Communicative & Integrative Biology 6:3, e23570; May/June 2013; © 2013 Landes Bioscience

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

Theerthankar Das* and Mike Manefield

Centre for Marine BioInnovation (CMB); School of Biotechnology and Biomolecular Sciences (BABS); University of New South Wales (UNSW); Sydney, NSW Australia

Keywords: *Pseudomonas aeruginosa,* phenazines, hydrogen peroxide, extracellular DNA

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 Δ*phzA-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 (H_2O_2) generation and subsequent H_2O_2 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 autolysin proteins such as AtlE in *S. epidermidis*[9] and gelatinase and serine protease by *Enterococcus faecalis*.[10] Additionally phage and hydrogen peroxide (H_2O_2) 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 Δ*phzS* 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 Δ*phzH* mutant appears light green because it produces 1-OHPHZ and PYO but lacks production of PCN.[18] The double mutant Δ*phzSH* 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 PAO1 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 Δ*phzA-G* mutant of *P. aeruginosa* strain PA14 incapable of producing phenazines. In this case, significantly lower eDNA production

*Correspondence to: Theerthankar Das; Email: theerthankar_das11@rediffmail.com
Submitted: 11/01/12; Revised: 01/09/13; Accepted: 01/10/13
Citation: Das T, Manefield M. Phenazine production enhances extracellular DNA release via hydrogen peroxide generation in Pseudomonas aeruginosa. Commun Integr Biol 2013; 6: e23570; http://dx.doi.org/10.4161/cib.23570

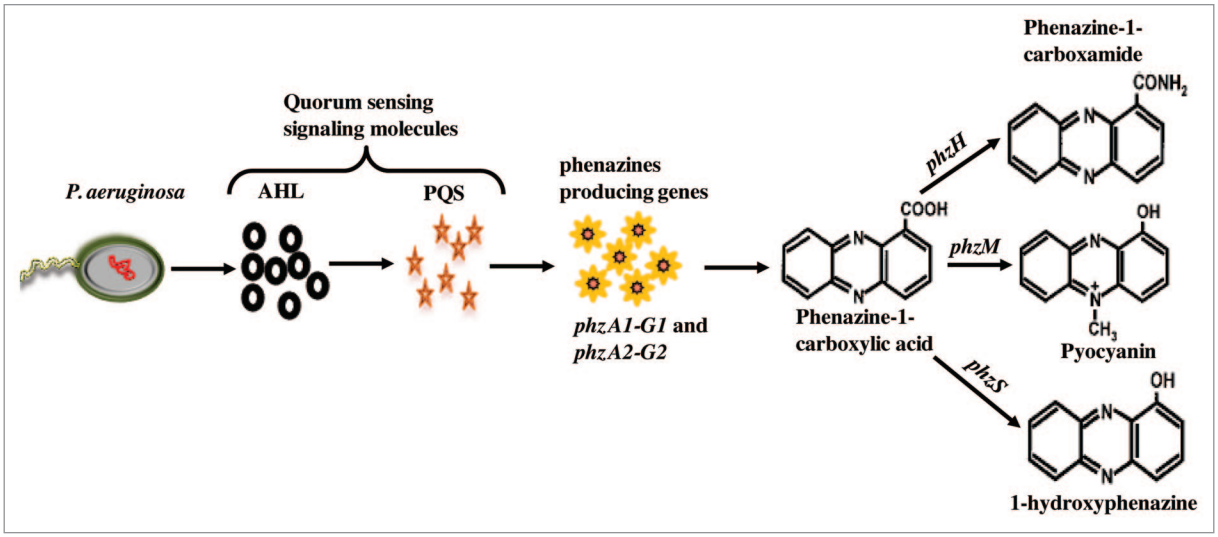

Figure 1. Schematic represents quorum sensing controlled expression of genes for phenazine production. *P. aeruginosa* synthesizes acylated homoserine lactones (AHLs) and Pseudomonas quinone signaling (PQS) as their primary and secondary quorum sensing signaling molecules. PQS regulates the synthesis 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.

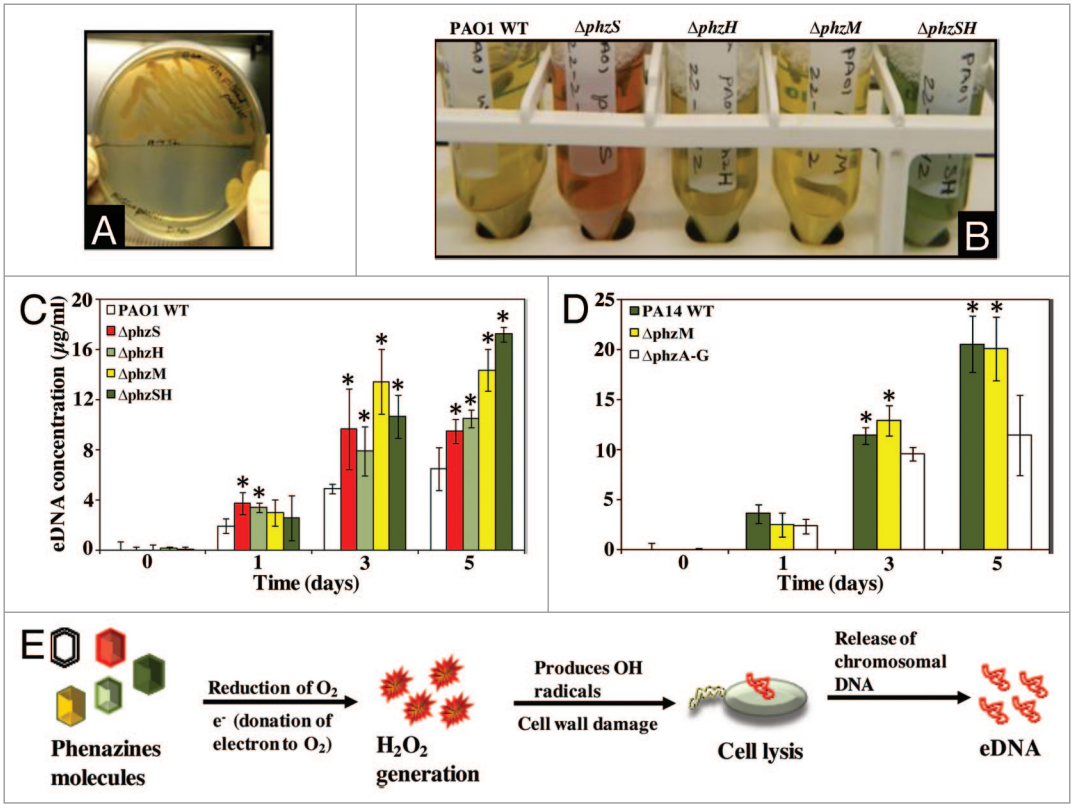

Figure 2. Different kinds of phenazine production and its influence in eDNA release. Comparison of bacterial colony formation on LB agar plate after 48 h incubation with non-filtered (upper half) and filtered (bottom half) bacterial supernatant (**A**). Production of various kinds of phenazine molecules 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 Δ*phzA-G* (**D**). Schematic represents the relationship between eDNA release and phenazine production is bridged via H_2O_2 generation and subsequent H_2O_2 mediated cell lysis (**E**).

was observed in comparison to PA14 wildtype that predominantly produces pyocyanin[15] and a PA14 Δ*phzM* 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 H_2O_2 generation as an intermediate agent. H_2O_2 generation occurs when phenazines, which are electrochemically active, accept electrons from NADH in the biofilm or cell culture and subsequently transfers that electrons it to molecular oxygen. Phenazines are thus involved in reduction of molecular oxygen to form reactive oxygen species like O_2^-, H_2O_2.[1] H_2O_2 can react with metals to produce highly reactive hydroxyl radicals that damage bacterial cell walls resulting in lysis of cells[22] 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 H_2O_2 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[1,23] 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.[24] 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).[25] 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 overnight under aerobic conditions at 37°C. Single colonies from the agar plates were used to inoculate 20 ml cultures in LB medium (ph 7) for 0, 1, 3 and 5 d at 30°C, 150 rpm. After growth, the *P. aeruginosa* strains were harvested and pelleted out by centrifugation at 6,500 rpm (4,912 × *g*) for 5 min at 10°C. After centrifugation, supernatants were separated from bacterial pellets. In order to remove remaining bacteria, supernatants were again filtered using 0.22 μm Millipore filter units (Millipore). To further ensure the filtered supernatants were free of bacterial cells, 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).[15]

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

***P. aeruginosa* strains**	**Phenazine production**					**Source**
	PCA	PCN	5-MCA	1-OHPHZ	PYO	
PAO1 Wildtype	+	+	+	+	+	15
Δ*phzS*	+	+	+	-	-	18
Δ*phzH*	+	-	+	+	+	18
Δ*phzM*	+	+	-	+	-	18
phzSH	+	-	-	-	++	15
PA14 Wildtype	+	+	+	+	+	15
PA14 Δ*phzM*	+	+	-	+	-	D.K. Newman lab
PA14 Δ*phzA-G*	-	-	-	-	-	19

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

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.

Acknowledgments

We thank Professor Dianne K. Newman, Department of Biology, California Institute of Technology for providing us with *P. aeruginosa* Δ*phzA-G* and Δ*phzM* strains. We thank Professor Korneel Rabaey, Laboratory of Microbial Ecology and Technology (LabMET), Ghent University for providing us with Δ*phzS,* Δ*phzH* and Δ*phzM* strains.

Funding

This work was funded by Australian Research Council (ARC) Future Fellowship Project ID FT100100078 (www.arc.gov.au). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

References

1. Price-Whelan A, Dietrich LEP, Newman DK. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nat Chem Biol 2006; 2:71-8; PMID:16421586; http://dx.doi.org/10.1038/nchembio764.
2. Tsuneda S, Aikawa H, Hayashi H, Yuasa A, Hirata A. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. FEMS Microbiol Lett 2003; 223:287-92; PMID:12829300; http://dx.doi.org/10.1016/S0378-1097(03)00399-9.
3. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010; 8:623-33; PMID:20676145.
4. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science 2002; 295:1487; PMID:11859186; http://dx.doi.org/10.1126/science.295.5559.1487.
5. Das T, Sharma PK, Busscher HJ, van der Mei HC, Krom BP. Role of extracellular DNA in initial bacterial adhesion and surface aggregation. Appl Environ Microbiol 2010; 76:3405-8; PMID:20363802; http://dx.doi.org/10.1128/AEM.03119-09.
6. Das T, Krom BP, Van der Mei HC, Busscher HJ, Sharma PK. DNA-mediated bacterial aggregation is dictated by acid-base interactions. Soft Matter 2011; 7:2927-35; http://dx.doi.org/10.1039/c0sm01142h.
7. Das T, Sharma PK, Krom BP, van der Mei HC, Busscher HJ. Role of eDNA on the adhesion forces between *Streptococcus mutans* and substratum surfaces: influence of ionic strength and substratum hydrophobicity. Langmuir 2011; 27:10113-8; PMID:21740034; http://dx.doi.org/10.1021/la202013m.
8. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog 2008; 4:e1000213; PMID:19023416; http://dx.doi.org/10.1371/journal.ppat.1000213.
9. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, et al. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology 2007; 153:2083-92; PMID:17600053; http://dx.doi.org/10.1099/mic.0.2007/006031-0.
10. Thomas VC, Thurlow LR, Boyle D, Hancock LE. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J Bacteriol 2008; 190:5690-8; PMID:18556793; http://dx.doi.org/10.1128/JB.00314-08.
11. Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. PLoS ONE 2010; 5:e15678; PMID:21187931; http://dx.doi.org/10.1371/journal.pone.0015678.
12. Zheng L, Chen Z, Itzek A, Ashby M, Kreth J. Catabolite control protein A controls hydrogen peroxide production and cell death in Streptococcus sanguinis. J Bacteriol 2011; 193:516-26; PMID:21036992; http://dx.doi.org/10.1128/JB.01131-10.
13. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol 2006; 59:1114-28; PMID:16430688; http://dx.doi.org/10.1111/j.1365-2958.2005.05008.x.
14. Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, et al. Cell death in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 2003; 185:4585-92; PMID:12867469; http://dx.doi.org/10.1128/JB.185.15.4585-4592.2003.
15. Das T, Manefield M. Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*. PLoS ONE 2012; 7:e46718; PMID:23056420; http://dx.doi.org/10.1371/journal.pone.0046718.
16. Venkataraman A, Rosenbaum M, Arends jan BA, Halitschke R, Angenent LT. Quorum sensing regulates electric current generations of *Pseudomonas aeruginosa* PA14 in bioelectrochemical systems. Electrochem Commun 2010; 12:459-62; http://dx.doi.org/10.1016/j.elecom.2010.01.019.
17. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J Bacteriol 2001; 183:6454-65; PMID:11591691; http://dx.doi.org/10.1128/JB.183.21.6454-6465.2001.
18. Rabaey K, Boon N, Höfte M, Verstraete W. Microbial phenazine production enhances electron transfer in biofuel cells. Environ Sci Technol 2005; 39:3401-8; PMID:15926596; http://dx.doi.org/10.1021/es048563o.
19. Dietrich LEP, Teal TK, Price-Whelan A, Newman DK. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. Science 2008; 321:1203-6; PMID:18755976; http://dx.doi.org/10.1126/science.1160619.
20. Maddula VSRK, Pierson EA, Pierson LS 3rd. Altering the ratio of phenazines in *Pseudomonas chlororaphis* (aureofaciens) strain 30-84: effects on biofilm formation and pathogen inhibition. J Bacteriol 2008; 190:2759-66; PMID:18263718; http://dx.doi.org/10.1128/JB.01587-07.
21. Chin-A-Woeng TFC, Thomas-Oates JE, Lugtenberg BJJ, Bloemberg GV. Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. Mol Plant Microbe Interact 2001; 14:1006-15; PMID:11497461; http://dx.doi.org/10.1094/MPMI.2001.14.8.1006.
22. Infection Control Today website. Available: http://www.infectioncontroltoday.com/articles/2001/09/infection-control-today-09-2001-the-effects-of-ge.aspx. Accessed 2012 October 30
23. Bakker EM, Tiddens HAWM. Pharmacology, clinical efficacy and safety of recombinant human DNase in cystic fibrosis. Expert Rev Respir Med 2007; 1:317-29; PMID:20477171.
24. Medina E. Neutrophil extracellular traps: a strategic tactic to defeat pathogens with potential consequences for the host. J Innate Immun 2009; 1:176-80; PMID:20375575; http://dx.doi.org/10.1159/000203699.
25. Su KY, Pisetsky DS. The role of extracellular DNA in autoimmunity in SLE. Scand J Immunol 2009; 70:175-83; PMID:19703007; http://dx.doi.org/10.1111/j.1365-3083.2009.02300.x.