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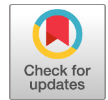

Pseudomonas aeruginosa Requires the DNA-Specific Endonuclease EndA To Degrade Extracellular Genomic DNA To Disperse from the Biofilm

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ABSTRACT The dispersion of biofilms is an active process resulting in the release of planktonic cells from the biofilm structure. While much is known about the process of dispersion cue perception and the subsequent modulation of the c-di-GMP pool, little is known about subsequent events resulting in the release of cells from the biofilm. Given that dispersion coincides with void formation and an overall erosion of the biofilm structure, we asked whether dispersion involves degradation of the biofilm matrix. Here, we focused on extracellular genomic DNA (eDNA) due to its almost universal presence in the matrix of biofilm-forming species. We identified two probable nucleases, *endA* and *eddB*, and *eddA* encoding a phosphatase that were significantly increased in transcript abundance in dispersed cells. However, only inactivation of *endA* but not *eddA* or *eddB* impaired dispersion by *Pseudomonas aeruginosa* biofilms in response to glutamate and nitric oxide (NO). Heterologously produced EndA was found to be secreted and active in degrading genomic DNA. While *endA* inactivation had little effect on biofilm formation and the presence of eDNA in biofilms, eDNA degradation upon induction of dispersion was impaired. In contrast, induction of *endA* expression coincided with eDNA degradation and resulted in biofilm dispersion. Thus, released cells demonstrated a hyperattaching phenotype but remained as resistant to tobramycin as biofilm cells from which they egress, indicating EndA-dispersed cells adopted some but not all of the phenotypes associated with dispersed cells. Our findings indicate for the first time a role of DNase EndA in dispersion and suggest weakening of the biofilm matrix is a requisite for biofilm dispersion.

IMPORTANCE The finding that exposure to DNase I impairs biofilm formation or leads to the dispersal of early stage biofilms has led to the realization of extracellular genomic DNA (eDNA) as a structural component of the biofilm matrix. However, little is known about the contribution of intrinsic DNases to the weakening of the biofilm matrix and dispersion of established biofilms. Here, we demonstrate for the first time that nucleases are induced in dispersed *Pseudomonas aeruginosa* cells and are essential to the dispersion response and that degradation of matrix eDNA by endogenously produced/secreted EndA is required for *P. aeruginosa* biofilm dispersion. Our findings suggest that dispersing cells mediate their active release from the biofilm matrix via the induction of nucleases.

KEYWORDS DNA-specific endonuclease I, EddB, EndA, biofilm, dispersion, endonuclease, nuclease

Surface-associated communities of bacteria, encased in a polymeric matrix of their own matrix, are referred to as biofilms. The surface association of biofilms can be to inert or living surfaces (1). Biofilms form as part of a developmental life cycle that is initiated by planktonic cells attaching to surfaces and turns full circle when biofilm cells

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liberate themselves from the biofilm structure and disperse. Dispersion is apparent by the hollowing of biofilm microcolonies, with central voids generated by single cells breaking free from the biofilm, or by an overall erosion of the three-dimensional biofilm structure (2–12). Dispersion likely occurs in response to the accumulation of waste products or oxygen depletion in the interior of biofilms, making dispersion a process by which bacteria avoid stress associated with biofilm growth and seek nutrient rich environments better suited for survival at new locales (3, 5, 8, 12). Dispersion, furthermore, can be induced in response to heavy metals and nitric oxide (NO), upon exposure to elevated concentrations of carbon sources, amino acids, and ammonium chloride, or by the sensing of *cis*-2-decenoic acid, an interkingdom fatty acid signaling molecule belonging to the (B)DSF family (5, 13).

While dispersed cells revert to the planktonic mode of growth and phenotypically resemble planktonic cells, it is now apparent that while dispersed cells actively escape as single cells from the biofilm, dispersed cells differ from planktonic cells. Dispersed cells were found to exhibit protein production and gene expression profiles that are distinct from those of planktonic cells and biofilms from which they escaped (3, 14–17). Moreover, dispersed cells appear to be more primed to reattach following egress from the biofilm (14, 18), to produce more matrix-degrading enzymes (16), to be more virulent than planktonic cells when tested using various acute and chronic virulence models (15, 16, 19), and depending on the antibiotic used, to be as susceptible as planktonic cells or resistant to antimicrobial agent (14, 20). These differences have led to dispersed cells being referred to as a third phenotype that is distinct from the phenotypes of both planktonic and biofilm cells (3, 15, 16). The distinct phenotype of dispersed cells, however, was found to be reversible and somewhat short lived. Using quantitative reverse transcriptase PCR (qRT-PCR) and antimicrobial susceptibility assays, Chambers et al. (14) demonstrated that in *Pseudomonas aeruginosa*, differences between planktonic and dispersed cells remained for 2 h postdispersion, with additional time being required for dispersed cells to express genes serving as signs of exponential growth.

Levels of the intracellular signaling molecule c-di-GMP have important implications for the bacterial mode of growth, with low c-di-GMP levels fostering the motile lifestyle and high intracellular levels promoting aggregative behavior, including attachment and biofilm formation (21, 22). It is thus not surprising that the induction of dispersion by *P. aeruginosa* biofilms has been linked to increased expression of *fliC* (encoding flagellin type B) (23), with cells actively leaving the biofilms, the activation of phosphodiesterases, and reduced levels of c-di-GMP present in dispersed cells relative to that in biofilm cells from which they egressed (6, 7, 9, 24). Additional support for c-di-GMP playing an important role in dispersion stems from the finding that planktonic cells expressing *yhjH* (also known as *pdeH*) encoding an *Escherichia coli* phosphodiesterase are similar with respect to gene expression, virulence, and susceptibility to antibiotics to dispersed cells obtained in response to NO (19, 25). The findings further underscored that dispersed cells are characterized by c-di-GMP levels that are significantly reduced relative to that in biofilm or even planktonic cells (26, 27).

Low c-di-GMP levels present in dispersed cells have led to the notion of dispersion being a consequence of reduced intracellular c-di-GMP levels, likely through the activation of phosphodiesterases (6, 7, 19). However, little is known about the subsequent events leading to biofilm dispersion. Considering dispersion coincides with a reduction in c-di-GMP levels, it is likely that dispersion coincides with phenotypes associated with low c-di-GMP levels, such as motility and the planktonic mode of growth, and/or a reversion of high c-di-GMP-associated phenotypes, such as reduced adhesiveness and decreased production of biofilm matrix components (21, 22, 28–33). The latter include the Psl and Pel polysaccharides by *P. aeruginosa*. In addition, localization of the large adhesin LapA has been linked to c-di-GMP. LapA is localized at the outer membrane at high c-di-GMP levels but released from the cell surface upon proteolytic cleavage by the protease LapG at low c-di-GMP (34). Not surprisingly, the release of the large adhesin LapA from the cell surface via cleavage by protease LapG

TABLE 1 Genes encoding potential and confirmed nucleases in the genome of *P. aeruginosa* PAO1[a]

PA number	Gene name	Localization	N-terminal signal	Description
PA0965	*ruvC*	Cytoplasmic	None	Holliday junction resolvase
PA2545	*xthA*	Cytoplasmic	None	Exonuclease III
PA2585	*uvrC*	Cytoplasmic	None	Endonuclease
PA2749	*endA*	Extracellular	Type I	Endonuclease, DNA-specific endonuclease I
PA3232	PA3232	Cytoplasmic	None	Probable nuclease
PA3495	Nth	Cytoplasmic	None	Endonuclease III
PA3725	*recJ*	Cytoplasmic	None	Single-stranded-DNA-specific exonuclease
PA3909	*eddB*	Extracellular	Type I	Extracellular DNA degradation protein
PA3910	*eddA*	Extracellular	Type I	Alkaline phosphatase, PhoD-type phosphatase[b]
PA4172	PA4172	Cytoplasmic	None	Probable nuclease, endonuclease III-like
PA4281	*sbcD*	Cytoplasmic	None	Exonuclease
PA4316	*scbB*	Cytoplasmic	None	Exodeoxyribonuclease I, exonuclease I
PA4424	PA4424	Unknown	None	Endonuclease II-like (*yraN* homolog)
PA5048	PA5048	Unknown	None	Probable nuclease, SNase-like
PA5147	*mutY*	Cytoplasmic		Endonuclease, endonuclease III-like

[a]Unless otherwise noted, localizations and descriptions were adopted from pseudomonas.com (41), while predictions of N-terminal signals were obtained from Lewenza et al. (42).
[b]Description adopted from references 45 and 46.

has been shown to coincide in a dispersion response by *Pseudomonas putida* and *Pseudomonas fluorescens* biofilms (24).

Given the impact of c-di-GMP modulation on biofilm matrix components and considering that dispersion coincides with biofilm erosion and single cells escaping the biofilm structure, with dispersed cells producing more matrix-degrading enzymes than planktonic or biofilm cells (16), it is likely that dispersion relies on factors that degrade or at least compromise the integrity of the biofilm matrix. Matrix degradation playing a role in dispersion is supported by the plethora of matrix-degrading factors such as proteases, deoxyribonucleases, and glycoside hydrolases having been linked to biofilm dispersal (15, 16, 35–38). However, most studies have relied on inducing dispersion by the exogenous addition of these factors, and specific matrix-degrading factors that are intrinsic to the biofilm dispersion response remain uncharacterized.

The goal of this study was to identify specific matrix-degrading factors that contribute to dispersion. The biofilm matrix is composed of a variety of polysaccharides, proteins, adhesins, and extracellular genomic DNA (eDNA) (39, 40). While biofilm dispersion is common among biofilm-forming species, the compositions of the biofilm matrix differ significantly among biofilm-forming species. More specifically, the polysaccharides and adhesins present in the biofilm matrix appear to be species or even strain specific. However, one matrix component that appears to be common to all biofilm matrices is eDNA. We therefore asked whether dispersion relies on eDNA degradation to weaken or compromise the matrix encasing biofilms for bacteria to escape.

RESULTS

Identification of probable or predicted nucleases. To determine whether the degradation of eDNA plays a role in the dispersion response, we surmised that eDNA degradation would involve nucleases that are active in the periplasmic or extracellular space and that the respective genes are induced upon induction of the dispersion response. To identify potential extracellular enzymes with nucleolytic activity, we made use of the biofilm model organism *Pseudomonas aeruginosa* PAO1 and screened the genome sequence for the presence of genes encoding nucleases using the search term "nuclease" (41). The screen revealed 15 genes encoding probable or predicted nucleases (Table 1). Of the 15 "nucleases," according to https://www.pseudomonas.com/, 10 were predicted to be localized in the cytoplasm, while EndA (PA2749) and EddA (PA3910) were predicted to be localized in the extracellular space (Table 1). The respective localizations were confirmed by Lewenza et al. (42) using a consensus computational strategy combined with a laboratory-based PhoA fusion screen. While subcellular prediction tools place EddB (PA3909) with equal likelihood in the cytoplas-

mic membrane, periplasm, outer membrane, or extracellular space, https://www.pseudomonas.com/ indicates EddB is localized in the extracellular space (Table 1). This subcellular localization is based on EddB harboring cleavable type I signal peptides (42) and having been experimentally confirmed to be secreted (43). An additional two "nucleases," PA4424 and PA5048, were listed as unknown (Table 1). The amino acid sequences of PA5048 and PA4424 lack N-terminal signaling peptides enabling translocation across membranes (42), likely suggesting a cytoplasmic location.

As we anticipated nucleases to act on eDNA to weaken the biofilm matrix, with eDNA degradation likely occurring extracellularly, all candidates predicted to be located in the cytoplasm were excluded. We likewise excluded PA4424 and PA5048 due to localization. This reduced the number of potential candidates to three: EndA, EddA, and EddB.

We furthermore asked whether there are indications that the three remaining candidates indeed encoded potential nucleases. Based on sequence analysis, *endA* likely encodes a probable DNA-specific endonuclease I. EddB, encoded by PA3909, was previously reported to be required for DNA degradation and to promote *P. aeruginosa* growth when DNA was supplied as the sole phosphate source (43, 44). *eddB* is downstream of *eddA* (PA3910), with *eddA* encoding a PhoD-like alkaline phosphatase that possesses both monoesterase and phosphodiesterase activities (45). Despite EddA having been shown to harbor alkaline phosphatase activity, we included EddA in our further investigation, as EndA has been speculated to be required for phosphorus acquisition from DNA (46) and EddA homologs, such as the *Zymomonas mobilis* CP4 PhoD alkaline phosphatase, have been shown to hydrolyze nucleotides (47).

Dispersion coincides with increased transcript abundance of genes in the *endA* and *eddA* operons. Having identified three potential candidates, we next determined their transcript abundance in response to dispersion cues. We reasoned that if EndA, EddA, or EddB plays a role in the dispersion response, then induction of dispersion would coincide with increased transcript abundance. To address this question, we determined the transcript abundance by qRT-PCR using RNA isolated from biofilms by two mutants, the Δ*bdlA* and PAO1/*bdlA*_G31A strains. By doing so, we took advantage of the finding that BdlA is central to the dispersion response to obtain homogenous populations of dispersing and nondispersing cells. This is supported by the fact that Δ*bdlA* biofilms are impaired in the dispersion response to a large variety of dispersion cues (9, 10), while biofilms overproducing BdlA_G31A, which transmits a constant signal-on bias for dispersion, are hyperdispersive (48). Previous findings indicated the hyperdispersive phenotype correlates with reduced biofilm biomass accumulation, but with a 2- to 3-fold increase of bacteria present in biofilm effluents compared to that in wild-type biofilms over the course of 5 days of biofilm growth (48). Moreover, we determined the transcript abundance of only *eddA*, not *eddB*, as the genes *eddA* and *eddB* were previously reported to be part of a 2-gene operon and to be coregulated (43, 45).

Relative to nondispersing biofilms, hyperdispersive biofilms were characterized by a significant 11-fold increase in *endA* transcript abundance and a 58-fold increase in the transcript abundance of *eddA* (Fig. 1A). The findings suggested the expression of *endA* and *eddA* was induced under dispersion conditions. It is of interest to note that based on quantification cycle (C_q) values, *endA* and *eddA* were transcribed in nondispersing Δ*bdlA* biofilm cells.

Insertional inactivation of *endA, eddA*, and *eddB* has no apparent effect on the biofilm architecture under flowing conditions. Previous findings indicated that nucleases contribute to biofilm formation and dynamics (43, 44, 49). As our qRT-PCR analysis indicated that *endA* and *eddA* (and indirectly, *eddB*) are transcribed in biofilms, we next determined whether inactivation of *endA*, *eddA*, and *eddB* affected biofilm formation. We therefore made use of mutant strains harboring transposon insertions in *endA*, *eddA*, and *eddB*. The respective mutant strains are referred to as *endA*::IS, *eddA*::IS, and *eddB*::IS strains. Biofilms of the strains were grown for 5 days under flowing conditions in flow cells after which, the biofilm architecture was viewed by confocal

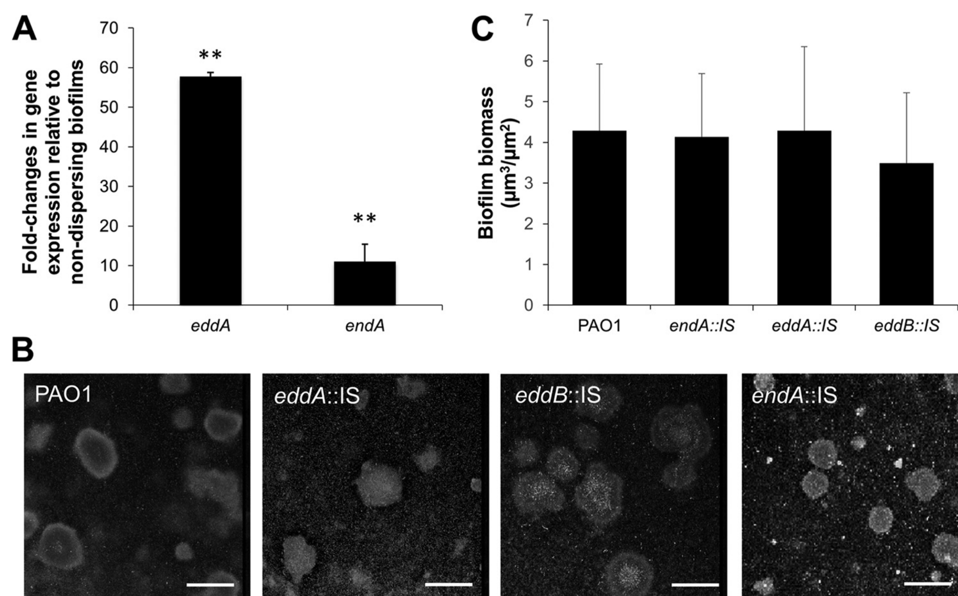
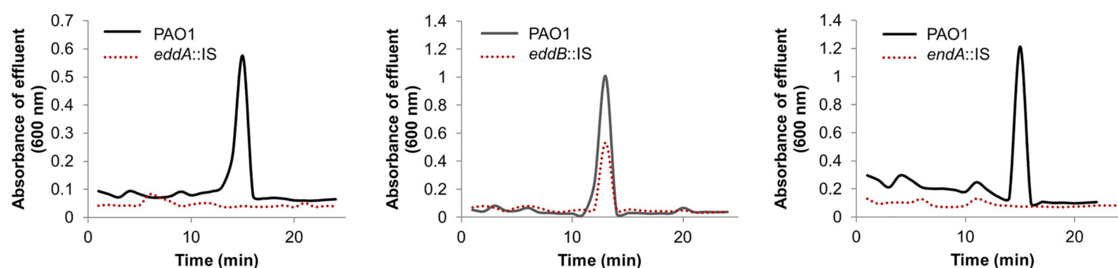


FIG 1 Transcript abundance of *endA* and *eddA* in hyperdispersive biofilms relative to that in nondispersing biofilms cells, and contribution of *endA*, *eddA*, and *eddB* to biofilm formation. (A) Fold changes of *endA* and *eddA* transcript abundance in hyperdispersive biofilms relative to that in nondispersing biofilms, as determined using qRT-PCR. Biofilms by the hyperdispersive strain PAO1/pJN-*bdlA*-G31A and the nondispersive Δ*bdlA* cells were grown for 3 days under flowing conditions in tube reactors using 5-fold diluted VBMM. *cysD* was used as a housekeeping control. Error bars indicate standard deviations. **, $P < 0.05$ relative to Δ*bdlA* biofilms. (B) Representative confocal images of the biofilm architecture by *P. aeruginosa* PAO1 and *endA*::IS, *eddA*::IS, and *eddB*::IS mutant strains. Biofilms were grown for 5 days under flowing conditions in flow cells and stained prior to microscopy using the Live/Dead BacLight viability stain. Bars, 100 μm. (C) COMSTAT analysis of the biofilm biomass. All experiments were performed in triplicate. Error bars indicate standard deviations.

microscopy. Visual inspection of biofilms by *endA*::IS, *eddA*::IS, and *eddB*::IS strains revealed an overall architecture characterized by the presence of large microcolonies that resembled biofilms formed by *P. aeruginosa* PAO1 (Fig. 1B). Quantitative analysis of the biofilm biomass using COMSTAT furthermore indicated insertional inactivation of *endA*, *eddA*, and *eddB* had apparent effect on the biofilm biomass accumulation under flowing conditions (Fig. 1C).

Inactivation of *endA* impairs biofilm dispersion in response to multiple dispersion cues. Considering the increased expression of *endA*, *eddA*, and *eddB* under dispersion-inducing conditions, we next asked whether the nucleases EndA and EddB or phosphatase EddA contributes to the dispersion response by *P. aeruginosa* biofilms. Previous findings indicated that dispersion can be induced by exposure to various exogenous dispersion cues (5, 13), including nitric oxide (NO) and glutamate (6, 9, 18, 23, 50). Furthermore, it has been demonstrated that dispersion can be detected by a decrease in the biofilm biomass and void formation, as determined using flow-cell-grown biofilms in conjunction with microscopy, as well as by a sharp increase in the absorbance (600 nm) in the effluent within 15 to 20 min upon induction of dispersion, as determined using tube reactors (6, 9, 10, 23, 48, 51). We made use of the latter approach to ease the collection of dispersed cells. Biofilms by the *P. aeruginosa* mutant (*endA*::IS, *eddA*::IS, and *eddB*::IS) strains were first grown for 5 days under flowing conditions in a tube reactor and then exposed to two different dispersion cues, glutamate or NO, to induce dispersion. While exposure of wild-type PAO1 biofilms to the dispersion cue glutamate coincided with increased turbidity of the medium effluent of biofilm tube reactors, no such increase was noted upon challenging biofilms by *endA*::IS and *eddA*::IS cells with glutamate (Fig. 2A). However, biofilms by *eddB*::IS cells dispersed in response to glutamate (Fig. 2A). Likewise, challenging biofilms by *endA*::IS and *eddB*::IS cells with NO failed to induce a dispersion response (Fig. 2B). In contrast, biofilms by *eddA*::IS cells dispersed in response to NO in a manner similar to that of biofilms by wild-type *P. aeruginosa* PAO1 (Fig. 2B).

A Glutamate-induced dispersion



B NO-induced dispersion

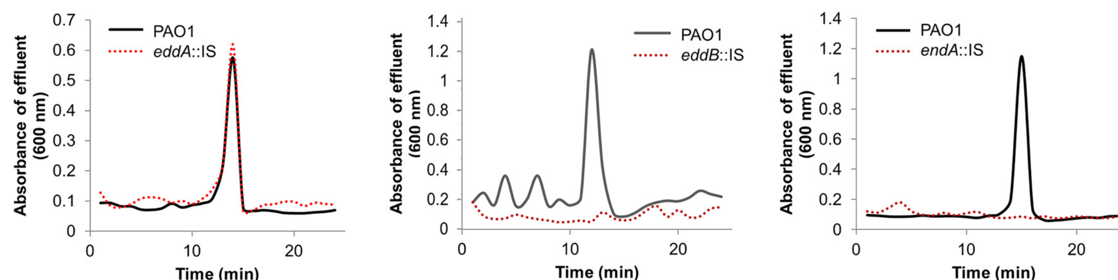


FIG 2 Insertional inactivation of *endA* impairs the dispersion response by *P. aeruginosa* when exposed to glutamate or nitric oxide (NO), while insertional inactivation of *eddA* or *eddB* impairs dispersion in a manner dependent on the dispersion cue used. Biofilms were grown for 5 days in 5-fold diluted VBMM in tube reactors. Dispersion was induced by the addition of glutamate (A) or nitric oxide (NO) (B) to the growth medium. Sodium nitroprusside served as a source of NO. Absorbance of biofilm tube reactor effluents after induction of dispersion is shown. Dispersion assays were performed at least three times, with each biological replicate consisting of 4 technical replicates. Representative dispersion profiles are shown.

Given that *eddA* and *eddB* were previously reported to be part of a 2-gene operon and to be coregulated (43, 45), our finding of EddA only contributing to dispersion to glutamate while EddB only contributed to dispersion to NO was surprising. To exclude polar effects of the transposon inserts, we determined whether complementation restored the dispersion response. Interestingly, multicopy expression of *eddB* restored dispersion by the *eddB*::IS biofilm in response to NO, while multicopy expression of *eddA* restored dispersion by the *eddA*::IS biofilm in response to glutamate (see Fig. S1 in the supplemental material). The findings indicated that the difference in dispersion phenotypes was not due to polar effects and that EddA and EddB likely have to work in concert to induce dispersion to multiple dispersion cues. In contrast, EndA alone contributed to dispersion in response to both glutamate and NO.

***endA* encodes a secreted protein capable of degrading genomic DNA derived from *P. aeruginosa*.** Given that, of the three candidates, only EndA contributed to biofilm dispersion in response to two dispersion cues, we selected EndA for further investigation. As indicated above, *endA* likely encodes a probable DNA-specific endonuclease I. Moreover, the deduced amino acid sequence of EndA shares ~50% identity to the periplasmic nucleases EndA from *E. coli* and Vvn from *Vibrio vulnificus*, as well as to the extracellular nucleases Dns from *Vibrio cholerae* and *Aeromonas hydrophila*, and EndA from *Shewanella oneidensis* MR-1 (52–56). In addition, Gnanadhas et al. (44) reported that exposure of *P. aeruginosa* biofilms to L-methionine results in increased DNase activity in culture supernatants, with L-Met treatment coinciding with upregulated expression of four DNase genes (*sbcB*, *endA*, *eddB*, and *recJ*). While the findings suggested EndA is an active nuclease and likely to be secreted, the DNase activity of EndA or its location was not confirmed in the study.

To determine whether EndA is secreted, we overproduced the protein in *E. coli* (BL21/pET-*endA*_V5/6×His) under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. The gene *endA* was modified to harbor C-terminal V5 and 6×His tags, resulting in a calculated molecular mass of EndA of 29.5 kDa. Uninduced cells were

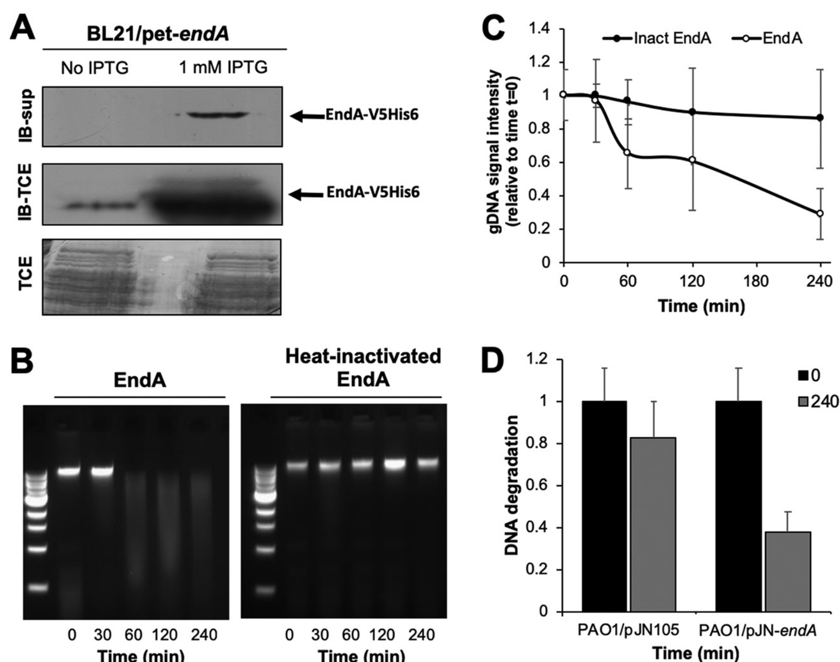


FIG 3 EndA is an extracellular nuclease. (A) Immunoblot analysis of EndA produced by *E. coli* BL21/pet-*endA*_V5/His in the absence and presence of IPTG in culture supernatants (IB-sup) and total extracts (IB-TCE). A total of 20 μg of total cell extracts and 1.5 μg of BL21/pet-*endA* supernatants were loaded. Coomassie-stained SDS gels showing total cell extracts (TCE) after transfer were used as loading controls. (B) Representative agarose gel images demonstrating EndA-dependent degradation of genomic DNA over a period of 240 min. EndA purified from *E. coli* culture supernatants was used. (C) Quantitative analysis of the nuclease activity of purified EndA (EndA) and purified EndA that was inactivated by heat (Inact EndA). (D) Quantitative analysis of the nuclease activity of supernatants obtained from *P. aeruginosa* PAO1 overexpressing *endA* relative to that in PAO1 harboring the empty plasmid pJN105. Experiments were performed in triplicate. Error bars indicate standard deviations.

used as a control. Relative to uninduced cells, a protein having an apparent mass of ~28 kDa was detected in total cell extracts after IPTG induction, as determined using immunoblot analysis and anti-V5 antibodies (Fig. 3A). More importantly, however, a protein of similar size was detected in culture supernatants of induced but not uninduced cells (Fig. 3A; see Fig. S2), suggesting that EndA is indeed secreted.

To characterize the activity of the V5/6×His-tagged EndA protein, we determined the level of DNA degradation using genomic DNA (gDNA) obtained from *P. aeruginosa* upon the addition of purified EndA. EndA was purified from culture supernatants to apparent homogeneity (Fig. S2). To this end, we measured gDNA degradation by visualizing the residual DNA by separation on agarose gels after incubation with EndA. As a negative control, we used heat-inactivated EndA. The purified enzyme was capable of degrading gDNA within less than 60 min (Fig. 3B and C). In contrast, heat-inactivated EndA was unable to do so (Fig. 3B and C). These assays demonstrated that gDNA was readily degraded by EndA, while no DNA degradation occurred when heat-inactivated EndA was added.

Dispersion coincides with EndA-dependent reduction in the eDNA content present in biofilms. Similar to the *E. coli*-produced EndA, EndA produced by *P. aeruginosa* was found to be secreted (Fig. S2) and capable of degrading gDNA (Fig. 3D). Therefore, we next asked whether EndA is capable of degrading eDNA present in *P. aeruginosa* biofilms. To do so, we first determined whether we can visualize eDNA by confocal microscopy, by staining eDNA with propidium iodide as previously reported (57). We made use of strain PAO1 carrying plasmid pMRP9-1, in which the green fluorescent protein (GFP) is constitutively expressed, and established biofilms in 24-well plates under static conditions. Established biofilms were either left untreated or exposed to salmon sperm DNA and then subsequently stained with propidium iodide.

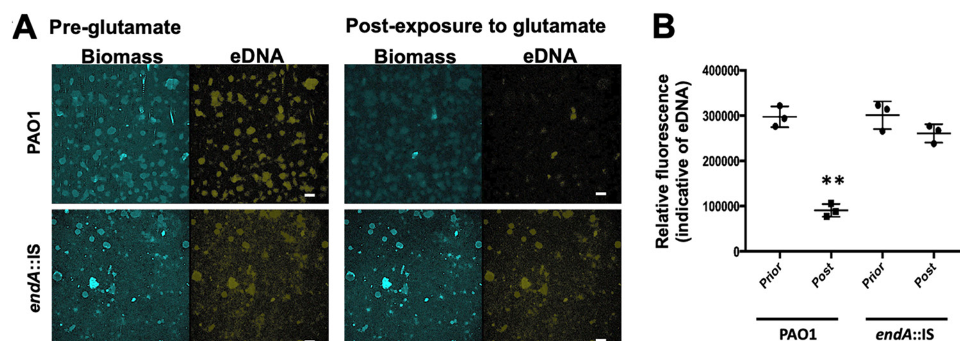


FIG 4 EndA contributes to eDNA degradation in biofilm upon induction of dispersion. (A) Representative confocal images of 5-day-old biofilms by PAO1 and *endA*::IS strains prior to and after addition of the dispersion cue glutamate. eDNA was visualized using propidium iodide. Bars, 100 μm. (B) Quantitative analysis of the relative fluorescence associated with eDNA. Experiments were performed in triplicate, with each biological replicate consisting of 4 technical replicates. **, $P < 0.05$ relative to biofilms prior to exposure to glutamate (pre-glutamate).

While untreated biofilms were stained by propidium iodide throughout (see Fig. S3A), biofilms that were exposed to exogenously added DNA primarily stained at the periphery of biofilm microcolonies (Fig. S3B). COMSTAT analysis furthermore suggested that while addition of exogenous DNA had no effect on the biofilm biomass (visualized via GFP), the propidium iodide-stained eDNA significantly increased relative to that in untreated control biofilms (Fig. S3C and D). The same settings used for eDNA detection were used to visualize eDNA prior to and after induction of the dispersion response. To do so, biofilms by PAO1 and *endA*::IS cells were grown in flow cells for 5 days. Prior to the induction of dispersion by glutamate, biofilms were stained with propidium iodide and images were acquired (Fig. 4A, "pre-glutamate"). No significant difference in the biofilm architecture was noted, with biofilms by PAO1 and *endA*::IS cells characterized by the presence of microcolonies. Moreover, staining of eDNA by propidium iodide revealed no differences between biofilms formed by the wild type and the *endA*::IS mutant (Fig. 4A). The lack of a significant biofilm phenotype under hydrodynamic conditions upon insertional inactivation of *endA* strongly indicated that this nuclease has only a minor role, if any, in biofilm formation.

Then, biofilms were exposed to the dispersion cue glutamate, and 30 min after exposure to glutamate, additional images of the same biofilm were acquired (Fig. 4A, "post-exposure to glutamate"). A comparison of the propidium iodide-stained eDNA detectable in biofilms prior to and after addition of glutamate suggests a reduction in eDNA in biofilms by *P. aeruginosa* PAO1. Quantitation of the fluorescence indicative of eDNA confirmed a significant reduction in eDNA after induction of dispersion in response to glutamate (Fig. 4B). In contrast, little to no difference in eDNA was noticed in *endA*::IS biofilms prior to and after addition of glutamate (Fig. 4A). The finding was confirmed by quantitative analysis of propidium iodide-based fluorescence (Fig. 4B). Overall, our findings suggested that while dispersion by the *P. aeruginosa* wild type results in a significant reduction in the overall detectable eDNA content, impaired dispersion coincides with a lack of eDNA modulation. The findings furthermore suggested that EndA likely contributes to the degradation of eDNA present in biofilms after induction under dispersion-inducing conditions.

Induction of *endA* coincides with a reduction of eDNA present in biofilms. To further confirm a role of EndA in eDNA degradation, we next determined the fate of eDNA upon overexpression of *endA*. We therefore made use of a complemented *endA*::IS/pJN-*endA* strain which harbored *endA* under the control of the arabinose-inducible P_{BAD} promoter. The *endA*::IS/pJN105 strain was used as a control. Biofilms by the mutant strains were grown for 5 days in the absence of arabinose to ensure the establishment of biofilms. Following 5 days of growth, arabinose was added to the growth medium for a period of 90 min to induce the transcription of *endA*. Prior to and following arabinose supplementation, the biofilm architecture and eDNA were moni-

tored by confocal microscopy (Fig. 5A). Relative to uninduced *endA*::IS/pJN105 biofilms, exposure to arabinose appeared to have no effect on the propidium stainable eDNA content of *endA*::IS/pJN105 biofilms (Fig. 5A). Moreover, no apparent change in the biofilm biomass was noted (Fig. 5A) The findings were supported by the quantitative analysis of fluorescence indicative of the biofilm biomass (Fig. 5B) and eDNA (Fig. 5C) prior to and after addition of arabinose. In contrast, induction of gene expression, upon exposure to arabinose, resulted in a reduction of the biofilm biomass and detectable eDNA present in biofilms by the *endA*::IS/pJN-*endA* strain (Fig. 5A). Analysis of the detectable fluorescence obtained following image analysis confirmed the induction of *endA* gene expression results in a significant reduction of both the biofilm biomass (Fig. 5B) and eDNA (Fig. 5C) relative to that in untreated *endA*::IS/pJN-*endA* biofilms.

Induction of *endA* results in biofilm dispersion. Our findings suggested not only that EndA contributes to eDNA degradation but also that induction of *endA* gene expression coincides with a significant reduction in the biofilm biomass. As biofilm biomass loss is indicative of dispersion, we wanted to confirm whether induction of *endA* gene expression indeed results in biofilm dispersion. We therefore made use of tube-reactor-grown *endA*::IS/pJN-*endA* biofilms harboring *endA* under the control of the arabinose-inducible P_{BAD} promoter. The *endA*::IS/pJN105 strain was used as a control. Mutant biofilms were allowed to form biofilms for 5 days in the absence of arabinose. Then, biofilms were exposed to arabinose to induce *endA* gene expression, and biofilm effluents were collected for a period of 90 min after addition of arabinose to the growth medium. Effluents by *endA*::IS/pJN105 biofilms appeared to have an average absorbance of ~0.1 over the entire 90 min period (Fig. 5D). The addition of arabinose to the growth medium resulted in repeated dispersion events of various intensities by *endA*::IS/pJN-*endA* biofilms over the course of 90 min (Fig. 5E), a response that was absent in Δ*endA*::IS/pJN105 biofilms used as a control (Fig. 5D). Our findings strongly suggested that induction of *endA* gene expression results in dispersion. It is of interest to note that the dispersion events noted upon induction of *endA* gene expression were similar to those previously observed upon induction of *bdlA*_G31A gene expression by *P. aeruginosa* biofilms (48). Moreover, the findings are in agreement with the reduction of the biofilm biomass noted upon induction of *endA* gene expression in flow-cell-grown biofilms (Fig. 5A and B).

Cells generated upon induction of *endA* gene expression share characteristics with dispersed cells. Chambers et al. (14) reported dispersed cells are more primed to reattach following egress from the biofilm and are more susceptible to tobramycin than biofilm cells. We therefore asked if dispersed cells obtained in response to *endA* gene expression adopt phenotypes generally associated with dispersed cells. As the induction of *endA* gene expression to induce dispersion resulted in cells dispersing at various time points (Fig. 5E) that makes it difficult to collect a sufficient number of dispersed cells, we instead made use of biofilms continuously overexpressing *endA*, to mimic hyperdispersive biofilms. The system is similar to the one making use of the overexpression of *bdlA*_G31A to generate hyperdispersive biofilms (Fig. 1A).

To assess attachment, biofilms (PAO1/pJN105 and PAO1/pJN-*endA*) were grown for 3 days in the presence of arabinose (0.1%) to ensure *endA* gene expression. Then, biofilms were harvested, homogenized to disrupt cell aggregates, diluted to an optical density at 600 nm (OD_{600}) of 0.2, and used as inoculum in attachment assays. Attachment was assessed by crystal violet (CV) staining following 2 h of incubation. Planktonic cells grown to exponential and stationary phases, and dispersed cells obtained in response to glutamate, were used as controls. In agreement with previous findings (14), dispersed cells attached more efficiently than cells grown to stationary phase (Fig. 6A). Attachment by biofilm cells by PAO1/pJN105 was comparable to that noted for planktonic cells grown to stationary phase (Fig. 6A). In contrast, biofilm cells overexpressing *endA* (PAO1/pJN-*endA*) attached as efficiently as dispersed cells (Fig. 6A).

We furthermore assessed the susceptibility phenotype. Biofilms (PAO1/pJN105, PAO1/pJN-*endA*, and *endA*::IS/pJN105) were grown as described above, harvested,

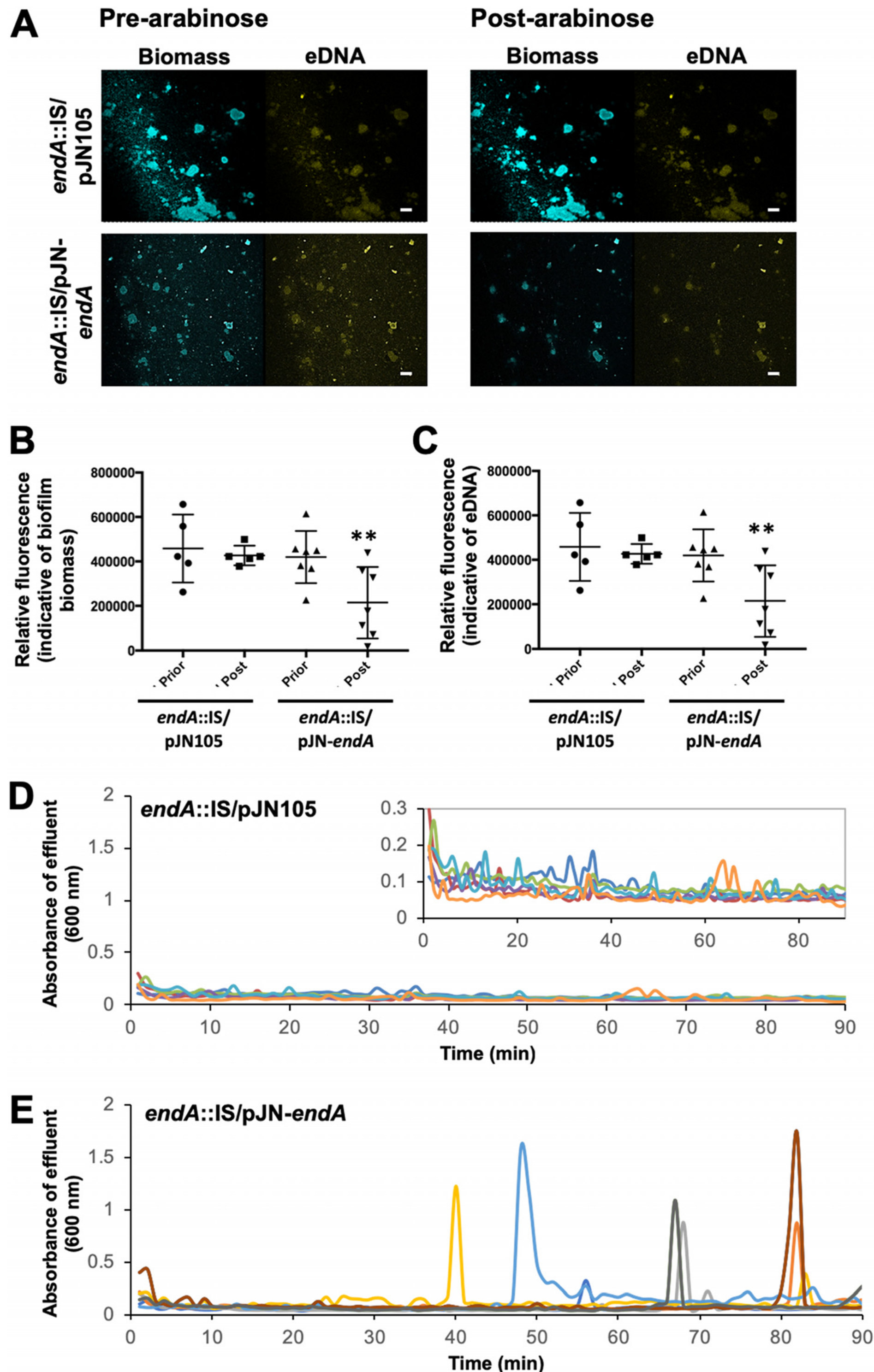


FIG 5 Induction of *endA* gene expression coincides with eDNA degradation and dispersion by *P. aeruginosa* biofilms. (A) Representative confocal images of 5-day-old biofilms by *endA*::IS/pJN105 and *endA*::IS/pJN-*endA* strains prior to and after addition of 0.8% arabinose. eDNA was visualized using propidium iodide. Bars, 100 μm. (B) Quantitative analysis of the relative fluorescence associated with the biofilm biomass. (C) Quantitative analysis of the relative fluorescence associated with eDNA. Experiments were performed in triplicate, with each biological replicate consisting of 4 technical replicates. **, $P < 0.05$ versus uninduced (prior) biofilms. Dispersion upon induction of *endA* gene expression. Graphs shown are representative of three independent biofilm replicates. (D) Dispersion profiles of biofilms by *P. aeruginosa endA*::IS harboring the empty plasmid pJN105 upon exposure to arabinose. Inset, zoomed-in graph to show biological replicates. (E) Detection of dispersion following induction of *endA* gene expression, with gene expression induced upon addition of 0.8% arabinose to the growth medium. Different colors represent biological replicates.

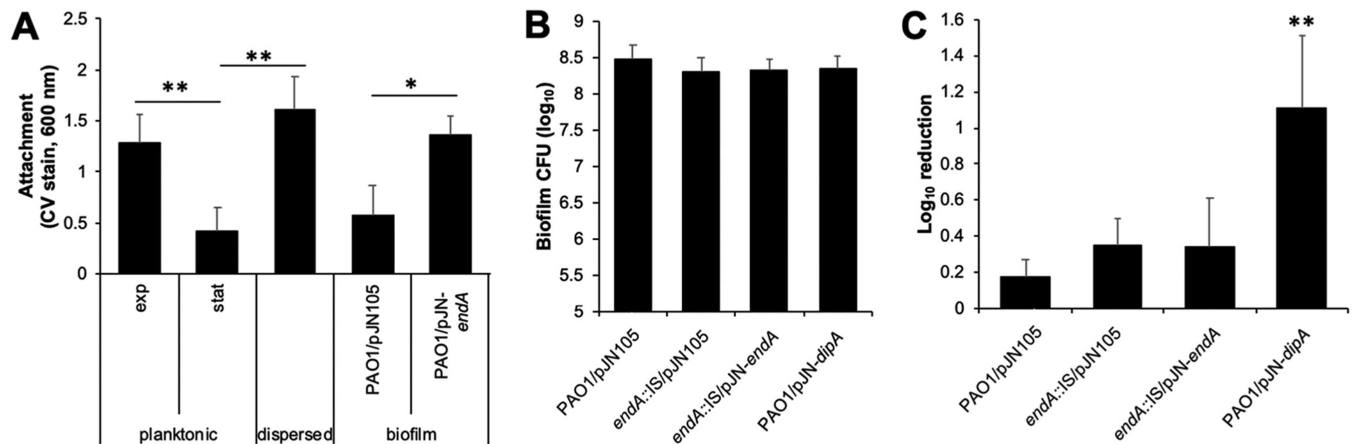


FIG 6 Induction of *endA* gene expression results in cells that more readily attach but are as susceptible to tobramycin as biofilms. For all experiments, biofilms were grown under flowing conditions in tube reactors in the presence of 0.1% arabinose. Following 3 days of growth, biofilms were then harvested and the resulting cells were subjected to attachment and susceptibility assays. (A) Attachment assays were carried out in 96-well plates, with each well inoculated with 200 μl of bacterial culture adjusted to an OD of 0.2. The adhering biomass was determined 2 h postinoculation using crystal violet (CV) staining. Absorbance was determined at 600 nm. Experiments were carried out at least in triplicate, with each repeat comprising 12 technical replicates. Error bars denote standard deviations. *, significantly different from PAO1/pJN105 biofilms; **, significantly different from *P. aeruginosa* grown planktonically to stationary phase; $P < 0.01$ as determined by ANOVA and SigmaStat. exp, exponential phase; stat, stationary phase. (B) Average numbers of viable cells present in biofilms, tube reactor grown, as determined using CFU count prior to exposure to tobramycin. Biofilm CFU is expressed as $\log_{10}$. Error bars represent standard deviations. Experiments were performed in triplicate, with each repeat comprising a technical duplicate. (C) Susceptibility phenotype of indicated strains to tobramycin (50 μg/ml) was determined using viability plate assays, calculated as CFU counts. Susceptibility is expressed as $\log_{10}$ reduction in viability. Experiments were performed in triplicate, with each repeat comprising technical duplicates. Error bars represent standard deviations. **, $P < 0.005$ versus PAO1/pJN105 by ANOVA.

homogenized to disrupt cell aggregates, and diluted to an OD_{600} of 0.2. The obtained cells were subsequently exposed to tobramycin (50 μg/ml) for 1 h. Homogenized biofilm cells not treated with tobramycin were used as a control. Under the conditions tested, no significant difference in the biofilm biomass was noted (Fig. 6B), apparent by biofilms being composed on average of $\sim 2.5 \times 10^8$ CFU/biofilm. Moreover, no difference in susceptibility to tobramycin, as determined using $\log_{10}$ reduction, was noted (Fig. 6C). Using a similar approach, we furthermore determined the susceptibility of biofilms overexpressing *dipA*, encoding the phosphodiesterase DipA (PAO1/pJN-*dipA*). This strain was chosen as DipA was previously demonstrated to be required for dispersion (7) by contributing to the relay of dispersion cue sensing into the modulation of c-di-GMP levels (6). The exposure of biofilms by PAO1/pJN-*dipA* to tobramycin coincided with significantly increased susceptibility (Fig. 6C). Taken together, our findings suggested that induction of *endA* gene expression coincides with *P. aeruginosa* adopting some but not all phenotypes generally associated with dispersed cells.

DISCUSSION

The goal of this study was to identify specific matrix-degrading factors that contribute to dispersion. Specifically, we were interested in determining whether *P. aeruginosa* employs nucleases to weaken the biofilm matrix to ultimately disperse from the biofilm. But why focus on nucleases and why eDNA? eDNA has long been known as a nutrient and a source of phosphorus and nitrogen (43). In 2002, however, Whitchurch and coworkers demonstrated an additional function of eDNA, that of a structural element, a factor mediating cell-cell as well as cell-surface interactions in *Pseudomonas aeruginosa* biofilms (58). Since then, eDNA has been shown to be deposited on the stalk of biofilms, enabling the formation of mushroom-shaped microcolonies (59), to enhance biofilm formation by various bacterial species (60–63), likely by enabling direct or indirect interactions with the bacterial cell surface (64, 65), and to cross-link matrix components such as the Pel polysaccharide present in the *P. aeruginosa* biofilm matrix (57). eDNA serving a structural role has been confirmed for several biofilm-forming species, as DNase I treatment strongly decreased the ability of various bacteria to attach to a surface and the subsequent formation of 3-dimensional structures was severely

negatively affected (39, 40, 49, 66). Given that eDNA is a structural element of the biofilm matrix, eDNA degradation has likewise been shown to weaken the biofilm matrix. In fact, several studies have reported that treatment with DNase I results in the release of large amounts of biomass in a large number of other biofilm-forming species, including *P. putida*, *Staphylococcus aureus*, *S. oneidensis*, and *Bacillus licheniformis* (40, 49, 52, 66–69). However, DNase treatment leading to the detachment of biofilms was found to be limited to young, but not mature, flow chamber-grown *P. aeruginosa* biofilms (58), probably due to mature biofilms harboring increasing amounts of matrix material other than extracellular DNA, and speculated to occur to improve nutrient supply.

Here, we identified a phosphatase, EddA, and two nucleases, EndA and EddB, as being induced upon induction of dispersion. Both EndA and EddB were found to be required for *P. aeruginosa* biofilm dispersion, with the role of EddB limited to dispersion in response to NO. The previously characterized phosphatase EddA likewise contributed to dispersion, but its role appeared to be limited to dispersion in response to glutamate. Our findings suggested the roles of EddA and EddB are complementary, with EddA and EddB likely working in concert to induce dispersion to multiple dispersion cues.

In contrast, EndA alone contributed to multiple dispersion cues. Further characterization indicated EndA is highly homologous to other bacterial nucleases, including EndA from *E. coli*, Vvn from *Vibrio vulnificus*, Dns from *Vibrio cholerae*, and EndA from *S. oneidensis* MR-1 (52–56). All of these enzymes are exported from the cytoplasm and either remain in the periplasm, as do Vvn and *E. coli* EndA, or are released into the medium, as are Dns and the *S. oneidensis* EndA (52–56). Our findings here strongly suggest that the *P. aeruginosa* EndA, like Dns and the *S. oneidensis* EndA, is an extracellular enzyme. Similar to other EndA orthologs, the *P. aeruginosa* EndA belongs to the family of $\beta\beta\alpha$-metal endonucleases, also known as His-Me finger endonucleases, that form a large and diverse protein superfamily (70). The classification is based on a conserved structural motif, a β-hairpin followed by an α-helix, with a single metal ion being crucial for function (71). Based on sequence alignments (not shown), *P. aeruginosa* EndA harbors all the highly conserved amino acid residues that were previously identified as critical for activity, including the eight cysteine residues that form four disulfide bonds required for proper folding. The findings are in agreement with *P. aeruginosa* EndA having DNase activity.

Insertional inactivation of *endA* had no significant effect on biofilm development or eDNA accumulation in the matrix. Likewise, *endA* deletion had no effect on growth in liquid. This is in contrast to previously described nucleases belonging to the $\beta\beta\alpha$-metal endonucleases that affected attachment and/or biofilm formation or displayed an aggregative phenotype in liquid. The difference in phenotypes indicated EndA by *P. aeruginosa* contributes to eDNA degradation in a manner independent of eDNA being a nutrient. Instead, our findings suggested EndA is involved in eDNA degradation to weaken the biofilm matrix and thus enables the escape from the biofilm matrix. This is supported by inactivation of *endA* resulting in impaired dispersion response, while induction of *endA* gene expression coincided with dispersion events. The noted dispersion events were similar to those previously observed upon induction of *bdlA*_G31A gene expression by *P. aeruginosa* biofilms (48). In addition, while dispersion by wild-type biofilms coincided with a reduction in the stainable eDNA content, *endA*::IS biofilms failed to demonstrate a reduction in eDNA. In contrast, induction of *endA* coincided with a significant reduction in eDNA present in biofilms. The findings furthermore demonstrated that EndA is sufficient to disperse established biofilms.

To our knowledge, this is the first report of DNase EndA being required for dispersion, with induction of *endA* gene expression resulting in biofilm dispersion and reduction in eDNA. Our findings raise several questions. First, if eDNA degradation results in weakening of the biofilm matrix to enable dispersion, is eDNA the only matrix component that is being degraded to enable dispersion? Second, are additional matrix

components degraded? The biofilm matrix by the nonmucoid lab strain *P. aeruginosa* PAO1 is primarily composed of eDNA, protein, and the two polysaccharides Pel and Psl. However, four classes of nonmucoid *P. aeruginosa* matrix-producing strains have been identified, with the classification based on the type and amount of exopolysaccharides present in the biofilm matrix. Class I and II strains primarily utilize Pel and Psl, respectively, as the biofilm matrix polysaccharides, with PAO1 belonging to class II. Class III strains utilize both Pel and Psl, while class IV strains overproduce both polysaccharides (72). The different classes of matrix producers in *P. aeruginosa* alone suggest that if polysaccharide degradation is involved in the dispersion response, each matrix producer is expected to have a unique set of matrix-degrading enzymes. These questions are subject to future investigations. An additional line of inquiry will address the question of how endogenously produced DNases differ from exogenously added DNases when it comes to inducing dispersion. This is based on DNase treatment only leading to the detachment of young biofilms (58), while *endA* induction coincided with dispersion of established biofilms. One explanation could be that the access to eDNA becomes more limited to exogenously added DNases as the biofilm, and the extent of the biofilm matrix, matures.

We also asked how similar EndA induction-derived dispersed cells are to dispersed cells obtained in response to the dispersion cues glutamate or NO. In agreement with previous findings (14), EndA induction-derived cells were as hyperadhesive as dispersed cells and attached more readily than biofilm cells. In contrast, EndA induction-derived cells were as susceptible as biofilm cells. The findings suggested that while induction of *endA* coincided with dispersion, *endA* induction was not sufficient to mimic all phenotypes ascribed to dispersed cells. Recent findings suggested a link between c-di-GMP in drug susceptibility. Gupta et al. (73) demonstrated that *P. aeruginosa* planktonic cells were rendered more resistant to antimicrobial agents by increasing intracellular c-di-GMP levels to those more commonly found in biofilm cells, while biofilm cells were rendered susceptible upon decreasing c-di-GMP levels. It is thus likely that EndA-induced dispersion is not affecting c-di-GMP. Considering that dispersion has been reported to be the result of an overall reduction in the intracellular c-di-GMP content (6, 7, 19), our finding furthermore suggests *endA* induction is likely a response of c-di-GMP modulation. But how can this be? While direct evidence is lacking, indirect evidence suggests a role of AmrZ. Originally described to inversely regulate alginate production and swimming motility in *P. aeruginosa*, AmrZ is now recognized as a global regulator of multiple virulence factors, including c-di-GMP, extracellular polysaccharide production, including of Pel and Psl polysaccharides, and flagella (74). Support for AmrZ playing a role in dispersion stems from AmrZ affecting *gcbA* expression and inversely regulating exopolysaccharide production and motility (74). Additionally, Chua et al. (15) demonstrated that *amrZ* is differentially expressed in dispersed cells relative to that in planktonic cells by using whole-transcriptome shotgun sequencing (RNA-seq). However, to address this question, future experiments will be required to elucidate c-di-GMP-dependent pathways leading to dispersion and, more specifically, *endA* induction.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 2. *P. aeruginosa* strain PAO1 was used as the parental strain. All planktonic cultures were grown in flasks at 220 rpm at 37°C using Vogel and Bonner citrate minimal medium (VBMM) (75). For plasmid maintenance, antibiotics were used at the following concentrations: 250 μg/ml carbenicillin and 50 μg/ml gentamicin for *P. aeruginosa* and 50 μg/ml ampicillin for *E. coli*. Unless indicated otherwise, arabinose was used at 1% to induce gene expression.

Strain construction. C-terminal V5/6×His tagging of EndA was accomplished by subcloning into pET101D (Life Technologies, Carlsbad, CA). The tagged constructs were then cloned into pJN105. The identity of all vector inserts was confirmed by PCR and sequencing. Plasmids were introduced into *P. aeruginosa* via conjugation. Additionally, transposon insertional inactivation of *endA* and *eddA* was confirmed by PCR and sequencing. Primers used for strain construction and confirmation are listed in Table 3.

Biofilm growth. Biofilms were grown for 5 days under continuous flow conditions in biofilm tube reactors or flow cells. The flow rate was 0.2 ml/min using 5-fold diluted VBMM medium. For plasmid maintenance, 10 μg/ml carbenicillin and 2 μg/ml gentamicin were added. Where indicated, the growth

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
Escherichia coli		
DH5α	F^- *ϕ80lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1 tonA*	Life Technologies
BL21	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm rne131* (DE3)	Life Technologies
P. aeruginosa		
PAO1	Wild-type strain PAO1	B. H. Holloway
Δ*bdlA* strain	Δ*bdlA* in PAO1, Km^r	9
endA::IS strain	PAO1 PA2749::IS*lacZ* Tet^r	82
eddA::IS strain	PAO1 PA3910::IS*lacZ* Tet^r	82
eddB::IS strain	PAO1 PA3910::IS*lacZ* Tet^r	82
Plasmids		
pET101D	Vector for directional cloning and V5/6×His fusion protein expression, Amp^r	Life Technologies
pET-*endA*	IPTG-inducible expression of C-terminal V5/6×His-tagged *endA* cloned into pET101D, Amp^r	This study
pJN105	Arabinose-inducible gene expression vector; pBRR-1 MCS; *araC*-P_{BAD} Gm^r	83
pJN-*bdlA*-G31A	Arabinose-inducible expression of C-terminal 6×His-tagged *bdlA* with G31A mutation cloned into pJN105, Gm^r	48
pJN-*dipA*_V5	Arabinose-inducible expression of C-terminal V5/6×His-tagged *dipA* (PA5017) cloned in pJN105, Gm^r	7
pJN-*endA*_V5	Arabinose-inducible expression of C-terminal V5/6×His-tagged *endA* (PA2749) cloned in pJN105, Gm^r	This study
pMJT1	Arabinose-inducible gene expression vector; pBRR-1 MCS; *araC*-P_{BAD} Gm^r	84
pMJT-*eddA*_V5	C-terminal V5-tagged PA3910 cloned into pMJT-1 *XbaI* and *SacI*; *araC*-P_{BAD} $Carb^r$	This study
pMJT-*eddB*_V5	C-terminal V5-tagged PA3909 cloned into pMJT-1 *XbaI* and *SacI*; *araC*-P_{BAD} $Carb^r$	This study
pMRP9-1	Enables continuous expression of *gfp*, $Carb^r$	85

medium was supplemented with 0.1% arabinose to induce expression of genes of interest. Flow-cell-grown biofilms were stained using propidium iodide or a Live/Dead BacLight viability stain kit (Invitrogen, Carlsbad, CA) to visualize eDNA. The biofilm architecture was visualized via confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 confocal microscope. The CLSM images were processed using LAS AF software. Quantitative analysis of biofilm architecture was accomplished using MATLAB with the COMSTAT software package (76).

TABLE 3 Primers used

Oligonucleotide	Sequence
qRT-PCR	
endA_FqPCR	GCTTTCCCGTTTGTTTGT
endA_RqPCR	TAGAGCTTCCAGCCGATT
cysD_FOR qPCR	CTGGACATCTGGCAATACAT
cysD_REV qPCR	TCTCTTCGTCAGAGAGATGC
eddA_FORqPCR	CCGACCAGTCGATCTTCTA
eddA_REVqPCR	TCCAGACGAAACGGATATT
Transposon insertional inactivation check primers	
endA_FOR	TTTCCCGTTTGTTTGTAGGC
endA_REV	CAGGGTATGTCCGCAGGT
eddA_FOR	ATGAGTGGGATGGACCTCAAGCGCCGC
eddA_REV	TCAGGCGCCGTCGGGCTG
eddB_FOR	AAGACCTTCGTCCTCGCCAAC
eddB_REV	ATAGATCAGCCCCACGGCAAT
Cloning[a]	
pJN105 MCS_FOR	TAGCGGATCCTACCTGACGC
pJN105 MCS_REV	CCATTCGCCATTCAGGCTG
endA_FpET	CACCCGCATGCTTTCCCGTTTGTTT
endA_RpET	GCGTCGACGCGAGGATAG
endA_FpETNheI	gctacgCGCATGCTTTCCCGTTTGTTT
pETHis_XbaI_rev	GCGCGCtctagaTCAATGGTGATGGTGATG
eddB_XbaI_FOR	GCGCGCGCtctagaATGCACCCCTTGCGTAACGCC
eddB_SacI-V5_REV	GCGCGCGCgagctcTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCCTGCGGTGCTTCTT CATCGC
eddA_XbaI_FOR_FOR	GCGCGCGCtctagaATGAGTGGGATGGACCTCAAG
eddA_SacI-V5_REV	GCGCGCGCgagctcTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGGCGCCGTCGGGCTG

[a]Restriction sites are in lowercase letters. The sequence of the V5 tag is underlined.

RNA extraction and quantitative reverse transcriptase PCR. To obtain RNA from dispersing and nondispersing biofilms, Δ*bdlA* and PAO1/*bdlA*_G31A mutant biofilms were grown in biofilm tube reactors in 5-fold diluted VBMM medium supplemented with 0.1% arabinose to induce *bdlA*_G31A gene expression. Following 5 days of growth, biofilm cells were collected directly into equal volumes of RNA Protect (Qiagen, Hilden, Germany). Isolation of mRNA and cDNA synthesis were carried out as previously described (77–79). qRT-PCR was performed using the Bio-Rad CFX Connect Real-Time PCR detection system and SsoAdvanced SYBR green supermix (Bio-Rad, Hercules, CA) with oligonucleotides listed in Table 3. *cysD* was used as a control. Relative transcript quantitation was accomplished using the CFX Manager software (Bio-Rad, Hercules, CA), by first normalizing transcript abundance (based on the threshold cycle [C_T] value) to *cysD* followed by determining transcript abundance ratios. Melting curve analyses were employed to verify specific single product amplification.

Biofilm dispersion. Dispersion assays were performed using biofilms grown for 5 days in flow cells or tube reactors. Image acquisition using flow-cell-grown biofilms was performed so that the same biofilm microcolonies were observed prior to and after addition of dispersion cues or 0.8% arabinose. For tube-reactor-grown biofilms, dispersion was induced by the sudden addition of L-glutamate (18 mM) or (500 μM) sodium nitroprusside to the growth medium, as previously described (9, 50). Sodium nitroprusside was used as a source of NO. In addition, biofilms were exposed to 0.8% arabinose to induce *endA* gene expression to determine whether induction of gene expression resulted in dispersion events. Regardless of the dispersion cue used, dispersed cells were collected from the tube reactor effluents at 1-min intervals for a total of 24 min or 90 min, using 96-well microtiter plates. The absorbance of the biofilm effluents was assessed by spectrophotometry at 600 nm. Dispersion events were characterized by an increase in the effluent optical density (OD), with the OD being at least two times greater than the baseline.

Fluorescence analysis of the biofilm biomass and eDNA prior to and after induction of dispersion. To quantify the amount of biomass and eDNA of biofilms prior to and after dispersion, the relative fluorescence intensities of confocal images that were acquired prior to and after addition of dispersion cues or arabinose were determined. Each image was analyzed for the relative fluorescence intensity indicative of biofilm biomass (green channel) and eDNA (red channel) using the Intensity Luminance V1 software (80).

Immunoblot analysis. Confirmation of V5/6×His-tagged EndA production was assessed by SDS-PAGE and immunoblot analysis. *E. coli* BL21 (BL21/pET-*endA*_V5/6×His) was grown planktonically in LB medium containing ampicillin for plasmid maintenance to exponential phase. Then, *endA* gene expression was induced by IPTG for 2 h. Uninduced cells were used as controls. Cells were subsequently harvested by centrifugation for 5 min at 16,000 × *g*, resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.3 mg phenylmethanesulfonyl fluoride, sonicated on ice with six 10-s bursts at 4 W, and then centrifuged for 5 min at 21,200 × *g* to pellet cell debris and unbroken cells. Furthermore, culture supernatants were collected. The protein concentrations were determined using a modified Lowry assay (Thermo Scientific, Waltham, MA) and bovine serum albumin as a standard. The samples (1.5 μg) were resolved on an 11% polyacrylamide gel and subsequently transferred onto polyvinylidene difluoride (PVDF) membrane using a Turbo Trans-Blot apparatus (Bio-Rad, Hercules, CA). Western blots were first probed with anti-V5 antibody followed by a secondary anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA). The blots were subsequently developed using Immun-Star WesternC chemiluminescence reagents (Bio-Rad, Hercules, CA). Following transfer, SDS-PAGE gels were Coomassie stained to ensure equal loading. Likewise, the presence of V5/6×His-tagged EndA production in culture supernatants of PAO1/pJN-*endA*_V5/6×His, grown planktonically in LB medium containing gentamicin for plasmid maintenance to exponential phase, was assessed as described above. Culture supernatants by strain PAO1/pJN105 harboring the empty pJN105 vector were used as a control.

Purification of 6×His-tagged proteins. EndA V5/6×His-tagged protein was purified from culture supernatants. First, culture supernatants were concentrated using Vivaspin 2 columns (Sartorius Stedim Biotech, Göttingen, Germany) according to the manufacturer's protocol, and the concentrated supernatants were loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Qiagen, Hilden, Germany). After washing to remove unbound protein, the resin was then washed with buffer, and EndA was eluted using an imidazole gradient according to the manufacturer's instructions for native protein purification. Fractions containing EndA were confirmed by SDS-PAGE. EndA-containing fractions were pooled and then desalted and concentrated using Vivaspin 2 columns.

DNA degradation assays. The DNA degradation assays were adapted from those described by Heun et al. (52) in order to confirm the nuclease activity of EndA. Briefly, purified EndA, obtained from *E. coli* culture supernatants, at a concentration of 1.5 μg was used and incubated with 250 ng of *P. aeruginosa* gDNA in 10 mM Tris-HCl (pH 7.6) buffer supplemented with dithiothreitol (DTT) (10 mM). Samples were incubated at 37°C, and aliquots (20 μl) were removed at 0, 30, 60, 120, and 240 min. Samples were stored at −20°C until analyzed by DNA gel electrophoresis using a 1% agarose gel. The integrity and quantity of the gDNA were analyzed by ImageJ (81). We likewise carried out DNA degradation assays using culture supernatants (1.5 μg) obtained from *P. aeruginosa* PAO1/pJN-*endA*. Culture supernatants by strain PAO1/pJN105 harboring an empty vector were used as a control.

Assessment of attachment. Initial attachment to a polystyrene surface was measured using the polystyrene microtiter dish assay system (96-well) as previously described (14) with the following modifications. Biofilms by *endA*::IS and PAO1 strains harboring empty vectors or overexpressing *endA* or *dipA* were grown for 3 days in 5-fold-diluted VBMM in the presence of 0.1% arabinose to induce gene expression. This was performed to obtain populations with homogenous phenotypes. Biofilms were harvested and homogenized using a Tissue-Tearor. Each well was inoculated with 200 μl of biofilms,

adjusted to an optical density at 600 nm of ~0.2. In addition, OD-adjusted planktonic cells grown to exponential and stationary phases, as well as dispersed cells obtained in response to glutamate, were used. The 96-well plates were then incubated for 2 h at 37°C with shaking at 220 rpm to ensure proper aeration. All experiments were carried out at least in triplicate, with each repeat comprising six technical replicates.

Biofilm antibiotic susceptibility testing. To determine whether induction of *endA* gene expression mimicking dispersion events rendered biofilm cells more susceptible to antimicrobial agents, biofilms were grown for 3 days with arabinose (0.1%). Then, biofilms were harvested, homogenized using a Tissue-Tearor, and centrifuged at 16,000 × *g* for 1 min at 22°C, and cell pellets were resuspended in fresh VBMM to an OD_{600} of 0.2. The resulting suspension was exposed tobramycin (50 μg/ml) for 1 h at 37°C. The suspension was subsequently serially diluted and plated on LB agar. Viability was determined via CFU counts, and susceptibility was expressed as log reduction.

Statistical analysis. All experiments were carried out at least in triplicate. Student's *t* test was performed for pairwise comparisons of groups, and multivariate analyses were performed with a one-way analysis of variance (ANOVA), followed by a Tukey's test to compare the means of all treatment groups. All statistical analyses were performed using the Prism 5 software (GraphPad Software, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00059-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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