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Nickel and cadmium ions inhibit quorum sensing and biofilm formation without affecting viability in *Burkholderia multivorans*

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ABSTRACT

Microbial influenced corrosion (MIC) and biofouling are multi-billion dollar challenges typically associated with bacterial biofilms. Using microtiter plate and quantitative reverse transcriptase polymerase chain reaction (q-PCR) assays, we show that sub-millimolar concentrations of nickel (Ni^{2+}) and cadmium (Cd^{2+}) inhibit biofilm formation by the bacterium *Burkholderia multivorans* through the inhibition (at the transcriptional level) of acyl-homoserine lactone quorum sensing (QS). These results advance the understanding of environmental factors influencing the establishment and subsequent development of biofilms, as this is the first paper linking heavy metals with quorum sensing and biofilm inhibition, and may guide strategies to mitigate MIC and biofouling of infrastructure surfaces.

1. Introduction

A study conducted by the U.S. government and the National Association of Corrosion Engineers (NACE) in 2001 estimated that the economic impact of corrosion was approximately 3.1% of the nation's Gross Domestic Product (GDP) (Koch, 2001). In today's (2014) dollars, this cost approaches $500 billion per year for the repair or replacement of infrastructure such as bridges and water distribution networks. A portion of this "corrosion cost" is directly or indirectly caused by biofilms and their associated products through a process known as microbial influenced corrosion (MIC).

Biofilms, or surface attached communities of microorganisms, cause corrosion through processes such as the production of organic acids, cathodic depolarization, and/or depassivation of a surface. Biofilms are difficult to treat once established because they have a number of defense mechanisms that make them resistant to physical and chemical attack (Stewart, 2001). Several strategies have been employed to control biofilm formation and associated biofouling. Traditional approaches include the use of a variety of antimicrobial agents (i.e., biocides) and modification of surface chemistry (reviewed in (van Loosdrecht, 1990; Hall-Stoodley et al., 2004). Many of these approaches have often been only marginally effective, or, as in the case of tributyltin (TBT), lead to unintended adverse environmental impacts (Antizar-Ladislao, 2008). This underscores the need for alternative preventive strategies such as quorum sensing interruption.

Quorum sensing refers to cell–cell communication between microorganisms through the production and reception of signal molecules. These biochemical signals allow the coordination of a number of mechanisms between individual cells, and in the case of biofilm formation, influence each step in the process from establishment to maturity (Davies et al., 1998; Gonzalez Barrios et al., 2006). Quorum sensing genes have been identified in archaeal, bacterial and fungal species, and these genes are known to play a critical role in biofilm formation in the latter two (Paggi et al., 2003; Chen et al., 2004). Accordingly, disruption of bacterial quorum sensing has shown promise for controlling biofilms (Hentzer et al., 2002). The majority of quorum disrupting compounds identified to date include furanones and other organic molecules that competitively bind to receptor molecules (De Nys et al., 2006), some antibiotics (Nalca et al., 2006), and quorum signal degrading enzymes

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(Thomas et al., 2005). If cell–cell signaling can be interfered with, biofilm formation may be delayed or the biofilm may not be as robust and therefore more susceptible to removal by various treatments.

This paper identifies a new class of quorum sensing inhibitors, divalent heavy metal ions, specifically nickel (Ni^{2+}) and cadmium (Cd^{2+}). We report that micromolar concentrations of Ni^{2+} and Cd^{2+} inhibit acyl-homoserine lactone quorum sensing at the transcriptional level, as well as inhibit biofilm formation by *Burkholderia multivorans*.

2. Materials and methods

2.1. Bacterial cultures and media

B. multivorans ATCC 17616 was grown on LB (Lennox) agar 35 ± 2 °C for 24 h. A single colony was selected then streaked onto a fresh LB plate and grown overnight for testing.

2.2. Evaluation of divalent metals on planktonic viability and biofilm formation

B. multivorans was suspended in sterile LB broth and mixed for 5 min. The suspension was added to LB in vials containing nickel, cadmium or cobalt (Co^{2+}) at a final concentration of 0–0.5 mM at a targeted absorbance (600 nm) of 0.01. The broth was added to a 96-well microtiter plate at a final test volume of 200 μL. Each plate row contained a minimum of 5 replicates per test condition. The plates were incubated in a shaker/incubator at 35 ± 2 °C and 250 rpm for 24 ± 2 h. After the incubation period, the plate was read using a Spectromax MX plate reader at 600 nm to evaluate the effect of nickel on planktonic viability via absorbance. Viability was confirmed by monitoring bacterial growth in the presence of the metal, by quantifying the increase in absorbance every 1–2 h over a period of 25 h.

The method used to characterize biofilm formation was as described elsewhere with minor modifications (Stepanovic et al., 2000). Briefly, the liquid from the wells was removed and then each well was rinsed with PBS to remove the loose bacteria. The biofilm in the wells was fixed with methanol, dried, and then stained with a 2% Hucker crystal violet solution. The crystal violet was removed and the wells were rinsed with water twice to remove the excess dye and dried again. The remaining dye in the wells after drying was re-solubilized with 160 μL of 70% ethanol and was read again using the plate reader at a setting of 570 nm. In order to determine whether the addition of homoserine lactone would attenuate the effect of nickel, 400 nM C8-HSL (Cayman Chemical) was added to a separate volume of broth prior to inoculation and assessed for biofilm formation as described.

2.3. Effect of divalent metals on acyl-homoserine lactone gene expression

Cell culture flasks containing *B. multivorans* suspended in LB and with 0–0.5 mM of Ni^{2+}, Cd^{2+}, or Co^{2+} were incubated for 12 h at 35 ± 2 °C and 250 rpm. Cells and RNA were harvested using the RNA protect solution and RNeasy mini kit with the RNase-Free DNase Set to remove genomic DNA (Qiagen). The QuantiTect® reverse transcription and SYBR® green PCR kits (Qiagen) were used for cDNA synthesis and PCR preparation per manufacturer's specifications. Real time PCR primers for N-acyl homoserine lactone synthase and transcriptional activator genes (*bmuIR*) to evaluate expression of acyl-homoserine lactones as compared to the expression of an endogenous control gene (*recA*) (Table S-1) were designed using the PrimerQuest PCR tool (IDT DNA) using information from Yao et al. (2002).

An Applied Biosystems™ 7500 Real Time PCR system was used for q-PCR. The cycler conditions were as follows: 2 min at 50 °C for UNG carryover prevention, 15 min at 95 °C for PCR initiation, and 40 cycles of 95 °C for 15 s (denaturation), 53.5 °C for 30 s (annealing), and 72 °C for 40 s (extension and data collection).

2.4. Data analysis

Means and standard errors for all experiments were collected at each test point. Data generated from the biofilm experiments were analyzed using a single factor analysis of variance (ANOVA) with a significance level of 95% (i.e., $p < 0.05$).

3. Results and discussion

3.1. Sub-lethal nickel concentrations decrease the expression of acyl-homoserine lactone genes in B. multivorans and subsequently hinder biofilm formation

Fig. 1 shows the results from a series of microtiter plate tests with the bacterium *B. multivorans* (ATCC 17616). This bacterium was selected because it is a member of the *Burkholderia cepacia* complex (BCC) that makes a relatively robust biofilm compared to other members of the BCC, and has known quorum sensing genes (Yao et al., 2002). Planktonic viability was assessed after 24 h by measuring absorbance at 600 nm. *B. cepacia* grew from an initial absorbance of 0.01 to about 0.8 over 24 h with nickel concentrations ranging from 0 (control) to 0.5 mM (Fig. 1(A)), indicating that the tested nickel concentrations were below lethal levels. To confirm that these nickel concentrations were sub-lethal, we measured their effect on bacterial growth over a 25-h period. With the exception of the wells containing 0.5 mM nickel, all concentrations resulted in the same growth pattern over the 24 h period (Figure S-1(A)).

In contrast to the planktonic cell results, attachment (i.e., biofilm formation) decreased from an absorbance of 0.22 (control) to 0.12 (at 0.4 mM) with increasing nickel concentrations (Fig. 1(B)). This decrease in biofilm formation is consistent with the down-regulation of quorum sensing genes (Fig. 2), in this instance homoserine lactone synthase and the transcriptional activator gene (*bmuIR*), for the production of N-octanoyl-L-homoserine lactone (C8-HSL) relative to the expression of an endogenous control gene (*recA*). Such quorum sensing genes have been identified in many members of the BCC (Lutter et al., 2001). Huber et al. (2001) demonstrated the importance of *cepI* and *cepR* genes (responsible for C8-HSL production in *B. cepacia* H111) for biofilm formation. Deletion of these genes resulted in flat and patchy biofilms; however, when 200 nM C8-HSL was added to a suspension of a *cepI* deletion mutant, the biofilm architecture returned back to its wild-type (Huber et al., 2001).

Following a similar approach, we conducted additional tests to establish a more clear etiology between the production of C8-HSL and biofilm formation. Addition of C8-HSL significantly ($p < 0.05$) reduced the inhibitory effect of nickel on biofilm formation (Fig. 3). These results were also observed in preliminary assays using another BCC strain, *B. cepacia* ATCC 25416 (Figure S-2), which forms less robust biofilms (Conway et al., 2002) but responds in a similar manner in the presence of C8-HSL. Note that the C8-HSL concentration used in these tests (400 nM) is consistent with levels observed in nature (Boettcher and Ruby, 1995), and is orders of magnitude lower than the concentration of nickel, which corroborates that nickel can inhibit cell–cell communication by inhibiting the production of the signal molecules.

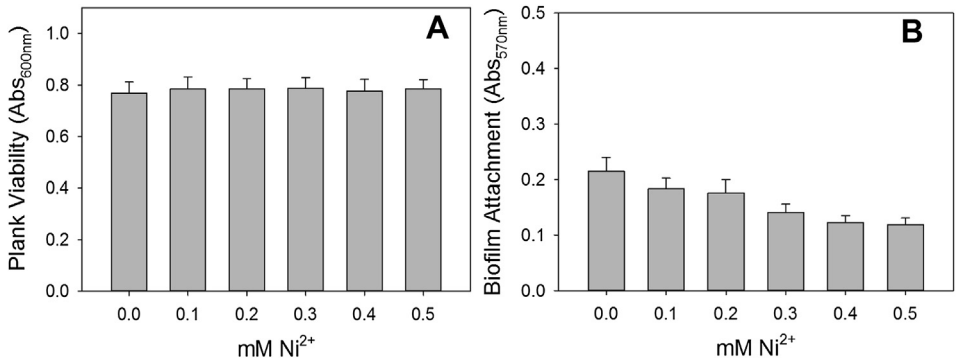

Fig. 1. Planktonic viability (A) and biofilm attachment (B) of *Burkholderia multivorans* 17616 in the presence of nickel, measured by absorbance at 24 h. Each bar represents the average of five experiments with a minimum of four replicates per experiment. Error bars in all figures represent standard errors. There is little change in planktonic viability after 24 h; however there is a marked difference in attachment from the control (one way ANOVA $p < 0.05$).

We also observed up-regulation of a gene encoding for a heavy metal efflux pump (*czcA*), although there was no correlation between its up-regulation and the added nickel concentration (Figure S-3). Note that up-regulation of efflux pumps could affect the intracellular nickel concentration and confound the effect of added (extracellular) nickel concentration on the extent to which *bmuIR* is down-regulated (Fig. 2).

3.2. Cadmium as another potential quorum sensing antagonist

Since nickel was able to down-regulate quorum sensing genes and inhibit biofilm formation without affecting overall viability, we investigated whether other divalent metals had a similar effect. Microtiter plate and qPCR analyses with *B. multivorans* targeting *bmuIR* were also performed with cadmium and cobalt salts (Fig. 4). While cadmium inhibited biofilm formation and the expression of quorum sensing genes at concentrations that do not affect planktonic viability (Fig. 4(A), (C) and (E)), cobalt's inhibition appears to be related to toxicity rather than QS inhibition at the transcriptional level (Fig. 4(B) and (F)). A growth curve was generated for cadmium as an additional comparison to nickel to evaluate potential toxicity. Although there was a 2-h lag in growth in the wells containing cadmium, all but the samples containing 0.5 mM cadmium reached the same optical density by 23 h (Figure S-1(B)) indicating that the tested cadmium concentrations were also sub-lethal.

3.3. Implications

This is the first study to report that nickel and cadmium decrease the expression of genes responsible for acyl-homoserine lactone quorum sensing and subsequently affect traits influenced by quorum sensing, such as biofilm formation, without affecting general (planktonic) cell viability. Heavy metals are typically used as enzyme co-factors for cellular metabolism in nanomolar concentrations, but are generally toxic at millimolar concentrations (Scheller et al., 2010; Srivastava et al., 2011). One of the most ubiquitous enzymes, urease, contains nickel as a co-factor (Karplus et al., 1997). Heavy metals have also been shown to stimulate the production of AHLs; for example, the *las* system in *Pseudomonas aeruginosa* and the *afe* system in *Acidithiobacillus ferrooxidans* are up-regulated by copper as a stress response (Ruiz et al., 2008; Thaden et al., 2010). In addition, the *afe* system stimulates biofilm formation to allow the biomining of Cu, Fe and U by *A. ferrooxidans* (Wenbin et al., 2011). This study shows that nickel and cadmium can inhibit non-essential cellular processes, such as quorum sensing, between concentrations necessary for survival and those that are toxic.

The pathway from signal production to the expression of a given trait can be impaired in several ways. These include the presence of multiple signals in the medium which "drown" out the target signal, competitive binding of receptor molecules by structurally similar molecules, or degradation of the molecule by other

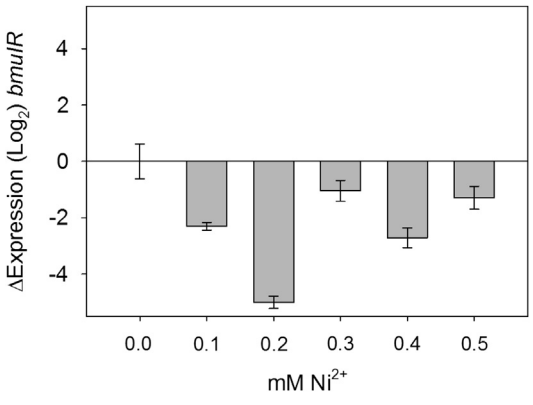

Fig. 2. Change in expression of acyl-homoserine lactone genes (*bmuIR*) in *Burkholderia multivorans* at different nickel concentrations compared to an endogenous control gene (*recA*). Each bar represents three experiments with a minimum of four replicates per experiment.

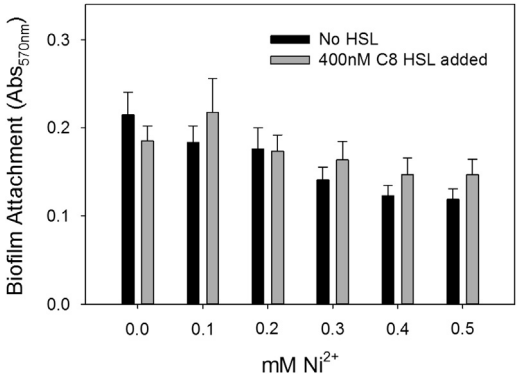

Fig. 3. Biofilm attachment of *B. multivorans* 17616 in the presence of 0–0.5 mM nickel alone (black bars) and with 400 nM C8-HSL added at inoculation (gray bars). Each bar represents a series of five experiments with a minimum of four replicates per experiment. The difference between the control lacking nickel and the samples containing nickel is significant (one way ANOVA $p < 0.05$); however, the addition of 400 nM HSL attenuates the effect ($p > 0.1$).

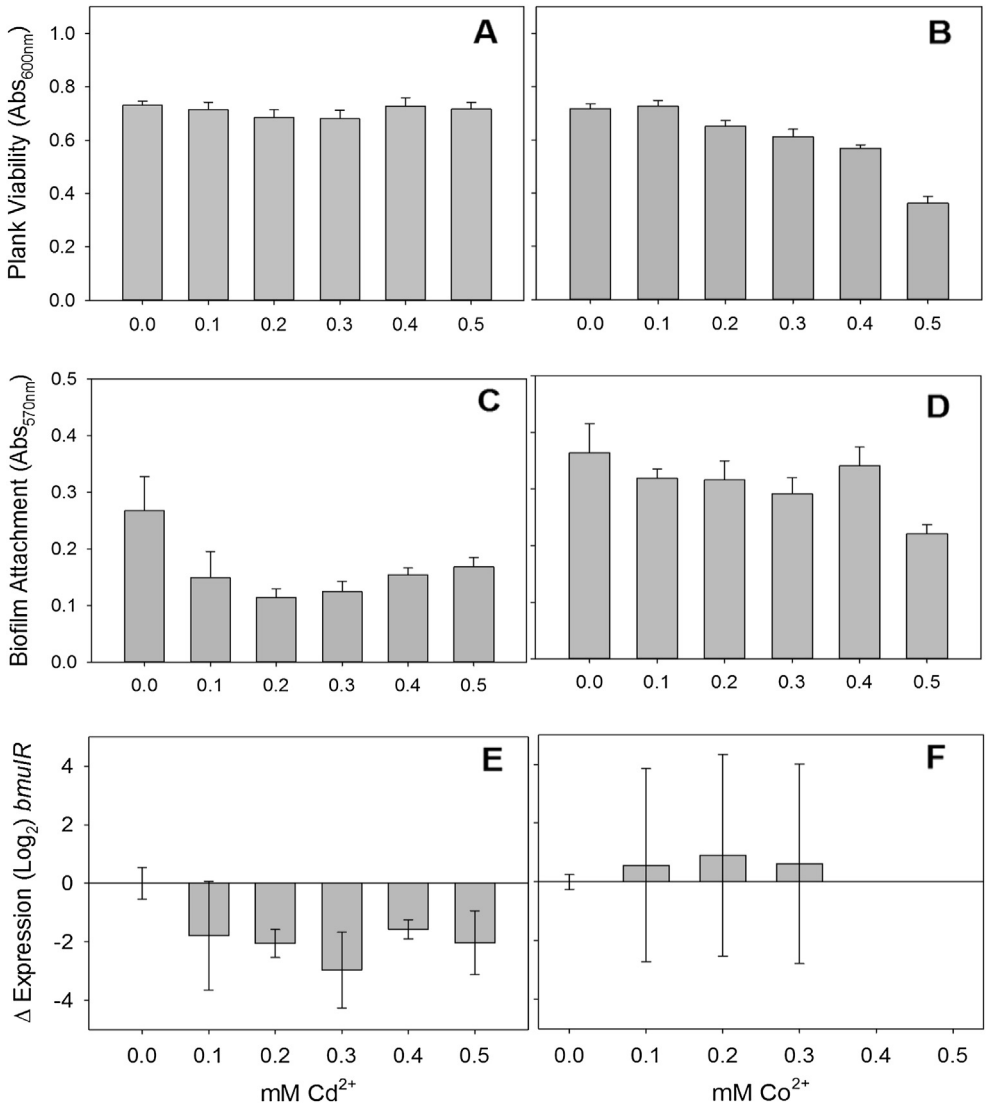

Fig. 4. Planktonic viability (A, B), biofilm attachment (C, D), and expression of *bmulR* genes (E, F) for *Burkholderia multivorans* 17616 in the presence of cadmium (left) and cobalt (right). Similar to nickel, cadmium decreases biofilm attachment (one way ANOVA $p < 0.05$) and down regulates acyl-homoserine lactone expression without affecting viability, while cobalt shows no clear correlation between metal concentration, viability and attachment.

organisms (Leadbetter and Greenberg, 2000; Lin et al., 2003). In this instance, how nickel and cadmium influence the signal in *B. multivorans* is unique; the expression of genes responsible for the signal, N-octanoyl-L-Homoserine lactone (C8-HSL), is reduced in the presence of the metal ions, thereby reducing the signal "volume" outside the cell, inhibiting cell–cell signaling, and subsequently biofilm formation.

Although further studies are required to identify the specific mechanisms by which nickel and cadmium inhibit quorum sensing at the transcriptional level, it is known that Ni^{2+}, Co^{2+}, and Cd^{2+} are competitive antagonists of both Mg^{2+} and Ca^{2+}(Abelson and Aldous, 1950; Waalkes and Poirier, 1984; Kasprzak et al., 1986; Littlefield et al., 1994). Magnesium is typically the most abundant divalent cation inside a prokaryotic cell, and is an essential cofactor for many enzymes, including DNA and RNA polymerases (Wackett et al., 2004). Nickel, cobalt, and cadmium have been demonstrated to inhibit polymerases, and conversely, it has been determined that increased concentrations of magnesium can attenuate this inhibition, a result predicted by the biotic ligand model (Miyaki et al., 1977; Snow et al., 1993; Di Toro et al., 2001). Additionally, zinc is a cofactor of several transcription factors, and is susceptible to competitive antagonism by other divalent cations (Bouhouche et al., 2000). Therefore, there are several potential mechanisms through which divalent cations may impact gene expression when they are present at sufficient levels. Notably, a strong interrelationship exists between magnesium uptake and that of other divalent cations; low concentrations of magnesium have been found to result in rapid uptake of both nickel and cobalt (Abelson and Aldous, 1950).

Besides their potential effects on transcription of quorum sensing genes, divalent cations may be directly involved in other mechanisms influencing bacterial surface adhesion. Competitive antagonism may play a role in biofilm inhibition. Both magnesium and calcium enhance prokaryotic adhesion through cation bridging interactions (De Kerchove and Elimelech, 2008; Orgad et al., 2011; Mi et al., 2012; Robertson et al., 2012). In addition, nickel and cadmium reduce biofilm mass (Hill et al., 2000; Lawrence et al., 2004; Morin et al., 2008). Paradoxically, however, nickel was found to increase biofilm formation in *Escherichia coli* K12 strains over-expressing the cell-surface protein Curli (Perrin et al., 2009).

This implies that the impact nickel has on biofilm formation may not be universal. How strongly the bacteria are affected can also be metal dependent. There was a gradual decrease in attachment as the concentration of nickel increased (Fig. 1(B)), while attachment decreased more abruptly in the presence of cadmium (Fig. 4(C)). Nickel is a micronutrient necessary for cellular metabolism and cells have an uptake pathway for nickel, in contrast to cadmium which is not typically used for cellular function. Since the biofilm lifestyle is preferred over a planktonic existence, there are multiple cellular mechanisms in place to ensure that biofilms can be formed; therefore, it is also possible that these metals affect other process influencing biofilm development besides quorum sensing.

In this study we demonstrate that nickel and cadmium can inhibit biofilm formation, though the inhibition of homoserine lactone quorum sensing, without affecting cellular viability. This should encourage further research on approaches for controlled release (e.g., coatings that modulate metal dissolution to ensure sufficient metal ion concentrations for biofilm control while preventing rapid metal depletion and exceedance of applicable water quality standards). Other important areas for further research include determining whether quorum sensing and biofilm inhibition can be exerted by less toxic substances and whether these compounds are as effective on multispecies biofilms. These research venues may enable the development of novel strategies to mitigate biofouling of surfaces and extend the life of infrastructure.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2014.03.013.

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