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Chelation of Membrane-Bound Cations by Extracellular DNA Activates the Type VI Secretion System in *Pseudomonas aeruginosa*

Mike Wilton,[a,b] Megan J. Q. Wong,[a,c] Le Tang,[a,c] Xiaoye Liang,[a,c] Richard Moore,[a,c] Michael D. Parkins,[a,b] Shawn Lewenza,[a,b,e] Tao G. Dong[a,b,c,d]

Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB, Canada[a]; Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada[b]; Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada[c]; Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada[d]; Faculty of Science and Technology, Athabasca University, Athabasca, AB, Canada[e]

***Pseudomonas aeruginosa* employs its type VI secretion system (T6SS) as a highly effective and tightly regulated weapon to deliver toxic molecules to target cells. T6SS-secreted proteins of *P. aeruginosa* can be detected in the sputum of cystic fibrosis (CF) patients, who typically present a chronic and polymicrobial lung infection. However, the mechanism of T6SS activation in the CF lung is not fully understood. Here we demonstrate that extracellular DNA (eDNA), abundant within the CF airways, stimulates the dynamics of the H1-T6SS cluster apparatus in *Pseudomonas aeruginosa* PAO1. Addition of Mg^{2+} or DNase with eDNA abolished such activation, while treatment with EDTA mimicked the eDNA effect, suggesting that the eDNA-mediated effect is due to chelation of outer membrane-bound cations. DNA-activated H1-T6SS enables *P. aeruginosa* to nonselectively attack neighboring species regardless of whether or not it was provoked. Because of the importance of the T6SS in interspecies interactions and the prevalence of eDNA in the environments that *P. aeruginosa* inhabits, our report reveals an important adaptation strategy that likely contributes to the competitive fitness of *P. aeruginosa* in polymicrobial communities.**

Cystic fibrosis (CF) is a common lethal inherited disease in Caucasians (1). Disease symptoms are caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that result in pleiotropic effects, including the accumulation of a dehydrated, viscous mucus layer in the patient's respiratory tract (2–4). This thickened mucus impairs the normal bronchopulmonary clearance and permits chronic microbial colonization of the respiratory tract (5, 6). The microbial clearance defect leads to an intense, sustained recruitment of the neutrophilic inflammatory response, resulting in tissue destruction, promotion of the decline of lung function, and patient mortality (7–9).

Although a large diversity of species comprise a dynamic CF microbiome, which varies through disease progression and between patients (10–18), *Pseudomonas aeruginosa* is one of the predominant pathogens present in CF patients according to the 2013 CF Foundation patient registry report (https://www.cff.org/). Chronic infection with *P. aeruginosa* and its conversion to an alginate-hyperproducing, mucoid phenotype is associated with progressive decline in lung function, increased risk of hospitalization, and reduced survival (19–22). *P. aeruginosa* can also cause serious infections to patients suffering from burns and immunocompromised diseases (23).

P. aeruginosa employs the type 6 secretion system (T6SS) to deliver toxic antimicrobial and antieukaryote effectors to target cells (24–28). Commonly found in Gram-negative bacteria, the T6SS is an organelle consisting of a cytosolic contractile outer sheath, a membrane complex, and an inner tube (27, 29). Sheath contraction ejects the inner tube and its associated effector proteins to the extracellular environment (30–32). The inner tube is composed of stacks of hemolysin coregulated proteins (Hcp), which have been found in the sputum of CF patients infected with *P. aeruginosa* (27). After contraction, the sheath is recognized and disassembled by an ATPase ClpV (31, 33). The dynamic activities of T6SS assembly, contraction, and disassembly can be visualized using fluorescence tags to ClpV or sheath proteins (31). *P. aeruginosa* model strain PAO1 possesses three T6SS clusters that are tightly regulated at transcriptional and posttranslational levels (24, 27, 34–36). For example, RetS (regulator of exopolysaccharide and type III secretion) negatively regulates the transcription of the H1-T6SS cluster (27, 37); thus, the *retS* mutant displays enhanced H1-T6SS activities (31). Although the H1-T6SS is dormant in the wild-type *P. aeruginosa* strain, its dynamic activity is rapidly induced when cells sense extracellular assaults, including the T6SS activity (31, 34), conjugation (38), and polymyxin B activity (38). A multicomponent signaling cascade, TagQRST-PpkA-PppA-Fha1, is required for the precise retaliatory response that activates the firing of H1-T6SS at the exact site of external assault (34, 38). Although the H2-T6SS and the H3-T6SS clusters are implicated in both antimicrobial and antieukaryote activities (26, 39, 40), their contribution to bacterial killing is substantially less effective than that of the H1-T6SS, as exhibited by prey cell tolerance of the *P. aeruginosa* mutant lacking the H1-T6SS (25, 38).

Extracellular DNA (eDNA) is present in many different environments, including soil, water, sediment, and gastrointestinal and respiratory tracts (41–44). The physiological functions of eDNA include horizontal gene transfer, nutrient source, and bio-

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Address correspondence to Tao G. Dong, tdong@ucalgary.ca.
M.W. and M.J.Q.W. contributed equally to this article.
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film formation (43, 45, 46). As a negatively charged macromolecule, eDNA in excess amounts also displays antimicrobial effects by chelating membrane-bound cations (47, 48). In the viscous CF lung mucus, eDNA is accumulated to a considerable level (up to 20 mg/ml) (1, 41, 42, 44). The DNA in CF sputum is largely derived from neutrophils, including the presence of neutrophil extracellular traps (NETs) that are deployed to combat infection of the lung (47, 49). However, eDNA in the matrix of laboratory-grown biofilms is microbial in origin and results from cell lysis or excretion of membrane vesicles (46, 50).

Interestingly, *P. aeruginosa* can sense some unknown signals released by killed *P. aeruginosa* cells during interspecies competition and can respond by increasing the expression of the H1-T6SS, the response termed *P. aeruginosa* response to antagonism (PARA) (51). Similarly, the H1-T6SS is transcriptionally induced when *P. aeruginosa* is cultivated in CF mucus (52) and secretion of Hcp is readily detectable in clinical CF sputum samples (27). However, the signals that trigger T6SS activation in CF are not well understood. Here we provide evidence that sputum isolated from adult CF patients enhances the dynamic activities of the H1-T6SS of *P. aeruginosa* within seconds of exposure, suggesting a posttranslational response. This response is mechanistically and temporarily distinct from the PARA (51), which also stimulates the expression of the H1-T6SS. We determined that H1-T6SS activation results from perturbation of the bacterial outer membrane caused by extracellular DNA (eDNA), a common macromolecule enriched in the CF sputum. We demonstrated that eDNA-mediated chelation of membrane-bound cations was sufficient to trigger H1-T6SS assembly. The addition of excess magnesium cations suppresses eDNA-mediated T6SS activation. Importantly, we demonstrate that deletion of the TagQRST signal-sensing pathway abolishes activation of the H1-T6SS. We further show that the eDNA-induced T6SS activities result in nonselective killing of neighboring bacterial species. Given the prevalence of eDNA in different environments, our results suggest that eDNA may function as a signaling molecule that contributes to the competitive fitness of *P. aeruginosa*.

MATERIALS AND METHODS

Strains and growth conditions. *P. aeruginosa* PAO1 and its derivative ClpV1-green fluorescent protein (ClpV1-GFP), *tagT*, *retS*, and T6SS mutants, and the *vasK* mutant of *Vibrio cholerae* V52 were described previously (27, 34, 53). *Burkholderia cepacia* ATCC 17759 and ATCC 25416 were from laboratory stock. The V52 *vasK* mutant was transformed with an mCherry2-expressing pBAD24 plasmid to differentiate V52 *vasK* genes from *P. aeruginosa* GFP-labeled cells. *Escherichia coli* PIR1 (Invitrogen) and Sm10 λpir were used for cloning and conjugation, respectively. Chromosomal fusions of TssB1, TssB2, and TssB3 to superfolder GFP (sfGFP) were constructed using crossover PCR and homologous recombination, as previously described (31). The stop codon of *tssB1*, *tssB2*, or *tssB3* was replaced by *sfgfp* in frame. Allelic replacement in PAO1 was performed using the pEXG2 suicide plasmid (54). All constructs were verified by sequencing.

Bacteria were routinely cultured aerobically at 37°C in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl). The following antibiotics and chemicals were used when appropriate: carbenicillin (100 μg/ml), streptomycin (100 μg/ml), irgasan (25 μg/ml), gentamicin (30 μg/ml), polymyxin B (50 μg/ml), and arabinose (0.1% [wt/vol]).

Extracellular DNA isolation. Upon enrollment in the Calgary Adult Cystic Fibrosis Clinic (CACFC), all patients consent to the regular submission and banking of sputum and bacterial isolates from each and every encounter. Ethical approval for this study was granted (Conjoint Health Research Ethics Board [CHREB] REB15-0854 and REB15-2744).

Fresh sputum samples from 5 unrelated CF adults who visited the CACFC were isolated, resuspended separately, and thoroughly mixed in 0.5× phosphate-buffered saline (PBS) buffer (Invitrogen) (pH 7.4) to obtain a final extracellular DNA concentration of approximately 300 to 500 μg/ml per sputum. Each resuspended sputum sample was heat treated at 100°C for 20 min to remove any heat-labile components. DNA was extracted from each of the sputum samples, *P. aeruginosa*, or neutrophils using a Wizard Genomic DNA purification kit (Promega) following the kit instructions. DNA was eluted to a final concentration of 500 μg/ml. Salmon sperm DNA (USB, MJS BioLynx) was used at a concentration of 0.4% (wt/vol) in 0.5× PBS.

Extracellular DNA and bacterial competition assay. *P. aeruginosa* (killer) and *B. cepacia* (prey) were grown aerobically overnight in LB. Killer cells were subcultured in fresh LB at a 1:50 dilution and grown to an optical density at 600 nm (OD_{600}) of 0.8. Cells were centrifuged and resuspended in LB or LB with 0.4% DNA medium. Killer cells and prey cells were then mixed at a 10:1 ratio and incubated on LB or LB with 0.4% DNA plates at 37°C for 3 h and 6 h. At each time point, cells were recovered in 0.5 ml LB solution, serially diluted, and plated on LB medium containing polymyxin B (50 μg/ml) to select against *P. aeruginosa* and for *B. cepacia* survival.

Fluorescence microscopy and data analysis. Overnight cultures were subcultured into fresh LB and cultivated aerobically at 37°C to an OD_{600} of 0.6. Cells were collected and concentrated 10 times by centrifugation. Cell pellets were resuspended in 50 μl of 0.5× PBS containing 0.4% (wt/vol) salmon sperm DNA or 10 ng/μl bacterial/neutrophil genomic DNA. Resuspended bacteria were spotted on a thin pad of 1% agarose–0.5× PBS and were covered with a glass coverslip. Cells were imaged at room temperature immediately to assess fluorescence foci. For the interaction between the *V. cholerae vasK* mutant and PAO1, round *V. cholerae* cells were quantified after 45 min of coincubation. A total of 36 30-by-30-μm fields of view of *P. aeruginosa* and *V. cholerae* were captured for the production of round cells and at least 24 fields of view for the quantification of ClpV-GFP foci. Data were generated through the analysis of the fields of view from three separate biological replicates.

Static images were captured by a Leica DMI 4000B inverted microscope equipped with an Orca R2 digital camera and Metamorph software for image acquisition using the 100× objective. The following excitation and emission filters were used for green fluorescence (excitation [Ex], 490-nm wavelength/20; emission [Em], 525/36). Images were formatted and analyzed using Imaris 7.0.0 imaging software. Time course image series were captured by a Nikon Ti-E inverted motorized microscope with a Perfect Focus system and Plan Apo 100× oil Ph3 DM (numerical aperture [NA], 1.4) objective lens. Spectra X light engine (Lumencore), ET-GFP (Chroma 49002), and ET-mCherry (Chroma 49008) filter sets were used to excite and filter fluorescence. A Photometrics CoolSNAP HQ2 camera (pixel size, 60 nm) and NIS Elements 4.0 were used to record images. For time course experiments, images were captured every 10 s for 5 min. The Fiji platform was used for all image analysis and manipulations (55). The image intensity and contrast were normalized across the time course by the Fiji autocorrectional macros. Image movement over the time course was corrected using StackReg transformation when necessary. The Fiji macro "Spectrum" temporal-color code was used to visualize localization of fluorescent foci over a 5-min time course. Merged phase-contrast and fluorescence images are presented.

RESULTS

Extracellular DNA in CF sputum elicits T6SS activation. Although substrates of the normally dormant H1-T6SS can be detected and T6SS genes are upregulated in the CF sputum (27, 52), the regulatory signals are unknown. To visually confirm T6SS activation in the sputum and differentiate transcriptional and posttranscriptional effects, we used a previously constructed

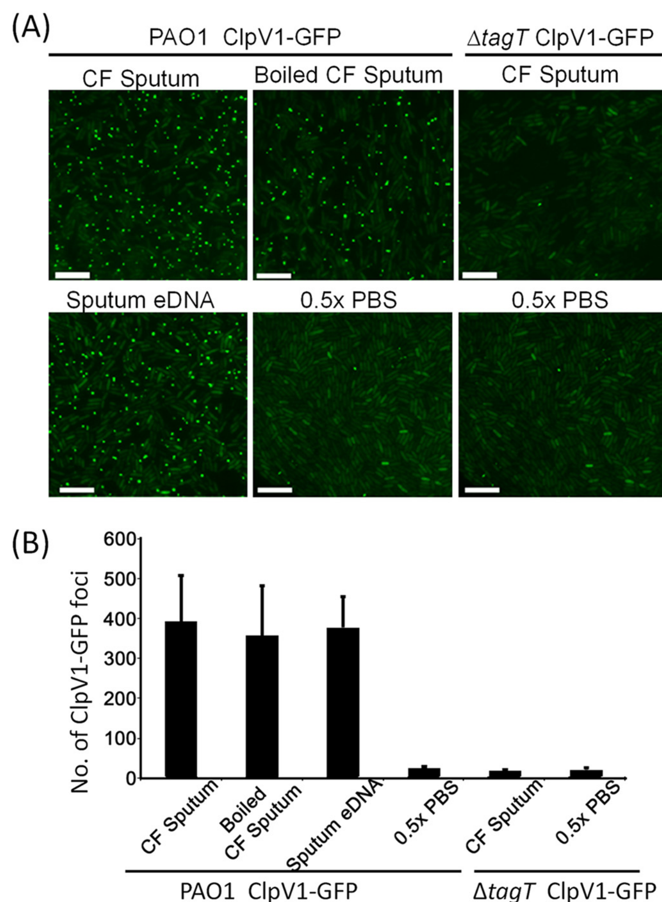

FIG 1 Cystic fibrosis sputum induces T6SS dynamics in *P. aeruginosa*. (A) T6SS activation of the *P. aeruginosa* wild-type strain or a Δ*tagT* mutant expressing ClpV1-GFP exposed to crude or heat-treated cystic fibrosis sputum or to sputum-derived eDNA. Results are representative of sputum samples derived from five adult cystic fibrosis patients. Imaging experiments were repeated independently three times per CF sputum sample, and representative images are presented (bar, 10 μm). (B) Quantification of active *P. aeruginosa* T6SS events as measured by the number of ClpV1-GFP foci present in the field of view (FOV). Each bar represents the mean of results from 15 fields of view with 350 to 600 *P. aeruginosa* cells in each field ± standard deviation (SD). Data are pooled from five independent cystic fibrosis sputum samples.

ClpV1-GFP strain to monitor the dynamics of H1-T6SS (27, 31). When cells were exposed to sputum samples from five adult CF patients (Fig. 1A), the activity of the H1-T6SS was induced, as measured by the formation of ClpV1-GFP foci (Fig. 1). Sputum derived from the CF lung is a complex mixture of proteoglycans, lipids, extracellular DNA (eDNA), microbial and host cells, and debris (41, 44, 49). Therefore, to determine the activating signal of T6SS, CF sputum was boiled to denature any protein components and kill any microbial or eukaryotic cells persisting in the milieu. Boiled sputum maintained its capacity to induce the formation of ClpV1-GFP foci, indicating that at least a portion of the contributing molecules responsible for T6SS activation were not heat labile (Fig. 1A). Given that total eDNA is present in considerable proportions (up to 20 mg/ml) in the CF lung sputum (42) and is heat stable, we extracted sputum-derived eDNA and challenged *P. aeruginosa* by the use of this purified component. Sputum-derived eDNA was capable of inducing ClpV1-GFP foci to a level comparable to that seen with the untreated and heat-treated CF sputum, suggesting that eDNA is a major component of sputum that elicits T6SS activation in *P. aeruginosa* (Fig. 1).

Because the H1-T6SS is known to be activated when cells sense external attacks and induction is dependent on the cell envelope-associated TagQRST signal transducing cascade (34, 38), we tested whether CF sputum-induced activation of the H1-T6SS is controlled similarly. Using the *tagT* mutant that cannot sense attacks (24, 34), we found that the ClpV1-GFP foci were occasionally formed in the CF sputum but that the number of foci was substantially reduced in the *tagT* mutant (Fig. 1).

DNA-mediated chelation of outer membrane-bound cations activates the T6SS. We next sought to characterize the mechanism by which eDNA elicits the T6SS activation. Previous research has shown that eDNA exhibits dose-dependent killing of *P. aeruginosa* by acting as a cation chelator that removes membrane-stabilizing divalent cations from the bacterial cell surface (42, 47, 48). Addition of excess divalent cations, including Mg^{2+} and Ca^{2+}, protects cells from eDNA-mediated lethality (48). Therefore, we postulated that removal of outer membrane-bound cations by eDNA triggers the T6SS activation. To test this, we treated cells with eDNA and excess Mg^{2+} divalent cations (Fig. 2). Consistent with the cation-chelating hypothesis, treatment with excess cations abolished the eDNA-induced T6SS activation in *P. aeruginosa*. Importantly, divalent cations did not suppress T6SS activities in the H1-T6SS hyperactive *P. aeruginosa* Δ*retS* ClpV1-GFP strain (31) (Fig. 2B). In addition, DNA that was degraded due to DNase treatment also failed to activate the H1-T6SS (Fig. 2).

Because eDNA is thought to perturb the bacterial cell surfaces in a fashion analogous to that seen with the cation chelator EDTA (47, 48, 56), we then tested whether EDTA could induce the T6SS activity in *P. aeruginosa* (Fig. 2). Indeed, EDTA treatment resulted in activation of the H1-T6SS, within seconds, to a level comparable to that seen with eDNA (Fig. 2). Exposure to polymyxin B, a cation-independent pore-forming antibiotic, was equally capable of inducing formation of *P. aeruginosa* ClpV1-GFP foci, consistent with previous results (38). The rapid response upon exposure to eDNA, EDTA, and polymyxin B highlights the importance of membrane integrity in regulating the *P. aeruginosa* T6SS activation through posttranslational control (Fig. 2).

Activation by eDNA is specific to H1-T6SS. *P. aeruginosa* possesses three T6SS clusters (H1, H2, and H3) that have been shown to be involved in targeting prokaryotic and eukaryotic organisms and to be triggered in response to various environmental cues (26, 34, 40). Therefore, we sought to determine whether the other two T6SS clusters (H2 and H3) of *P. aeruginosa* are activated by eDNA treatment. We individually labeled the TssB1, TssB2, and TssB3 sheath proteins with sfGFP to monitor sheath dynamics. We found that T6SS foci were occasionally formed but that exposure to eDNA failed to activate formation of TssB2-sfGFP or TssB3-sfGFP foci (Fig. 3A).

Activation of H1-T6SS by prokaryotic and eukaryotic DNA. The CF lung is highly enriched in DNA derived primarily from neutrophils, including the presence of NETs that trap and kill bacteria (47, 49, 57). It was recently shown that *P. aeruginosa* lysates induce the H1-T6SS of kin cells but that the signal is unknown (51). Therefore, we sought to decipher whether DNA isolated from human neutrophil cells or *P. aeruginosa* is capable of inducing *P. aeruginosa* H1-T6SS dynamics. We also included DNA from salmon sperm to test if the activation is specific to

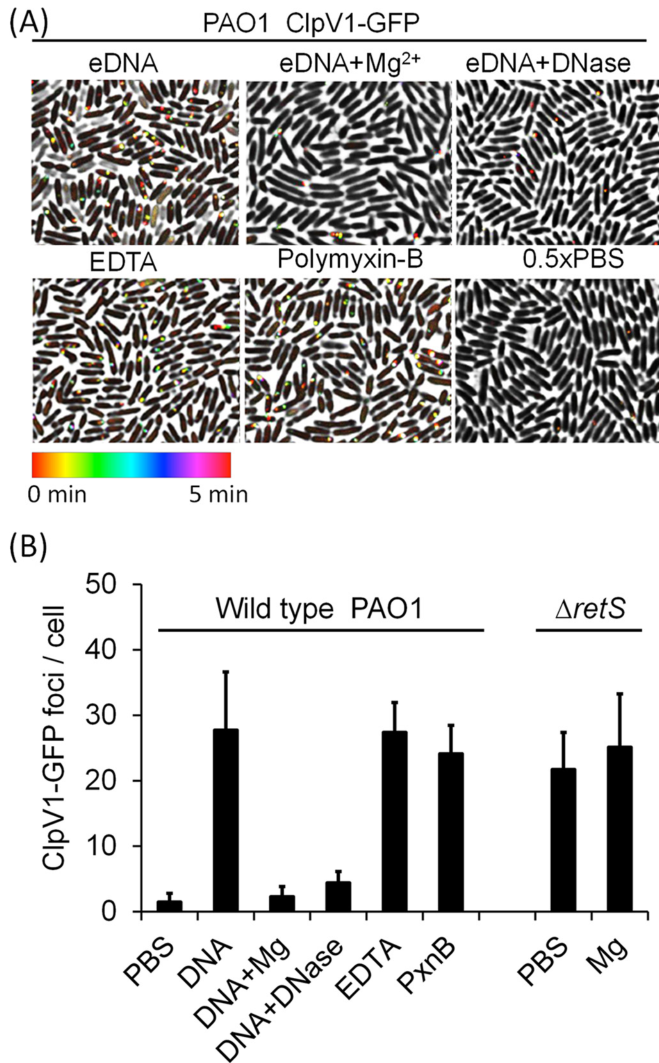

FIG 2 T6SS activation occurs through extracellular DNA-induced cation chelation. (A) Activation of formation of T6SS ClpV1-GFP foci in response to eDNA or to eDNA treated with excess divalent Mg^{2+} or DNase, EDTA, or polymyxin B (PxnB). Wild-type (WT) *P. aeruginosa* cells were imaged every 10 s starting immediately after they were spotted onto 0.5× PBS agarose pads. Bacteria were resuspended in 0.5× PBS containing 0.4% (wt/vol) eDNA or were resuspended in 0.5× PBS and subsequently spotted onto pads containing 0.2 mM EDTA or 2 μg/ml polymyxin B. ClpV1-GFP localization was followed every 10 s for 5 min, and the results were temporally color coded. (B) The 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 in a 5-min period. Each bar represents the mean of results from 10 fields with 400 to 600 cells in each field ± SD. To test whether the repression of T6SS activity by Mg^{2+} is independent of membrane damage, the PAO1 *retS* mutant, known to exhibit activated T6SS, was tested in the presence or absence of excess Mg^{2+}. The T6SS activity in the *retS* mutant was not affected by Mg^{2+} treatment.

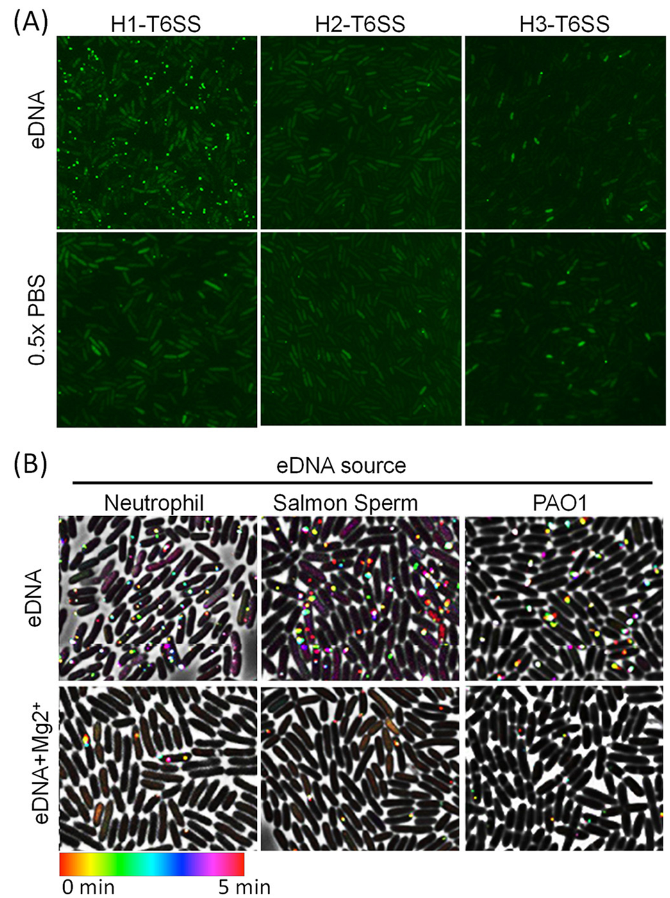

FIG 3 Prokaryotic and eukaryotic extracellular DNA activates H1-T6SS. (A) eDNA promotes activation of H1-T6SS but not H2 or H3 in *P. aeruginosa*. Mid-log-phase *P. aeruginosa* cells were imaged immediately following the addition of 0.4% (wt/vol) salmon sperm DNA or 0.5× PBS. For each sample or condition, 15 images were taken. Representative images are presented. (B) eDNA derived from human neutrophils, salmon sperm, or *P. aeruginosa* elicits activation of H1-T6SS. Mid-log-phase *P. aeruginosa* ClpV1-GFP was imaged every 10 s for 5 min immediately following the addition of 10 μg/ml of eDNA or eDNA supplemented with 10 mM Mg^{2+}.

CF-related DNA or is due to the intrinsic cation-chelating nature of DNA. Indeed, we found that eDNA, regardless of the source, activated the H1-T6SS in *P. aeruginosa* (Fig. 3B).

Extracellular DNA induces nonselective attack in *P. aeruginosa*. The H1-T6SS is tightly regulated to stay dormant and is able to carry out precise retaliatory attacks only when *P. aeruginosa* is provoked by a neighboring T6SS active species (31, 34). Considering that eDNA is also present in many diverse environments, including soil and water (43), eDNA-mediated activation of H1-T6SS would allow *P. aeruginosa* to nonselectively attack neighboring species regardless of being or not being provoked.

To test this prediction, we used the *vasK* mutant of *V. cholerae*, a T6SS null mutant previously demonstrated to be incapable of activating the H1-T6SS response and thus to be able to coexist with *P. aeruginosa* (34). We found that coincubation of *P. aeruginosa*, *V. cholerae vasK*, and eDNA led to a significant increase in *V. cholerae* cell rounding, suggesting that eDNA was a sufficient trigger to promote *P. aeruginosa* T6SS-H1 attack (Fig. 4A). Importantly, coincubation of the two bacterial species in the absence of eDNA or incubation of *V. cholerae vasK* with eDNA alone resulted in few round *V. cholerae* cells (Fig. 4A).

We then examined the effect of eDNA on the interaction between *P. aeruginosa* and *B. cepacia*, another frequent opportunistic pathogen that can coinhabit the CF lung (58). We tested the survival of two *Burkholderia cepacia* strains after coincubation on solid LB medium in the presence or absence of eDNA. Results showed that the eDNA treatment substantially reduced the sur-

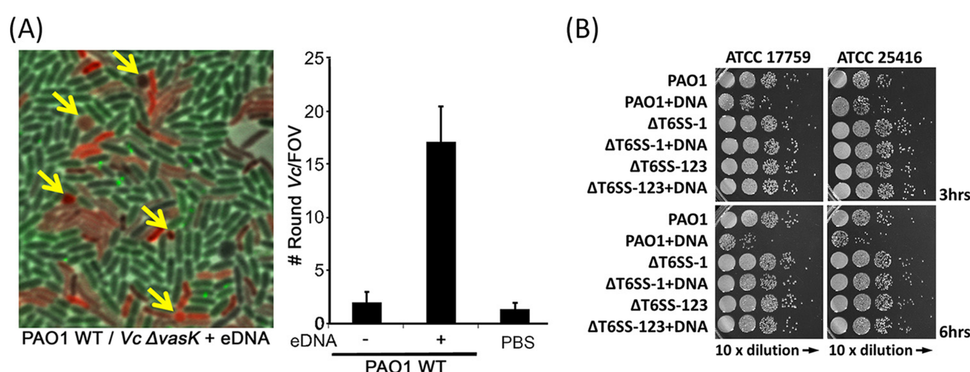

FIG 4 Extracellular DNA triggers *P. aeruginosa* T6SS attack. (A) Coincubation of 0.4% (wt/vol) salmon sperm DNA and *P. aeruginosa* ClpV1-GFP leads to rounding of the *V. cholerae* V52 Δ*vasK* strain (T6SS null mutant). Arrows point to examples of round *V. cholerae* cells. The average number of round *V. cholerae* cells per field of view (FOV; error bars represent ± standard deviations) is shown for each mixture from 35 images. (B) Competition between *P. aeruginosa* and *B. cepacia* strains (ATCC 17759 and ATCC 25416). Cultures of killer and prey cells were mixed at a ratio of 10:1 on LB medium with or without 0.4% (wt/vol) DNA. Survival of *B. cepacia* was examined by serial plating on LB polymyxin B plates. ΔT6SS-1, the *tssB1* deletion mutant; ΔT6SS-123, the *tssB1 tssB2 tssB3* triple deletion mutant.

vival of *B. cepacia* when it was mixed with wild-type PAO1 but not when it was mixed with the T6SS mutants (Fig. 4B). These data suggest that *P. aeruginosa* could nonselectively attack other species regardless of being or not being provoked in eDNA-abundant environments.

DISCUSSION

P. aeruginosa has been shown to adapt to the complex polymicrobial environment in the CF lung through genetic mutations and regulatory changes, but characterization of the molecular mechanism for *P. aeruginosa* persistence in the context of CF multispecies interaction remains elusive. Here we demonstrated that eDNA, abundant in the CF lung, promotes *P. aeruginosa* H1-T6SS firing through chelating surface-bound cations and causing outer membrane instability. This activation enables *P. aeruginosa* to nonselectively attack neighboring microbial species, thereby expanding its target range. Because eDNA is ubiquitously present in diverse environments, including biofilms and bodily fluids, eDNA-mediated H1-T6SS activation likely occurs under many other DNA-enriched conditions.

Gram-negative bacteria possessing lipopolysaccharide (LPS) on the cell outer surface are negatively charged, and the stability of the outer membrane requires the presence of divalent cations to neutralize the highly charged environment (42, 56). We found that treatment with eDNA and EDTA activates the H1-T6SS dynamics and that addition of excess magnesium cations abolishes such activation. We also confirmed previous findings indicating that treatment with the membrane-targeting antibiotic polymyxin B induces the H1-T6SS dynamics (38). Collectively, these results support the contention of the current model that outer membrane instability is a major signal perceived by *P. aeruginosa*, leading to orchestration of an immediate retaliatory H1-T6SS attack (24, 34).

A recent report indicated that *P. aeruginosa* lysates, prepared by microbial interaction or sonication, can trigger a defensive response in the remaining *P. aeruginosa* kin population, termed the *P. aeruginosa* response to antagonism (PARA), but that the signaling molecule(s) involved remains unknown (51). However, it is important to delineate that eDNA exposure results in rapid (occurring on the order of seconds to minutes) firing of the H1-T6SS whereas PARA occurs over hours (51) independently of the TagQRST pathway. While a diffusible unknown signal of kin lysis leads to a longer-term, sustained PARA response, our results obtained using eDNA and EDTA suggest that external insults promoting outer membrane instability result in an immediate retaliatory response. This is consistent with previous observations from studies using the T6SS attack and polymyxin B (34, 38).

The DNA present in the CF lung contributes to the high viscosity of CF sputum by forming a gel-like matrix with other macromolecules that affects lung function and antibiotic efficacy (48, 59). Recombinant human DNase 1 (rhDNase) has been used to treat CF patients to reduce the viscosity and increase mucus clearance (60). This treatment results in moderate to significant improvements in lung function and reductions in exacerbation frequency (61–63). Interestingly, rhDNase treatment does not affect the overall bacterial load but does causes a mild decrease in the amount of *P. aeruginosa* present in the CF lung (61, 64). Our findings with respect to DNA-mediated H1-T6SS activation suggest that rhDNase treatment may reduce the fitness of *P. aeruginosa* in competing with other microbes, thereby shifting the microbial community. A systematic evaluation of microbiota changes during rhDNase treatment may help clinicians to fully understand the variation in rhDNase treatment efficacies.

In conclusion, our study demonstrated that the H1-T6SS of *P. aeruginosa* can be activated by native and heat-treated CF sputum, eDNA, and EDTA. In comparison with the previously described precise retaliatory response to aggressive competing species (34), eDNA-mediated activation may represent an important nonselective strategy that promotes the competitive fitness of *P. aeruginosa* against passive neighboring species in environments enriched with membrane-targeting agents, including divalent-cation chelators and polymyxin B.

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We declare that we have no conflicts of interest.

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