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Role of Pyocyanin and Extracellular DNA in Facilitating *Pseudomonas aeruginosa* Biofilm Formation

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Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that is primarily responsible for infections related to cystic fibrosis (CF) airways, burn wounds, urinary tract infections, surgery-associated infections, and HIV-related illness. Pyocyanin and extracellular DNA (eDNA) are the major factors dictating the progression of biofilm formation and infection. Pyocyanin is a potent virulence factor causing cell death in infected CF patients and is associated with high mortality. eDNA is a key player in *P. aeruginosa* biofilm formation and is also responsible for the high viscosity of CF sputum that blocks the respiratory airway passages. In this chapter, we summarize our recent findings on the role of pyocyanin in facilitating *P. aeruginosa* biofilm formation. Pyocyanin promotes eDNA release in *P. aeruginosa* by inducing cell lysis mediated via hydrogen peroxide (H_2O_2) production. Pyocyanin intercalates with the nitrogenous bases of DNA and creates structural perturbation on the double-helix structure. Pyocyanin-eDNA binding significantly influences *P. aeruginosa* cell surface hydrophobicity and influences the physicochemical interactions facilitating bacterial cell-to-cell interaction (aggregation) and ultimately facilitates robust biofilm formation. A pyocyanin knockout (Δ*phzA-G*) mutant is shown to have significantly reduced eDNA release and biofilm formation in comparison to its wild-type. To this end, we discover that antioxidant glutathione directly binds to pyocyanin and modulates pyocyanin structure and function, thus inhibiting pyocyanin-eDNA binding and consequently hampering biofilm development.

Keywords: *Pseudomonas aeruginosa*, Pyocyanin, extracellular DNA, glutathione, biofilm

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that readily forms biofilms and causes life-threatening infections, particularly in immunocompromised persons. *P. aeruginosa* is primarily responsible for infections related to airways in cystic fibrosis (CF) and bronchiectasis, burns wounds, urinary tract infections (UTIs), and nosocomial infections. For instance, *P. aeruginosa* infection and subsequent biofilm formation is implicated in 80% of CF deaths worldwide [1]. In CF patients, *P. aeruginosa* infection is responsible for lung tissue damage, impaired lung function, respiratory failure, and premature death [1, 2]. In spite of intensive antibiotic therapy against infections, the mean life expectancy of CF patients is short (~45 years) [1]. A recent study suggests that *P. aeruginosa* and its associated infections are more persistent and dominant in CF patients aged over 18 years (91%) than in patients less than 18 years (39%) [3]. In the case of burn injury infections, *P. aeruginosa* along with *Staphylococcus aureus* are recognized as principal pathogens responsible for serious complications in ~20.9% and 22.9% of patients, respectively [4]. Studies suggest that, in the United States, approximately 2.5 million patients with burn injury requires medical attention in which it is estimated more than 100,000 hospitalizations result every year, causing approximately 12,000 deaths from associated infections [5]. In hospital-acquired UTIs, *P. aeruginosa* is the third most common pathogen next to *Escherichia coli* and *Proteus mirabilis* [6]. In United States, UTI-associated hospital admission is estimated to be 300,000 patients every year, and urological disease costs more than 3.5 billion dollars annually in the United States alone [6].

Persistent *P. aeruginosa* infections that culminate in biofilm formation are attributed to a matrix of extracellular polymeric substances (EPS) in which bacterial cells are embedded [7, 8]. The EPS matrix of *P. aeruginosa* primarily consists of biomolecules, such as polysaccharides (alginate, lipopolysaccharides), proteins (protease, elastase), extracellular DNA (eDNA), metabolites (phenazines), exotoxin, and siderophores [6, 7]. These molecules present in the matrix play a significant role in the pathogenesis of *P. aeruginosa* infections. In addition, in many bacterial species, the matrix as a whole provides an inherent protection against both host immunity and traditional antibiotic therapy, which makes eradication extremely difficult [7, 9]. Previous studies acknowledge that the production of phenazines such as pyocyanin and the release of DNA from cells, providing freely available eDNA by *P. aeruginosa*, are the major factors dictating the formation of a biofilm and the persistent infection within the host [7, 8, 10, 11]. For instance, *P. aeruginosa*-infected CF and bronchitis sputum contains a significant amount of pyocyanin (up to 16 and 23 μg/ml, respectively) and eDNA (3–14 mg/ml) compared to none in non-CF patients [11, 12]. Pyocyanin is a highly versatile molecule recognized for its effect as a potent virulence factor, causing cell death in chronically infected CF patients and directly associated with decreased lung function and high mortality [11, 13]. eDNA is similarly a key factor in *P. aeruginosa* biofilm formation and protecting bacteria by inducing antibiotic resistance [8, 14, 15] and is also a contributing factor to the high viscosity of CF sputum that blocks the respiratory airway passages [12].

In this chapter, we focus our discussion on very recent findings that elucidate the essential role of pyocyanin in promoting eDNA production and interacting with eDNA to facilitate biofilm

formation in *P. aeruginosa*. To this end, we highlight novel strategies targeting the interaction of pyocyanin with eDNA to prevent *P. aeruginosa* biofilm formation.

2. Pyocyanin production by *P. aeruginosa*

P. aeruginosa synthesizes a variety of phenazines; however, the most abundantly produced is pyocyanin [5-methyl-1(5H)-phenazinone]. Up to 95% of *P. aeruginosa* isolates preferentially produce pyocyanin [16], which at a neutral pH is a distinctive blue in color and turns red in acidic pH conditions. It is a small and highly diffusible nitrogen-containing aromatic compound with a multitude of biological activities [16, 17]. In *P. aeruginosa*, pyocyanin production involves a stepwise process, beginning with the synthesis of the primary quorum sensing (QS)
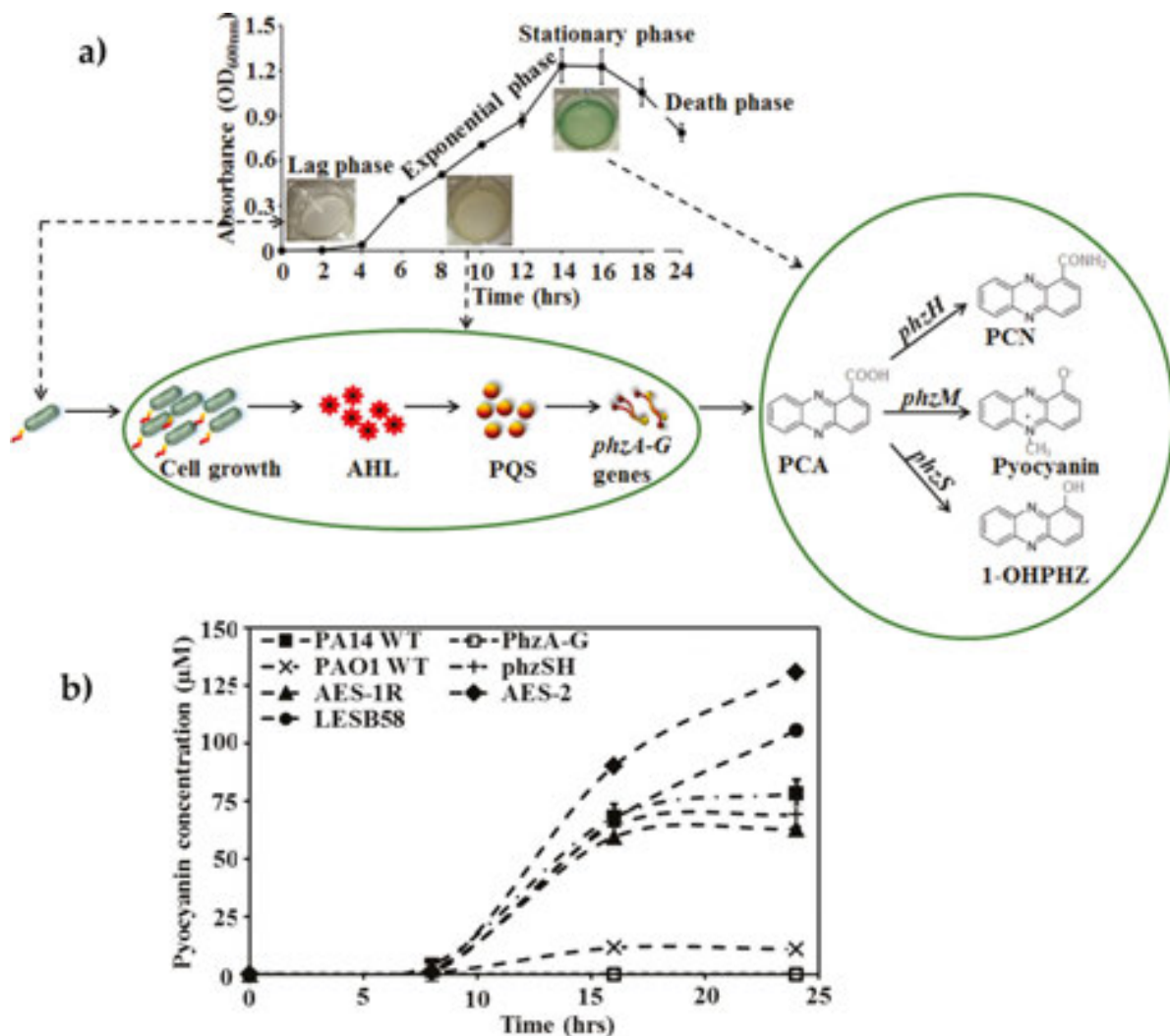
Figure 1. (a) Schematic of phenazine (pyocyanin) production by *P. aeruginosa*. Pyocyanin production is triggered at the early stationary phase through QS molecules (AHL and PQS), phenazine-producing genes (*phzA-G*), and finally by gene *phzM*. (b) Pyocyanin production by various *P. aeruginosa* clinical (AES-1, AES-2, and LESB58) and laboratory (PA14 wild-type, Δ*phzA-G*, PAO1 wild-type, and Δ*phzSH*) strains at different (0, 8, 16, and 24 h) time points. Error bars represent standard deviations from the mean (*n*=4).

molecule N-acyl-L-homoserine lactone (AHL) during the exponential growth phase followed by the secondary QS molecule *Pseudomonas* quinolone signaling (PQS) during the late exponential phase. PQS directly controls the expression of *phzA-G* operons resulting in the production of phenazine-1-carboxylic acid (PCA) from its precursor chorismic acid. PCA is then modified to produce three metabolites during the early stationary phase of which pyocyanin is the predominant product and is regulated by the *phzM* gene. The two other types of phenazine products are phenazine-1-carboxamide (PCN; encoded by *phzH*) and 1-hydroxy-phenazine (1-OHPHZ; encoded by *phzS*) [16, 18] (**Figure 1a**).

Figure 1b shows pyocyanin production by various *P. aeruginosa* clinical (AES-1R, AES-2, and LESB58) and laboratory (PA14 wild-type, $\Delta phzA$-G, PAO1 wild-type, and PAO1 *phzSH*) strains at different time points (0, 8, 16, and 24 h) grown under planktonic conditions in Luria broth (LB) at 37°C and 150 rpm (unpublished data). The cell-free supernatant of various bacterial strains was used to quantify pyocyanin by absorbance at 691 nm. A standard curve for pyocyanin was generated by addition of a known concentration (0–150 μM) of purified pyocyanin (Sigma-Aldrich, USA) to LB medium in a 1 ml cuvette, and absorbance was assayed at 691 nm (λ_{max} of pyocyanin) using Bio-Rad Smartspec 3000 (Bio-Rad Laboratories, Australia). This curve was used to calibrate pyocyanin concentration in bacterial supernatants. For most of the *P. aeruginosa* strains (AES-1R, AES-2, LESB58, PA14 wild-type, PAO1 wild-type, and PAO1 *phzSH*) used in this study, at initial growth stage (8 h postinoculation), the concentration of pyocyanin produced was less than 5 μM followed by a significant increase in pyocyanin production recorded at ≥16 h of growth and reaching between 75 and 100 μM pyocyanin for AES-1R, LESB58, PA14 wild-type, and PAO1 *phzSH* strains and ~140 μM pyocyanin for AES-2 strain after 24 h growth, whereas PAO1 wild-type produces less than (<) 15 μM pyocyanin. In contrast, the *phzSH* mutant of PAO1, which is deficient of *phzS* and *phzH* genes, was unable to convert PCA to PCN or 1-OHPHZ [16, 19] and consequently overproduced pyocyanin, whereas, after 24 h, phenazine-deficient mutant ($\Delta phzA$-G) does not produce any pyocyanin.

3. Pyocyanin a virulence factor

Pyocyanin was formerly disregarded as a bacterial secondary metabolite but has recently been ascribed a variety of roles in microbial ecology, and importantly a relationship with the severity of *P. aeruginosa* infections [20]. Previous research has shown a correlation between pyocyanin concentration in CF sputum and severity of infection [20]. The role of pyocyanin has been intensively studied due to its electrochemical and redox activity. The diffusible nature of pyocyanin means that it can easily diffuse through the host cell membrane and undergo redox reactions with other molecules [2]. For instance, it accepts electrons from NADH and subsequently donates electrons to molecular oxygen to form reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [13]. The H_2O_2 generated via redox reactions has been identified as a potent inhibitor of other bacterial and fungal species found within the microbiome of the CF lung as well as significantly altering host cell functions [2, 21]. In CF patients, pyocyanin-mediated ROS oxidize host intracellular and extracellular reduced glutathione (GSH) to form glutathione disulfide or oxidized glutathione (GSSG; **Figure 2**) [13].
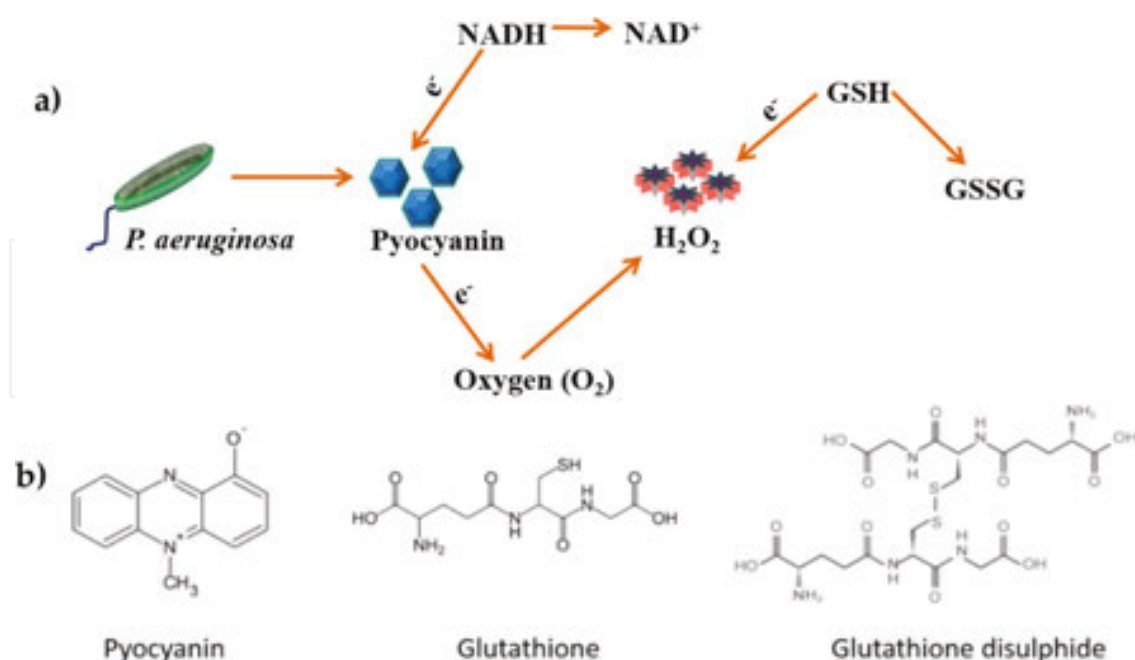
Figure 2. (a) Oxidation of GSH to GSSG by pyocyanin. *P. aeruginosa* produces pyocyanin, and pyocyanin accepts electrons (e⁻) from NADH and donates these electrons to oxygen to generate H_2O_2. Glutathione also donates electrons to H_2O_2 forming GSSG. (b) Structure of pyocyanin, GSH, and GSSG.

Depleted GSH levels during the chronic stage of CF infection leads to widespread epithelial cell death and consequently lung damage, respiratory failure, and mortality [21, 22]. Pyocyanin also inhibits catalase activity in the airway epithelial cells and thus aggravates oxidative stress in lung epithelial cells [23].

4. Pyocyanin promotes eDNA release in *P. aeruginosa* via H_2O_2 generation

In bacteria, eDNA release is mediated by both lytic and nonlytic mechanisms. The lytic mechanism involves the controlled lysis of a small number of bacterial cells via the production of various QS-mediated cell lysing agents, such as lytic prophages, autolysin proteins, enzymes, and H_2O_2. In nonlytic mechanisms, eDNA release is through bacterial outer membrane blebs/vesicles that contain large amounts of DNA [24–26]. QS-dependent mechanisms involve the AHL and PQS systems, whereas QS-independent mechanisms operate via release through flagellum and type IV pili [27, 28]. PQS in *P. aeruginosa* PAO1, a virulent laboratory strain, triggers eDNA release in the early phase of planktonic culture through the induction of prophages [27]. In accordance, a mutant for QS synthesis in *pqsA* and a *pqsL* mutant that overproduces PQS show low and high amounts of eDNA release, respectively [27]. QS-independent mechanisms, which also include phage induction [28], are responsible for eDNA release only in well-established PAO1 biofilms (>10 days) and not in planktonic cultures.

A recent work in this field by Das and Manefield has shown that pyocyanin production in *P. aeruginosa* laboratory strains PAO1 and PA14 triggers a significant increase in eDNA release

[19]. Quantitative analysis of eDNA in PA14 wild-type (120 h grown in LB medium) cell-free supernatant showed ~20 to 25 μg eDNA/ml. In contrast, cell-free supernatant of pyocyanin-deficient mutant of PA14 (ΔphzA-G) produced 50% less eDNA. Interestingly, ΔphzA-G culture grown in the presence of exogenous addition of pyocyanin showed significant increase in eDNA release [19]. In another study, Steinberger and Holden recorded up to 220 and 500 mg eDNA/g of cellular DNA in 5-day-old biofilms of *P. aeruginosa* and *Pseudomonas putida*, respectively; however, their study did not compare the link between pyocyanin and eDNA production in *P. aeruginosa* and *P. putida* [29].

In general, phenazines such as pyocyanin induce H_2O_2 production and subsequently trigger cell death in host (mammalian) and competing fungal and bacterial cells are well documented. A recent study showed concrete evidence of *P. aeruginosa* strains producing the highest concentrations of pyocyanin generated the highest concentrations of H_2O_2 [19]. The production of H_2O_2 initiates ~7 to 14% increase in cell lysis in planktonic cultures of *P. aeruginosa* and consequently promotes eDNA release. **Figure 3** shows a schematic of pyocyanin-mediated eDNA release via H_2O_2 in *P. aeruginosa*. H_2O_2-mediated eDNA release is not limited to *P. aeruginosa* strains; for instance, in oral Gram-positive bacteria (*Streptococcus sanguinis*), pyruvate oxidase activity induces ~10% increase in cell death in its own population via H_2O_2 production and consequently facilitates eDNA release [30].
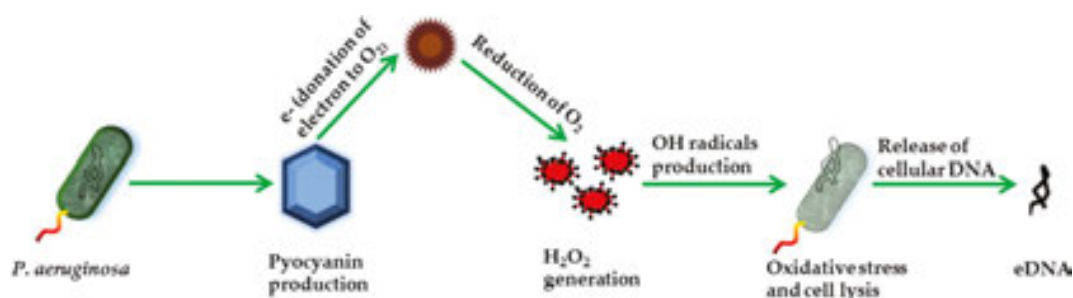
**Figure 3.** Schematic showing pyocyanin-mediated release of eDNA via H_2O_2 production.

By examining pyocyanin production in different strains of *P. aeruginosa* in relation to H_2O_2 generation, cell lysis, and eDNA concentration, a clear relationship emerged. The findings in this study demonstrate that pyocyanin is involved in eDNA release in growing *P. aeruginosa* planktonic cultures, raising interesting questions about its role in biofilm biology. Pyocyanin-mediated eDNA production, which likely happens as a consequence of cell lysis via H_2O_2 generation, could possibly assist *P. aeruginosa* biofilm formation in several ways, as eDNA has been shown to be an essential biomolecule that necessitates the establishment of *P. aeruginosa* biofilm [7, 8]. Previous studies suggest that the production of phenazines enhance bacterial adhesion, microcolony formation, and increased biomass in *P. aeruginosa* biofilms and current generations of *P. aeruginosa* PA14 in bioelectrochemical systems [31, 32]. From previous findings in concurrence with the current results, we hypothesized that the phenazine pyocyanin may promote biofilm formation in *P. aeruginosa* via eDNA release through H_2O_2-mediated cell lysis. However, the exact mechanism of how pyocyanin facilitates *P. aeruginosa* biofilm formation is yet to be elucidated.

5. Pyocyanin binding to eDNA

Previous studies have demonstrated that eDNA is a key constituent in the construction and structural integrity of the biofilm matrix in many bacterial species, and the cleaving of eDNA by nuclease enzymes such as DNase I disintegrates the biofilm matrix, thereby increasing the susceptibility of bacterial cells within the biofilm matrix to antimicrobial agents such as detergents and antibiotics [33, 34]. eDNA acts as a scaffold for the whole biofilm by binding with other biomolecules such as peptides/enzymes/proteins and polysaccharides. For exam‐ ple, in *Streptococcus mutans*, an oral pathogen responsible for dental plaques, the competence stimulating peptide (CSP) and DNA-binding protein ComGB interact with eDNA, and this interaction is essential for the uptake of eDNA by *S. mutans*, which facilitates bacterial cell-to- cell interaction and biofilm formation [35]. In another example, HU and IHF (DNA-binding proteins) produced by human pathogenic bacterium *E. coli* strain U93 specifically bind to double-stranded DNA (dsDNA) in the EPS, and by binding with eDNA, such proteins generate specific structures within eDNA. The enzymatic digestion of these proteins makes eDNA lose its structural stability and thereby disrupts *E. coli* biofilms [36]. In *Listeria monocytogenes*, a food- borne pathogen, eDNA binding with *N*-acetylglucosamine, a type of peptidoglycan, has been shown to be an essential molecular interaction that initiates *L. monocytogenes* adhesion to surfaces [37]. To our knowledge, our discovery was the first to demonstrate that, apart from biopolymers such as proteins and polysaccharides, a secondary metabolite such as pyocyanin strongly binds with DNA to dictate the establishment of *P. aeruginosa* biofilm.

5.1. Mechanism of pyocyanin-DNA binding

The mechanism of pyocyanin-eDNA binding was elucidated using different types of spectro‐ scopic techniques by Das et al. [10]. In this study, we used *P. aeruginosa* pyocyanin (Sigma- Aldrich, USA) and calf thymus DNA sodium salt (type I fibers; 42% GC content; Sigma-
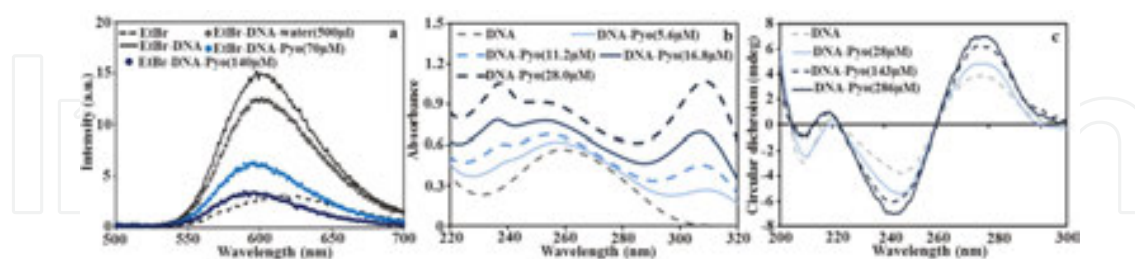
Figure 4. Mechanism of pyocyanin-DNA binding using spectroscopic techniques. (a) Fluorescence emission spectro‐ scopy techniques show that pyocyanin displaces EtBr bound to dsDNA, indicating that pyocyanin is an intercalating agent. (b) UV-vis spectra of DNA with pyocyanin showed that hyperchromic (increase in absorbance intensity) and hypochromic (shift in wavelength of DNA peak from 259 to 253 nm) effects are indicative of the intercalation of pyo‐ cyanin between nitrogenous base pairs of DNA and exposure of nitrogenous base pairs due to the unwinding of the DNA helix. (c) CD spectra of DNA-pyocyanin mixtures confirm that DNA binds to pyocyanin. The increase in mdeg at 277 nm in DNA peak confirms that pyocyanin intercalates into the nitrogenous bases of DNA, whereas the shift in DNA peak at 247 nm to 244, 243, and 242 nm in the presence of 28, 143, and 286 μM pyocyanin, respectively, confirms that pyocyanin also binds to sugar-phosphate backbone of DNA.

Aldrich, USA), which has predominantly dsDNA (~90%) quantified using Qubit fluorescent dye assay and Qubit 2.0 Fluorometer (Invitrogen, USA) as mentioned previously.

Using fluorescence emission spectroscopy (Varian Cary Eclipse Fluorescence Spectrophotometer, USA), it was found that pyocyanin displaces ethidium bromide (EtBr) bound to dsDNA. All experiments were done in SHE buffer (2 mM HEPES, 10 µM EDTA, and 9.4 mM NaCl in Milli-Q water adjusted to pH 7 with NaOH). Light emission at 615 nm (λ_{ex}=480 nm) was quantified at room temperature in 1 ml quartz cuvette. The fluorescence emission spectra suggest that the addition of pyocyanin (70 or 140 µM) reduced the DNA (6 ng/µl)-EtBr (4 µM) peak maxima to that of an EtBr solution without DNA (**Figure 4a**). It is well known that EtBr is a classic intercalating agent that strongly binds to DNA via intercalation and the displacement of EtBr by pyocyanin suggests that pyocyanin can bind to DNA. However, the mechanism of pyocyanin-DNA binding was not immediately clear.

To determine the binding mechanism between pyocyanin and DNA, a Varian Cary 100 Bio UV-visible (UV-vis) spectrophotometer technique was used [10]. UV-vis spectroscopic scans from 200 to 800 nm were performed in 1 ml quartz cuvette on DNA, pyocyanin, and the DNA-pyocyanin complex in Milli-Q water. **Figure 4b** shows the UV-vis range spectra of DNA (50 ng/µl) in the presence of increasing concentrations of pyocyanin (5.6, 11.2, 16.8, or 28.0 µM). The spectra of DNA with pyocyanin showed a gradual increase in absorbance intensity of the DNA peak and a shift of the DNA peak from 259 to 253 nm with increasing pyocyanin concentrations. The observed hyperchromic (due to the increase in absorbance intensity) and hypochromic (due to the shift in wavelength of DNA peak) effects are indicative of the intercalation of pyocyanin between nitrogenous base pairs of DNA and exposure of nitrogenous base pairs due to the unwinding of the DNA helix [38].

The pyocyanin-DNA binding mechanism was further confirmed using a Chirascan circular dichroism (CD) spectrophotometer (Applied Photophysics, UK). The experiments were conducted using 1 mm path length quartz cuvette, and mixtures of dsDNA at 135 ng/µl with varying pyocyanin (0, 28, 143, and 286 µM) concentrations in 350 µl Milli-Q water were scanned from 200 to 320 nm after a 15-min static incubation at 25°C. The spectra of DNA-pyocyanin mixtures confirm that DNA binds to pyocyanin with statistically significant changes in peak intensity (P<0.05) at all four characteristic DNA peaks (209, 221, 247, and 277 nm) achieved with pyocyanin concentrations above 28 µM. The significant increase in mdeg at 277 nm in DNA peak is a clear confirmation that pyocyanin intercalates into the nitrogenous bases of DNA. Additionally, the shift in DNA peak at 247 nm to 244, 243, and 242 nm in the presence of 143 and 286 µM pyocyanin, respectively (**Figure 4c**) show that pyocyanin also binds to sugar-phosphate backbone of DNA and therein creates local perturbations in the DNA double-helix structure but, however, does not cause any significant transition in form (i.e. B-DNA to A or Z form).

5.2. Pyocyanin-eDNA binding influences *P. aeruginosa* cell surface hydrophobicity and physicochemical interactions

Pyocyanin is well known as an electron shuttle [18], and our recent investigation revealed that pyocyanin intercalates with DNA [10]. In line with this, previous studies revealed that eDNA

promotes bacterial aggregation through acid-base interactions involving electron-donating and electron-accepting groups [39]. Contact angle measurements on various Gram-positive and Gram-negative bacterial cell surfaces revealed that eDNA significantly influences modulation in bacterial cell surface hydrophobicity [8, 39]. For instance, the cell surface of *Streptococcus epidermidis* 1457 wild-type is more hydrophobic in the presence of eDNA (average water contact angle of 85°), whereas *S. epidermidis* 1457 Δ*atlE* (eDNA-deficient mutant) and DNase I (DNA cleaving enzyme)-treated *S. epidermidis* 1457 wild-type showed significant reduction in the water contact angle (39–44°) [39]. Based on these findings, we hypothesized that pyocyanin is involved in facilitating eDNA binding to *P. aeruginosa* cells and thus possibly influences *P. aeruginosa* cell surface properties such as hydrophobicity and surface energies and consequently influences physicochemical interactions.

The hypothesis was tested by measuring contact angles of *P. aeruginosa* PA14 and PAO1 lawn growths prepared by the deposition of bacteria, from a planktonic suspension, onto a 0.2 μm pore diameter filter (nitrocellulose membrane filter; Millipore, USA) using negative pressure [39, 40]. Contact angles were measured with standard polar (water and formamide) and nonpolar (diiodomethane) liquids using goniometer (KSV model 200; KSV Instrumentation Pvt. Ltd., Finland) following the sessile drop technique [39]. To remove eDNA, *P. aeruginosa* strains were treated with DNase I before lawn preparation and its contact angle was measured as above. DNase I untreated *P. aeruginosa* PA14 and PAO1 wild-type showed significantly higher degree of water contact angle in comparison to untreated Δ*phzA-G*. After DNase I treatment only, the wild-type strains showed a significant decrease in the water contact angle and ultimately equivalent to the water contact angle of the Δ*phzA-G* mutant (**Figure 5a**) [40]. This result demonstrates that the interaction of pyocyanin with eDNA modulates cell surface hydrophobicity in *P. aeruginosa*.
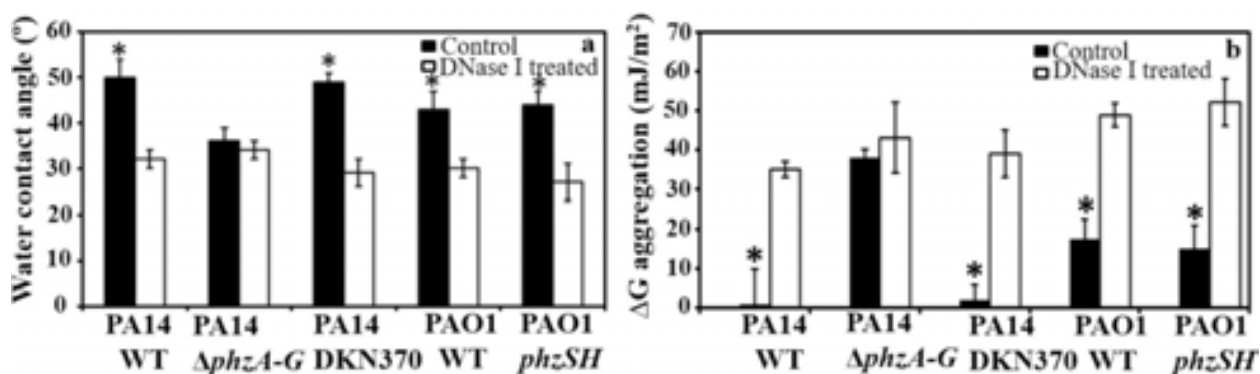

Figure 5. (a) Effect of DNase I and pyocyanin on *P. aeruginosa* cell surface hydrophobicity. The removal of eDNA by DNase I treatment shows a significant decrease in cell surface hydrophobicity (water contact angle) on all pyocyanin-producing strains, whereas pyocyanin-deficient strain (Δ*phzA-G*) showed no change in the water contact angle regardless of DNase I treatment. (b) Gibbs free energy of aggregation indicates that the removal of eDNA or the absence of pyocyanin significantly declines the efficiency of *P. aeruginosa* cell-to-cell aggregation. Error bars represent standard deviations from the mean (*n*=3). Asterisks indicate statistical significance (Student's *t*-test *P*<.05) in comparison to DNase I treatment. PA14 strain data are taken from Das et al. [40], whereas PAO1 contact angle and Gibbs free energy of aggregation results are unpublished.

The modulation in *P. aeruginosa* cell surface hydrophobicity has shown a direct impact on bacterial surface energy components as determined using theoretical surface thermodynamics (**Figure 5b**). Surface thermodynamics elucidated the physicochemical interactions that are responsible for bacterial cell-to-cell interactions at close approach. Physicochemical interactions are nonspecific interactions forces that derive from molecules that are present on the bacterial cell surface. In our study, pyocyanin and eDNA are the molecules that facilitate attractive physicochemical interactions such as short-range acid-base and long-range Lifshitz-van der Waals interactions. The removal of eDNA from *P. aeruginosa* wild-type cell surface or the absence of pyocyanin in Δ*phzA-G* mutant strain showed significant impact (i.e. nonattractive interaction, especially on the short-range acid-base interaction, which includes electron-donating and electron-accepting parameters). However, the long-range Lifshitz-van der Waals interactions remained unaffected between wild-type and Δ*phzA-G* regardless of DNase I treatment [40]. Another important parameter that commonly exists and drives bacterial interactions includes the presence of biopolymers (DNA, polysaccharides, and proteins) and cell appendages (pili, fimbriae) that extend hundreds of nanometers from the cell surface [41]. These biopolymers/appendages initiate hydrogen bonding by colliding with its neighboring cells and thereby help bacterial cells to overcome small physicochemical energy barrier and promote bacterial cell-to-cell interactions [7, 41].

5.3. Pyocyanin-eDNA binding is essential for *P. aeruginosa* biofilm formation

eDNA was previously acknowledged as a biofilm-promoting factor, whereas pyocyanin was mainly considered as a secondary metabolite essential for the persistence of *P. aeruginosa* cells in highly dense biofilm by enabling maintenance of a basal rate of respiration for energy harvesting and to maintain cytoplasmic redox homeostasis [18]. From our previous investigation, it was revealed that pyocyanin-eDNA binding influences essential physicochemical interactions that drive bacterial cell-to-cell interactions. Such findings prompted the hypotheses that pyocyanin-eDNA binding is essential for *P. aeruginosa* biofilm formation.

P. aeruginosa PA14 wild-type and Δ*phzA-G* mutant biofilm formation was investigated on a glass substratum using confocal laser scan microscopy (CLSM) technique complemented using ImageJ software to quantify biofilm characteristic properties (**Figure 6**). Biofilm growth, staining, imaging, and analysis were performed using the protocol described previously [10]. Comparative studies were undertaken between biofilms (grown for 24 h in LB medium, 37°C, 150 rpm) of wild-type (**Figure 6a** and **b**) and Δ*phzA-G* (**Figure 6c** and **d**) grown in the absence and presence of DNase I. Findings indicated that *P. aeruginosa* PA14 biofilm architecture, thickness, and biofilm biomass in wild-type biofilms grown in the presence of DNase I are similar in biofilms of a phenazine-deficient mutant strain (**Table 1**). The removal of eDNA reduced wild-type biofilm thickness and biomass by ~40% and 65%, respectively, whereas phenazine-deficient mutant also showed ~40% reduction in biofilm thickness and up to 80% reduction in biomass. This result clearly indicates that the influence of eDNA on developing a three-dimensional and structurally integrated biofilm is equivalent to the influence of pyocyanin on biofilm.
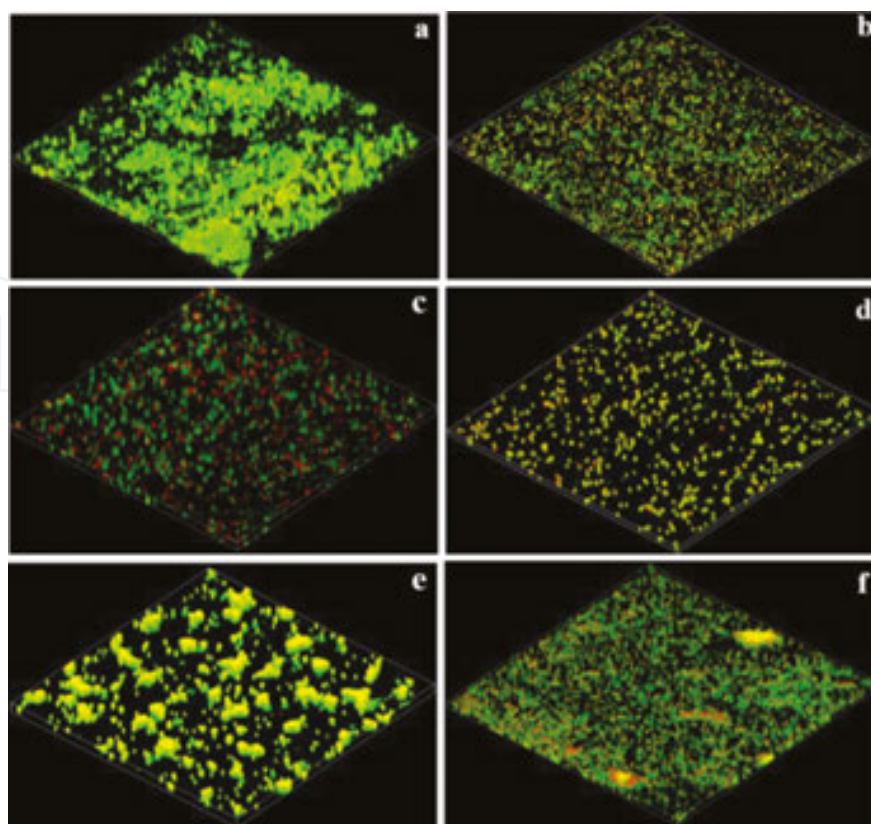
Figure 6. Confocal microscopy imaging of *P. aeruginosa* PA14 strains. (a) PA14 wild-type, (b) PA14 wild-type grown in the presence of DNase I (40 U), (c) Δ*phzA-G*, (d) Δ*phzA-G* in the presence of DNase I (40 U), (e) Δ*phzA-G* in the presence of exogenous pyocyanin (200 μM), and (f) Δ*phzA-G* in the presence of exogenous DNA (1 ng/μl) and exogenous pyocyanin (200 μM).

P. aeruginosa	Thickness (μm)	Biomass (μm³/μm²)
Wild-type	8.3±0.3	1.4±0.4
Wild-type+DNase I (40 U)	**5.2±1.5**	**0.5±0.1**
Δ*phzA-G*	**4.9±0.4**	**0.3±0.1**
Δ*phzA-G*+DNase I (40 U)	**3.9±0.5**	**0.3±0.3**
Δ*phzA-G*+pyocyanin (200 μM)	***7.2±1.0***	***0.6±0.2***
Δ*phzA-G*+pyocyanin (200 μM)+DNA (1 ng/μl)	***7.9±0.4***	***1.0±0.1***

Table 1. Quantification of *P. aeruginosa* (nonestablished) biofilm properties using ImageJ software. Significant differences were observed in biofilm thickness and biomass between control (PA14 wild-type) and wild-type grown in the presence of DNase I (40 U) and Δ*phzA-G* regardless of whether they were grown in the presence of DNase I. Mean ±standard deviations (*n*=3). Boldfaced data indicate that the differences were statistically significant (Student's *t*-test *P*<0.05) in comparison to control (wild-type), whereas boldfaced italicized data indicate that the thickness and biomass of Δ*phzA-G* grown in the presence of pyocyanin and the combination of pyocyanin+DNA is significantly (Student's *t*-test *P*<0.05) higher than the biofilm of Δ*phzA-G* alone (control).

A previous study by Ramos et al. also showed similar results with phenazine-deficient mutant but could not elucidate the mechanism behind the influence of phenazine on biofilm formation in *P. aeruginosa* [30]. This finding is compatible with the hypothesis that eDNA and pyocyanin act in concert to influence *P. aeruginosa* biofilm formation, with a direct interaction between pyocyanin and eDNA likely underlying the mechanism of enhanced biofilm integrity. The fact that biofilm formation in the phenazine-deficient mutant is partly restored by the addition of exogenous pyocyanin (**Figure 6e**), with the addition of both exogenous pyocyanin and DNA showed further enhancement in biofilms in comparison to the pyocyanin only treatment (**Figure 6f**). This suggests that this particular phenazine is partly, but not wholly, responsible for the phenomenon, and that interaction with eDNA is an essential factor to facilitate biofilm formation.

6. Glutathione disrupts *P. aeruginosa* biofilm formation

With the increased tolerance of bacteria to existing antibiotic therapies [1] and the necessity to use high doses of antibiotics with their related side effects [42, 43], there is an urgent public health priority to identify new methods and targets for the control of *P. aeruginosa* biofilms.

### 6.1. Glutathione interacts with pyocyanin and inhibits its binding with eDNA

GSH is a thiol tripeptide (γ-glutamylcysteinylglycine) found in all human cells and in some bacterial species. GSH is considered to be a master antioxidant and its primary functions include defense against ROS and free radicals and maintaining a healthy immune system [13]. In humans, intracellular GSH levels vary from 2 to 10 mM, whereas, in the extracellular lung lining fluid (ELF), levels range from 250 to 800 μM [44]. In contrast, intracellular GSH concentration in bacterial cell differs significantly from species to species [45–47]. For instance, in *E. coli*, GSH is an essential molecule and exists in the millimolar range [47], whereas, in *P. aeruginosa* PAO1, intracellular GSH concentration is reported in the nanomolar range (70 nM) [46]. However, in many Gram-positive bacteria such as *Bacillus cereus*, GSH is not found [45, 48].

Pyocyanin undergoes oxidation by donating electron to molecular oxygen to form superoxides and H_2O_2 [49]. GSH, being a thiol antioxidant, will donate electron/protons to pyocyanin directly through the -SH group from cysteine [50], thereby interfering in the pyocyanin oxidation process by inhibiting H_2O_2 generation [50]. The antioxidant property of GSH makes it a potential inhibitor of pyocyanin toxicity.

Our recent investigation using CD and UV-vis suggests that pyocyanin-GSH complex interferes with pyocyanin binding to DNA (**Figure 7a** and **b**) [10], whereas nuclear magnetic resonance (NMR; Bruker Avance 400 spectrometer) of the pyocyanin-GSH complex at various pyocyanin-GSH ratios clearly indicates that GSH (with at least fivefold higher concentration than pyocyanin) is required to modulate pyocyanin aromatic structure (**Figure 7c**; unpublished data). As discussed earlier, pyocyanin is a planar molecule that intercalates into the nitrogenous base of DNA. By instead conjugating with GSH, intercalation with DNA is restricted. However, it is interesting to observe that with the increases in GSH concentration, the

inhibition of pyocyanin binding to DNA is increased; almost complete inhibition was observed at a molar ratio of pyocyanin/GSH of ~1:6. A similar observation was reported recently by Muller and Merrett [50] demonstrating that thiol concentration needs to be available in the millimolar range to neutralize pyocyanin toxicity. Their work also suggests that GSH forms a cell-impermanent conjugate with pyocyanin. This suggests that thiol antioxidants could be a potential clinical target against *P. aeruginosa* toxicity by preventing pyocyanin entry into host cells and prohibiting pyocyanin-mediated cell lysis.
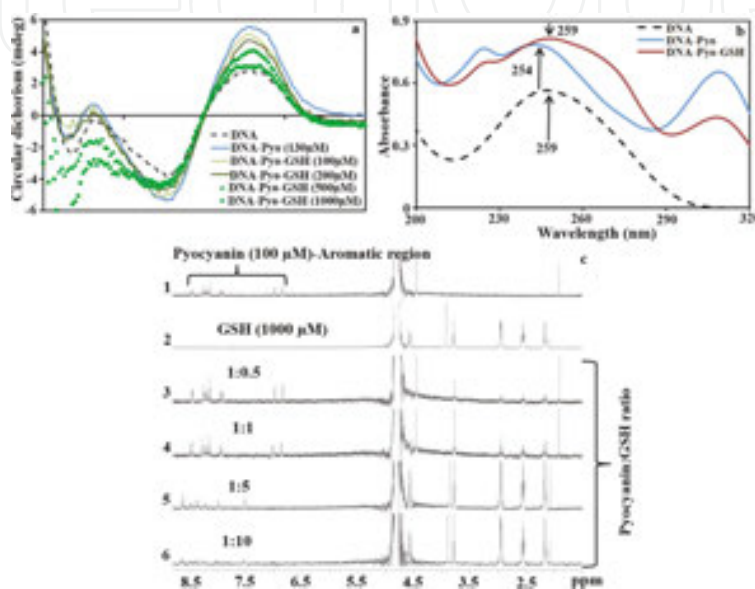

Figure 7. Inhibition of pyocyanin intercalation with DNA. (a) CD spectra of DNA-pyocyanin-GSH mixtures confirm that GSH inhibits pyocyanin intercalation with DNA; however, the inhibition is GSH concentration dependent. At above 1000 μM, GSH (which is ~1:9 ratio to pyocyanin concentration used in this study) successfully inhibits pyocyanin intercalation to DNA nitrogenous base (peak 277 nm) and binding to DNA backbone (peak at 244 nm). (b) UV-vis spectra of DNA with pyocyanin-GSH complex inhibit the shift of wavelength of DNA peak (i.e. retained DNA peak at 259 nm) indicative of the absence of hypochromic effect, which indicates no intercalation. (c) Proton NMR spectra of pyocyanin (100 μM)-GSH (50, 100, 500, or 1000 μM) mixtures indicated a considerable downfield shift of the pyocyanin aromatic peaks. However, this shift was observed only at higher GSH (i.e. at 500 and 1000 μM) concentration ratios (i.e. at pyocyanin/GSH 1:5 and 1:10), whereas, at low GSH (i.e. at 50 and 100 μM) concentrations, pyocyanin aromatic peaks remain stable.

6.2. Glutathione disrupts clinical strains of *P. aeruginosa* biofilms and facilitates bactericidal activity

Biophysical techniques confirmed that GSH inhibits pyocyanin-eDNA intercalation. This made us to hypothesize that GSH could disrupt biofilms, as pyocyanin and eDNA are the crucial factors that initiate biofilm formation. Our recent investigation confirmed that interrupting the pyocyanin-eDNA intercalation using GSH results in a significant disruption of the biofilms of pathogenic Australian epidemic strain (AES-1R and AES-1M isolated from a CF patient; unpublished data). **Figure 8** shows the effect of GSH, DNase I treatment (12 h, 37°C, 150 rpm) on preestablished biofilms grown in LB medium (24 h, 37°C, 150 rpm) imaged using CLSM.

An analysis of biofilm image using ImageJ software revealed that GSH or DNase I-treated AES-1R and AES-1M biofilm showed significant difference (Student's *t*-test *P*>0.05) of ~30 to 40% and 60 to 80% reduction in its biofilm thickness and total biofilm biomass, respectively (**Table 2**). This shows that eDNA and pyocyanin are vital molecules in the building of biofilms of clinical strains of *P. aeruginosa*, and by modulating pyocyanin structure and function, biofilm formation can be restrained while concomitantly reducing the toxicity of pyocyanin.
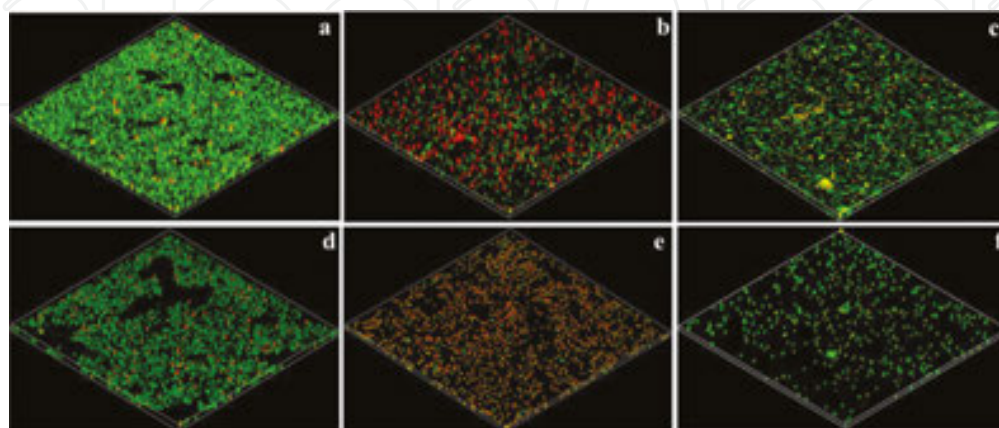
Figure 8. Confocal microscopy imaging of 24 h preestablished biofilms of *P. aeruginosa* clinical strains AES-1R and AES-1M. (a) AES-1R (control), (b) AES-1R treated with 2 mM GSH, (c) AES-1R treated with DNase I (40 U), (d) AES-1M (control), (e) AES-1M treated with 2 mM GSH, and (f) AES-1M treated with DNase I (40 U).

P. aeruginosa	Treatment	Thickness (µm)	Biomass (µm³/µm²)	Average Live/Dead (%)
AES-1R	—	8.6±1.1	1.8±0.4	83/17
AES-1R	GSH (2 mM)	**5.1±0.8**	**0.5±0.2**	37/**63**
AES-1R	DNase I (40 U)	**4.9±1.0**	**0.7±0.3**	84/16
AES-1M	—	7.4±1.4	1.1±0.3	79/21
AES-1M	GSH (2 mM)	**4.1±0.7**	0.7±0.4	12/**88**
AES-1M	DNase I (40 U)	**3.9±0.6**	0.2±0.1	93/7

Table 2. Quantification of *P. aeruginosa* (24 h preestablished) biofilm properties using ImageJ software. Significant differences were observed in biofilm thickness, biomass, and average percentage of live and dead biomass between control (AES-1R and AES-1M) and GSH (2 mM) or DNase I (40 U)-treated biofilms. Mean±standard deviations (*n*=3). Boldfaced data indicate that the differences were statistically significant (Student's *t*-test *P*<0.05) in comparison to control.

Biofilm image (**Figure 8**) and quantification of live (green) and dead (red) biofilm biomass (**Table 2**) clearly show significant increase in dead biomass when biofilm exposed to GSH treatment. GSH-mediated bactericidal activity in *P. aeruginosa* is proposed through the generation of H_2O_2 via the auto-oxidation of GSH (**Figure 8**). H_2O_2 generation by GSH was quantified using Amplex red H_2O_2 assay kit (Thermo Fisher Scientific, USA) as per manufacturer's instruction using multi-well plate reader (Perkin-Elmer, USA). About 11.5 µM H_2O_2

was generated by 2 mM GSH after 4 h; this finding supports previous published work that suggested that GSH undergoes auto-oxidation to produce H_2O_2 [51–53]. In biological system of healthy individuals, the production of catalase and other chelating agents inhibits the oxidation stress generated by the auto-oxidation of GSH [52]; however, in infected patients, pyocyanin inhibits catalase activity in lung epithelial cells [23] and consequently inhibits the protection against oxidative stress generated by all kinds of endogenous and exogenous molecules.

7. Conclusion and perspective

Bacterial biofilms form a highly protective biological matrix that enables persisting popula-tions of bacteria to survive in otherwise highly hostile environments. These matrices vary highly between species; however, they share a common structural element (eDNA). Within *P. aeruginosa*, eDNA is both necessary and sufficient for structured biofilm formation and maintenance. We have demonstrated that eDNA enhances intercellular aggregation by reducing the Gibbs free energy between cells while also enhancing hydrophobicity by increasing the water contact angle. Furthermore, these properties are enhanced by the presence of the virulence factor pyocyanin, which is able to directly bind to nitrogenous base pairs within dsDNA by mode of intercalation. Pyocyanin is uniquely produced by *P. aeruginosa* and as such confers a unique method by which *P. aeruginosa* is able to strengthen its biofilm.

Biofilm formation is associated with increased resistance to antibiotic therapies and persistence of bacterial colonization within the CF lung. Novel treatment strategies seek to act on molecules that are essential for bacterial persistence such as biofilm constituents. Biofilm disruption is associated with increased antibiotic susceptibility and the clearance of bacteria. We have shown that, by disrupting the biofilm association with thiol-based antioxidants, namely GSH, which directly binds and clears freely available pyocyanin, intercellular aggregation and overall biofilm structure are perturbed. This is enhanced by the activity of nucleases such as DNase I, which remove the underlying eDNA scaffold, resulting in a complete disruption of the biofilm structure by decreasing the water contact angle of bacterial cells and increasing the Gibbs free energy between cells.

Thus, the intercalation between pyocyanin and eDNA is both a unique and highly exploitable interaction in *P. aeruginosa* biofilms, and this interaction is necessary for any kind of structured biofilm architecture in *P. aeruginosa*. By exploring the disruption of this interaction, both GSH and DNase I have arisen as potential therapeutic candidates for the elimination of persistent infections of *P. aeruginosa* existing as a biofilm. This is particularly useful for long-term host-adapted strains of *P. aeruginosa*, such as those persisting in the CF lung, which typically develop a multidrug-resistant profile as the result of repeated antibiotic therapies.

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