Although this is lethal to the individual cell in which it occurs, we find it benefits the population as a whole during infection of a mammalian host. Thus, host and pathogen each may use PCD as a survival-promoting strategy. We suggest that activation of the Alp cell lysis pathway is a disease-enhancing response to bacterial DNA damage inflicted by the host immune system.

Pseudomonas aeruginosa | essential regulator | cell lysis | lung colonization | programmed cell death

Programmed cell death (PCD) refers to cell suicide that results from a genetically encoded program (1). It is well recognized that PCD occurs in mammalian cells, where it plays important roles in development, in the removal of damaged cells, and in fighting bacterial infections (1–4). In contrast to the situation in multicellular organisms, the proposal that PCD occurs in bacteria has been controversial, largely because the evolutionary advantage of PCD to a single-celled organism is unclear (5, 6). In bacteria, PCD appears to have roles in limiting the spread of bacteriophage (7), in the formation of biofilms (8, 9), and has been suggested to prevent the proliferation of compromised cells (10, 11). Whether PCD of pathogenic bacteria influences the ability of these organisms to cause disease has remained unclear.

Pseudomonas aeruginosa is a Gram-negative bacterium and an important opportunistic pathogen of humans (12). The organism is one of the most common causes of ventilator-associated and hospital-acquired pneumonia (13) and it is notorious as the principal cause of morbidity and mortality in cystic fibrosis (CF) patients (12). Chronic colonization of the CF lung by *P. aeruginosa* typically leads to progressive lung damage and, eventually, respiratory failure and death (12). A distinctive feature of *P. aeruginosa* is that it encodes a large number of putative transcription regulators (14). These regulators are speculated to facilitate adaptation of the organism to varied environments, including those within the human host.

Here we identify an essential transcription regulator in *P. aeruginosa* that we call AlpR. We show that AlpR is essential because it represses a previously undocumented PCD pathway. We present evidence that the AlpR-regulated PCD pathway can be activated in a subset of cells in response to DNA damage and promotes colonization of the murine lung. Our findings suggest that bacterial PCD can enhance the virulence of *P. aeruginosa* and that PCD functions altruistically during the course of an infection. In addition, these findings have implications for the role of PCD pathways in other pathogens. PCD may represent a survival strategy common to both host and pathogen that each mounts during the course of an infection.

Results

Loss of AlpR Results in Cell Lysis. Analysis of the *P. aeruginosa* strain PAO1 genome revealed a putative transcription regulator encoded by the *PA0906* gene that is highly conserved among different strains of *P. aeruginosa* and exhibits homology to the CI repressor protein from bacteriophage λ (λCI), and the LexA protein from *Escherichia coli* (Fig. S1*A*) (15). Although no transcription regulator has been definitively shown to be essential for growth of *P. aeruginosa* under standard laboratory conditions, *PA0906* is among the set of candidate essential genes in *P. aeruginosa* strains PAO1 and PA14 (16, 17). We therefore reasoned PA0906, which we refer to here as AlpR, might be essential in *P. aeruginosa* because it represses the expression of genes whose products can be lethal. We first used a ClpXP-based protein depletion system to explicitly test whether or not AlpR is essential in *P. aeruginosa* strain PAO1 (18). Depletion of AlpR resulted in at least a 10^5-fold decrease in CFUs (Fig. 1*A*). Thus, loss of AlpR results in a loss of viability or inhibition of cell growth in PAO1. AlpR therefore appears to be essential in this strain of *P. aeruginosa*.

Proteins related to AlpR, such as λCI and LexA, must be dimeric to bind the DNA and contain dimerization determinants in their C-terminal domains (CTDs) (15, 19). We found that the predicted CTD of AlpR could (*i*) interact with itself in a bacterial

Significance

The programmed cell death (PCD) of mammalian cells plays important roles in fighting bacterial infections. Relatively little is known about the adaptive role of PCD in bacteria. Here we report the discovery of a potential PCD pathway in *Pseudomonas aeruginosa*. We show that activation of the system can occur in a subset of cells in response to DNA damage through cleavage of an essential transcription regulator that controls a cell lysis program. Although this is lethal to the individual cell in which it occurs, we find that PCD enhances the ability of the bacterium to cause disease. Our findings suggest that PCD is a strategy used by both host and pathogen to promote survival during an infection.

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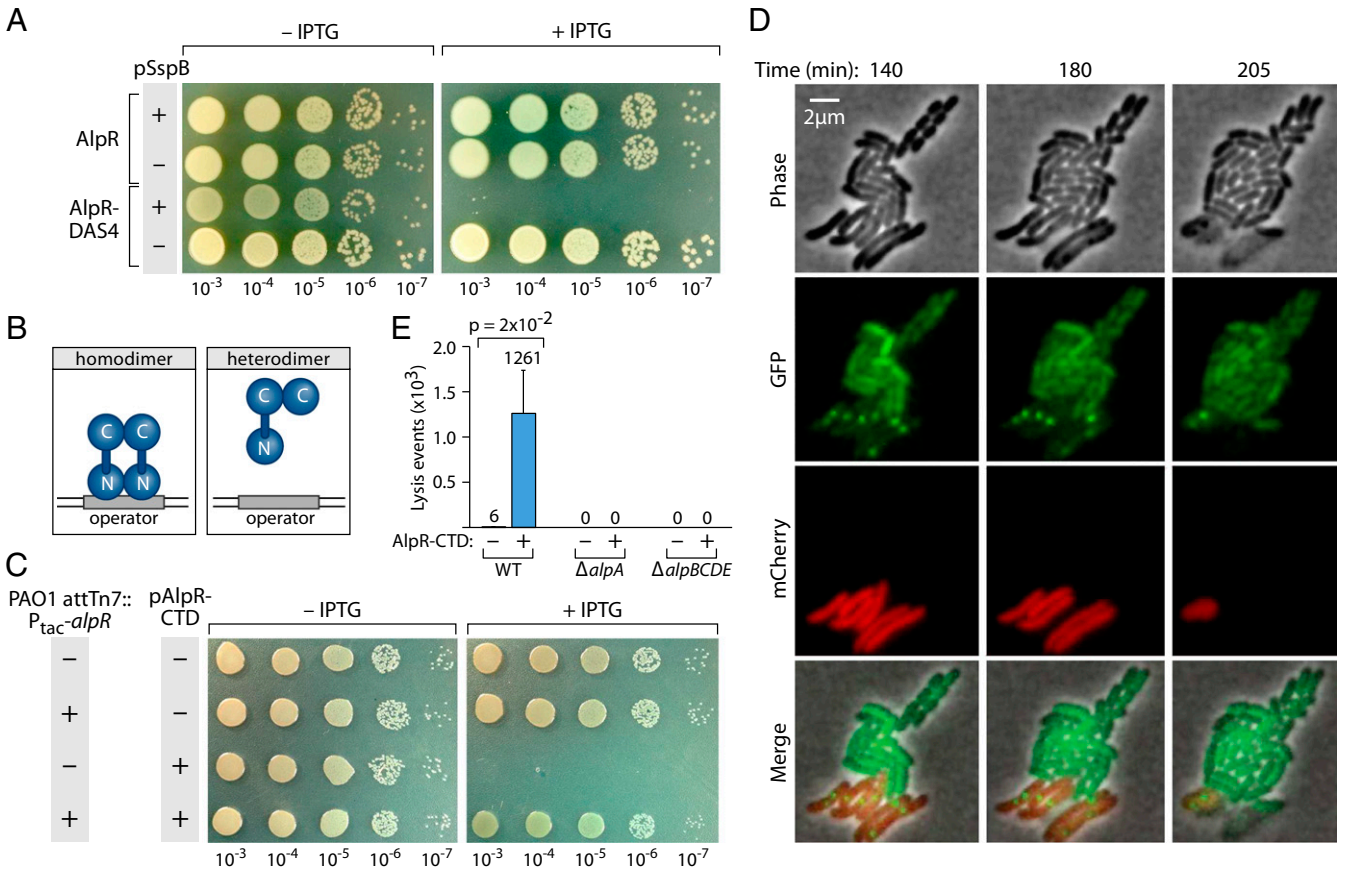

Fig. 1. Loss of AlpR function results in cell lysis. (*A*) Depletion of AlpR with a ClpXP-based system. AlpR with a C-terminal DAS4 epitope tag is degraded by the ClpXP protease complex when the ClpXP adaptor protein SspB is supplied (18). IPTG-induced synthesis of SspB (supplied by pSspB) resulted in inhibition of growth or in cell death only in cells that synthesized AlpR-DAS4. (*B*) Genetic strategy for inhibition of AlpR function with the AlpR-CTD; the AlpR-CTD sequesters full-length AlpR into inactive heterodimers. (*C*) Ectopic synthesis of the AlpR-CTD results in cell death or the inhibition of cell growth in wild-type PAO1 cells but not in cells that contain an additional copy of *alpR* (PAO1 *att*Tn*7*::P$_{tac}$-*alpR*). Plasmid pAlpR-CTD directs the synthesis of the AlpR-CTD in an IPTG-inducible manner. Wild-type PAO1 cells and PAO1 *att*Tn*7*::P$_{tac}$-*alpR* cells containing pAlpR-CTD (pAlpR-CTD$^+$) or the empty control vector (pAlpR-CTD$^-$) were serially diluted and incubated on media lacking or containing IPTG. (*D*) Ectopic synthesis of the AlpR-CTD results in cell lysis. TLFM image sequence of mCherry-labeled cells (PAO1 *att*Tn*7*::*mCherry*) synthesizing the AlpR-CTD and GFP-labeled control cells (PAO1 *att*Tn*7*::*gfp*) that do not synthesize the AlpR-CTD. Images were acquired at 5-min intervals. (*E*) Quantification of the effect of the AlpR-CTD on the lysis of wild-type, Δ*alpA* mutant, and Δ*alpBCDE* mutant cells. Counting of lysis events in TLFM image sequences of mCherry-labeled cells that do or do not synthesize the AlpR-CTD.

two-hybrid assay (Fig. S2*A*), and (*ii*) functionally substitute for the CTD of λCI (Fig. S2*B*), suggesting that the AlpR-CTD contains a dimerization determinant. We thought we might be able to interfere with the activity of AlpR in wild-type cells of *P. aeruginosa* by synthesizing the AlpR-CTD; our expectation was that the AlpR-CTD would sequester an AlpR monomer into an inactive heterodimer and function as a dominant-negative mutant (Fig. 1*B*). Indeed, synthesis of the AlpR-CTD in *E. coli* interfered with the binding of a hybrid repressor, in which the CTD of λCI had been replaced with the CTD of AlpR, to a λ operator (Fig. S2*C*). Consistent with the idea that AlpR is essential, ectopic synthesis of the AlpR-CTD appeared to be lethal to wild-type cells of PAO1 (Fig. 1*C*); similar results were observed when the AlpR-CTD was ectopically synthesized in *P. aeruginosa* strains CF18, PA14, and in the CF epidemic strain LESB58 (Fig. S3 *A* and *B*). Furthermore, ectopic synthesis of the AlpR-CTD did not result in lethality or impair growth in cells that contained a second copy of *alpR* (Fig. 1*C*), suggesting that the deleterious effect of the AlpR-CTD observed in wild-type cells is mediated by sequestration of AlpR.

We next sought to determine whether ectopic synthesis of the AlpR-CTD results in lethality or prevents cell growth. In wild-type PAO1 cells grown in liquid culture, induction of the AlpR-CTD resulted in a decrease in the OD_{600} of the culture and an increase in the abundance of DNA in the culture supernatant (Fig. S3*C*). To directly observe the phenotypic consequences of AlpR sequestration, we used time-lapse fluorescence microscopy (TLFM) (20). The growth of cells that synthesized mCherry and the AlpR-CTD was visualized alongside control cells that synthesized GFP alone. Following induction of the AlpR-CTD, most red fluorescent cells lysed, with some noticeably losing structural integrity or forming spheroplasts before lysis (Fig. 1*D* and Movie S1). Before cell lysis, green fluorescent foci appeared despite the absence of GFP in these cells (Fig. 1*D* and Movie S1). These fluorescent foci that appeared as a prelude to cell lysis might represent a marker for cell death, or may form in a manner that is mechanistically unrelated to the onset of cell death. Cell lysis did not occur in control cells that synthesized GFP (Fig. 1 *D* and *E* and Movie S1). Time-lapse microscopy revealed that the ClpXP-based depletion of AlpR also resulted in cell lysis (Movies S2 and S3). These findings indicate that the loss of AlpR function, either through ectopic synthesis of the AlpR-CTD or through depletion of AlpR, results in cell death through cell lysis. The name AlpR stands for "*P. aeruginosa* lysis phenotype repressor."

AlpR Undergoes Autocleavage in Response to DNA Damage and Functions as a Repressor. Like the related λCI and LexA proteins, AlpR contains a so-called Ser-Lys dyad that in LexA and in

λCI mediates autocleavage (19). The autocleavage of LexA and λCI occurs in response to DNA damage, leading to inactivation of these regulators and the de-repression of target genes. We therefore asked whether AlpR undergoes autocleavage in response to DNA damage. Exposure of *P. aeruginosa* cells to the antibiotic ciprofloxacin, which leads to DNA damage, resulted in cleavage of wild-type AlpR but not the AlpR mutant AlpR (S153A) that contained amino acid substitution S153A that is predicted to prevent autocleavage (19) (Fig. 2*B*). Exposure of cells to hydrogen peroxide, which also damages DNA, resulted in cleavage of AlpR but did not result in cleavage of AlpR(S153A) (Fig. S4). These findings suggest that AlpR undergoes cleavage in response to DNA damage and establish that substitution S153A results in a mutant version of AlpR that cannot undergo autocleavage.

Exposure of PAO1 cells to ciprofloxacin was shown previously to result in an increase in expression of *alpR* and the *PA0907–PA0911* genes (named here *alpABCDE*) (21), which are in a putative operon that is located adjacent to and transcribed divergently from *alpR* (Fig. 2*A*). Exposure to ciprofloxacin induced expression of the *alpR*, *alpA*, *alpB*, *alpC*, and *alpE* genes in wild-type cells but not in cells that synthesized AlpR(S153A) (Fig. 2*C*). These findings suggest that AlpR, either directly or indirectly, represses expression of the *alpR* and *alpABCDE* genes.

ChIP-Seq Reveals That AlpR Regulates the *alp* Genes Directly. To determine whether the effect of AlpR on expression of the *alp* genes might be direct or indirect we used chromatin immunoprecipitation coupled with high-throughput DNA-sequencing (ChIP-Seq) to identify those regions of the PAO1 chromosome that AlpR associates with. ChIP-Seq with an epitope-tagged version of AlpR revealed that AlpR associates with only two regions of the PAO1 chromosome: the *alpR-alpA* and *alpA-alpB* intergenic regions (Fig. 2*D*). Global transcription start-site mapping experiments in PAO1 (22) reveal the existence of promoters in each of these regions, and the expression of *alpR* promoter–, *alpA* promoter–, and *alpB* promoter–*lacZ* fusions was repressed by ectopic synthesis of AlpR (Fig. S5 *A* and *B*). AlpR therefore exerts its effect on expression of these target genes directly.

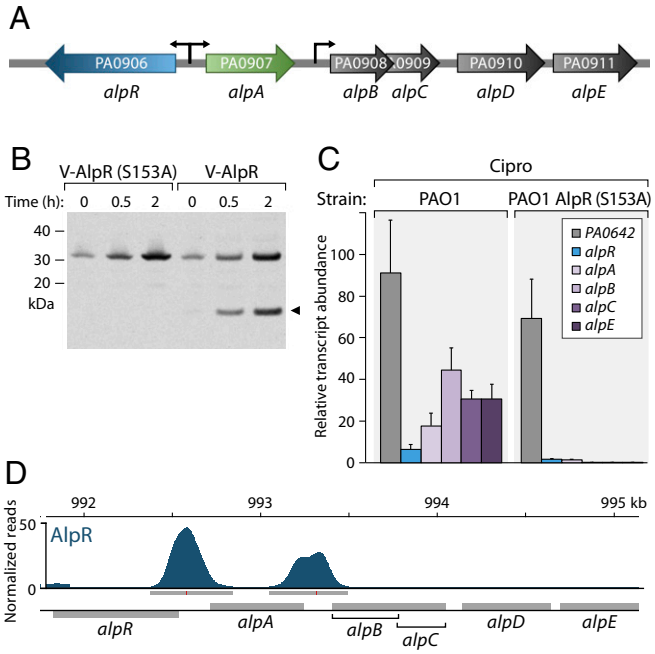

Fig. 2. AlpR is a repressor that undergoes cleavage in response to DNA damage. (*A*) Schematic of *alp* gene cluster. (*B*) AlpR is cleaved in cells exposed to ciprofloxacin. Western blot analysis of AlpR with a vesicular stomatitis virus-glycoprotein (VSV-G) epitope tag fused to its N terminus (V-AlpR) and V-AlpR(S153A) following exposure of cells to ciprofloxacin for the indicated amounts of time. Arrow indicates AlpR cleavage product. (*C*) AlpR represses expression of the *alp* gene cluster. Quantification of transcript abundance by qRT-PCR in cells of the indicated strains following exposure to ciprofloxacin. Relative transcript abundance is shown in cells treated with ciprofloxacin compared with untreated cells. Expression of the *PA0642* gene was induced in both the wild-type and AlpR(S153A) mutant cells, indicating that AlpR does not control the expression of all genes that are induced upon exposure to ciprofloxacin. (*D*) ChIP-Seq reveals that AlpR regulates the *alp* genes directly. ChIP-Seq with V-AlpR shows AlpR associates with the *alpR-alpA* and *alpA-alpB* intergenic regions.

The Essential Function of AlpR Is to Repress Expression of *alpA*. Having established that AlpR represses expression of the *alpABCDE* genes, we wondered whether this might be the essential function of AlpR. That is, we wondered whether de-repression of *alpABCDE* results in cell lysis. Although the *alp* genes have not previously been shown to influence cell lysis, AlpB is annotated as a holin (14), which are small pore-forming proteins typically produced by bacteriophage that facilitate cell wall degradation and cell lysis at the end of a phage lytic cycle (23). Consistent with this idea, we found that AlpB could functionally substitute for the holin from bacteriophage λ in *E. coli* (Fig. S6) (24).

AlpC may be an antiholin, a protein that counters the activity of a holin. In keeping with this notion, genes that encode antiholins can be found immediately downstream of those encoding the cognate holin, and AlpC is predicted to contain transmembrane domains, which are a feature common to antiholins (23). AlpD and AlpE are hypothetical proteins of unknown function, and AlpB, AlpC, and AlpD homologs are encoded by *P. aeruginosa* phage DE3 (25). De-repression of *alpABCDE* might therefore influence cell lysis through a holin-dependent mechanism and the *alp* genes, which are highly conserved in strains of *P. aeruginosa*, may have originated from a phage.

If the essential function of AlpR is to repress expression of the *alpABCDE* genes then a strain that lacked these genes would be expected to tolerate ectopic synthesis of the AlpR-CTD. Synthesis of the AlpR-CTD did not result in lethality in cells that lacked the *alpABCDE* cluster (Fig. 3*A*), suggesting that AlpR is essential because it represses expression of one or more of the *alpABCDE* genes. To assess the individual contribution of each of the genes in this operon to the lethality that results upon ectopic synthesis of the AlpR-CTD we constructed strains in which each of these genes (except *alpB* alone) was deleted. The results depicted in Fig. 3*A* suggest that *alpA* is essential for the cell lysis that occurs upon ectopic synthesis of the AlpR-CTD and that genes *alpB*, *alpD*, and *alpE* also contribute. Consistent with the possibility that AlpC might serve to limit the holin-like activity of AlpB, cells that lack *alpC* were found to be more sensitive to the lethal effects of the AlpR-CTD than wild-type cells (Fig. 3*A*).

AlpA Is a Positive Regulator of the *alpBCDE* Genes. Structural prediction algorithms suggest that AlpA may contain a winged-helix DNA-binding domain. We therefore asked whether AlpA might be a positive regulator that in turn is required for expression of the *alpBCDE* genes. Ectopic expression of *alpA* strongly activated expression of an *alpB* promoter-*lacZ* fusion in PAO1 cells, demonstrating that AlpA is a positive regulator of the *alpB* promoter (Fig. 3*B*), and in cells of *E. coli*, suggesting that AlpA regulates the *alpB* promoter directly (Fig. S5*C*). Furthermore, comparing the effects of ciprofloxacin on the abundance of transcripts in cells of the PAO1 AlpR(S153A) mutant strain to those in wild-type cells and to those in cells that contain a premature stop codon early on in the *alpA* ORF, indicates that genes in the putative *alpBCDE* operon are positively regulated by AlpA (Fig. 3*C*). Consistent with the hypothesis that the principal role of AlpA in promoting cell lysis is to positively regulate expression of the *alpBCDE* genes, we found that cells lacking either *alpBCDE* or *alpA* did not lyse following ectopic synthesis of the AlpR-CTD

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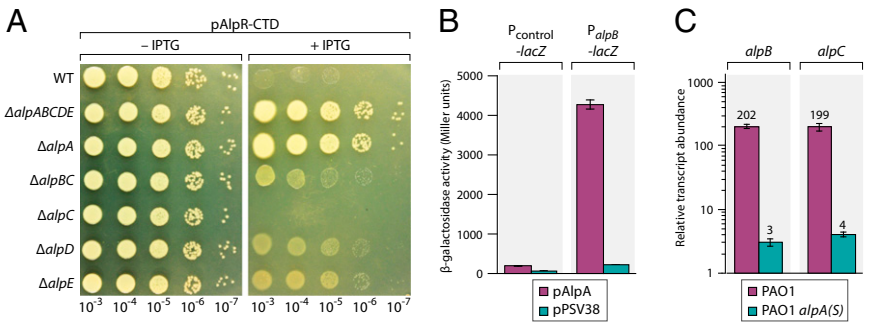

Fig. 3. The essential function of AlpR is to repress expression of *alpA*, which encodes a positive regulator of the *alpBCDE* lysis genes. (*A*) Effect of ectopic synthesis of the AlpR-CTD. Wild-type and mutant PAO1 cells containing plasmid pAlpR-CTD were serially diluted and incubated on media lacking or containing IPTG. (*B*) AlpA positively regulates expression of an *alpB* promoter–*lacZ* fusion. Cells harboring the indicated promoter–*lacZ* fusions and containing plasmid pAlpA or the empty control plasmid pPSV38 were assayed for β-galactosidase activity. (*C*) AlpA positively regulates expression of the *alpBC* genes. Quantification of transcript abundance by qRT-PCR in cells following exposure to ciprofloxacin. [Cells of the PAO1 *alpA(S)* mutant strain contain a premature stop codon early in the *alpA* ORF.] Relative transcript abundance is shown in cells treated with ciprofloxacin compared with cells of the PAO1 AlpR(S153A) mutant strain treated with ciprofloxacin.

(Fig. 1*E* and Fig. S7*A*). In addition, we found that ectopic expression of the *alpBCDE* cassette resulted in lethality (Fig. S7*B*). Inactivation of AlpR therefore initiates a regulatory cascade that de-represses *alpA* expression resulting in the production of a positive regulator that then goes on to drive expression of the *alpBCDE* genes.

DNA Damage Induces the Alp System in a Subset of Cells and Results in Cell Lysis. We next asked whether treatment of PAO1 cells with ciprofloxacin resulted in a homogeneous or heterogeneous response. The analysis of cells containing an *alpB* promoter-*yfp* fusion by TLFM revealed that the addition of ciprofloxacin to cells for 1 h resulted in induction of *alpB* promoter activity in a subset of cells (Fig. 4 and Movies S4–S11). *P. aeruginosa* possesses a pyocin-associated cell death mechanism that can respond to DNA damage (26). Because we sought to define the causal link between *alpBCDE* induction and cell death upon DNA damage, we inactivated this potentially confounding pathway by deletion of *prtN*, a gene required for its activity (27). Importantly, essentially all of the Δ*prtN* mutant cells in which the *alpB* promoter-*yfp* reporter was induced lysed (Fig. 4 and Movie S8), whereas few of the Δ*prtN* Δ*alpBCDE* mutant cells in which the *yfp* reporter was induced lysed (Fig. 4 and Movie S10). These findings indicate that DNA damage-dependent induction of the Alp system results in cell lysis. Moreover, the strong association of *alp* induction and cell death indicates that triggering the Alp system commits a cell to death. Indeed, even in the presence of the pyocin system, we see that following DNA damage a significantly greater fraction of Alp-induced cells survive when *alpBCDE* are absent, implying that the Alp system contributes to the lysis of wild-type cells (Fig. S8 and Movies S4 and S6). In total, our findings suggest that the Alp system represents a PCD pathway that can be activated in a subset of cells in response to DNA damage.

The AlpR-Regulated PCD Pathway Enhances Colonization of the Host Lung. *P. aeruginosa* is a notorious lung pathogen and is the principal cause of chronic and intractable pulmonary infections in individuals with CF, as well as a major cause of acute pneumonias in compromised individuals, such as those on mechanical ventilation (12, 13). *P. aeruginosa* elicits a strong neutrophil response, which includes the generation of reactive oxygen species including H_2O_2 as an antimicrobial defense (28, 29). We posited that *P. aeruginosa* present in the lung would experience DNA damage resulting in cleavage of AlpR and induction of the Alp PCD pathway in a subset of cells. We therefore asked whether the *alp* system influences the ability of *P. aeruginosa* to colonize the murine lung in a model of acute infection. Fig. 5*A* indicates that cells of the Δ*alpA* mutant strain, cells of the Δ*alpBCDE* mutant strain, and cells of the AlpR(S153A) mutant strain were unable to colonize the murine lung as well as wild-type cells. Furthermore, cells of a derivative of the Δ*alpA* mutant strain in which the *alpA* gene was restored (PAO1 *alpA*-IN), colonized the murine lung similarly to wild-type cells, demonstrating that the effect of the Δ*alpA* deletion on lung colonization was not the result of a secondary mutation introduced during construction of the Δ*alpA* mutant strain (Fig. 5*A*). Note that cells containing deletions of *alpA* or *alpBCDE*, or that synthesized AlpR(S153A), grew similarly to wild-type cells when grown in vitro (Fig. S9), ruling out the possibility that the colonization defects observed in vivo could be attributed to any differences in growth rate observed in vitro. These findings suggest that *alpA*, the *alpBCDE* genes, and the ability of AlpR to undergo autocleavage are all important for lung colonization.

We hypothesized that the virulence defect in the Δ*alpA* strain was because of the fact that PCD-mediated lysis of a subset of the *P. aeruginosa* cells in the lung benefitted the remaining cell population rather than because of intrinsic defects in the virulence of individual cells upon the loss of this regulator. To test this, we compared lung colonization by tagged wild-type and

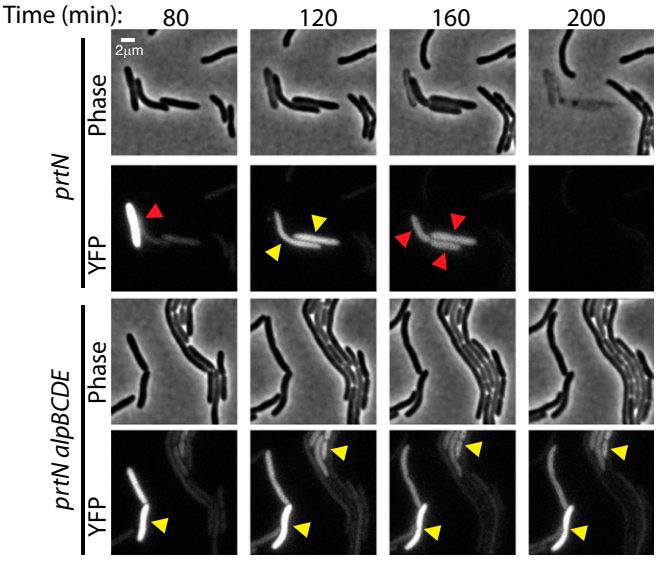

Fig. 4. Induction of the Alp system occurs in a subset of cells treated with a DNA-damaging agent and results in *alpBCDE*-dependent cell lysis. Representative cropped regions from TLFM image sequences of Δ*prtN* mutant cells and Δ*prtN* Δ*alpBCDE* mutant cells containing an *alpB* promoter–*yfp* fusion following exposure to ciprofloxacin. The addition of ciprofloxacin resulted in induction of *alpB* promoter activity in a subset of cells. Expression of the *alpB* promoter–*yfp* fusion was not induced in cells that were not exposed to ciprofloxacin (Movies S9 and S11). Yellow arrowheads point to *alp*-induced cells; red arrowheads point to cells that undergo lysis in the subsequent frame. Cells of the Δ*prtN* mutant strain in which the *yfp* reporter was induced lysed (*Upper*). Cells of the Δ*prtN* Δ*alpBCDE* mutant strain in which the *yfp* reporter was induced did not lyse (*Lower*). Images were normalized for background fluorescence. (Scale bar, 2 μm.)

tagged Δ*alpA* mutant cells in an excess of wild-type cells. When wild-type PAO1 with an intact PCD pathway dominated the population, colonization by cells of the tagged wild-type and tagged Δ*alpA* strains was indistinguishable (Fig. 5*B*). Taken together, these findings strongly suggest that in the acute lung infection model used here, PCD that occurs in a subset of cells through the de-repression of AlpR-controlled genes promotes colonization by *P. aeruginosa*. Although both the Alp and pyocin systems can contribute to the lysis of cells that occurs in response to DNA damage in vitro, we do not know their relative contributions to the lysis of *P. aeruginosa* that appears to occur in the host lung. Nonetheless, our findings demonstrate that the Alp system is functionally relevant in this context.

Discussion

The findings we have described reveal a possible PCD pathway in *P. aeruginosa* that can be activated in response to DNA damage in a subset of cells. DNA damage results in de-repression of *alpA* expression. In turn, AlpA positively regulates expression of the *alpBCDE* lysis genes. The cell lysis that ensues could provide nutrients or liberate toxins or other bacterial factors that might facilitate lung colonization by the remaining *P. aeruginosa* cells in the population (30, 31). PCD pathways in other pathogenic bacteria could enhance virulence in the same fashion.

The PCD of mammalian cells is thought to control bacterial infections through a variety of mechanisms. In particular, neutrophil extracellular traps that promote the killing of pathogenic bacteria are generated through apoptosis (3). In addition, eradication of infected cells through death-receptor–induced apoptosis is thought to limit disease caused by attaching and effacing pathogens, such as enteropathogenic *E. coli* (4). Our findings with the Alp system suggest that the PCD of a subpopulation of *P. aeruginosa* cells can enhance colonization of the host by the remainder of the cell population. PCD may therefore represent a survival-promoting strategy common to both pathogen and host during infection.

The Alp pathway has distinct similarities to—as well as distinct differences from—other PCD pathways that have been defined in both eukaryotes and prokaryotes. In *Staphylococcus aureus*, a holin encoded by *cidA* is thought to be responsible for mediating cell lysis in a PCD pathway that contributes to biofilm formation and might play a role in virulence (8, 32). We have found that AlpB can functionally substitute for the λ holin, suggesting that in *P. aeruginosa* AlpB might contribute to PCD in a manner related to CidA or holin-like proapoptotic factors of the BCL-2 family in eukaryotes (24). In *E. coli*, LexA has been shown to not only mediate the classic SOS response, but also to mediate a PCD pathway referred to as apoptosis-like death (ALD) in response to extensive DNA damage (10, 11). The Alp system is analogous to the ALD system in *E. coli* in that both are under the control of a repressor that becomes inactivated in response to DNA damage through autocleavage. However, in *P. aeruginosa*, LexA does not appear to control expression of the *alp* genes (21). Furthermore, in *E. coli*, ALD is not mediated by genes analogous to *alpABCDE*, and LexA does not exclusively control the expression of a dedicated death cassette like AlpR does in *P. aeruginosa*. The Alp system is therefore distinct from the ALD response with respect to both the genes that directly mediate PCD and the specific DNA damage-responsive regulator used to control PCD.

We cannot exclude the possibility that in the host lung, expression of the *alpABCDE* genes becomes induced, but to a degree that is insufficient to promote cell lysis. In this case, induction of the *alp* genes in a subset of cells would facilitate colonization of the lung by a lysis-independent mechanism. In relation to this, it is noteworthy that in *Serratia marcescens*, a protein with features similar to that of a holin has been implicated in promoting the secretion of chitinases (33). It is therefore conceivable that expression of the *alp* genes might promote colonization of the host lung through an effect on protein secretion. However, because we find that the vast majority of cells in which the *alp* system is induced in response to DNA damage undergo lysis, we favor the hypothesis that the lysis of Alp-induced cells plays a key role in facilitating infection of the host lung by the remainder of the cells in the population.

Pathogens present in the host can experience DNA damage as a result of the host immune response (28). Although host processes that generate DNA-damaging agents are principally thought to limit infections, our findings suggest that they can also act to promote the virulence of *P. aeruginosa* by activating the *alp* genes. Conceivably, treatment of *P. aeruginosa* infections with antibiotics, such as ciprofloxacin, that damage the DNA may also inadvertently promote the virulence of any surviving cells through the activation of the Alp system. Control of the *alp* genes by a DNA damage-responsive transcription regulator may provide a facile way for *P. aeruginosa* to respond to these insults. In addition, the Alp system may facilitate the survival of *P. aeruginosa* in environments other than those of the host. Damage of *P. aeruginosa* DNA resulting from phage infection or from the actions of competing microbes could result in Alp-mediated lysis in a subset of cells, thereby limiting the spread of phage or promoting biofilm formation, and in turn enhancing the survival of the population as a whole (5–9).

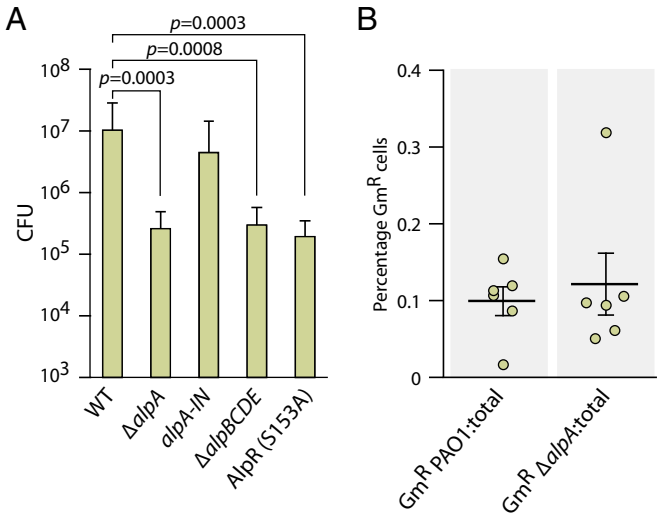

Fig. 5. The AlpR-regulated PCD pathway promotes *P. aeruginosa* colonization of the murine lung. (*A*) Ability of the indicated strains to colonize the murine lung. Male C57BL/6 mice were inoculated via the oropharyngeal route with equivalent numbers of wild-type PAO1 cells and the indicated mutant derivatives. Graphs indicate the mean and error bars indicate SD. After 24 h mice were killed and CFUs in the lungs were enumerated. (*B*) Ability of the indicated marked strains to colonize the murine lung when mixed with an excess of wild-type cells. PAO1 cells marked with a gentamicin-resistance cassette (Gm^R PAO1) and PAO1 Δ*alpA* cells marked with a gentamicin resistance cassette (Gm^R Δ*alpA*) were each mixed with an excess of unmarked wild-type PAO1 cells and inoculated into male C57BL/6 mice via the oropharyngeal route. After 24 h mice were killed and the percent gentamicin-resistant (Gm^R) cells compared with the total number of cells in the lungs was determined. Experiments were performed twice with similar results. A representative dataset is shown.

Materials and Methods

Plasmids and Strains. All bacterial strains and plasmids used in this study are described in *SI Materials and Methods*.

AlpR Sequestration Assays on LB Agar Plates. The pAlpR-CTD plasmid, synthesizing the AlpR-CTD, was introduced into the indicated strains by electroporation. Colonies of plasmid-containing cells were selected on LB agar containing gentamicin and resuspended in PBS to an OD_{600} of 0.01 (considered the 10^{-2} dilution). Tenfold serial dilutions of cells (10 μL) were spotted onto LB agar plates containing gentamicin with or without 10 mM

isopropyl-β-D-thiogalactopyranoside (IPTG). Plates were incubated overnight at 37 °C before being photographed.

Time-Lapse Microscopy. Time-lapse microscopy sequences were acquired on a Nikon Ti-E inverted microscope with a 60× oil objective, automated focusing (Perfect Focus System, Nikon), a Xenon light source (Sutter Instruments), a CCD camera (Clara series, Andor), and image acquisition software (NIS Elements, Nikon), essentially as described previously (20). Additional experimental details are provided in *SI Materials and Methods*.

Western Blot Analyses. Equal numbers of cells were lysed by sonication and separated by SDS/PAGE on 12% Bis-Tris NuPAGE gels (Invitrogen). Western blotting was performed essentially as described previously (18).

Quantitative PCR. Triplicate cultures were grown from separate single colonies. Ciprofloxacin was added to cultures at OD_{600} 0.5, and samples were harvested 120 min later. TriReagent was used for RNA isolation (Molecular Research Center) and cDNA synthesis was conducted as described previously for *P. aeruginosa* (22). Transcript abundances were determined relative to the 23S rRNA transcript by quantitative RT-PCR (qRT-PCR) with the iTaq SYBR Green Supermix (Bio-Rad) and an Applied Biosystems StepOnePlus detection system. The specificity of the PCR primers was verified by melting curve analyses. Relative transcript abundances are the average of three biological replicates. Error bars represent SD.

ChIP-Seq. Strains PAO1 V-AlpR and PAO1 (the mock control) were grown in LB to an OD_{600} of 0.5. ChIP, library preparation, DNA sequencing, read mapping and peak calling is detailed in *SI Materials and Methods*.

β-Galactosidase Assays. Cells were permeabilized with SDS and $CHCl_3$ and assayed for β-galactosidase activity, as described previously (34). Assays were performed in triplicate. Values are averages based on three independent measurements from one experiment. Error bars represent the SD from the mean.

***P. aeruginosa* in Vivo Infection.** All animal experiments were performed as approved by the Dartmouth College Institutional Animal Care and Use Committee. Male C57BL/6 mice (Jackson Labs) 8–10 wk of age were inoculated with 2×10^7 CFU of *P. aeruginosa* suspended in sterile PBS via oropharyngeal aspiration essentially as described previously (35). Additional details are provided in *SI Materials and Methods*.

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