Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*

Shawn Lewenza1,2*

1 Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB, Canada
2 Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada

Edited by: Franca Walsh, Agroscope, Changins-Wädenswil, Switzerland
Reviewed by: Elaine Allan, University College London, UK

*Correspondence:* Shawn Lewenza, Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada T2N 4N1. E-mail: slewenza@ucalgary.ca

**SOURCE AND FUNCTIONS OF EXTRACELLULAR DNA**

Extracellular DNA (eDNA) is released from dead plant or microbial organisms and accumulates in soil, aquatic, and sediment environments (Dell’Anno and Danovaro, 2005; Vlassov et al., 2007; Pietramellaro et al., 2009). Bacteria actively release or secrete DNA, or it is released during bacterial lysis and outer membrane vesicle formation (Chiang and Tolker-Nielsen, 2010). eDNA is known to accumulate in many Gram-negative and Gram-positive bacterial biofilms (Tett et al., 2009; Chiang and Tolker-Nielsen, 2010).

Extracellular DNA is present in healthy body sites and fluids, such as the gastrointestinal tract, blood, milk, secretions, and likely on mucosal surfaces (Vlassov et al., 2007). During infection, eDNA can accumulate due to the heavy recruitment of host immune cells and the production of neutrophil extracellular traps (NETs), as discussed later. Chronic lung infections in persons challenged with *Pseudomonas aeruginosa* have accumulated antibiotic resistance phenotypes to APs and aminoglycosides. These dual antibiotic resistance and immune evasion strategies may be expressed in DNA-rich environments and contribute to long-term survival.

**DNA IS A NUTRIENT SOURCE**

Given the abundance of eDNA in the environment, it is not surprising that DNA has a significant influence on bacterial physiology and serves many functions for bacteria. eDNA has been shown to serve as a sole nutrient source of phosphate, nitrogen, and carbon for *Pseudomonas aeruginosa*, *Escherichia coli*, and *Shewanella* spp. (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006; Panchuk et al., 2008). We identified a secreted DNase (EddB) that is produced in the presence of low DNA concentrations and under limiting phosphate conditions (Mukhaly et al., 2010). The EddB DNase is required for degradation of eDNA and utilization of DNA fragments or nucleotides as a sole source of carbon, nitrogen, and phosphate (Mukhaly et al., 2010). There is an alkaline phosphatase expressed upstream of the DNase, EddA, which may also be required for phosphorus acquisition from DNA. In *Shewanella oneidensis*, a secreted Dnase (ExeM) with significant homology to EddB (34% identity) is also required for utilization of DNA as a nutrient source (Goddeke et al., 2011). A number of intracellular ssDNA exonucleases have also been shown to be required for growth using DNA as a sole carbon source (Palchevskiy and Finkel, 2006). DNA uptake also facilitates lateral gene transfer (LGT) and integration of foreign DNA sequences into the genome. Palchevskiy and Finkel (2006) proposed that dsDNA was taken into the cell, similar to the process of DNA uptake for LGT, converted to ssDNA and then degraded by intracellular exonucleases upon entry into the cytoplasm.

**DNA IS A BIOFILM MATRIX POLYMER**

Extracellular DNA is required and primarily acts to facilitate attachment, aggregation, stabilization, and maturation of biofilm
formation (Chiang and Tølker-Nielsen, 2010). DNase treatment of young *P. aeruginosa* biofilms results in biofilm dissolution, but mature biofilms resist DNase treatment, indicating a role in early biofilm formation (Whitchurch et al., 2002). Accumulation of exopolysaccharide (EPS) in mature biofilms probably accounts for the inability to degrade mature biofilms with exogenous DNase. Mutant strains that accumulated less eDNA during biofilm formation were more destabilized by treatment with sodium dodecyl sulfate (SDS) (Allesen-Holm et al., 2016), providing further evidence for a role in biofilm stabilization. Treatment of young biofilms with DNase impaired the development of the cap structures of mushroom-shaped biofilms (Barken et al., 2008). DNase treatment of biofilms formed by Gram-negative or Gram-positive bacteria reduces the biomass, which suggests that eDNA is a ubiquitous DNA polymer (Tetz et al., 2009). The exception to the rule is in *Caulobacter crescentus* where eDNA blocks biofilm formation by binding to the polar holdfast structure, which is required for irreversible attachment (Berne et al., 2010). eDNA has been shown to localize to specific regions of mushroom-shaped microcolonies formed by *P. aeruginosa* in flow-chamber biofilms. In mature microcolonies, eDNA localizes primarily to the stalk structure, at the boundary of the stalk and cap (Allesen-Holm et al., 2006). In unstructured peg-adhered biofilms, eDNA can be visualized throughout thin biofilms with no particular organization (Mulcahy et al., 2008). eDNA has also been shown to be present as a matrix component in biofilms formed in vivo during infection with *P. aeruginosa* (Mulcahy et al., 2011). NET formation has been demonstrated that DNA efficiently binds divalent metal cations that including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (Mulcahy et al., 2008). In addition, DNA has a rapid antimicrobial killing activity that can be neutralized by pre-incubating DNA with excess cations before exposure to bacteria (Mulcahy et al., 2008). As bacterial surfaces are highly negatively charged and consequently have high levels of Mg$^{2+}$ and Ca$^{2+}$ bound to the surface (Nicas and Hancock, 1980), we suspected that DNA chelated cations from surfaces and disrupted membrane integrity. Using fluorescence microscopy to monitor membrane integrity, we demonstrated that DNA causes major perturbations to the outer and inner bacterial membranes, leading to rapid cell lysis and death. In addition, cells treated with antimicrobial concentrations of DNA released small outer membrane vesicles. This result indicated that DNA can strip sections of outer membrane from the envelope, disrupting outer and inner membrane integrity, resulting in cell lysis. The membrane destabilizing effects of DNA are similar to that of known cation chelator ethylenediaminetetraacetic acid (EDTA). DNA appears to have a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Mulcahy et al., 2008).

### EXTRACELLULAR DNA TRAPS

Neutrophil extracellular traps were first described in neutrophils, but have since been identified in other immune cell types including eosinophils and mast cells (Brinkmann and Zychlinsky, 2012). NETs can kill Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses (Brinkmann et al., 2004; Urban et al., 2006, 2009; Guimarães-Costa et al., 2009; Saitoh et al., 2012). Although there are numerous antimicrobial neutrophil components embedded in NETs (Urban et al., 2009), bacterial killing is largely attributed to the antimicrobial activity of histones (Brinkmann et al., 2004). NET killing can be blocked by either dissolving the NET structure with DNase, or by the addition of neutralizing anti-histone antibodies, which block histone antimicrobial activity. The process of NETosis is a novel mechanism of trapping and killing bacteria, as well as limiting bacterial dissemination (Brinkmann and Zychlinsky, 2012; McDonald et al., 2012; Yipp et al., 2012). For the purpose of this review, it is important to note that NET formation during infection is likely a major contribution of DNA accumulation at the site of infection. NET formation has been observed in CF sputum and likely contributes to the accumulation of eDNA during chronic CF lung infections (Marcos et al., 2010; Manzenreiter et al., 2012). Neutrophils are among the first immune cells recruited to the infection site and most of the DNA in the CF lung is derived from neutrophils (Lethem et al., 1996). In plant roots, an eDNA barrier is produced that protects the root from infection and is analogous to eDNA traps of human immune cells (Hawes et al., 2011).

CATION CHELATION AND ANTIMICROBIAL ACTIVITIES OF DNA

The focus of our initial work was to test the hypothesis that the matrix polymers influence bacterial gene expression. While biofilm polymers are known to have several protective immune evasion functions, we wondered if the matrix polymers also drive unique gene expression profiles that contribute to the phenotypes of cells in biofilms. Our initial observation upon addition of purified DNA exogenously to planktonic cultures was that bacterial growth was inhibited at DNA concentrations greater than 5 mg/ml (Mulcahy et al., 2008). Due to the highly anionic character of DNA, we hypothesized that DNA was a cation chelator and indeed demonstrated that DNA efficiently binds divalent metal cations that including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (Mulcahy et al., 2008). The exception to the rule is in *Caulobacter crescentus* where eDNA blocks biofilm formation by binding to the polar holdfast structure, which is required for irreversible attachment (Berne et al., 2010). eDNA has been shown to localize to specific regions of mushroom-shaped microcolonies formed by *P. aeruginosa* in flow-chamber biofilms. In mature microcolonies, eDNA localizes primarily to the stalk structure, at the boundary of the stalk and cap (Allesen-Holm et al., 2006). In unstructured peg-adhered biofilms, eDNA can be visualized throughout thin biofilms with no particular organization (Mulcahy et al., 2008). eDNA has also been shown to be present as a matrix component in biofilms formed in vivo during infection with *P. aeruginosa* (Mulcahy et al., 2011). NET formation has been demonstrated that DNA efficiently binds divalent metal cations that including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (Mulcahy et al., 2008). In addition, DNA has a rapid antimicrobial killing activity that can be neutralized by pre-incubating DNA with excess cations before exposure to bacteria (Mulcahy et al., 2008). As bacterial surfaces are highly negatively charged and consequently have high levels of Mg$^{2+}$ and Ca$^{2+}$ bound to the surface (Nicas and Hancock, 1980), we suspected that DNA chelated cations from surfaces and disrupted membrane integrity. Using fluorescence microscopy to monitor membrane integrity, we demonstrated that DNA causes major perturbations to the outer and inner bacterial membranes, leading to rapid cell lysis and death. In addition, cells treated with antimicrobial concentrations of DNA released small outer membrane vesicles. This result indicated that DNA can strip sections of outer membrane from the envelope, disrupting outer and inner membrane integrity, resulting in cell lysis. The membrane destabilizing effects of DNA are similar to that of known cation chelator ethylenediaminetetraacetic acid (EDTA). DNA appears to have a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Mulcahy et al., 2008).

### ANTIMICROBIAL PEPTIDE KILLING AND RESISTANCE MECHANISMS

Cationic antimicrobial peptides (APs) are short, amphipathic peptides with broad-spectrum antimicrobial activity produced by the immune systems of most forms of life (Hancock and Sahl, 2006). The mechanism of killing is primarily through membrane binding and disruption, although they also disrupt cytoplasmic processes (Hancock and Sahl, 2006; Kraus and Peschel, 2006). Host defense peptides are another class of short peptides that may not have direct antimicrobial activities, but are protective due to their ability to modulate the innate immune response (Hancock and Sahl, 2006). APs are positively charged and therefore interact with the negatively charged lipopolysaccharide (LPS) in the Gram-negative outer membrane surface. The hydrophobic character permits membrane integration, disruption, and ultimately cell lysis and death. Gram-negative and Gram-positive bacteria alter their membrane charge to resist peptide killing by producing modified phospholipids, LPS, or teichoic acid structures, whose negative charges are masked (Kraus and Peschel, 2006; Anaia-Lopez et al., 2012). Surface modifications that contribute to AP resistance include alanine-modified teichoic acids, highly acylated lipid A, as well as phosphoethanolamine and aminouracilomodified lipid A species (Kraus and Peschel, 2006; Moskovitz and Ernst, 2010; Anaia-Lopez et al., 2012). Collectively, these modifications prevent or limit peptide binding or entry and disruption of bacterial membranes. CF isolates of *P. aeruginosa* are known to
produce highly acylated lipid A species and aminosugar biosynthesis modifications on the 1- and 4′-phosphates of lipid A (Moskowitz and Ernst, 2010).

**DNA-INDUCED EXPRESSION OF THE pmr OPERON**

The pmr genes are required for the covalent addition of aminosugar to the 1- and 4′-phosphates of lipid A (Moskowitz and Ernst, 2010), which protects the outer membrane from AP treatment (Johnson et al., 2012), and is required for peptide resistance (Moskowitz et al., 2004; Lewenza et al., 2005). The *pmr* genes are regulated by the PhoPQ and PmrAB systems in *P. aeruginosa*, and in many other Gram-negative organisms including *Salmonella enterica*, Klebsiella pneumoniae, and Yersinia pestis (Macfarlane et al., 1999; Grosman, 2001; McPhee et al., 2006; Cheng et al., 2010; O’Loughlin et al., 2010). The *P. aeruginosa* PhoQ sensor responds to Mg2+ levels and is activated under Mg2+ limiting conditions, leading to increased expression of the *pmr* operon. In Mg2+-rich conditions, the presence of sub-lethal exposure to APs also induces expression of the *pmr* operon (McPhee et al., 2003), although this adaptive resistance is controlled by the CprRS and ParRS two-component systems (Fernández et al., 2012).

Although DNA prevented growth at higher concentrations, we examined the influence of sub-lethal concentrations of DNA on *pmr* gene expression. In planktonic cultures grown in Mg2+-rich media supplemented with DNA, we showed that DNA caused a concentration-dependent induction of the *pmr* operon (PA3552–PA3559) in *P. aeruginosa* (Mulcahy et al., 2008). DNA induction of this operon can be explained by cation sequestration by DNA, and subsequent activation of the PhoPQ/PmrAB systems. Increased amounts of DNA resulted in more Mg2+ sequestered and therefore increasingly higher levels of *pmr* gene expression. Figure 1 depicts the cation chelating effects of DNA on the structure of LPS in *P. aeruginosa*. Gene induction by DNA can be prevented by the addition of excess cations in combination with DNA, confirming that the cation chelating activity of DNA can be neutralized. We have recently shown that DNA can also induce expression of the *Salmonella enterica* serovar Typhimurium pmr operon and causes increased AP resistance (Submitted), indicating that DNA may play a general role in activating the PhoPQ system in DNA-rich environments.

**DNA-INDUCED EXPRESSION OF SPERMIDINE SYNTHESIS GENES**

**CONCENTRATION OF eDNA IN BIOFILMS AND INFECTION SITES**

A large number of *P. aeruginosa* genes are regulated under Mg2+ limiting conditions; some exclusively by PhoPQ and others are controlled by a second Mg2+ sensing two-component system PmrAB (McPhee et al., 2006). While the *pmr* operon is directly controlled by both PmrA and PhoP (McPhee et al., 2003, 2006), we identified a three-gene cluster upstream of PmrAB with homology to spermidine synthesis genes PA4773 (speD) and PA4774 (speE) that is controlled exclusively by PmrAB (McPhee et al., 2003). The addition of DNA to planktonic cultures also induced the expression of PA4773–PA4775 in a concentration-dependent manner (Johnson et al., 2012). Mutants in the PA4773–PA4775 genes were sensitive to APs, indicating a potential role in resistance to APs (Lewenza et al., 2005). We confirmed that PA4773–PA4774 were required for spermidine synthesis, which is localized on the bacterial surface (Johnson et al., 2012). Surface and exogenous spermidine protects the outer membrane from APs polymyxin B and CF10A, but also from treatment with other cationic antibiotics including the aminoglycoside gentamicin (Johnson et al., 2012). Polymamines are typically found in the cytoplasm but here we have identified a novel role for polyamines on the bacterial surface. In the presence of eDNA, we proposed that *P. aeruginosa* produces spermidine as an organic polycation replacement for the divalent metal cation Mg2+ that functions to mask the negative surface charge and block AP binding (Figure 1). Magnesium ions are essential to cross-bridge the core phosphates of lipid A, so it is not surprising that *P. aeruginosa* produces a replacement polycation in the presence of DNA or under Mg2+ limiting conditions. Surface polyamines also act as antioxidants and quench reactive oxygen species, thereby protecting the outer membrane from oxidative stress damage to lipids (Johnson et al., 2012).

**DNA-INDUCED ANTIMICROBIAL RESISTANCE IN BIOFILMS**

To test for a role of DNA-induced expression of the *pmr* genes in biofilm-specific antibiotic resistance, we determined the minimum biofilm eradication concentration (MBEC) in wild type biofilms and in biofilms formed the presence or absence of exogenous DNA (Mulcahy et al., 2008). DNA-enriched biofilms were shown to be eightfold more tolerant to the APs polymyxin B and colistin, and 64- to 128-fold more tolerant to the aminoglycosides gentamicin and tobramycin. Interestingly, planktonic cultures containing exogenous DNA also demonstrated DNA-induced resistance to aminoglycosides and APs (Mulcahy et al., 2008). Exogenous DNA did not have an effect on β-lactam or fluoroquinolones resistance. A mutant in the *pmr* cluster did not exhibit any DNA-induced resistance to APs, indicating that these genes were expressed and required for resistance in DNA-enriched biofilms (Mulcahy et al., 2008). The *pmr* mutant showed an intermediate aminoglycoside resistance phenotype, indicating that the *pmr* aminosugar biosynthesis modification also contributed partially to DNA-induced aminoglycoside resistance. It is possible that the anionic eDNA bound positively charged aminoglycosides and provided some protection as a matrix barrier, thus explaining the residual level of resistance in the presence of DNA. It is known that DNA is capable of binding to aminoglycosides (Rampal et al., 1988; Purdy Drew et al., 2009) and APs (Bucki et al., 2007). Therefore it is possible that DNA can induce specific resistance mechanisms and also act as a protective matrix absorbing and limiting antimicrobial exposure.

**CONCENTRATION OF eDNA IN BIOFILMS AND INFECTION SITES**

An important question that has not been fully answered is to determine if sufficient DNA accumulates in biofilms or during infections, to induce the expression of those protective, AP resistance phenotypes. In microarray studies comparing the gene expression profiles of biofilm to planktonic cultures, the PhoPQ/PmrAB-controlled genes are not among the biofilm-induced genes (Whiteley et al., 2001; Wüth et al., 2003). This may be due to an insufficient accumulation of DNA in these particular biofilm model systems, and/or the presence of high Mg2+ levels in the growth media used, which can neutralize eDNA and prevent activation of the Mg2+ sensing PhoPQ and PmrAB systems. However, a recent paper described a novel regulator of biofilm
Antimicrobial peptides (green) can displace cations and disrupt membrane integrity, leading to cell lysis and death. Extracellular DNA binds and sequesters cations from the environment and the membrane. This results in Mg$^{2+}$ or cation chelation, the PhoPQ/PmrAB systems are activated, leading to the production of covalently attached aminoribosamine to the phosphates of lipid A (red) and the production of polycation spermidine (charge, +3) on the surface, which may bind electrostatically to negative charges in the core oligosaccharide (dark blue) of the O antigen. Both modifications mask the negative charges and protect the outer membrane from peptide damage.

FIGURE 1 | Lipopolysaccharide (LPS) modifications in the presence of extracellular DNA that contribute to antimicrobial peptide resistance. (A) Divalent metal cations, including Mg$^{2+}$ (orange) bind to the negatively charged phosphates of the lipid A moiety of LPS and act to stabilize LPS. Antimicrobial peptides (green) can displace cations and disrupt membrane integrity, leading to cell lysis and death. (B) Extracellular DNA binds and sequesters cations from the environment and the membrane. (C) In response to limiting Mg$^{2+}$ or cation chelation, the PhoPQ/PmrAB systems are activated leading to the production of covalently attached aminoribosamine to the phosphates of lipid A (red) and the production of polycation spermidine (charge, +3) on the surface, which may bind electrostatically to negative charges in the core oligosaccharide (dark blue) of the O antigen. Both modifications mask the negative charges and protect the outer membrane from peptide damage.

Although the total concentration of eDNA can be quantitated in biofilms (Wu and Xi, 2009), the localized concentration may be more important than the overall concentrations. The accumulation of DNA at infection sites is not well documented but sputum from the lungs of persons challenged with CF is known to accumulate DNA at concentrations ranging from <1 to 20 mg/ml (Shak et al., 1990; Ranasinha et al., 1993). There are relatively low Mg$^{2+}$ concentrations in the CF lung (0.88–2 mM; Palmer et al., 2005; Sanders et al., 2006), not high enough to neutralize the cation chelating potential of such high DNA concentrations. Based on the known concentration of DNA and Mg$^{2+}$ in the CF lung, it is probable that the PhoPQ/PmrAB-controlled genes are expressed in the CF lung and may contribute to long-term survival in the CF lung. Recently, colistin-resistant mutants have been characterized from CF patients and shown to contain gain-of-function PhoQ and PmrB sensor mutations, leading to increased expression of the pmr genes (Miller et al., 2011; Moskowitz et al., 2012). This result underscores the importance of these genes in the CF lung, particularly in those patients treated with colistin.

FUTURE WORK

To date, we have shown that eDNA influences the expression of several genes including a secreted DNase, and at least two operons controlled by the PhoPQ and PmrAB two-component systems. We are currently exploring the global effect of eDNA on bacterial gene expression using a genome-wide transcriptomic method and screening a library of transcription lux fusions (Lewenza et al., 2005) to identify novel DNA-induced or repressed genes. While aminoribosamine-modified LPS and surface spermidine both protect the outer membrane and contribute to AP resistance in vitro, these genes may also protect P. aeruginosa from APs produced by the innate immune system. It will be important to examine the role of DNA accumulation in biofilms.
We identified a new property of eDNA as a divalent metal cation chelator, which is required to induce expression of multi-ple operons that contribute to decreasing the permeability of the outer membrane to APs and aminoglycosides. Extracellular DNA is essential for maintaining Bordetella bacteriuria integrity on abiotic surfaces and in the upper respiratory tract of mice. PLoS ONE 6:e18886. doi:10.1371/journal.pone.0018886

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