Phenazine production enhances extracellular DNA release via hydrogen peroxide generation in Pseudomonas aeruginosa

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In Pseudomonas aeruginosa eDNA is a crucial component essential for biofilm formation and stability. In this study we report that release of eDNA is influenced by the production of phenazine in P. aeruginosa. A ΔphzA-G mutant of P. aeruginosa PA14 deficient in phenazine production generated significantly less eDNA in comparison with the phenazine producing strains. The relationship between eDNA release and phenazine production is bridged via hydrogen peroxide (H₂O₂) generation and subsequent H₂O₂ mediated cell lysis and ultimately release of chromosomal DNA into the extracellular environment as eDNA.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogenic bacterium well known for causing chronic lung infections in cystic fibrosis patients.1 Similar to many other bacterial species, biofilm formation of P. aeruginosa is facilitated by self-produced extracellular polymeric substances primarily composed of polysaccharides, proteins, lipids and extracellular DNA (eDNA).2,3 eDNA is crucial in various bacterial species for adhesion, cell-to-cell interaction/aggregation, biofilm formation and stability and protection of biofilms against antibiotics and detergents.4-8 In Gram positive bacteria release of eDNA involves lysis of a small population of bacterial cells, mediated through various autolysins such as AtlE in S. epidermidis9 and gelatinase and serine protease of Enterococcus faecalis.10 Additionally phage and hydrogen peroxide (H₂O₂) mediated eDNA release at the cost of cell lysis have also been reported in diverse Streptococcus species.11,12

In P. aeruginosa, eDNA release is mediated through quorum sensing (QS) molecules such as N-acyl-L-homoserine lactones and the Pseudomonas quinolone signal by inducing phage production.13 eDNA release via non-QS pathways has also been documented in P. aeruginosa biofilms involving flagella and type IV pili mediated cell lysis.13,14 Recently Das and Manefield exposed that eDNA release in P. aeruginosa also happens through production of the phenazine pyocyanin.15 Phenazine production is mediated via QS controlled expression of phzA-G operons resulting in production of the primary phenazine phenazine-1-carboxylic acid (PCA). PCA is then modified to produce a variety of secondary phenazine molecules such as pyocyanin (PYO) through action of the phzM gene, phenazine-1-carboxamide (PCN), encoded by phzH and 1-hydroxy phenazine (1-OHPHZ) encoded by phzS as shown in Figure 1.16,17 In relation to our recently published paper,15 where we showed pyocyanin enhances eDNA release, in this addendum we demonstrated that like pyocyanin other phenazine molecules also promote eDNA production in P. aeruginosa.

Results and Discussion

By disrupting the activity of specific phenazine genes in wildtype PA01 the ratio of production of the various phenazine molecules is altered as indicated by the change in bacterial cell free supernatant color (Fig. 2B). For instance the mutant ΔphzS appears red because it lacks the gene responsible for 1-OHPHZ production and also responsible for conversion of 5-methylphenazine-1-carboxylic acid (5-MCA), an immediate precursor of PYO (which is red colored compound), into PYO.18 The ΔphzH mutant appears light green because it produces 1-OHPHZ and PYO but lacks production of PCN.18 The double mutant ΔphzSH is unable to produce PCN or 1-OHPHZ and consequently overproduces PYO giving the supernatant a dark green appearance.15

Figure 2C demonstrates that mutant strains over-producing specific phenazines released significantly more eDNA than the PA01 wildtype strain especially after 3 and 5 d of batch culture growth. In support of this observation it has been reported previously that altering the proportion of specific phenazine production, via activating or deleting specific phz genes, has significant impacts on Pseudomonas adhesion, biofilm formation and biocontrol activity.19-21 Similar observations were made using a ΔphzA-G mutant of P. aeruginosa strain PA14 incapable of producing phenazines. In this case, significantly lower eDNA production...
**Figure 1.** Schematic represents quorum sensing controlled expression of genes for phenazine production. *P. aeruginosa* synthesizes acylated homoserine lactones (AHLs) and Pseudomonas quinone signaling (PQS) as their primary and secondary quorum sensing signaling molecules. PQS regulates the synthesis of phenazine-1-carboxylic acid (PCA) through a set of primary phenazine producing genes *phzA1-G1* and *phzA2-G2*. PCA then converts into various kinds of specific phenazine encoded by specific genes.

**Figure 2.** Different kinds of phenazine production and its influence in eDNA release. Comparison of bacterial colony formation on LB agar plate after 48 h incubation with non-filtered (upper half) and filtered (bottom half) bacterial supernatant (A). Production of various kinds of phenazine molecules by *P. aeruginosa* PAO1 strains (B). Quantification of eDNA release in supernatants of various kinds of phenazine producing *P. aeruginosa* PAO1 (C) and PA14 (D) strains. Error bars represents standard deviations from the mean (n = 3). Asterisks indicate statistically significant (p < 0.05) differences in eDNA concentration in comparison to the PAO1 wildtype (C) and mutant strain ΔphzA-G (D). Schematic represents the relationship between eDNA release and phenazine production is bridged via H₂O₂ generation and subsequent H₂O₂ mediated cell lysis (E).
was observed in comparison to PA14 wildtype that predominantly produces pyocyanin and a PA14 ΔphzM mutant that is deficient in pyocyanin production but producing PCN and 1-OHPHZ (Fig. 2D).16

The relationship between eDNA release and phenazine production is bridged via H2O2 generation as an intermediate agent. H2O2 generation occurs when phenazines, which are electrochemically active, accept electrons from NADH in the biofilm or cell culture and subsequently transfers that electrons to molecular oxygen. Phenazines are thus involved in reduction of molecular oxygen to form reactive oxygen species like O2-, H2O2. H2O2 can react with metals to produce highly reactive hydroxyl radicals that damage bacterial cell walls resulting in lysis of cells22 and ultimately release of chromosomal DNA forming eDNA (Fig. 2E).15 Linking the results from the previous and current study, we propose that phenazines may have significant ecological impact not only on P. aeruginosa biofilm formation but also on other bacterial species that persist in mixed biofilms along with P. aeruginosa. Phenazine influenced H2O2 generation and lysis of competing bacterial cells in mixed biofilms and subsequent eDNA release may give it a competitive edge.

Moreover, large quantities of pyocyanin as well as DNA are known to exist in the sputum of cystic fibrosis (CF) patients and these factors could promote biofilm formation and subsequently enhance mortality in CF patients through deterioration of the host immune system. Host immune cells called neutrophils kill infecting microbes by trapping them in the neutrophil extracellular matrix which mainly consists of DNA and antimicrobial peptides. However, killing of microbes may further encourage release of microbial eDNA and excess of DNA is responsible for failure of host immune systems by inciting autoimmune diseases (systemic lupus erythematosus). This makes the biological significance of eDNA of greater interest for further research since it not only encourages biofilm formation but also damages host immune systems.

### Materials and Methods

**Bacterial species, culture conditions and quantification of eDNA.** All P. aeruginosa strains used in this study are listed in Table 1. Strains were plated onto LB agar plates and incubated aerobically for 48 h at 37°C. No bacterial colony formation on LB agar plates was observed (Fig. 2A). To further ensure the filtered supernatants were free of bacterial contamination, 50 μl of filtered supernatant was used to inoculate LB agar plates and incubated aerobically for 48 h at 37°C. No bacterial colony formation on LB agar plates was observed (Fig. 2A). The concentration of eDNA present in the filtered supernatant of various P. aeruginosa strains at various growth days was quantified by using Qubit 2.0 Fluorometer (Invitrogen, Life Technologies). Statistical analysis. The amount of eDNA release by various phenazine producing P. aeruginosa strains was analyzed using a two-tailed Student’s t-test. Differences were considered significant if p < 0.05.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Table 1. P. aeruginosa strains used in this study and their relevant phenazine producing characteristics

| P. aeruginosa strains | Phenazine production | Source |
|-----------------------|----------------------|--------|
| PA01 Wildtype         | + + + + + | 15     |
| ΔphzS                 | + + - + + | 18     |
| ΔphzH                 | - - + + + | 18     |
| ΔphzM                 | + + - + - | 18     |
| phzSH                 | + + - + + | 15     |
| PA14 Wildtype         | + + - + + | 15     |
| PA14 ΔphzM            | + + - + - | D.K. Newman lab |
| PA14 ΔphzA-G          | - - - - - | 19     |

Note: +, produce basal level of phenazine; ++, produce elevated amount of phenazine.
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