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Type 6 Secretion System-Mediated Immunity to Type 4 Secretion System-Mediated Horizontal Gene Transfer

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Abstract

Gram-negative bacteria use the Type VI secretion system (T6SS) to translocate toxic effector proteins into adjacent cells. The *Pseudomonas aeruginosa* H1-locus T6SS assembles in response to exogenous T6SS attack by other bacteria. Here, we find that this lethal T6SS counterattack also occurs in response to the mating pair formation (Mpf) system encoded by broad-host-range IncPa conjugative plasmid RP4 present in adjacent donor cells. This T6SS response was eliminated by disruption of Mpf structural genes but not components required only for DNA transfer. Because T6SS activity was also strongly induced by membrane-disrupting natural product polymyxin B, we conclude that RP4 induces “donor-directed T6SS attacks” at sites corresponding to Mpf-mediated membrane perturbations in recipient *P. aeruginosa* cells to potentially block acquisition of parasitic foreign DNA.

Bacteria often exhibit antagonistic behaviors toward each other in microbial communities (1). One molecular mechanism mediating such behavior is the Type VI Secretion System (T6SS) (2). The T6SS is a widely conserved (3) dynamic multi-component nanomachine structurally related to contractile phage tails (4, 5). Gram-negative bacteria use the T6SS to kill prokaryotic and eukaryotic prey cells through contact-dependent delivery of toxic effectors (6, 7). In *P. aeruginosa* T6SS encoded by the H1-T6SS cluster (8) selectively targets T6SS⁺ bacteria that attack it by sensing these exogenous attacks and post-translationally activating its own T6SS at the precise location of the initial assaults (9, 10). We previously hypothesized that the signal triggering the T6SS counterattack was the perturbation of the cell envelope (10). Thus, we wondered whether other systems capable of breaching the cell envelope would trigger a similar T6SS response. One system capable of delivering macromolecules across the envelopes of other Gram-negative cells is the Type IV Secretion System (T4SS) (11). This secretion system is associated with conjugative elements such as the broad-host-range, IncPa plasmid RP4 (12) as well as virulence elements in several bacterial species (13). T4SS-mediated DNA conjugation involves 3 sets of proteins: (i) the core structure and pilus components comprising the mating pair formation

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Supplementary Materials:

Materials and Methods

Table S1

Movies S1-S2

References (30–31)

(Mpf) complex, (ii) the relaxosome complex, which initiates DNA transfer by binding to and nicking the origin of transfer, and (iii) a coupling protein that bridges the relaxosome and Mpf complexes (14). During conjugation, the pilus extends from donor cells to mediate close cell-cell contact with recipients, which allows transfer of the DNA-bound relaxosome components to occur (14).

If T4SS-mediated cell-cell interactions could trigger T6SS attack, donor cells of a heterologous conjugation-proficient T6SS⁻ species should be sensitive to killing by T6SS⁺ *P. aeruginosa*. Therefore, we determined whether carrying the RP4 plasmid affected survival of *E. coli* K12 strain MC1061 when grown in competition with *P. aeruginosa*. For consistency with previous studies (9, 10), a *retS* mutant with a transcriptionally up-regulated H1-T6SS locus was used. When mixed with *P. aeruginosa* we recovered approximately 30-fold fewer viable *E. coli* cells carrying RP4 compared to those lacking it (Figure 1A). This difference was not observed for *P. aeruginosa* mutants that were T6SS⁻ (*vipA*) but was still observed in a triple mutant lacking the three known *P. aeruginosa* T6SS effectors Tse1, Tse2, and Tse3 (7) (Figure 1A). Although a *pppA* mutant with a hyperactive but unregulated T6SS could slightly inhibit *E. coli* growth, there was no enhanced killing of *E. coli* cells carrying RP4 compared to those without it (Fig. 1A), and deletion of *tagTa* gene critical for sensing exogenous T6SS attack (10), completely abolished *E. coli* killing (Fig. 1A). Furthermore, in 3-strain mixture containing RP4⁺ and RP4⁻ *E. coli* with *P. aeruginosa* only RP4⁺ *E. coli* were killed (Fig 1B). Thus, T6SS-dependent killing of RP4⁺ *E. coli* involves the same attack-sensing mechanism implicated in the T6SS counterattack responses (10).

We next determined the genetic requirements for the RP4-dependent induction of the *P. aeruginosa* T6SS donor-directed attack. RP4 was subjected to transposon mutagenesis and transformed into *E. coli* strain MC1061. Individual mutants were sequenced to determine transposon insertion sites (Figure 1C). Conjugation efficiency into recipient *E. coli* strain MG1655 was then determined for each of these RP4 mutants, and T6SS activation efficiency was calculated from the survival rate of MC1061 *E. coli* with these mutant plasmids grown in competition with T6SS⁺ *P. aeruginosa* (Table S1). Plotting the data for each mutant revealed several different phenotype clusters (Figure 1D). Mutants in Cluster 1 maintained wild type levels of conjugation efficiency and induced T6SS killing at levels comparable to the wild type plasmid. Most of these mutants were insertions in genes outside of the *traI* or *tra2* loci, the exceptions being a disruption in the RP4 entry exclusion factor *trbK* (15), and a disruption of *traEa* topoisomerase III homolog (16). Neither of these genes are required for the Mpf system or DNA transfer (17) (Table S1). Mutants in Cluster 2 were completely defective in their ability to transfer DNA and did not induce the T6SS donor-directed killing response in *P. aeruginosa*. All of these insertions disrupted genes encoding Mpf structural components (Table 1). There were two outliers not quite in Cluster 1 or 2 that were still able to transfer DNA, but did not induce a significant T6SS response (Table S1), insertions in *trbH*, a lipoprotein believed to connect the pilus to the core complex (14), and *trbN*, a periplasmic transglycosylase that remodels the donor peptidoglycan and is required for pilus synthesis (14). Similar transposon disruptions of homologs of *trbH* (18) and *trbN* (19) in heterologous T4SSs affect the formation and stability of the Mpf pili. Mutants in Cluster 3 induced a greater donor-directed T6SS response than wild type RP4 but were

defective in DNA conjugation (Figure 2B). These mutants included disruptions of relaxosome components *traI* and *traJ* as well as coupling protein *traG* (Table S1). Like those in cluster 3, mutants in cluster 4 also induced more T6SS killing than wild type but exhibited no defect in conjugation. Although it remains unclear why cluster 3 and 4 mutants induce more efficient T6SS-mediated killing, it is clear that successful DNA transfer is not required to trigger a T6SS attack by *P. aeruginosa*. We next determined whether other conjugative plasmids were also able to induce donor-directed T6SS attack. IncN compatibility group plasmid pKM101 (20) induced a T6SS attack comparable to RP4 (Figure 2A), while *E. coli* carrying the sex factor F plasmid was unaffected by T6SS⁺ *P. aeruginosa* (Figure 2B). It is not known why the *E. coli* F factor cannot be successfully transferred into *P. aeruginosa* (21), but this observation suggests that T6SS activation correlates to some degree with whether the host range of a given plasmid includes *P. aeruginosa*.

If the *P. aeruginosa* donor-directed T6SS attack could be triggered by the Mpf system of donor species, then this attack might suppress plasmid transfer into a population of T6SS⁺ *P. aeruginosa* cells. Accordingly, we measured the frequency with which the plasmid pPSV35 (22) could be transferred into T6SS⁺ or T6SS⁻ *P. aeruginosa* from the *E. coli* donor strain SM10 (23), which carries a chromosomally-integrated RP4 plasmid. Because pPSV35 does not encode its own transfer machinery but can be mobilized by the SM10 encoded conjugation system (22), the frequency with which *P. aeruginosa* cells acquired pPSV35 reflects the efficiency at which this plasmid is transferred into but not between *P. aeruginosa* cells. When donor *E. coli* and recipient *P. aeruginosa* were mixed at a 1:1 ratio, we observed an approximately an 86% decrease in conjugation efficiency into a T6SS⁺ strain compared to its isogenic T6SS⁻ *vipA* mutant (Figure 3A). This reduction in transfer efficiency did not match the observed magnitude of killing of RP4⁺ MC1061 (Figure 1A) probably because of intrinsic differences in the ability of various donor strains to promote Mpf and T6SS activation with *P. aeruginosa* similar to what we observed in our RP4 mutant analysis (Figure 1D). *P. aeruginosa* mutants defective in the attack-sensing pathway genes *tagT* and *pppA* (10), also exhibited greater conjugation efficiency as recipient strains (Figure 3A). Examination of mixtures of T6SS⁺ *P. aeruginosa* and *E. coli* RP4⁺ donor cells by fluorescence microscopy revealed rounding and blebbing of *E. coli* cells, a response typical of T6SS-mediated bacterial killing (Figure 3B). Thus inhibition of the conjugative transfer of pPSV35 was likely due to killing of *E. coli* cells through a donor-directed T6SS attack by *P. aeruginosa*.

The fact that multiple secretion systems can induce a T6SS counterattack suggested that the signal initiating this response really is a generalized disruption of the *P. aeruginosa* membrane. Accordingly, we asked whether polymyxin B, an antibiotic known to disrupt Gram-negative bacterial membranes by binding the lipid A component of lipopolysaccharides (24–26), could induce T6SS activity in *P. aeruginosa*. We used a *P. aeruginosa* strain carrying a ClpV1-GFP and fluorescent time-lapse microscopy to monitor T6SS organelle formation and dynamics (9, 10) after exposure to polymyxin B. Cells exhibited a 6-fold increase in the average number of visible ClpV1-GFP foci per cell within 90 seconds of being spotted onto agar pads containing 20 µg/mL of polymyxin B (Figure

4A, 4C, Movie S1). Following this increase in T6SS activity, most ClpV1-GFP foci disappeared over the next 3 minutes with the remaining foci becoming non-dynamic (Figure 4A, Movie S1). The loss of dynamics likely reflects consumption of intracellular ATP pools after prolonged exposure to polymyxin B intoxication. This increase in T6SS activity was not observed when cells were spotted onto agar pads lacking polymyxin B (Figure 4A, 4D, Movie S1). Additionally, this increase in ClpV1-GFP foci was not observed in *tagT* mutants even in the presence of polymyxin B (Figure 4A, 4E-F Movie S2), suggesting that the same attack-sensing pathway that senses T4SS and T6SS attacks is responding to this antibiotic and mediates activation of the T6SS.

These studies support a model in which the donor-directed T6SS attack response in *P. aeruginosa* likely involves detection of perturbations in the cell envelope caused by the invasive components of the T4SS conjugation machinery. T6SS may represent a type of bacterial “innate immune system” that can detect and attack invading infectious elements not by recognizing their molecular patterns (e.g., nucleic acid sequences as do the CRISPR elements (27, 28), or methylated DNA as do restriction enzymes (29)) but rather by recognizing “Transfer Associated Patterns” (TAPs) including membrane disruptions that occur during interactions with other cells deploying T6SS and T4SS translocation machines. Broad-host-range conjugative elements represent infectious bacterial “diseases” that can cause metabolic stress on their newly acquired hosts and thus represent a fitness burden to bacterial populations unable to combat their acquisition. The donor-directed T6SS attack paradigm may represent a strategy for suppressing the movement of horizontally transferred genetic elements in bacterial populations regardless of their signature molecular patterns (e.g., nucleic acid chemical structures or primary sequences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supporting Movies and Table can be found in the Supplementary Materials. This work was supported by NIAID grants AI-018045 and AI-26289 to J.J.M.

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Fig. 1. Mating pair formation induces a donor-directed T6SS attack in *P. aeruginosa*. (A) Summary of competition assays between either MC1061 (grey) or MC1061 RP4 (black) and the indicated strains of *P. aeruginosa*. Reported are the numbers of colony forming units (CFU) of surviving *E. coli*. Data are mean $\pm$ SD with $N = 4$ to 8. (B) Summary of 3-strain competitions between MC1061, MC1061 RP4 *traG* (Tra⁻but Mpf⁺), and *P. aeruginosa*. Surviving CFUs of each *E. coli* strain determined by plating on media selective for each strain. $N = 6$. (C) Map of the RP4 plasmid indicating positions of transposon insertions. Labels with two genes separated by a slash (e.g., '*traE/traF*') represent insertions into the intergenic region between the two genes. (D) Plot of T6SS activation efficiency versus

conjugation efficiency for each transposon mutant. The indicated clusters. Efficiencies are scaled so that values for WT RP4 are 100% and the RP4⁻ parent strain are 0%. The cyan dot represents wild type RP4. The lower limit of detection for our assay was ~200 conjugants; mutants where the conjugation efficiency was below this number are reported as 0% in the graph.

A **B** **Fig. 2.**

IncN but not IncF induces donor-directed T6SS attacks. (**A** and **B**) Summary of *E. coli* survival after competition with T6SS⁺ (black bars) or T6SS⁻ (grey bars) *P. aeruginosa*. Data are mean $\pm$ SD, $N = 3$. (**A**) Competition assays between *P. aeruginosa* and *E. coli* MG1655 carrying no plasmid, RP4, or pKM101. pKM101 confers streptomycin resistance so MG1655 rather than MC1061 was used. (**B**) Competition assays between *P. aeruginosa* PAO1 and *E. coli* MC1061 carrying no plasmid, RP4, RP4 hyper-inducer Tra⁻Mpf⁺ mutant (*traG*), or F'. F' was confirmed to be functional by successfully mating into several different *E. coli* strains (data not shown).

Fig. 3.

Donor-directed T6SS attack blocks heterologous transfer of DNA. **(A)** The conjugation efficiency into different *P. aeruginosa* mutants. Data are mean $\pm$ SD, $N = 7$. **(B)** Representative field of cells containing a mixture of *P. aeruginosa* PAO1 *retS clpV1-gfp* (green) and *E. coli* S17-1 RP4⁺ donor cells (non-fluorescent). *E. coli* cells exhibit cell rounding characteristic of T6SS-mediated killing (arrows). Larger magnification of rounding cells are shown in the insets.

Fig. 4.

Activation of T6SS organelle formation in response to polymyxin B treatment requires TagT. **(A)** Wild type (WT) or *tagT* mutant ($\Delta tagT$) *P. aeruginosa* cells were imaged every 10 seconds starting immediately after being spotted onto agar pads containing 0 (untreated, UT) or 20 μ g/mL polymyxin B (PB). Total number of ClpV1-GFP foci was divided by the number of cells for each field of cells to determine the average number of foci per cell. Each curve represents the mean of 12–16 fields with 250–600 cells in each field $\pm$ SD. **(B)** Color scale used to temporal-color code ClpV1-GFP signal. **(C–F)** ClpV1-GFP localization was followed for 5 minutes and temporally color-coded.